Serum- and Stromal Cell-Free Hypoxic Generation of Embryonic Stem Cell-Derived Hematopoietic Cells In Vitro, Capable of Multilineage Repopulation of Immunocompetent Mice

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ABSTRACT

Induced pluripotent stem cells (iPSCs) may become a promising source for the generation of patient-specific hematopoietic stem cells (HSCs) in vitro. A crucial prerequisite will be the availability of reliable protocols for the directed and efficient differentiation toward HSCs. So far, the most robust strategy for generating HSCs from pluripotent cells in vitro has been established in the mouse model involving ectopic expression of the human transcription factor HOXB4. However, most differentiation protocols include coculture on a xenogenic stroma cell line and the use of animal serum. Involvement of any of both would pose a major barrier to the translation of those protocols to human autologous iPSCs intended for clinical use. Therefore, we asked whether long-term repopulating HSCs can, in principle, be generated from embryonic stem cells without stroma cells or serum. Here, we showed that long-term multilineage engraftment could be accomplished in immunocompetent mice when HSCs were generated in serum-free medium without stroma cell support and when hypoxic conditions were used. Under those conditions, HOXB4+ embryonic stem cell-derived hematopoietic stem and progenitor cells were immunophenotypically similar to definitive bone marrow resident E-SLAM+ (CD150−CD48−CD45−/H11545 CD201−) HSCs. Thus, our findings may ease the development of definitive, adult-type HSCs from pluripotent stem cells, entirely in vitro.

INTRODUCTION

Pluripotent stem cells are able to self-renew indefinitely and can be differentiated into all mature cell types of the three germ layers. Given this unique ability, embryonic stem cells (ESCs) and especially autologous induced pluripotent stem cells (iPSCs) are regarded as an invaluable unlimited resource for cell replacement therapies in regenerative medicine. The hematopoietic system is of particular interest for induced pluripotent stem-based therapies because of its unique ability to self-organize after transplantation of stem and progenitor cells. iPSC-derived, patient-specific hematopoietic stem cells (HSCs) could be generated, as well as a broad range of mature effector cells for which a strong clinical demand exists, such as erythrocytes, granulocytes, and thrombocytes. Proof of principle for pluripotent stem cell-based cell therapies was recently demonstrated in a mouse model of sickle cell anemia. HSCs were generated from genetically corrected iPSCs that cured the disease after transplantation [1] and in human patient-derived iPSCs in which the mutation had been corrected with zinc-finger endonucleases [2]. In most cases, stable engraftment has been achieved only after depletion of natural killer (NK) cells, which were reported to reject nuclear transfer ESC-derived hematopoietic grafts [3]. Therefore, improved protocols have to be developed for the robust generation of completely mature, bone marrow resident-type definitive HSCs. To achieve this goal, a better understanding of the instructive cues during embryonic hematopoiesis is crucial.

During embryonic development, hematopoiesis is initiated at different time points and multiple anatomical sites, such as the yolk sac (YS), the aorta-gonad-mesonephros (AGM) region, and the placenta, with final subsequent expansion of HSCs in the fetal liver [4]. So far, the first definitive HSCs known to organize long-term, multilineage engraftment in lethally irradiated recipients can be detected from embryonic day (E) 10.5 on in mice, in the ventral part of the large
vessels, the AGM region, the vitelline, the umbilical arteries, and the placenta [5–7]. This appears to occur through the direct transition of so-called hemogenic endothelial cells within the ventral wall of the dorsal aorta into the very first HSCs [8–11]. Different developmental stages of hematopoietic stem and progenitor cells (HSPCs) can be recognized by their differential expression of certain surface molecules: nascent HSCs in the AGM and the placenta express the surface antigens CD41 and CD34 and lack CD45, CD150, and CD48, whereas more mature fetal liver HSCs are devoid of CD41 expression but display CD150 and CD45 on their surface [12–15].

Numerous reports have shown that hematopoietic commitment and differentiation in differentiating mouse ESCs recapitulate the early developmental processes occurring in the embryo [16]. Existing protocols for hematopoietic differentiation of ESCs rely on induction of cell aggregates termed embryoid bodies (EBs) or on monolayer cultivation on extracellular matrices, stroma cell lines, or a combination of both. Whether and to what extent definitive (i.e., adult) hematopoiesis can be achieved without genetic manipulation of the cells is unclear. It has been possible to generate T- and B-cells by extended coculture of ESCs with OP9 stromal cells [17, 18]. Moreover, Iorion et al. recently identified two distinct Flk1-1 + hematopoietic cell populations in differentiating ESCs in vitro that sequentially emerge on day 3.25 and day 5.25 of EB differentiation [19]. In contrast to the 3.25 Flk1+ cell population, the later-appearing Flk1+ cells express higher levels of endogenous HOXB4 and Sox17 and can produce B- and T-cells on OP9 and OP9-DL1 stromal cells, thus suggesting that the early Flk1+ population may recapitulate YS hematopoiesis, whereas the later 5.25 Flk1+ population cells may correspond to a definitive hematopoietic population of the AGM. In agreement with this, all definitive HSCs emerging in the AGM of the mouse embryo have been demonstrated to express Hoxb4 [20].

So far, robust production of definitive HSCs from mouse ESCs capable of long-term multilineage reconstitution of adult recipient animals has been achieved only by enforced ectopic expression of either human HOXB4 alone or human HOXB4 in combination with Cdx4 [21–24]. In this experimental system, ESC-derived HSCs exhibit a unique mixture of expression cell surface markers characteristic of both embryonic and adult, mature HSCs. ESC-derived HSCs are CD41+, ckit+, and CD150+ but promiscuous for CD48 and CD45 expression [15].

Most of the current protocols for generation of hematopoietic progenitors and HSCs from murine and human ESCs use fetal calf serum and neonatal bone marrow- or AGM-derived stromal cell lines such as OP9 and AM20.1B4, respectively [22, 25]. The poorly defined factors and composition of xenogenic serum, its batch-to-batch variability, and the difficulty of controlling stromal cell quality prohibit a tight control of the complex hematopoietic differentiation steps. Although serum- and stromal cell-free conditions that allow efficient generation of mouse ESC-derived hematopoietic cells have been described that include the stepwise addition of key factors for hematopoietic differentiation, such as bone morphogenetic protein 4 (BMP4), activin A, and vascular endothelial growth factor (VEGF) [26], none of these previously published studies demonstrated the in vitro production of transplantable HSCs from ESCs under completely defined serum- and stromal cell-free conditions from the very first steps of ESC differentiation on.

In this study, we showed that long-term reconstituting HSCs can be efficiently generated in the absence of serum and without stromal cell support under hypoxic conditions by the stepwise addition of cytokines to promote hematopoietic specification and expansion of differentiating mouse ESCs.

### Materials and Methods

#### Embryonic Stem Cell Culture, Retroviral Transduction, and Embryoid Body Differentiation

Mouse embryonic stem cells (CCE) were grown without feeders in knockout Dulbecco’s modified Eagle’s medium (DMEM) under previously described conditions [23]. DMEM was exchanged for Iscove’s modified Dulbecco’s medium (IMDM) 2 days prior to initiation of in vitro differentiation. Transduction of ESCs with ecotropic virus particles was carried out as described [23]. Clones were isolated by flow cytometrical sorting of enhanced green fluorescence protein (eGFP)-positive cells. For embryoid body formation, 5,000 ESCs per milliliter were plated in StemPro34 medium plus nutrient supplement (Gibco, Grand Island, NY, http://www.invitrogen.com), 2 mM l-glutamine (l-Gln), penicillin/streptomycin (Gibco), 50 μg/ml ascorbic acid, 200 μg/ml iron saturated transferrin, 4 ng/ml recombinant human BMP4, and 4 × 10^-4 monothioglycerol. After 2.5 days, 5 ng/ml recombinant human fibroblast growth factor 2 (rhFGF2; basic fibroblast growth factor [bFGF]), 5 ng/ml recombinant human activin A, 5 ng/ml recombinant human VEGF (rhVEGF), 20 ng/ml recombinant murine thrombopoietin (TPO), and 100 ng/ml recombinant murine stem cell factor (mSCF) were added to the cultures. Cytokines were obtained from either R&D Systems Inc. (Minneapolis, MN, http://www.rndsystems.com) or Peprotech (Hamburg, Germany, http://www.peprotech.com). EBs were dissociated at day 6 by 0.05% trypsin-EDTA treatment at 37°C for 2–5 minutes.

#### Culture/Expansion of ESC-Derived Hematopoietic Cells

Dissociated EBs [ESC-derived hematopoietic cells [ESC-HCs]] were cultivated under various conditions. In our previously described standard serum-free (SF) conditions, cells were grown in serum-free StemPro34 medium plus nutrient supplement (Gibco) at a density of 2 × 10^5/ml, containing 100 ng/ml rmSCF, 20 ng/ml recombinant human Flt3-ligand (rhFlt3-L), 4 ng/ml recombinant murine interleukin (IL)-3, 10 ng/ml recombinant human IL-6, 40 ng/ml insulin-like growth factor-I (IGF-I), and 10^-6 M dexamethasone [23]. Stroma cell-free cultivation or coculture of EB-derived cells with OP9 or AFT024 stromal cells was performed in IMDM plus 2 mM l-Gln with or without 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing the following cytokines: 10 ng/ml rmSCF, 20 ng/ml recombinant murine TPO, 100 ng/ml rhFlt3, 40 ng/ml rhVEGF (stem cell factor [SCF], TPO, Flt3-L, and VEGF [STFV]-serum [S]/STFV-SF). OP9 stroma cells were plated at a density of 25,000 cells per six-well plate 24 hours prior to cocultivation. AFT024 cells were seeded at 1 × 10^5 cells per six-well plate 3 days ahead of initiation of the cocultures and treated with mitomycin (10 ng/ml) immediately before addition of the EB-derived cells. At day 7 of coculture on OP9 or AFT024 stroma cells were transferred into a T-75 tissue culture flask with fresh IMDM supplemented with or without serum and cytokines and cultivated for additional 3 days. Cell numbers and volumes were determined using an electronic cell counter (CASY; Roche Diagnostics Deutschland GmbH, Mannheim, Germany, http://www.roche-applied-science.com/sis/innovatis/
index.jsp). Cells were kept at a density of 2–4 × 10^6 cells per milliliter. For morphological analysis of hematopoietic cultures, cells were spun onto glass slides and stained with May-Grünwald/Giemsa according to standard protocols. OP9 stroma cells were maintained in α-minimal essential medium (Invitrogen) supplemented with 20% FCS and 2 mM l-Glu. AFT024 cells were propagated in DMEM plus 10% FCS at 33°C.

**Colony Assays**

For the growth of hematopoietic precursor cells, ESC-HCs were plated in semisolid methylcellulose supplemented with murine SCF, murine IL-3, human IL-6, and human erythropoietin (M3434 MethoCult; Stemcell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) in 35-mm dishes by seeding 1,000 ESC-HCs that had been grown under the different conditions for 7–21 days. All assays were done in triplicate, and colonies were evaluated after 8–10 days.

**Fluorescence-Activated Cell Sorting Analysis**

The developmental status of expanding ESC-HCs was periodically analyzed by flow cytometry assessment of the following surface markers: CD41, CD45, c-kit, CD34, CD150, CD48, CD201, and Tie-2. (Antibodies were purchased from eBioscience Inc., San Diego, CA, http://www.ebioscience.com). 4′,6-Diamidino-2-phenylindole was used for exclusion of dead cells. Gates were set on the basis of control samples stained according to the method called Fluorescence Minus One. Flow cytometry measurements were performed on an LSRII instrument (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) using FACSDiva software, and data analyses were performed with FlowJo software (Tree Star, Ashland, OR, http://www.treestar.com).

**Transplantation and Peripheral Blood Analysis**

Cells were transplanted into 12–32-week-old 129S6/SvEvTac mice via tail vein injection. Unsorted ESC-HCs (3 × 10^6, 2 × 10^6, 1 × 10^6, 3 × 10^5, or 1 × 10^5 cells) were cotransplanted with 2 × 10^6 freshly isolated, Sca-1-depleted bone marrow cells (129S6/SvEvTac) per recipient mouse. Sca-1^- cells were depleted using the Anti-Sca-1 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) according to the manufacturer’s guidelines. Peripheral blood samples were taken periodically by puncture of the retrobulbar plexus. Whole blood samples were counted on an animal blood counter system (Scil Animal Care Company, Vierenheim, Germany, http://www.scilvet.com). After erythrocyte lysis was performed, the remaining white blood cells were stained and analyzed by flow cytometry. All animal experiments were approved by the local ethical committee and performed according to its guidelines.

**RESULTS**

**Serum-Free, Cytokine-Mediated Instruction Enhances Hematopoietic Differentiation in HOXB4^- EBs**

To efficiently generate HSPCs from ESCs in vitro, optimal conditions should be determined for each step in the embryonic stem cell/embryoid body (ES-EB) culture model. In this study we used an ESC clone (CCE line) constitutively coexpressing eGFP and HOXB4 (termed HOXB4clone 8; HOXB4#8). We have previously shown that HOXB4#8 ESC-HCs express high amounts of human HOXB4, thus enabling long-term multilineage repopulation of Rag2^-/-γC^-/- immunodeficient mice [24] (S. Pilat, personal communication). As a first step, we analyzed the specification of hematopoietic cells from day 6 to day 10 of EB formation (EBd6–EBd10) under different conditions. Control and HOXB4#8 ESCs were allowed to differentiate “unsupervised” into EBs in the presence of serum or by directed differentiation in serum-free medium supplemented with BMP4 at day 1 and bFGF, activin A, VEGF, SCF, and TPO from day 2.5 of EB differentiation according to Pearson et al., with modifications [26]. Optical inspection of the EB differentiation cultures at day 6 revealed that CCE and HOXB4#8 ESCs had formed a somewhat higher number of EBs compared to Pearson et al., with modifications [26].
More mature CD45^+ cells were hardly detectable at that time point. However, their numbers progressively increased to approximately 40% at day 10 of EB differentiation, albeit the majority of those cells still coexpressed CD41. In the presence of serum, a substantially lower proportion of CD41^+ cells (~20%), as well as mature CD45^+ cells (~20%), developed at all analyzed time points. Notably, ectopic expression of HOXB4 delayed the peak of hematopoietic induction within the embryoid body by 2 days, to day 8. After dissociation of EBs at day 6, serum-free expansion cultures were initiated with single-cell suspensions using a cocktail of hematopoietic cytokines (SCF, Flt3-ligand, and vascular endothelial growth factor).

In line with the higher number of embryoid bodies in serum-containing medium, approximately 7.6 times as many cells were recovered from the HOXB4#8 EBs immediately after their dissociation than from those formed in serum-free medium (Fig. 1B). However, until day 10 of suspension culture under serum-free cytokine-supplemented conditions, HOXB4#8 ESC-HCs had expanded 10-fold. In contrast, the number of cells initially kept in serum dropped dramatically until day 2, after which it increased only six-fold. Fluorescence-activated cell sorting (FACS) analysis of day 10 suspension culture under serum-free cytokine-supplemented conditions revealed no significant differences in the overall content of CD41^+ cells (Fig. 1C). However, HOXB4#8 ESC-HC cultures displayed a higher proportion of cells expressing CD41 at high levels (CD41^+hi) than CD41^+LO, as well as mature CD45^+ cells (~20%), which had been shown to contain in vivo repopulating ESC-derived HSPCs [15].

Reduced O2 Levels Support Maintenance of ESC-Derived Hematopoietic Progenitor Cells in Serum-Free Medium

To simulate more physiological conditions, we investigated the impact of reduced oxygen levels on the expansion of ESC-derived hematopoietic progenitor cells. Compared with atmospheric oxygen levels (normoxia; partial O2 pressure [pO2] 20%), reduced O2 levels (hypoxia; pO2 5%) led to a distinctive increase in the number of CFU-GEMMs in serum-free (STFV-SF) conditions now being comparable to the numbers obtained after stroma cell coculture.
under more physiological, hypoxic O2 levels in well-defined serum-free cultures (Fig. 2C). As observed in 20% O2, STFV-SF cultures contained a high proportion of CD45− cells. However, the proportion of CD41hickitCD34+ progenitor cells in serum-free cultures was much higher under hypoxia than under normoxic conditions (Fig. 2D). Hence, hematopoietic progenitors were efficiently generated under more physiological, hypoxic O2 levels in well-defined serum-free medium without the need for stromal cell support in vitro.

Hypoxic Conditions In Vitro Support ESC-HC Engraftment in Immunocompetent Mice In Vivo

So far, long-term multilineage repopulation with HOXB4-expressing ESC-derived hematopoietic cells has, in most cases, only been achieved in immunodeficient recipient mice lacking NK cells or after depletion of these cells. Because our growth conditions protected ESC-HCs from NK cell-mediated cell lysis in vitro (supplemental online Figure 3), we transplanted HOXB4#8-derived ESC-HCs into lethally irradiated immunocompetent syngeneic mice (129S6/SvEvTac) to assess whether more mature, definitive adult-type HSCs were generated under hypoxic O2 conditions in vitro. To also verify whether a correlation between the number of transplanted CD41hickitCD34+ cells and the level of long-term donor cell repopulation in vivo truly exists, we determined the proportion of this subpopulation in the graft immediately prior to transplantation. Green fluorescence protein-positive (GFP+) donor cell chimerisms were periodically determined by FACS analysis of peripheral blood. After EBd6 cells had been cocultured under hypoxic conditions on stromal cells in the presence of serum for 7 days, mice were transplanted with 3×10^5 ESC-HCs containing 5,700 (AFT024) and 14,400 (OP9) CD41hickitCD34+ cells, respectively. ESC-HCs from both stromal cocultures conferred substantial donor cell chimerism 4 weeks post-transplantation (AFT024: mean, 52.5%; OP9: mean, 75.6%), reflecting efficient homing and short-term engraftment of the injected cells (Fig. 3A). Hence, NK cell-mediated rejection of ESC-HCs obviously did not occur, being consistent with the results of the NK cell lysis assays obtained in vitro. Six months post-transplantation, donor cell chimerism in the transplanted groups (AFT024: mean, 11.4%; OP9: mean, 43.4%) appeared to correlate with the content of CD41hickitCD34+ progenitor cells in the cultures prior to transplantation. To estimate the minimal number of long-term repopulating HSPCs generated in vitro, we transplanted OP9 coculture-derived ESC-HCs into immunocompetent recipient mice in limiting dilution. Three of five mice receiving the lowest cell dose of OP9-cocultured ESC-HCs (1×10^4 cells per mouse) were not radioprotected beyond the second week after transplantation. However, a cell dosage of 3×10^5, 1×10^6, and 3×10^7 rescued 80% of the mice for at least 18 weeks after transplantation (Fig. 4C). At that time we detected high levels of donor cell chimerism, with averages ranging from 42% to 66% GFP+ cells in the mouse cohorts (Fig. 4A, 4B).

All of these mice showed multilineage repopulation over the whole observation period (Fig. 4B). Thus, a minimal cell number of 3×10^5 ESC-HCs cocultured on OP9 stroma at 5% O2 contained sufficient amounts of hematopoietic progenitor cells to rescue mice from lethal irradiation and to confer long-term multilineage repopulation in immunocompetent mice, in vivo. It is noteworthy that a bias toward myelopoiesis (GFP+CD11b+) and away from lymphopoiesis (GFP+CD3+) was observed in vivo (Fig. 4B), which is a well-known effect of HOXB4 activity [21–24]. Lymphoid cells, which develop from the HOXB4-expressing HSPCs after transplantation in vivo, commonly display a selective silencing of ectopic retroviral HOXB4 expression (as described in Discussion).

Stroma Cell-Free, Hypoxic Culture Conditions Are Sufficient for Development of Long-Term Repopulating Embryonic Stem Cell-Derived HSPCs In Vitro

We also evaluated the potency of hypoxic, stroma cell-free cultures grown only in cytokine-supplemented serum (STFV-S) in comparison with OP9 and AFT024 cocultures to support the generation of HSPCs capable of long-term repopulation in vivo. Embryonic stem cell-derived HSPCs (ES-HSPCs) from all three expansion cultures mediated high levels of short-term engraftment at 7 weeks post-transplantation (Fig. 5A). However, hypoxic, stroma-free ESC-HCs cultures appeared to confer an at least fourfold higher level of long-term donor cell chimerism than those cocultured on stroma cells (Fig. 5B). In line with this observation, STFV-S cultures contained around a twofold to fourfold higher number of CD41hickitCD34+ cells prior to transplantation than ESC-HCs cocultured on OP9 or AFT024 stroma, respectively (STFV-S: 5.5×10^5 vs. OP9: 2.5×10^6 cells vs. AFT024: 1.2×10^6).

Finally, to examine whether transplantable ESC-HCs could be generated under hypoxic conditions without any stroma or even serum support, we transplanted recipient mice with ESC-HCs grown for 23 days in the presence of cytokines with and without serum (STFV-S vs. STFV-SF). Donor cell chimerism analysis 12 weeks after transplantation suggested that both conditions mediated multilineage repopulation (Fig. 6A). This observation is in good agreement with their comparable maturation toward CD41hickitCD34+ cells when grown in hypoxic conditions in vitro (Fig. 2D). In mice, fetal liver and adult-type HSCs are highly enriched in the CD150+CD48−CD45−CD201+ cell fraction,
termed E-SLAM cells [29]. Both hypoxic in vitro cultures contained such a subpopulation, with the serum-free cultures containing a somewhat higher proportion of E-SLAM/H11001 cells (Fig. 6C). Whether or not this reflects a higher amount of mature repopulating ESC-HCs being more similar to adult HSPCs when generated in serum-free cultures in vitro remains to be determined.

Taken together, our results suggest that serum- and stroma cell-free hypoxic conditions allow for the conversion of ESCs toward multilineage repopulating HSCs in vitro.

DISCUSSION

We have previously shown that HOXB4 promotes the development of HSPCs capable of long-term multilineage repopulation in immunodeficient Rag2(H11002/)H11002/C(H11002/) mice in vivo from differentiating mouse ESCs, in vitro [23, 24]. For ESC-derived HSPC development to occur, certain threshold levels of HOXB4 expression need to be exceeded (S. Pilat, S. Carotta, B. Schiedlmeier et al., manuscript in preparation). Because such grafts can be rejected by NK cells, long-term multilineage repopulation has hardly been achieved in immunocompetent recipients so far, presumably because of the immature developmental state of the generated cells in vitro.

In vivo, full maturation toward definitive HSCs depends not only on the transcriptional program executed within the cells but also on the microenvironment, consisting of cell-cell contact, soluble factors, and physical parameters, such as shear stress or low oxygen partial pressure [30, 31]. It has been shown, for example, that yolk sac cells can mature to definitive HSCs if cocultured with AGM-derived stromal cells [32] after transplantation into the liver of newborn mice [33] or transplacentar injection into fetuses at days 11–15 of development [34]. Thus, environmental cues are able to instruct and support developing hematopoietic cells to mature to definitive long-term repopulating HSCs (LT-HSCs). Although it is not yet known in detail how HOXB4 mediates its hematopoiesis-promoting effects, results from our group strongly suggest that this homeodomain transcription factor does so by altering the sensitivity of hematopoietic cells to (micro)environmental cues [35, 36].

Because HSCs expand in the fetal liver before they seed the bone marrow niche around birth, we asked whether a fetal liver-derived cell line, AFT024, would be more suitable in promoting the maturation of HOXB4(H11001) ESC-derived hematopoietic progenitors than bone marrow-derived OP9 stromal cells, especially as these cells have been demonstrated to secrete BMP4, a factor necessary for mesoderm specification and the development of hemogenic endothelium cells from ESCs in vitro [37, 38]. Moreover, AFT024 cells have repeatedly been shown to support the maintenance and even expansion of HSCs from bone marrow.
and fetal liver and during prolonged coculture [39, 40]. To our surprise, there was no clear and consistent difference between the capabilities of AFT024 or OP9 cells to support hematopoietic development and maturation of dissociated day 6 embryoid bodies, in vitro (Fig. 2) as well as in vivo (Fig. 5). One possible explanation may be an ability of HOXB4 to intrinsically overcome the need for BMP4-mediated signaling during ESC differentiation, although we have not addressed this question in this study. However, our observations that ectopically expressed HOXB4 can support the development of ESC-derived HSPCs capable of engrafting immunodeficient Rag2−/−γc−/− [23] and immunocompetent 129S6/SvEvTac mice long-term, without the need of any coculture with stromal cells, may support the idea of a BMP4 independence of HOXB4-expressing cells. In agreement with this, others have also observed that coculture of HOXB4-expressing ESCs with AGM-derived stromal cells also did not further enhance their hematopoietic differentiation [41]. A recently published observation by Jackson et al. may explain the nonadditive effect of stromal cocultures on hematopoietic differentiation of HOXB4−/−-ESCs [42]. Induction of this transcription factor appears to intrinsically bias ESC differentiation toward paraxial mesoderm and also induces hematopoietic differentiation of nontransduced, HOXB4-negative cells by a paracrine mechanism, thus suggesting that HOXB4-expressing stem and progenitor cells may create their own niche.

During embryonic development, the appearance of the first definitive hematopoietic cells, being CD41+ (integrin αvβ3), is initiated in the ventral part of the dorsal aorta and depends on BMP4 signaling, which initiates the expression of the essential hematopoietic transcription factors SCL/Tal and Runx1/AML via SMAD1 and SMAD6 [43, 44]. In the ES-EB in vitro differentiation system, formation of the earliest, CD41+ cells was first detectable from day 4 of embryoid body differentiation on. Interestingly, their fraction was strongly reduced in the presence of fetal calf serum, although it is known to contain significant amounts of BMPs [45]. In the presence of HOXB4, not only was hematopoietic development delayed but the negative effect of serum was seemingly aggravated (Fig. 1). Mesoderm specification and its maintenance are known to require active FGF signaling [46]. However, continuation of FGF activity suppresses blood specification later on during development, presumably by antagonizing the BMP4 signaling pathway [47, 48]. Thus, FGFs present in serum may promote mesoderm specification, but because of their constant presence, they may also be responsible for the lower content of earliest CD41+ hematopoietic cells compared with serum-free, defined conditions. The activity of HOXB4...
counteracts FGF signaling, as we have shown previously [35].

Thus, inhibition of mesoderm formation by HOXB4 may explain
the delayed appearance and, as a result, the low content of
CD41/H11001 hematopoietic cells during embryoid body development
when serum was present (Fig. 1). These observations further
support our proposed model that HOXB4 integrates and modu-
lates incoming signals from the local microenvironment [35, 36].

Although the overall number of embryoid bodies that had
developed until day 6 was higher when cytokine-supplemented
serum was present, we observed an initial strong drop of the
total number of cells growing in suspension until day 2 after EB
dissociation (Fig. 1B). The most obvious explanation is that far
more cells grew as an adherent layer when prior EB differen-
tiation had taken place in the presence of serum, even though the
subsequent serum-free conditions selected for the growth of
hematopoietic cells in suspension. Serum contains a plethora of
yet undefined components that are supportive of the develop-
ment and proliferation of many different cell types that depend
on direct cell-cell interactions within the microenvironment of
the EB. Nonetheless, progenitors must have, at least in part, already
been present in the EBs, as the proportion of CD41/H11001 cells increased
within the EBs from day 6 on in serum-free conditions (Fig. 1A).

Under atmospheric oxygen partial pressure, the proportion
of CD41/H11001-Kit/H11001 CD34/H11002 cells that contains the population capable
of long-term repopulation in immunodeficient mice [15] was
strongly reduced under serum-free conditions. In line with this,
the frequency of early CFU-GEMM-forming progenitors was also
significantly diminished. Remarkably, the switch to hypoxia (i.e.,
5% O2) was sufficient to mediate a strong multiplication of CFU-
GEMM and total colony-forming cell frequencies, which outper-
formed those of serum-containing cultures. Concomitantly, the
percentage of CD41/H11001-Kit/H11001 CD34/H11002 cells also strongly increased
to levels comparable to those grown in serum with or without
stroma cells. Under these serum-free, hypoxic conditions, the

Figure 6. Donor cell chimerism in immunocompetent recipient mice (129S6/SvEvTac) of HOXB4/H11001 embryonic stem cell-derived hematopoietic stem and progenitor cells (ES-HSPCs) grown under stroma-free conditions. (A): Donor chimerism (percentage of GFP/H11001 cells) in the peripheral blood of individual mice transplanted with 3 x 10^6 HOXB4-expressing ES-HSPCs that had been expanded for 23 days under hypoxic conditions, stroma-free and with or without serum supplement (STFV+S or STFV-SF, respectively). Analysis was performed 12 weeks post-
transplantation. Median chimerism rates are shown. (B): Chimerism 12 weeks post-transplantation was predominantly myeloid (GFP/H11001 CD11b/H11001) with a lymphoid engraftment (GFP/H11001 CD3/H11001) <1%. Arithmetic means of the assigned animal numbers are shown. Error bars represent SD. (C): Flow-cytometric analysis for CD150, CD48, CD4, and CD201 expression after 23 days of cultivation. An E-SLAM/1 cell population, being CD150/H11001 CD48/H11001 CD45/H11001 CD201/H11001, was unambiguously detectable only in serum-free cultures, with an average frequency of approximately 0.8% of all nucleated cells. Abbreviations: ESC-HC, embryonic stem cell-derived hematopoietic cell; GFP, green fluorescence protein; PB, peripheral blood; pTx, post-transplantation; S, cytokine-supplemented serum; SF, serum-free; STFV, stem cell factor, thrombopoietin, Flt3-ligand, and vascular endothelial growth factor; w, week.

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HOXB4⁺ ES-HSPCs were even able to develop to a phenotype similar to that of adult long-term repopulating HSCs found in bone marrow, being CD45⁺ EPCR⁺ CD48⁻ CD150⁺ (E-SLAM⁺; Fig. 6B) [29]. Physiologic oxygen concentrations of 1%–5% O₂ (so-called physiologic hypoxia) are known to be of crucial importance for the maintenance of adult hematopoietic stem cells. As a matter of fact, quiescent, label-retaining LT-HSCs appear to locate to the most hypoxic areas within the bone marrow, the sinusoids [49]. Under those conditions, intracellular hypoxia leads to the stabilization of a master sensor of oxygen partial pressure, the basic helix-loop-helix transcription factor hypoxia-inducible factor-1α (HIF-1α), which regulates genes involved in cellular adaptation to hypoxia, such as erythropoietin, VEGF, and angiopoietin. HSCs in which the HIF-1α gene has been deleted by induced homologous recombination rapidly become exhausted under stress conditions, such as during serial transplantation or aging [50], thus underlying the tight relationship of oxygen-tension and cell identity. In fact, the ES-HSPCs obtained in vitro were even able to repopulate immunocompetent, lethally irradiated mice for at least 24 weeks after transplantation in vivo (Figs. 5, 6). Thus, in the ES-B system, hypoxia is a crucial factor that allows the maturation of differentiating ESCs toward a more adult-type HSPCs, entirely in vitro, when HOXB4 is present. It is possible that inhibition of FGF signaling again plays a crucial role during the maturation the ES-HSPCs under hypoxic conditions. Hypoxia has very recently been demonstrated to inhibit the transcription of genes directly regulated by FGF signaling, namely Sprouty4 and Dusp6, and also a key effector of FGF in the presomitic mesoderm of the E9.5 mouse embryo, double-phosphorylated ERK of the ERK/mitogen-activated protein kinase pathway [51].

A very low proportion of lymphoid cells expressing HOXB4 (bearing GFP⁺) was detected after transplantation of HOXB4 ESC-HCs in the immunocompetent mice (Figs. 4, 5), as well as in immunodeficient Rag2⁻/⁻; BYC⁻/⁻ mice [21–24], which do not possess any lymphoid cells per se. Because lymphoid cells are generated in the immunodeficient mice, HOXB4 must be able to promote the development of multipotent lymphomyeloid hematopoietic progenitors in vitro and at least allow development of common lymphoid progenitors in vivo. Thus, the incompatibility of HOXB4 expression and lymphoid development presumably occurs at a later stage. As a consequence, most of the few detectable CD3⁺ cells that still develop have silenced the retroviral vector [21–24]. Very recently, a plausible mechanistic explanation has been offered for the inhibition of lymphopoiesis by HOXB4, based on the results of global gene expression comparisons and HOXB4 ChiP-Seq experiments [52]. In that study, five different HSPC types were compared: bone marrow HSCs, myeloid-biased and lymphoid-biased HSC subpopulations [53], HOXB4 ESC-HCs derived from our clone 8 (used in the present study), and independently established HOXB4 ESC-HCs [54]. Their results strongly suggest that HOXB4 ES-HSPCs are much more similar to the adult myeloid-biased (My-) HSC subtype than to lymphoid-biased HSCs. In the My-HSCs, they observed a dysregulation of mitochondrial and lysosomal genes by HOXB4, which may explain the block of lymphoid differentiation mechanistically.

An improved understanding of the crucial signaling pathways altered by HOXB4 and exact timing of its activities promoting or inhibiting hematopoietic development and differentiation will presumably allow its replacement by (small molecule) drugs that mimic its activities. In light of the rapid progress made in the establishment of protocols for efficient, vector-free generation of patient-specific iPSC cells, our results may also prove valuable for the future generation of IPS-derived, patient-specific HSCs in vitro.

Of note, spectral karyotyping analysis of the ESC line used to determine its ploidy (unmanipulated and transduced CCE-cells) revealed that it carried a trisomy of chromosome 8 (supplemental online Figure 4). This is the most commonly observed chromosomal abnormality found in mouse ESC lines and is known to correlate with a low efficiency of germ line transmission [55]. However, this trisomy does not appear to negatively affect their differentiation potential as embryoid bodies, in vitro. Furthermore, teratomas, in vivo, formed from such ESCs behave similar to their counterparts with normal karyotypes [56]. Trisomy of the human syntenic chromosomes 8 and 16 has been associated with acute lymphoblastic leukemia [57]. However, the chromosomal aberration of this clone does not appear to promote a leukemogenic potential of these cells in vitro, as we and others have never observed any leukemias in mice transplanted with hematopoietic cells derived from our CCE lines, so far. Furthermore, independent trisomy 8 ESC clones containing an inducible eGFP-HOXB4 cassette proved that hematopoietic development of differentiating ESCs in vitro was enforced only after HOXB4 induction and was not promoted by the trisomy itself (supplemental online Figure 5).

**CONCLUSION**

When these data are taken together, we have established serum- and stroma-free, hypoxic culture conditions allowing the conversion of mouse embryonic stem cells ectopically expressing HOXB4 toward hematopoietic cells capable of multilineage engraftment of irradiated, immunocompetent recipient animals, entirely in vitro.

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**AUTHOR CONTRIBUTIONS**

D.A.L., N.H., S.P.-C., C.R., R.J., and B. Schlegelberger: conception and design of research, data collection, analysis and interpretation of data; H.K. and B. Schiedlmeier: conception and design of research, data collection, analysis and interpretation of data, manuscript writing and editing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

B. Schiedlmeier is a cofounder and stock holder of Morphogenesis Inc. (Tampa, FL).
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