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Running head: In vitro hESC hematopoiesis independent of MYB

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ABSTRACT

Although hematopoietic precursor activity can be generated in vitro from human embryonic stem cells, there is no solid evidence for the appearance of multipotent, self-renewing and transplantable hematopoietic stem cells. This could be due to short half-life of hematopoietic stem cells in culture or, alternatively, human embryonic stem cell-initiated hematopoiesis may be hematopoietic stem cell-independent similar to yolk sac hematopoiesis, generating multipotent progenitors with limited expansion capacity. Since Myb was reported to be an excellent marker for hematopoietic stem cell-dependent hematopoiesis, we generated a MYB-eGFP reporter human embryonic stem cell line to study formation of hematopoietic progenitor cells in vitro. We found CD34+ hemogenic endothelial cells rounding up and developing into CD43- hematopoietic cells without expression of MYB-eGFP. MYB-eGFP+ cells appeared relatively late in embryoid body cultures as CD34+CD43+CD45lo cells. These MYB-eGFP+ cells were CD33 positive, proliferated in IL-3 containing media and hematopoietic differentiation was restricted to the granulocytic lineage. In agreement with data obtained on murine Myb-/- embryonic stem cells, bright eGFP expression was observed in a subpopulation of cells, during directed myeloid differentiation, which again belonged to the granulocytic lineage. In contrast, CD14+ macrophage cells were consistently eGFP- and were derived from eGFP- precursors only. In summary, no evidence was obtained for in vitro generation of MYB+ hematopoietic stem cells during embryoid body cultures. The observed MYB expression appeared late in culture and was confined to the granulocytic lineage.
INTRODUCTION

During embryogenesis, hematopoietic development is spatiotemporally organized in different waves. During the first, so-called primitive wave of hematopoiesis, nucleated erythrocytes and macrophage-like cells are generated in the yolk sac (YS). Subsequent waves generate multipotent progenitor cells, first in the YS and, finally, in the aorta-gonado-mesonephros region (AGM) where hematopoietic stem cells (HSCs) are generated(1).

Members of the MYB transcription factor family are important transcriptional regulators throughout embryonic development. One of these three family members, MYB, is differentially expressed during embryonic hematopoietic development(2). While Myb transcripts have been detected at low levels during early waves of hematopoiesis, this process is not MYB-dependent. In contrast, HSC-dependent hematopoiesis appears to strictly rely on MYB as loss of function mutants of MYB lead to embryonic lethality due to failure of fetal liver hematopoiesis(3, 4). In the zebrafish, transgenic animals expressing eGFP under control of myb regulatory elements have been used for visualization and analysis of HSC generation from aortic blood forming hemogenic endothelium(5).

In postnatal life, long term repopulating hematopoietic stem cells (LT-HSC) were found to express the highest levels of Myb and levels decrease progressively in short term repopulating HSC (ST-HSC) and in the multipotent progenitor population (MPP)(6, 7).

MYB-independent hematopoiesis consists largely of short-lived precursors and mature blood cells. However, the mononuclear phagocyte system (MPS), consisting of brain microglial cells, hepatic Kupffer cells and skin Langerhans cells, seems to be derived from MYB-independent hematopoietic progenitor cells (HPC) rather than bone marrow-derived MYB-dependent HSC. Recently, Schulz and colleagues have shown that two parallel pathways of macrophage differentiation can be distinguished by their inherent dependence on MYB(8). MYB-independent cells originate in the yolk sac around E7.5-E8.5, possibly from an erythro-myeloid restricted precursor (EMP), while MYB-dependent macrophages originate from bone marrow HSC.

Gene therapeutic strategies for inherited immune deficiencies or other genetic diseases of the blood rely on the assumption that HSC can be generated in vitro from pluripotent stem cells(9). In these approaches, patient-specific induced pluripotent stem cells are generated and the defective gene is corrected by homologous recombination subsequent to targeting the gene defect by zinc-finger nucleases (ZFN) or TALENs. Once the genetic defect is corrected, “cured” HSC are generated by an appropriate in vitro differentiation protocol before infusion. However, current protocols have failed to convincingly demonstrate the generation of HSC in pluripotent stem cell differentiation cultures.

To investigate whether during in vitro human embryonic stem cell (hESC) derived hematopoiesis HSC are formed or rather, hematopoiesis from hESC depends on the emergence of a myb independent EMP-like cell, we generated a MYB reporter line using random integration of a bacterial artificial chromosome (BAC) reporter construct in which eGFP expression is under control of the MYB regulatory DNA sequences.
METHODS

Cell lines, culture of cell lines and isolation of primary cells

All experiments were approved by the Medical Ethical Committee of Ghent University Hospital (Belgium). The WA01 (National Institutes of Health code: WA01) human embryonic stem cell (hESC) line was used in all experiments. Further methods describing used cells and culture of cells can be found in supplemental materials.

Hematopoietic differentiation of hESC in spin embryoid bodies (EB)

To differentiate hESC into hematopoietic cells, the protocol from Ng et al. was used with minor modifications(10). In brief, 5x10³ single cell-adapted hESC were spun at 480 g into each well of a 96-well low attachment plates and subsequently cultured in APEL medium containing 10 µM Rock inhibitor Y-27632 (Selleckchem, Houston, TX, USA), 40 ng/ml SCF (Peprotech, Rocky Hill, NJ, USA), 2 ng/ml BMP4 (R&D, Minneapolis, MN, USA) and 20 ng/ml VEGF165 (Peprotech), further referred to as “EB mix”. After 4 days, spin EB were transferred on an OP9 cell layer and further cultured in EB mix for a total of 7-14 days. Half of the medium was changed on day 7, with APEL medium containing EB mix cytokines, unless a different combination of cytokines is specified. These cytokines were added at following concentrations: 50 ng/ml IL-3 (R&D), 50 ng/ml Flt3-L (R&D), 10 ng/ml TPO (Peprotech) and/or 50 ng/ml IL-6 (R&D).

For myeloid differentiation, spin EBs were dissociated at day 11 and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D) and 20 ng/ml IL-6 (R&D). For erythro-megakaryocytic differentiation, spin EBs were dissociated and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech) and 50 ng/ml EPO (eBioscience, San Diego, CA, USA).

Flow cytometry and cell sorting

Flow cytometric analysis was performed on a LSR II system (BD biosciences, San Jose, CA, USA). Cell sorting was performed with a FACS ARIA IIIU system (BD Biosciences). A list of antibodies used can be found in supplemental materials.

Real time RT-PCR

Cells were lysed and cDNA was synthesized using the SYBR power cells-to-Ct system (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Primer sequences can be found in supplemental Table S1. PCR reagents and SYBR GreenI master were obtained from Roche (Roche, Penzberg, Germany) and used according to the manufacturer’s instructions. The reactions were run on a lightcycler480, 384well system (Roche).
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**Statistical analysis**

All statistical analyses were performed using SPSS V22.0 (IBM, New York, USA). Significance was assessed using Mann-Whitney U statistical analysis with significance level set at $p \leq 0.05$.

Additional materials and methods are provided in supplemental materials and methods file.
RESULTS

Generation of MYB-eGFP transgenic reporter hESC.

To study MYB expression during hematopoietic differentiation, we generated a MYB-eGFP reporter hESC line using random insertion bacterial artificial chromosome (BAC) transgenesis (supplemental figure 1).

After nucleofection of hESC and neomycin selection, neomycin-resistant hESC colonies were obtained. In figure 1, data are shown of clone 5, which gave highest eGFP expression and was used in all experiments. Besides the endogenous MYB loci on chromosome 6q, a clear hybridization signal on chromosome 11q was detected on FISH analysis, confirming successful integration of the BAC reporter vector (Figure 1 A). High-resolution arrayCGH shows integration of the BAC plasmid from +84.6 kbp 5’ of the MYB start codon to -39.3 kbp 3’ of the start codon. This region encompasses the complete MYB gene and upstream regulatory elements (Figure 1 B).

Hematopoietic differentiation of the MYB-eGFP cell line, before removal of the selection cassette, showed eGFP expression by CD43+ hematopoietic cells as well as by CD34+CD43- endothelial cells (Figure 1 D). As no MYB transcripts could be found by qPCR analysis in the CD34+CD43- population (supplemental figure 2), aberrant expression due to interference of the pGK promotor was hypothesized. Therefore, the pGK promotor-driven selection cassette was removed through transient transfection with a Cre recombinase-encoding plasmid (Figure 1 C). After removal of the selection cassette, no eGFP expression was observed in non-hematopoietic cells (Figure1 D). Although the expression of eGFP found at this point in time was relatively weak, prolonged culture, clearly showed strong eGFP+ cells as determined by confocal microscopy analysis of d20 EB differentiation cultures (Figure 1 E).

Fidelity of the random integration reporter cell line was validated by qPCR analysis for MYB expression. eGFP-, eGFPlow and eGFPhi cells were sorted from d14 hematopoietic differentiation cultures in the presence of SCF, TPO, IL-3 and IL-6 and MYB mRNA levels were shown to correlate with eGFP protein expression in the sorted populations (Figure1 F and supplemental figure 2). Note that the eGFP cells are not completely negative for MYB mRNA. This indicates that the reporter line is not as sensitive as RT-qPCR for MYB expression. However, this does not preclude the detection of HSC as these cells are supposed to express high levels of Myb(6).

Endothelium-derived early hematopoietic precursors are MYB-eGFP negative.

Spin EB cultures were set up and screened daily for the appearance of hematopoietic eGFP+ cells. Expression of CD43, the earliest marker for hematopoietic cells, was first observed in our cultures at day 7. Few eGFP+ cells became apparent from day 11 onwards, forming a clear population on day 14. As shown in Figure2 A-B, these eGFP+ cells were contained within the CD34+CD43+ population, which has been reported to contain progenitors of multiple hematopoietic lineages. On the other hand the CD34+CD43- endothelial and CD34+CD43lo emerging HPC populations were consistently negative for
eGFP expression (Figure 2 B). As expression was also found highest in the CD34⁺CD43⁺ population within the CD45⁻ population (supplemental figure 2), we hypothesized this population to have the highest possibility of containing MYB⁺ multipotent progenitors.

To assess whether the eGFP⁺CD43⁺ hematopoietic cells were emerging directly from hemogenic endothelium, day 11 CD34⁺CD43⁻ endothelial cells were sorted and replated on OP9 stromal cells in medium containing SCF, BMP4 and VEGF. After 6 days of culture a distinct CD45⁺ population was apparent, however no eGFP⁺ cells were seen at this timepoint. After 11 days of culture, eGFP⁺ cells became apparent (supplemental figure 3 A). As this assay might miss the emergence of transient eGFP⁺ cells directly from hemogenic endothelium, we analyzed using a similar set-up, the emergence of hematopoietic cells from endothelium by live confocal imaging. During live confocal imaging, adherent eGFP⁺CD34⁺CD43⁻ endothelial cells were found to round up and form cells with hematopoietic appearance. These cells subsequently upregulated CD43 and proliferated extensively over the course of 72 hrs. We could not detect eGFP expression during the whole process of blood cell generation and subsequent proliferation, suggesting MYB-independent generation of HPC (Figure 3 and Supplemental Movie S1). Similar experiments initiated with sorted eGFP⁻CD34⁺CD43⁻ endothelial cells derived from earlier (day 7) or later time points (day 14) of EB cultures were much less potent in the generation of hematopoietic cells and generated only few blood cells.

To verify whether other or additional growth factors were required for the generation and/or expansion of the eGFP⁺CD34⁺CD43⁻CD45⁻/lo population, EB-derived hematopoietic cells were cultured in different cytokine combinations of SCF, TPO, Flt3L, IL-3 and IL-6. It was found that eGFP⁺CD34⁺CD43⁻CD45⁻/lo cells expanded most efficiently in conditions containing IL-3. As shown in Figure 4A, the mean of absolute numbers of eGFP⁺CD34⁺CD43⁻CD45⁻/lo per EB was about six fold higher than those in conditions without IL-3. This suggests that the eGFP⁺CD34⁺CD43⁻CD45⁻/lo cells are responsive to IL-3, as has been reported previously(11).

Characterization of eGFP negative and eGFP positive subsets.

To further define the eGFP⁺ cells generated in these cultures, we analyzed these cells for expression of the stem cell phenotype CD38⁻, CD90⁻, CD49f⁺ and CD45RA⁻ as previously described by Notta et al., the myeloid lineage markers CD33 and CD123, and erythro-megakaryocytic lineage markers CD41a and CD235a. As shown in figure 4B, eGFP⁺CD34⁺CD43⁻CD45⁻/lo cells expressed low levels of CD45RA, were negative for CD38 and CD90 and expressed CD49f. This CD34⁺CD45RA⁻CD38⁻CD49f⁺ phenotype was present in all conditions including the conditions containing IL-3, and is similar to the long term engraftable HSC described by Notta et al(12). However, the eGFP⁺ cells in our cultures also showed clear expression of CD33 and CD123, while erythroid lineage markers were negative, suggesting myeloid lineage commitment of these cells. The phenotype was very similar independent of the cytokines added, except for the condition in EBmix + TPO + Flt3-L, where CD38 was upregulated. CD235 and CD41a were consistently absent on these populations, arguing against erythroid commitment of eGFP⁺CD34⁺CD43⁻CD45⁻/lo cells. The eGFP CD34⁺CD43⁺CD45⁻ cells, on the other hand, contained mainly erythroid committed cells, while the CD45⁺ population, consisted of cells
expressing myeloid lineage markers (data not shown). The eGFP* cells derived from eGFP- hemogenic endothelium, also phenotyped as CD45^lo/CD33^+CD14^- cells, consistent with the data obtained on bulk cultures (supplemental figure 3 B).

We assessed myeloid, erythroid and megakaryocytic differentiation capacity of the different populations obtained in the cultures with various growth factor mixes to assess multipotency. Results were qualitatively similar for all growth factor conditions. In figure 5 A, the results are shown of sorted cell populations from d14 spin EB cultures expanded with EBmix plus IL-3 and Flt3-L, and subsequently assayed under conditions optimal for either myeloid, erythroid or megakaryocytic differentiation. The eGFP^+CD34^+CD43^+CD235a/CD41a^-CD45^- population clearly contained progenitors giving rise to myeloid, erythroid and megakaryocytic cells, while the eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^-lo population was shown to give rise to granulocytic lineage myeloid cells only, with complete absence of monocytic, erythroid and megakaryocytic precursor potential. On the other hand, eGFP^-CD34^-CD43^-CD235a/CD41a^+ cells gave rise mainly to erythroid and megakaryocytic cells, although some myeloid precursor activity was also observed. eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- cells were able to give rise to CD11b^-CD14^- granulocytic lineage cells, CD11b^-CD14^- macrophage lineage cells, CD71^-CD235^+ erythroid and CD41^- megakaryocytic cells (Figure 5 A).

To assess expression of genes associated or determining commitment towards the different lineages, RT-qPCR was performed for MPO, GATA1, PU.1 and MPL on the same sorted populations as in Figure 5 A. MYB was assessed as a control and showed high expression in the eGFP^+ population as expected (Figure 5 B). In line with the multipotent precursor capacity, the eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- cells expressed only low levels of the various genes analyzed. In contrast, the eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- cells had significantly higher levels of PU.1 suggesting that together with the acquisition of CD45, the cells become committed to the macrophage lineage. As expected, the eGFP^-CD34^-CD43^-CD235a/CD41a^- express high levels of GATA1 and MPL, emphasizing their erythro-megakaryocytic commitment. In contrast, the eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- population was shown to give rise to granulocytic lineage myeloid cells only, with complete absence of monocytic, erythroid and megakaryocytic precursor potential. On the other hand, eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- cells gave rise mainly to erythroid and megakaryocytic cells, although some myeloid precursor activity was also observed. eGFP^-CD34^-CD43^-CD235a/CD41a^-CD45^- cells were able to give rise to CD11b^-CD14^- granulocytic lineages, CD11b^-CD14^- macrophage lineages, CD71^-CD235^+ erythroid and CD41^- megakaryocytic cells (Figure 5 A).

To study the generation of the eGFP^+ precursor at the clonal level, CFU assays and single cell sorting followed by liquid culture were performed on d11 and d14 EB cultures (figure 6). The day11 eGFP^-CD34^-CD43^- cell population gave rise to CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E and CFU-E. These colonies were analyzed microscopically for eGFP expression and it was observed that the CFU-GM, CFU-G and CFU-GEMM contained eGFP positive cells whereas the CFU-M, CFU-E and BFU-E were consistently negative (figure 6 A). Similarly, using single cell culture and flow cytometric analysis, we found that d11 and d14 eGFP^-CD34^-CD43^- progenitors consisted of GEMM, GM, G, M, E precursors. A marked skewing towards the myeloid lineage was observed on d14 of culture. In agreement with the CFU assays, eGFP^+ cell containing wells were confined to the wells containing G, GM and GEMM precursor cells, whereas the wells lacking eGFP expression contained E and M.
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precursors (figure 6 B and 6 C). These data clearly show, that the MYB-eGFP⁺ precursors is a more committed myeloid precursor derived from a multipotent MYB-eGFP⁻ hematopoietic precursor cell.

While macrophage development is MYB-independent, MYB marks granulocytic lineage differentiation.

As MYB was described to be dispensable for the generation of YS derived macrophages, we studied the role of MYB during myeloid lineage choice. When EB cultures were grown under myeloid differentiation conditions, a homogenous population of CD45⁺CD33⁺ was obtained after 4 days of culture (Figure 7 A). To further define both eGFP⁺ and eGFP⁻ populations, we analyzed both populations by flow cytometry, cytospin and qPCR.

The eGFP⁺ population was found to be CD45lo and positive for CD33, weakly expressing the myeloid markers CD11c, CD11b, CD123 and CD13. The granulocytic marker CD15 was found absent. This phenotype is in line with the surface phenotype of myelocytes, a precursor of the granulocyte lineage. Macrophage markers CD14, CD16, CD115, HLA-DR and CD86 were consistently negative. On the other hand, the eGFP⁻ population was found to be CD45hi, CD33⁺. These cells were CD11c⁺hi, CD11b⁺hi, CD14⁺, HLA-DR⁺, CD86⁺ compatible with a activated macrophage phenotype. CD115, the receptor for M-CSF, was found only weakly positive. The cells expressed CD16, which is normally expressed on tissue macrophages (Figure 7 B).

On cytological analysis using May-Grünwald-Giemsa staining, eGFP⁺CD14⁺ cells show a granulocytic cytoplasm compatible with myelocytic granulocyte lineage cells, whereas the eGFP CD14⁺ cells have a morphology compatible with tissue macrophages (Figure 7 C). This was confirmed by qPCR analysis showing these eGFP⁺ cells to express the granule protein lactoferrin (LTF), eGFP⁻ cells on the other hand were devoid of lactoferrin expression (Figure 7 D). As expected, MYB expression levels were high in eGFP⁻CD14⁺ cells (Figure 7 D).

To analyze whether the generation of these macrophages from hematopoietic precursor cells was MYB-independent, we sorted eGFP CD34⁺CD43⁻CD45⁻ committed precursor cells, cultured these in myeloid conditions on OP9 stromal cells and analyzed them daily for the expression of eGFP. After 4 days, a population of CD14⁺ cells was already clearly visible. These cells were negative for eGFP as assessed by flow cytometry (Figure 8 A). Further culture of these cells for up to 11 days, showed clear eGFP positive populations at all analyzed time points. However, at no time point were eGFP⁺CD14⁺ cells observed. After 11 days of culture, virtually all eGFP⁻ cells were CD14 positive (Figure 8 A).

To determine whether an eGFP⁺CD14⁻ intermediate stage gave rise to the eGFP CD14⁺ macrophage cells, sorted day 11 eGFP⁺CD34⁺CD43⁻CD45⁻CD14⁻ committed HPC were cultured under myeloid conditions and analyzed using live confocal microscopy to track cells becoming CD14⁺. We did not observe eGFP expression in cells acquiring CD14 expression (Figure 8 B and Supplemental Movie S2). These data suggest that the generation of CD14⁺ macrophage cells from hESC is MYB-independent.
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Discussion

We here show evidence that multipotent HPCs expressing high levels of MYB are not generated in human EB cultures initiated with hESC. We therefore conclude that the hematopoietic cells generated in vitro from hESC using current in vitro protocols, emerge through an endothelial intermediate, and are precursors with limited stem cell activity that resemble yolk sac hematopoietic progenitors. In addition we show that the first progenitors derived from endothelial cells are eGFP CD34+CD43+ cells, which then develop into eGFP+CD34+CD43+ granulocyte committed progenitors.

Multipotency and self-renewal are two hallmark characteristics of HSC. With regard to multipotency: almost all hematopoietic cell types can be generated from hESC-derived hematopoietic precursors, including erythrocytes(13), megakaryocytes(14), granulocytes, monocytes(15), NK cells(16) and T cells(17, 18). Although, to our knowledge, it has not been demonstrated that a single hESC-derived hematopoietic precursor cell is able to form all of the aforementioned cell types, the absence of these reports may be due to technical issues associated with precursor cells of limited proliferative capacity. The fact that T cells, can be generated from fetal HPC does not in itself prove the presence of multipotent progenitors or HSC, since it has been shown that T cells can be generated from YS precursors that arise before HSC are generated(19, 20). Self-renewal of HSC is evaluated by their ability to reconstitute immune deficient mice. Previous studies have reported repopulation by in vitro hESC-derived HPC(21, 22). Wang et al. reported multilineage hematopoietic repopulation upon intrafemoral injection of HPC(22). However, hESC-derived HPCs had limited proliferative and migratory capacity compared with somatic HSCs. In another study by Ledran et al., whole hESC differentiation co-cultures with stromal cells derived from murine fetal tissues were injected intrafemorally in mice. These cells were reported to repopulate bone marrow of the non-injected femur as well as the spleen. In addition, secondary engraftment was reported. However, the engrafted cells were not fully defined. The phenotype shown is compatible with myeloid cells (CD33+CD13+). As it is known that tissue macrophages have a long lifespan, and are found lifelong in the adult, without a need for HSC to replenish them, chimerism may have been caused by non-HSC dependent cells. Evidence for engraftment of CD34+ cells in the bone marrow was lacking.

In a recent article by Amabile et al. hESC were injected in mice to form teratoma. The authors show that in these teratoma, also hESC derived CD34+ hematopoietic cells are formed. Upon isolation and transplantation of these CD34+ HPC into immunodeficient mice, engraftment of human cells was found, including CD34+ cells in the bone marrow. This in vivo differentiation model therefore suggests that HSC can be generated from human pluripotent stem cells (23).

In conclusion, based on these data it remains questionable whether HSC can be formed from ESC using currently available in vitro differentiation protocols, although it is clear that multipotent progenitors are formed. Changes to the culture conditions, such as other cytokine mixtures or the use of more appropriate feeder lines may result in HSC generation. The MYB-eGFP cell line that we generated will be very helpful in screening for such conditions. Which factor(s) are missing to generate
HSC is unknown to date. However, recent publications using reprogramming of specified cells are starting to shed light on this issue (24-26). Recently it was reported that overexpression of 5 factors in hESC-derived HPC confers short-term engraftment potential to these hESC-derived cells(24). One of the factors which was required for in vivo engraftment was characterized as MYB. A similar strategy was used for reprogramming murine hematopoietic cells toward transplantable HSC (26). Although the reprogramming factors needed for conferring HSC properties have not yet reached consensus, this approach may prove to be an alternative method for generating HSC from pluripotent stem cells.

An alternative hypothesis could be that HSC are continuously generated in vitro, but lack necessary signals that are present in vivo, and thus quickly degenerate to restricted precursor cells, losing their self-renewal capacity. Fetal, newborn and adult HSC are known to lose the capacity to self-renew within days of in vitro culture(27-29).

To study the emerging HPC in vitro, we isolated CD34+ CD43- endothelial cells and studied the characteristics of hematopoietic cells generated from them through time-lapse microscopy. We did not observe generation of MYB-eGFP expressing cells, that subsequently lose MYB expression in vitro. This is in line with the reported generation of hematopoietic cells directly from hemogenic endothelium from Myb-/- murine ESC, showing MYB to be dispensable for endothelial to hematopoietic transition(30). Instead, we observed either cells that remained MYB negative, or cells, which upregulated MYB over the course of several days and were further characterized as granulocyte lineage cells. In the murine system, strong evidence was provided that MYB is essential for HSC function. Transplantable HSC were shown to express high levels of Myb (6). This is strengthened by the fact that Myb-/- mice die around fetal day 15 due to lack of transplantable HSC (4), moreover, conditional deletion of Myb leads to exhaustion of the stem cell pool and failure to engraft upon transplantation of LSK cells (6). We can therefore conclude that it is unlikely that HSC are generated in these spin EB cultures.

Conditional MYB knockout models show a marked decrease in granulocytic development, suggesting an important role for MYB in granulocytic development(7). In addition, hematopoietic differentiation cultures set up with Myb+ murine ESC also showed defective granulocyte lineage differentiation (30, 31). Clarke et al. reported a similar potential to generate CFU-E and CFU-M between wildtype and Myb+ mESC in short term cultures, however, later on, the numbers of CFU-E and CFU-GM were decreased. Sakamoto et al. compared wild type with Myb-/- mES cells and knock out mES cells that expressed Myb under control of a tetracyclin-inducible promotor. They reported similar numbers of hematopoietic cells generated by wild type and knock out cells, whereas induction of Myb during hematopoietic differentiation resulted in vastly higher cell numbers. Similar to Clarke et al., they report a reduced differentiation towards erythroid and granulocytic lineages, whereas the monocytic differentiation is unaffected. The relative absence of these lineages in mESC cultures reflects the MYB dependency of a lineage restricted erythroid-megakaryocytic and erythro-myeloid precursor rather than the generation of MYB-dependent HSC. These data thus point towards a differential requirement of MYB during macrophage/granulocyte commitment, as is also apparent in our experiments.
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Recent studies suggest that tissue macrophages belong to a separate lineage derived from MYB-independent precursors. These are derived from YS derived EMP and persist by local proliferation of terminally differentiated cells or precursors into adult life independent from the bone marrow. The MYB-eGFP macrophage cells generated in our cultures, co-express CD14 and CD16, a phenotype previously described for tissue macrophages (32, 33).

In conclusion, we have shown that hematopoietic precursor cells that arise from endothelial cells in EB cultures are MYB negative. MYB-positive precursors arise later in the cultures and are granulocyte lineage restricted. These data therefore provide evidence that bona fide HSC are neither generated nor maintained in these cultures. Rather, yolk sac-like hematopoietic precursors are formed. We here describe that granulocytic lineage committed progenitor diverge thereof, and that this process is accompanied by the upregulation of MYB. In this light reinvestigation of Myb signal in YS precursors might be advised. In addition, we have described a Myb reporter cell line which may be helpful to screen for conditions which can generate HSC from hemogenic endothelium in vitro.
Author contributions

Stijn Vanhee: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Katrien De Mulder: conception and design, collection of data; Yasmine Van Caeneghem: conception and design, collection of data; Greet Verstichel: conception and design; Nadine Van Roy: collection and assembly of data, data analysis and interpretation; Björn Menten: collection and assembly of data, data analysis and interpretation; Imke Velghe: collection of data; Jan Philippé: collection and assembly of data, data analysis and interpretation; Dominique De Bleser: contributed vital new reagents; Bart N. Lambrecht: contributed vital new reagents; Tom Taghon: data analysis and interpretation; Georges Leclercq: data analysis and interpretation; Tessa Kerre: data analysis and interpretation; Bart Vandekerckhove: conception and design, data analysis and interpretation, manuscript writing;

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Conflict of Interest Disclosures

The authors declare no conflict of interest.
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Figure legends

Figure 1: Functional validation of MYB-eGFP reporter hESC.

A) MYB FISH analysis (green) on metaphase nuclei (DAPI stain in grey); inset shows chromosome 11 only; B) ArrayCGH showing amplification of the genomic region within the BAC plasmid. A relevant portion of chromosome 6 is shown; c) Validation of selection cassette removal, as assessed by PCR ranging from eGFP to the first exon of MYB (upper gel) or ranging from pPGK to the first exon of MYB (lower gel); D) MYB-eGFP reporter hESCs differentiated towards the hematopoietic lineage were analyzed for eGFP expression. CD34⁺CD43⁻ endothelial and CD43⁺ hematopoietic populations are depicted. Both floxed and non-floxed cell lines are shown; E) Confocal fluorescence microscopy of a day 20 EB culture in EB mix showing bright eGFP positive round cells, scale bare measures 100µM; F) MYB qPCR analysis of eGFP sorted hematopoietic cells from EB differentiation culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression. Error bars indicate standard deviation (SD) of the mean (n=4).

Figure 2: Early MYB-eGFP⁺ HPC are generated directly from hemogenic endothelium.

A) Gating strategy for assessment of eGFP expression, a representative plot of EB day 11 culture is shown; B) Flow cytometric analysis of day 11 and day 14 embryoid body cultures in EB mix. Wild type hESC are presented as control for eGFP signal in all populations. Percentages of cells in each quadrant are shown; C) Time-lapse confocal analysis of replated day 11 hemogenic endothelium. A single frame is depicted every two hours starting from the defined starting point. Cropped images are shown for every channel. Arrows depict a single endothelial cell at the starting point of analysis and the progeny thereof at later points in time. Scale bar measures 100 µM; At 72h, the cells have rapidly proliferated and generated two clusters of 8 cells. These cells have downregulated CD34 and upregulated CD43. Scale bar measures 100µM.

Figure 3: Early HPC show commitment towards the myeloid lineage.

A) Absolute cell number of eGFP⁺ cells within the CD34⁺CD43⁺ population of day 14 embryoid body cultures. Error bars indicate standard deviation (SD) of the mean (n=3); B) Flow cytometric analysis of day 14 MYB-eGFP EB, cultured in the presence of different cytokine mixes as indicated. Dot plots are gated on CD34⁺CD43⁺. Cells within the eGFP⁺ gate are depicted in the histograms. Shaded histograms show isotype control stained samples for the indicated marker. Representative plots of 3 independent experiments are shown; C) Flow cytometric analysis of differentiation cultures towards granulocytic, monocytic, megakaryocytic and erythroid cells. Day 14 MYB-eGFP hESC-derived precursors with indicated phenotypes were isolated from IL-3 and Flt3-L expanded EB; representative plots of at least 3 independent experiments are shown; D) qPCR analysis for lineage commitment genes on indicated populations, isolated from day 14 MYB-eGFP hESC EB expanded in presence in IL-3 and Flt3-L. Expression is shown relative to the expression of GAPDH. Error bars indicate standard deviation (SD) of the mean (n=3).
Figure 4: Clonal progeny analysis of CD34+CD43+ progenitors.

A) Representative microscopic images of eGFP CD34+CD43+ d11 EB derived progenitor CFU assay, analyzed after 14 days of liquid culture. A representative image for each colony type is depicted. Both eGFP and brightfield channels are shown; B) Representative analysis of single cell (clonal) cultured CD34+CD43+ EB derived progenitors, analyzed after 7 days of culture. CD235a/CD41a- cells are depicted in the center and right panels. Scoring gates are indicated with E (erythroid/megakaryocytic), M (monocytic) and G (granulocytic). Combination of these gates led us to determine colony type as G, M, E, GM or GEMM as indicated for the representative plots. Representative progeny of eGFP+CD34+CD43+ EB derived progenitors are depicted in the dotted box; C) Frequency of progenitor types within CD34+CD43+ EB derived progenitors at the indicated time points. Absolute numbers of colonies are indicated between brackets. The progeny of eGFP+CD34+CD43+ are depicted in the panels below as fractions of wells containing eGFP+ cells or wells lacking eGFP+ cells; all wells were scored as shown in panel B.

Figure 5: Myeloid cells show strong expression of MYB-eGFP upon granulocytic lineage differentiation.

A) Flow cytometric analysis of myeloid-directed differentiation of day 11 MYB-eGFP hESC embryoid bodies, after 5 days of myeloid culture. Representative plots of at least 3 independent experiments are shown; B) phenotypic analysis of eGFP+ (upper panels) and eGFP- (lower panels) populations in myeloid directed differentiation cultures of day 11 MYB-eGFP embryoid bodies after 5 days of myeloid culture. All cells shown in histograms are gated on CD45. Shaded histograms show control staining for the depicted marker. Representative plots of 3 independent experiments are shown; C) May-Grünwald-Giemsa staining of cytospin samples of myeloid-directed differentiation of day 11 MYB-eGFP hESC embryoid bodies, eGFP+CD14+CD11b+ and eGFP+CD14+ populations are depicted; D) qPCR analysis for lactoferrin (LTF) and MYB expression in indicated populations. Expression is shown relative to the mean of GAPDH and YWHAZ expression. Error bars indicate standard deviation (SD) of the mean (n=2).

Figure 6: Macrophages develop without expression of MYB-eGFP and show characteristics of yolk sac-derived tissue macrophages.

A) Flow cytometric analysis of myeloid-directed differentiation of day 11 MYB-eGFP hESC EB-derived CD34+CD43+CD45+CD14-eGFP precursors, after 4, 8 and 11 days of myeloid culture; B) Time-lapse confocal analysis of live stained cultures. CD34+CD43+CD45+eGFP+CD14- cells were cultured in myeloid differentiation cultures. Samples were live stained with CD14-PE for confocal analysis. eGFP+ myeloid cells are shown as reference. Single channel panels are shown every hour starting from 11.30 h after plating onwards. Scale bars measure 10 µM.
A. Absolute cell number of CD34+CD43+eGFP+ cells per EB

- SCF + VEGF + BMP4 (EBmix)
- EBmix + IL-3 + Flt3-L
- EBmix + TPO + Flt3-L
- EBmix + IL-3 + IL-6 + TPO + Flt3-L

B. Flow cytometry histograms for CD45 and eGFP in different conditions:

- EBmix
- EBmix + IL-3 + Flt3-L
- EBmix + TPO + Flt3-L
- EBmix + IL-3 + IL-6 + TPO + Flt3-L
A. Myeloid | Erythroid | Megakaryocytic | Input population

- eGFP^CD34^CD43^CD235a/CD41a^-CD45^-
- eGFP^CD34^CD43^-CD235a/CD41a^-CD45^-
- eGFP^CD34^CD43^-CD235a/CD41a^-CD45^-
- eGFP^CD34^CD43^-CD235a/CD41a^-CD45^-

B. MYB | MPO | PU1

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GATA1 | MPL

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Supplemental materials and methods

MATERIALS AND METHODS

Cell lines, culture of cell lines and isolation of primary cells

All experiments were approved by the Medical Ethical Committee of Ghent University Hospital (Belgium). The WA01 (National Institutes of Health code: WA01) human embryonic stem cell (hESC) line was used in all experiments. The hESC line was kept in an undifferentiated state on MEFs as described. Single cell adapted hESC were cultured as described (Costa et al. Nature Protocols. 2007, Vol 2, No 4, pp 792-796). Briefly, single cell suspensions were made from hESC cultures using TrypLE select (Gibco, Life Technologies, Carlsbad, CA, USA) and were subsequently cultured on MEFs at a density of 21,000/cm^2. Cultures were split every 3 days. OP9 cells, MOLT4 and K562 were purchased from ATCC (LGC Standards SARL, Molsheim, France). Cell lines were cultured in medium supplemented with L-glutamine, streptomycin and penicillin with 10-20% FCS (all from Invitrogen, Life Technologies, Carlsbad, CA, USA). Peripheral blood monocytes were isolated after hydroxyethyl starch (HES) sedimentation for 30’ at room temperature and subsequently sorted by FACS.

Hematopoietic differentiation of hESC in spin embryoid bodies (EB)

To differentiate hESC into hematopoietic cells, the protocol from Ng et al. was used with minor modifications (Ng et al. Nature Protocols. 2008, Vol 3, No 5, pp 768-776). In brief, 5x10^3 single cell-adapted hESC were spun at 480 g into each well of a 96-well low attachment plates and subsequently cultured in APEL medium containing 10 µM Rock inhibitor Y-27632 (Selleckchem, Houston, TX, USA), 40 ng/ml SCF (Peprotech, Rocky Hill, NJ, USA), 2 ng/ml BMP4 (R&D, Minneapolis, MN, USA) and 20 ng/ml VEGF165 (Peprotech), further referred to as “EB mix”. After 4 days, spin EB were transferred on an OP9 cell layer and further cultured in EB mix for a total of 7-14 days. Half of the medium was changed on day 7, with APEL medium containing EB mix cytokines, unless a different combination of cytokines is specified. These cytokines were added at following concentrations: 50 ng/ml IL-3 (R&D), 50 ng/ml Flt3-L (R&D), 10 ng/ml TPO (Peprotech) and/or 50 ng/ml IL-6 (R&D).

For myeloid differentiation, spin EBs were dissociated at day 11 and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D) and 20 ng/ml IL-6 (R&D). For erythro-megakaryocytic differentiation, spin EBs were dissociated and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech) and 50 ng/ml EPO (eBioscience, San Diego, CA, USA).
**Methocult assay**

Methocult assay was performed according the manufacturers protocol. In brief, 2x10^3 CD34+CD43+ cells were isolated using FACS at indicated time points. Cells were resuspended in Methocult GF H84434 medium (Stemcell technologies, Vancouver, British Columbia, Canada) and plated in 35mm tissue culture dishes. Colonies were scored after 14 days of culture.

**Single cell progeny assay**

For single cell progeny assay d11 or d14 embryoid body cultures were dissociated and stained for FACS sorting following standard procedures. Cells were deposited in Terasaki 60well format plates (Nunc, Roskilde, Denmark) using FACS ARIA III with ACDU. Cells were cultured on OP9 feeders in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D), 20 ng/ml IL-6 (R&D) and 50 ng/ml EPO (eBioscience). Cells were scored for growth by microscopy, and expanding clones were assayed by flow cytometry after 7 days of culture for indicated markers.

**Flow cytometry and cell sorting**

Before adding mAb, FcR blocking was performed using human IgG (Miltenyi Biotec). Flow cytometric analysis was performed on a LSR II system (BD biosciences, San Jose, California, United States of America). Cell sorting was performed with a FACS ARIA IIIU system (BD Biosciences). The following (conjugated) anti-human mAbs were used: CD10-PE (eBioscience), CD13-PE (eBioscience), CD11b-PE (Becton Dickinson); CD11c-PE (Becton Dickinson); CD14-PE (Miltenyi Biotec); CD15-PE (Miltenyi Biotec); CD16-PE (Coulter); CD33-PE (Miltenyi Biotec); CD34-APC (Miltenyi Biotec); CD41a-PE (Becton Dickinson); CD43-PE (Becton Dickinson), -V450 (Becton Dickinson), CD66-PE (eBioscience), CD86-PE (eBioscience), CD117-PECy7 (c-kit, eBioscience), CD123 (IL-3R)- PE (eBioscience); CD235A (GlycophorinA)-PE (Beckman-Coulter, Brea, CA, USA). Data were analyzed using F MarcDIVA software (Becton Dickinson) and Flowjo software (Treestar, Ashland, OR, USA).

**Real time RT-PCR**

Cells were lysed and cDNA was synthesized using the SYBR power cells-to-ct system (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Primers were designed and in silico specificity was determined using NCBI primer BLAST and manufactured by Biolegio (Biolegio, Nijmegen, The Netherlands). All PCRs were validated for efficiency and linearity on four point dilution series using a relevant control cDNA. All primers had efficiencies between 90 and 110%. Primer sequences can be found in supplemental TableS1. PCR reagents and SYBR GreenI master were obtained from Roche and used according to the manufacturer’s instructions. The
reactions were run on a LightCycler480 384well system (Roche, Penzberg, Germany). The following cycling conditions were used: 5 min at 95°C for initial denaturation, 40 cycles at 95°C for 15 s, and 60°C for 30 s. After amplification, a melting curve was generated for every PCR product and compared with melting curve of control cells. The relative expression was calculated for each gene by the dCt method, relative to the mean of reference genes used as indicated.

Generation of reporter Bacterial Artificial Chromosome

BAC plasmid clone CH17-400L19 containing the MYB gene flanked by approximately 100kbp of genomic sequence on either side of the start codon was obtained from CHORI BACPAC (Children’s Hospital Oakland Research Institute, Oakland, CA, USA). To generate reporter BAC plasmids, an eGFPpA-LoxP-PGK-Gb2-Neo-pA-LoxP vector was targeted to the first start codon of the MYB gene using recombination-mediated genetic engineering. In brief, CH17-400L19 BAC plasmid containing DH10b were grown under Chlor-Amphenicol antibiotic selection (Sigma-Aldrich). pSC101BADgabeA[tet] (Genebridges, Heidelberg, Germany) was transfected into electro competent BAC containing cells and grown at 32°C under tetracyclin antibiotic resistance (Sigma-Aldrich). Bacterial cells containing both plasmids were then transfected with PCR amplified targeting construct, with primer integrated 50bp homology arms, and grown overnight at 37°C under Chlor-Amphenicol and Kanamycin resistance (Sigma-Aldrich).

BAC plasmids were purified using an adapted miniprep protocol, briefly, overnight bacterial cultures were pelleted and resuspended in Qiagen (Qiagen, Venlo, The Netherlands) minelute buffer P1, lysed using Qiagen minelute buffer P2 and the reaction was neutralized using Qiagen minelute buffer N3. Cell lysate was then spun down for 10’ at 13200rpm in a chilled centrifuge (4°C), supernatant was transferred to a fresh tube and precipitated using isopropanol (Sigma-Aldrich). Isolated plasmid was then resuspended in nuclease free water (Ambion). For analysis of BAC plasmids, 50µg of plasmid DNA was digested for up to 16h with BamHI restriction enzyme (NEB, Ipswich, Massachusetts, USA). Restriction fragments were separated on a 0.8% agarose gel and visualized using UV light after incubation with Ethidium Bromide.

The MYBeGFP reporter construct was functionally validated by transfection in MOLT4 and K562, which express levels of MYB within a biologically relevant range. After transfection and neomycin selection, both cell lines expressed eGFP as expected. Subsequent transfection of the cell line with Cre recombinase induced an eGFP bright population in both cell lines suggesting that removal of the selection cassette caused increased activity of the MYB promoter (Figure S1).
**Generation of reporter hESC**

A total of $2 \times 10^6$ single cell adapted hESC were nucleofected with 5µg purified BAC reporter using Lonza Amaxa human stem cell nucleofector kit 2 (Lonza) with program F16. Cells were replated at high density $(1 \times 10^5c/cm^2)$ in single cell conditions on drug resistant DR4 MEFs (a kind gift of Dr. Hochepied, VIB, Ghent, Belgium). A total of 10µM of the Rock inhibitor Y-27632 (Selleckchem) was added. G418 (50µg/ml) (Gibco) was added from day three onwards and retained for three weeks. Single colonies were picked, expanded and screened for transgene integration. Colonies showing transgene integration were transfected using pCAGGS-NLS-Cre-PGK-Puro (plasmid 7779, BCCM/LBMP, Ghent, Belgium) and kept under puromycin (Sigma-Aldrich) selective pressure (300ng/ml) for three days to remove the neomycin selection cassette. Single colonies were expanded and screened by PCR. Genomic DNA was isolated using Genelute mammalian genomic DNA miniprep kit (Sigma-Aldrich) according to the manufacturer’s instructions. Colonies containing successfully floxed cells were cloned using single cell deposition on the FACS Aria.

**FISH analysis**

Metaphase slides were pretreated with RNase and pepsin. Slides were denatured with 70% formamide/2× sodium saline citrate phosphatase (SSCP) at 80°C for 5 min. Probe mix was denatured at 75°C for 5 min, incubated at 37°C for 30 min and subsequently applied to the slides under a 18 × 18 mm coverslip. After 2–4 days of hybridization, slides were washed with 50% formamide/2× sodium saline citrate (SSC) (pH 7.3–7.5) at 42°C (3 × 5 min), followed by 3 washes in 2 × SSC (42°C). Metaphases were stained using the Vysis MYB SpectrumAqua FISH Probe Kit (Abbott Molecular, North Chicago, Illinois, United States of America) Slides were mounted in Vectashield (Vector, Burlingame, California, United States of America) containing DAPI counterstain.

**High-resolution arrayCGH**

DNA was hybridized to 1M human Genome CGH Microarray slides from Agilent Technologies (Santa Clara, CA, USA) according to the manufacturer’s instructions with minor modifications. 1µg of genomic DNA was labeled with Cy3 and 1µg of reference DNA (Kreatech, Amsterdam, The Netherlands) was labeled with Cy5 using the BioPrime Array CGH Genomic Labeling System (Invitrogen) and subsequently purified by precipitation. After 24 h of hybridization at 65 °C, microarrays were washed and scanned at 5 µm resolution using a DNA microarray scanner (Agilent). The scan images were processed with Feature Extraction Software 10.1 (Agilent) and further analyzed and visualized with our in-house developed and freely available software tool ViVar (http://www.cmgg.be/ViVar/) (Sante et al. Submitted). Copy number variants (CNVs) were identified
by circular binary segmentation requiring a minimum of 5 consecutive oligonucleotides exceeding an absolute log$_2$-ratio threshold of 0.35 ($\approx 2.5 \times$ standard deviation).

**Live confocal analysis**

Live confocal analysis were performed on a Leica SPE confocal microscope with CO$_2$ and temperature control. All analysis were done at 37°C in MEM$\alpha$ containing 20% FCS and indicated cytokines. Images were made using an ACS APO 10.0x0.30 DRY lense. Fluorochrome-labeled mAbs were added directly to the cultured medium. Following mAbs were used: CD34-APC (Miltenyi biotec), CD43-PE (Beckton Dickinson), CD14-PE (Miltenyi biotec). Azide was removed from these antibodies using D-Tube™ Dialyzer Mini, MWCO 12-14 kDa (Novagen, EMD Millipore, Billerica, MA, USA). Images were acquired using LAS AF software (Leica microsystems, Wetzlar, Germany). Images were processed using Fiji (Fiji is Just imageJ) software. Adjustment of brightness and contrast and cropping of the images were performed on the complete image and all channels.

**Statistical analysis**

All statistical analyses were performed using SPSS V22.0 (IBM, New York, USA). Significance was assessed using Mann-Whitney U statistical analysis with significance level set at $p \leq 0.05$. 
Supplemental table I: primer sequences used in this study

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Figure S1: Generation of MYB-eGFP reporter hESC.

A) Schematic overview of BAC recombineering. Red squares indicate homology arms, grey triangles indicate LoxP sequences; B) Restriction digest of the targeted BAC vector shows successful integration of the selection cassette in between BamHI restriction sites; C) K562 and MOLT4 stably transfected with MYB-eGFP BAC plasmid were analyzed for eGFP expression, before and after transient Cre recombinase transfection. Mean Fluorescence Intensities (MFI) for gated populations are shown.
Figure S2: Expression profile of MYB in EB derived hematopoietic progenitors.

A) Kinetics of MYB expression in bulk hESC feeder differentiation culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression; B) MYB expression was analyzed in indicated populations from d14 EB. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver CD34+. Error bars indicate standard deviation of the mean (n=3); C) MYB expression was analyzed in indicated populations within the CD34⁺CD43⁺ population. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver CD34⁺. Error bars indicate standard deviation of the mean (n=3).
Figure S3: CD34^+CD43^−eGFP^− endothelial cells give rise to eGFP^− hematopoietic precursors, before emergence of eGFP^+CD34^+CD43^+ cells.

A) Analysis of progeny of CD34^+CD43^− hemogenic endothelial cells derived from d11 EB. Cells are analyzed after 6 and 11 days of culture respectively; B) Phenotypic analysis of CD45^+ cells from panel A at indicated time points.
**MovieS1: Time-lapse analysis of HPC generation from endothelial cells.**

Time-lapse confocal analysis of MYBeGFP hESC d11 EB differentiation sorted CD34⁺ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD34-APC and CD43-PE. Images were acquired every 30 minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 2h per second). Arrow indicates a single CD34⁺ sorted endothelial cell transforming into a hematopoietic progenitor cells.

**MovieS2: Time-lapse analysis of macrophage differentiation.**

Time-lapse confocal analysis of MYB-eGFP hESC d11 EB differentiation of sorted CD34⁺CD43⁻CD45⁻eGFP⁻CD14⁻ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD14-PE. Images were acquired every 15 minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 1h per second). Arrow indicates a single progenitor developing into a CD14⁺ macrophage.