Development of Hematopoietic Repopulating Cells from Embryonic Stem Cells

By Michael Kyba, Rita C. R. Perlingleiro, and George Q. Daley

Introduction

Embryonic stem cells are defined as totipotent by virtue of their potential to chimerize any tissue of a developing embryo. From the standpoint of the development of cellular therapies, this virtue is problematic, because in general only a single or limited number of cell types is required for a given therapy. There are two possible solutions to this problem: one either identifies the critical genetic elements that control the lineage choices along the path toward a given cell type and then enforces these instructions on as much of the culture as possible, or one defines conditions which select out and amplify the cell type of interest from among its unwanted cousins. The latter approach has proven highly effective in our work towards the derivation of a repopulating hematopoietic stem cell (HSC) from embryonic stem cell cultures, particularly when used in combination with an element of genetic instruction.

The hematopoietic potential of mouse ES cells is easily demonstrated. ES cells grown under the appropriate conditions (suspension culture and the removal of LIF) will spontaneously form proliferating and differentiating cellular clusters referred to as embryoid bodies (EBs). The EBs increase in both size and cellular variety with time, and within 8–10 days, superficial red islands containing hemoglobinized erythrocytes become

Hematopoiesis can be identified at earlier stages by disaggregating the embryoid bodies into single cells and plating these cells in suspension cultures with hematopoietic cytokines. Under these conditions, hematopoietic colonies will form containing differentiated cells of myeloid, erythroid, or mixed lineages. The types of colony-forming cells (CFCs) that can be identified in this manner change over the course of time, but the pattern of change is highly reproducible: at early time points (5 days of differentiation) primitive erythroid precursors predominate. The primitive erythrocyte (Ery-P) is peculiar to early embryogenesis and is distinguished from the adult-type, or definitive erythrocyte (Ery-D) by the fact that it is nucleated, and therefore larger, and expresses embryonic globin isoforms ($\beta$-H1 in the mouse) with a higher affinity for oxygen than adult globin ($\beta$-major). At later time points (8 days of differentiation and beyond) Ery-D, myeloid, and mixed colony types predominate.

Since hematopoietic CFCs are derived from a hematopoietic stem cell, one would predict that HSCs should be detectable prior to day 6 of EB differentiation. Many investigators, ourselves included, have looked for the presence of HSCs in early EBs by attempting to use their disaggregated cells as a substitute for bone marrow to rescue and reconstitute hematopoiesis in lethally irradiated adult mice. In our hands, neither rescue from the lethal effects of irradiation nor reconstitution of the hematopoietic system can be demonstrated. This would at first glance appear to present a paradox; however, there is a striking embryonic correlate to these results. In the early embryo, the first tissue to undergo hematopoietic differentiation is the extraembryonic mesoderm of the yolk sac. Like the EB, the yolk sac initially produces Ery-P, and at later time points myeloid and mixed CFC. However, prior to the onset of circulation, cells from the yolk sac will not reconstitute hematopoiesis in lethally irradiated adult hosts. Repopulating HSCs are detected in the yolk sac at around day 11, shortly after the establishment of

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circulation, however they are also detected in greater numbers at this time point at an intraembryonic site, the major vessels of the aorta-gonads-mesonephrous (AGM) region. Because autonomous culture of AGM tissue explanted before the onset of circulation can give rise to adult-repopulating cells, it is generally accepted that the AGM is the site of origin of the definitive HSC, while the yolk sac cells that initially give rise to Ery-P are specialized for that purpose and not capable of adult repopulation. Repopulating cells present in the yolk sac at day 11 are thus taken to have arrived there through the circulation. Although there are problems with this interpretation that are beyond the scope of this chapter, it should be apparent that hematopoiesis as described in EBs recapitulates the early stages of embryonic yolk sac hematopoiesis. It would therefore appear that the EB is incapable of producing cells corresponding to those of the AGM. The EB is certainly a smaller and much more chaotic structure than the embryo of equal developmental age.

Leukemic Engraftment

As a first step towards exploring the usefulness of EB culture systems for the generation of repopulating HSCs, we attempted to disconnect the repopulating activity of the putative HSC from its lymphoid–myeloid–erythroid differentiation potential. We reasoned that the ideal agent to accomplish this would be a transforming factor that acted either to block HSC apoptosis, or to drive HSC proliferation, or both, while at the same time leaving the natural ability of the HSC to differentiate along lymphoid, myeloid, and erythroid lineages unperturbed. Such a factor exists uniquely in the form of the oncogene, Bcr/Abl. This genetic fusion, the causative agent of chronic myeloid leukemia (CML), is encoded by the Philadelphia chromosome (a translocation between chromosomes 9 and 22) in CML patients. Bcr/Abl has the dual effect of driving HSC proliferation and blocking apoptosis. The fact that Bcr/Abl-transformed HSC can still undergo normal differentiation is

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demonstrated by the presence of the Philadelphia chromosome in both lymphoid and myeloid cells of patients with CML. Indeed, the presence of this unique marker in all hematopoietic lineages of these patients is taken as classical evidence for the existence of an HSC.12 Our strategy was to transduce Bcr/Abl into cells from day 5 EBs, the stage in which the stem cell for hematopoiesis is being specified as a derivative of the hemangioblast, the earliest progenitor defined for the hematopoietic lineage within EBs.13

Bcr/Abl-Induction of Hematopoietic Cultures

- Generate EBs by plating $10^4$ ES cells per ml in *differentiation medium* (IMDM (Sigma) with 15% fetal calf serum (FCS for differentiation; StemCell), 50 µg/ml ascorbic acid (Sigma), 200 µg/ml iron-saturated transferrin (Sigma), $4.5 \times 10^{-4} M$ monothioglycerol (MTG; Sigma)) supplemented with 0.9% methylcellulose (M3120, StemCell Technologies) in 35 mm petri dishes (StemCell).
- Harvest on day 5 (120 hr) by diluting the methylcellulose with PBS and centrifuging the EBs. Wash once with PBS, and dissociate with 0.25% collagenase for 60 min at 37°C followed by repeated passage through a 23 G needle.
- Spin-infect (1300 g for 90 min at 30°C in a Beckman GH-3.8 rotor) $3 \times 10^5$ cells with 10 ml of Bcr/Abl viral supernatant containing 4 µg/ml polybrene (Sigma) in three wells of a 6-well dish precoated with stromal cells (see below). Our viral vector expresses GFP from a downstream IRES, marking the transduced cells with green fluorescence.
- Culture on OP9 stroma at 37°C/5%CO₂. The growth conditions under which transduced populations are initiated are critical. We used a cytokine cocktail consisting of 0.5 ng/ml murine IL3 (interleukin 3; Peprotech), and 50 ng/ml each of human IL6 (interleukin 6; Peprotech), human SCF (stem cell factor; Peprotech), and human FL (FLT3 ligand; Peprotech), 50 µM β-mercaptoethanol, in IMDM/15% FCS, over OP9 stromal cells.
- Once the colonies have become dense, passage in the absence of stroma in the same growth medium. Under these conditions, cultures become dominated by immature hematopoietic blast cells.

We favor colony induction on the stromal cell line OP9, which is genetically null for M-CSF, based on our early experiments to optimize the system. Other stromal cell lines, including M2-10B4 and DAS104-4 gave an overabundance of macrophages and mast cells under these conditions, while OP9 promoted an expansion of hematopoietic blast cells (Fig. 1).

In addition to bulk cultures, clonal cell lines can be established by plating in methylcellulose suspension cultures and picking individual colonies. When expanded as above, the majority of our clonal cultures also consisted of blast cells. The in vitro differentiation potential of the clones was limited to producing Ery-P colonies or secondary blast cell colonies, however when we injected 4×10⁶ cells into sublethally irradiated (500 Rad) 129Sv/Ev (Taconic) or NOD/SCID (Jackson) mice via lateral tail vein, mice succumbed to a donor-derived multilineage leukemia 5–9 weeks posttransplant. Because the leukemias were initiated from cloned cells, and because they contained lymphoid, myeloid, and erythroid elements, we concluded that Bcr/Abl had targeted a hematopoietic stem cell, albeit one with an intrinsic defect in adult engraftment that rendered these differentiation potentials

![Fig. 1. Morphology of Bcr/Abl-transduced cells growing on different stromal cell lines. (See Color Insert.)](image-url)

latent. We named this putative target cell the embryonic (or primitive) HSC in accordance with its primitive differentiation potential \textit{in vitro}, and the primitive hematopoiesis to which it presumably would have contributed had it not been disaggregated from its EB environment and targeted with Bcr/Abl.\(^\text{17}\)

Non-oncogenic Engraftment

With the formal demonstration that it was possible to target and expand repopulating hematopoietic blast cells from EBs, we turned to inducible expression systems with the aim of enabling nononcogenic engraftment. We began by investigating conditional regulation of signaling from the c-Mpl receptor. In addition to lineage-specific effects on megakaryocytes,\(^\text{18,19}\) the cytokine thrombopoietin and its receptor, c-Mpl, had been shown to be required for proper HSC self-renewal,\(^\text{20,21}\) and had been used to expand immature hematopoietic progenitors from bone marrow.\(^\text{22}\) To enable inducible Mpl signaling in our model, CCE ES cells were infected with a retrovirus (provided by Anthony Blau, University of Washington, Seattle, WA) carrying the F36V mutant of the FKBP dimerization domain fused to the cytoplasmic domain of c-Mpl\(^\text{23,24}\) allowing for activation of Mpl signaling in response to a chemical inducer of dimerization (CID), AP20187. The virus carried an IRES-GFP


\(^{22}\) H. Ku, Y. Yonemura, K. Kaushansky, and M. Ogawa, Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice, \textit{Blood} \textbf{87}, 4544–4551 (1996).


reporter, which allowed us to select high-level, stable expressers. The top 10% of GFP-expressing cells were sorted by FACS, expanded, and sorted a second time in the same way. In the third round of FACS, the brightest 1% of cells was selected for single cell cloning by sorting directly into 96-well dishes. After expansion to clonal cell lines, FKBPMpl-expressing ES cells were tested for their ability to give rise to hematopoietic blast cells on OP9 in the presence of the CID. Day 5 EBs were disaggregated, and 10^5 cells were plated in a single well of a 6-well dish of OP9, in the same medium used to expand Bcr/Abl-induced cells, with or without 100 nM AP20187. Although all clones expressed high levels of GFP as undifferentiated ES cells, the majority of clones silenced the provirus with differentiation. Silencing with differentiation is a problem that confounds many ES cell gene expression systems (see below). Nevertheless, several clones were able to generate a hematopoietic cell population whose expansion was now dependent on the presence of the CID (Fig. 2), indicating that in these clones, the hematopoietic lineage is competent for expression of the provirus, perhaps due to a fortuitous integration site. Like the Bcr/Abl-induced blast cells, the FKBPMpl cells grew semi-attached to the OP9 stroma, with a rounded morphology and relatively scant basophilic cytoplasm (Fig. 2). By FACS analysis (Table I) they were also superficially similar to the Bcr/Abl-induced cells. We injected nine sublethally conditioned (500 Rad) mice with 5 x 10^6 cells each, and treated five of these with CID (2 mg/kg per day AP20187, by intraperitoneal injection). Unlike the results we observed for Bcr/Abl, neither the CID-treated mice, nor the untreated mice, were repopulated with these cells. Although this model was not exhaustively exploited, this result demonstrates that continual activation of signaling pathways that are sufficient to generate a blast cell outgrowth in vitro is not necessarily sufficient to enable cellular survival and maintenance in adult recipients. It also suggests that in spite of superficial similarities, the cells generated by activation of different regulatory pathways may be intrinsically different.

In order to test regulators other than dimerizable receptors, we sought a more generic conditional expression system. An attractive candidate system developed by Wutz et al.\textsuperscript{25} allowed for doxycycline-induced gene expression and coexpression of GFP from a bidirectional promoter. This system makes use of the reverse tetracycline transactivator (rtTA)\textsuperscript{26}


integrated into a constitutive locus, ROSA26, allowing for transactivator expression in all tissues and cell types. The inducible locus is 5' to the HPRT gene on the X-chromosome, and has a single loxP site, which allows for the

lox-in of a circular plasmid carrying any gene of interest. We tested this system with several candidate genes and found that although transgene expression was robust in ES cells, it was severely attenuated in differentiated EB cells. When a circular plasmid is integrated into a single lox-P site, the entire length of the plasmid separates the constitutive locus, HPRT, from the inducible locus (Fig. 3, original orientation). We reasoned that this extra distance may push the inducible locus out of the constitutively open transcriptional domain occupied by HPRT, subjecting the inducible locus to silencing with differentiation. We therefore reengineered the system, inverting its orientation such that the inducible locus stays proximal to HPRT, and eliminating the coinducible GFP reporter (Fig. 3, inverted orientation). This targeting ES cell line, which we named Ainv15, gave robust, inducible expression of transgenes even in highly differentiated cells.28

Lox-in to Derive Inducible ES Cell Lines from Ainv15 Targeting Cells

- Coelectroporate 20 μg each of the targeting plasmid carrying the inducible gene of interest and CRE-expression plasmid into 8×10^6 Ainv15 cells in 800 μl PBS. (We do this electroporation at room temperature on the BioRad gene pulser with capacitance extender, using the settings: 0.25 V, 500 μFD.)
- Plate the electroporated cells on 10 cm dishes with dense neo-resistant MEFs.
- Begin selection in G418 the next day (350 μg/ml) and maintain until colonies appear around day 10–14. In the first few days, cells will be very dense, feed twice per day. Thereafter, feed once per day.
- Pick colonies by flooding the dish with PBS, and extracting individual colonies with a P20 pipette. Transfer to 100 μl of 0.25% trypsin/EDTA and pipette to disrupt the colony. Incubate at 37°C for 2 min, and disrupt once again by pipetting. Add 900 μl of medium with 10% serum, and collect the cells of the colony by centrifugation. Replate the disrupted contents onto MEFs in 12-well dishes.
- Integration can be detected by using the following primers, which amplify across the loxP site to give band of approximately

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FIG. 3. Targeting constructs. Chromosome 6 carries the reverse tetracycline transactivator (rtTA) integrated into the ROSA26 locus. The X-chromosome carries modifications 5' to the HPRT gene. After lox-in (shown by the symbol chi) of the targeting plasmid, the formerly nonfunctional neo gene acquires a start codon and a promoter/enhancer, making it functional and enabling selection for integration of the plasmid. In addition, the gene of interest, formerly carried by the plasmid, is now integrated downstream of the tetracycline responsive element (TRE), enabling its doxycycline-regulated expression. In the original configuration, the TRE also drives expression of a GFP reporter gene. After integration of the plasmid, the neo gene remains proximal to HPRT, while the inducible gene is located distally, separated by several kilobases of plasmid sequence. In this configuration, the inducible gene is subjected to silencing with differentiation. In the inverted configuration, the GFP reporter is eliminated, and the target locus is inverted relative to HPRT. Lox-in places the inducible gene proximal to HPRT and the neo gene distal, separated by several kilobases of plasmid sequence. In this configuration, the inducible gene is not subject to silencing with differentiation. PGK, phospho-glycero-kinase enhancer; P RosA, ROSA26 enhancer/promoter; iGene, inducible gene; loxP, recognition sequence for Cre recombinase; Δ neo, deletion mutant of the neomycin (G418) resistance gene; ATG, start codon for the neo gene.
420 bp if integration has been successful: LoxinF: 5′-ctagatctcgaggagc-3′ LoxinR: 5′-atacctctctcggcagagca-3′ Cycle conditions: 45 sec at 95°C, 1 min at 60°C, 1 min at 72°C, repeat 29×, using Taq polymerase and Promega PCR buffer supplemented with 1.25 mM MgCl$_2$.

We selected HoxB4 as the first candidate on the basis of both information on its expression pattern (detected in the HSC but not in more differentiated hematopoietic progenitors,$^{29}$ nor in yolk sac$^{30}$) and the competitive engraftment advantage that it affords HSC after bone marrow transplantation.$^{31}$ We introduced HoxB4 into the inducible locus and began testing its effects on the hematopoiesis of embryoid bodies. One of the great advantages of our inducible system is that it enables control over cells within their native EB environment, however our standard methodology for generating EBs, suspension of ES cells in methylcellulose, was not amenable to adding or removing an inducing agent at different time points in EB development. We therefore switched to a strictly liquid culture system where EBs were initiated in hanging drops (Fig. 4).

**Hanging Drop EB Cultures**

- Use an 8-well multichannel pipettor to plate approximately 300 drops per 15 cm non-tissue culture-treated dish, with each drop containing 100 ES cells in 10 μl of differentiation medium (see above, nb. no methylcellulose).
- Invert the dishes and incubate for 2 days at 37°C in 5% CO$_2$. ES cells quickly descend to the nadir of the drop, aggregate, and form a single EB per drop.
- Collect EBs after 48 hr, by flushing the dish with PBS, transferring to a 15 ml tube, and allowing the EBs to sediment by gravity for 3 min. Aspirate the liquid and collect the EBs in 10 ml of fresh differentiation medium. Transfer to 10 cm bacterial-grade dishes, and culture under


slow swirling conditions on a rotating shaker (50 rpm) set up inside of a dedicated 37°C/5% CO₂ incubator.

- Feed the swirling cultures every two days by replacing half of the spent medium with fresh differentiation medium.

These techniques (hanging drops, use of bacterial-grade, non-tissue culture-treated dishes, and slow swirling) are all necessary to prevent attachment of the EBs to their dishes. We have observed that if EBs are allowed to attach, their complex structure quickly disintegrates, their cellular content spreads across the substrate, and they do not undergo hematopoietic differentiation.32

**HoxB4-Induction of Hematopoietic Cultures**

- Set up one dish of hanging drops using the iHoxB4 (inducible HoxB4) ES cells.
- Culture as described above, however induce HoxB4 expression at day 4 (96 hr) of EB development by adding doxycycline to the differentiation medium at a final concentration of 1 μg/ml.
- Allow the EBs to develop for two more days.

On day 6, collect EBs by gravity, wash once with PBS, and collect again by gravity. Dissociate the EB cells by adding 0.5 ml of 0.25% trypsin and incubating 2 min at 37°C. Add 5 ml of IMDM/10% FCS and passage repeatedly through a 5 ml pipette until the EBs have disintegrated, and collect by centrifugation. Plate 10^5 cells per well onto semi-confluent 6-well dishes of OP9, in IMDM/10% FCS with 40 ng/ml each of murine VEGF (vascular endothelial growth factor; Peprotech), and human TPO (thrombopoietin; Peprotech), and 100 ng/ml of each of human SCF (stem cell factor; Peprotech), and human FL (FLT3 ligand; Peprotech). Maintain induction of HoxB4 by including doxycycline at 1 µg/ml. This cytokine cocktail was selected based on the developing understanding of the cytokine requirements of the definitive HSC as well as those of the EB-derived hemangioblast.

After several days, colonies of semi-adherent cells arise on the OP9 stromal layer, and these can be passaged by trypsinization (collecting both adherent and nonadherent cells) onto fresh OP9 and expanded in the same medium.

HoxB4 cultures are rich in CFC, especially the CFU-GEMM (colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte) and by cytospin are dominated by immature blast cells similar in morphology to those produced by the Bcr/Abl experiment (Fig. 5). By antibody staining, the cells are prominently c-kit and PECAM-positive, however cultures contain both positive and negative cells for CD45, Sca-1, and CD41, and usually a small frequency of AA4.1-positive cells (Table I). The proportions

![Fig. 5. HoxB4-induced cells. (A) Morphology of HoxB4-induced cells grown on OP9 stromal cells. (B) FACS analysis of peripheral blood of a recipient mouse three weeks posttransplant. GFP expression, which marks donor cells, is measured on the X-axis. Antibody staining is measured on the Y-axis. The percentage of cells falling into each quadrant is shown in the upper right-hand corner. In the first FACS, cells were stained with a nonspecific antibody, which does not recognize any blood cell type. In the second FACS, a cocktail of myeloid-specific antibodies was used (Gr-1 and Mac-1) to label circulating granulocytes and monocytes. In the third FACS, a cocktail of lymphoid-specific antibodies was used (B220, CD4, and CD8) to label circulating lymphocytes. (See Color Insert.)](image-url)
of cells positive for these latter markers is somewhat variable from experiment to experiment, however we have observed that good adult engraftment potential correlates best with higher proportions of Sca-1 and AA4.1. Cells positive for lineage-specific markers are also often seen: cultures always contain some fraction Gr-1 and Mac-1 positive (myeloid-committed) cells, and may contain a smaller fraction of B220, CD4, or CD8 (lymphoid-committed) positive cells.

For the purposes of detecting donor contribution to adult recipients, cells were labeled by infection with a GFP-expressing retrovirus, sorted for GFP-positivity, and replated onto fresh OP9. After expansion, $2 \times 10^6$ cells were injected into lethally irradiated (2 doses of 500 Rad, separated by 4 hr) syngeneic mice (129 Ola, Harlan Laboratories). Cells home rapidly to the bone marrow, and we observe contribution to the peripheral blood almost immediately. Myeloid contribution is always more prevalent than lymphoid contribution, as shown in Fig. 5, and overall contributions vary from mouse to mouse, ranging, in the case of the iHoxB4 cells, from a few percent to a high of 50%, or higher with retroviral transduction of HoxB4 (see below). It is not necessary to maintain expression of HoxB4 in the recipients, although doing so (by adding doxycycline to the drinking water) correlates somewhat with higher levels of donor engraftment.

The hematopoietic progenitors of the early EB that we are targeting have a primitive embryonic hematopoietic fate specification. By causing ectopic expression of HoxB4 in vitro, this fate specification is perturbed, and certain aspects of definitive hematopoietic fate are conferred upon the cells, most importantly the ability to repopulate adult recipients. This reprogramming is also evident by comparing the expression of certain genes between the Bcr/Abl-induced and the HoxB4-induced in vitro populations. The HoxB4 cells downregulate primitive globin, $\beta$-H1, and upregulate definitive markers involved in bone marrow homing, CXCR4 and Tel, whereas the Bcr/Abl cells do not downregulate $\beta$-H1, and have low levels of CXCR4. However, the HoxB4 cells’ relative ineffectiveness at competing with endogenous bone marrow HSC (there is a very strong requirement for lethal donor conditioning, and even with the injection of $2 \times 10^6$ cells we still see fractional engraftment) as well as their propensity to contribute to myeloid over lymphoid lineages in transplanted recipients,


suggests that they have not been completely respecified as bone marrow HSC. Significant additional work remains to understand the relationship between HoxB4-induced, ES-derived HSCs compared to their counterparts in fetal liver, cord blood, and adult bone marrow.

While the inducible system for expression of HoxB4 described above has many advantages over the retroviral transduction approach described for Bcr/Abl, we have also used retroviral expression of HoxB4 to the same end. In order to facilitate hematopoietic contribution from ES cells derived via therapeutic cloning, with a minimal number of additional genetic manipulations, we infected day 6 EB cells with an MSCV-HoxB4-iresGFP retrovirus and expanded these cells under the same conditions used to expand the inducible HoxB4 cells, however without doxycycline. HoxB4 efficiently targets the cell with the ability to form semi-adherent colonies on OP9, and rapidly expands it in vitro. Because the recipient mice in this experiment carried a null mutation for Rag2, the ability of HoxB4-induced EB-derived hematopoietic blast cells to produce lymphoid progeny could be monitored sensitively in the complete absence of host-derived lymphocytes. Under these circumstances we clearly observed the presence of donor-derived lymphocytes, by measuring genomic rearrangement of the IgH and TCR loci, the presence of circulating antibody, and the presence of some circulating IgM-positive donor cells, however the levels of lymphoid repopulation were much lower than the levels of myeloid. While this may indicate that HoxB4 favors myeloid differentiation of EB-derived progenitors, it may also reflect an underlying differentiation bias of these formerly primitive hematopoietic progenitors.

Therapeutic Repopulation

Past work with genetically unmodified ES cells differentiated as EBs has shown that such cells are severely limited in their capacity to successfully chimerize the adult hematopoietic system. Interestingly, the most successful attempts, in which some lymphoid repopulation was seen in Rag1−/− immunodeficient mice, made use of cells from 3-week-old EBs. As discussed above, the origins of hematopoiesis in the EB occur in the first

36 A. M. Müller and E. A. Dzierzak, ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients, Development 118, 1343–1351 (1993).
week. This suggests that under some circumstances, the late stage EB may generate an environment conducive to a primitive-to-definitive respecification of hematopoietic progenitors, although as evidenced by the difficulty of repeating these results, the intrinsically variable nature of the differentiation process \textit{in vitro} makes these circumstances elusive. We have targeted EB day 6 hematopoietic progenitors due both to their abundance and to the consistency with which they can be generated \textit{in vitro}, however expression of HoxB4 at later times, when the environment may be more conducive to induction of definitive hematopoiesis, may give more completely respecified cells.

While genetic modification of murine ES cells has allowed us to model their differentiation to hematopoietic repopulating cells, less invasive methods will likely be needed before a hematopoietic therapy is available from human ES cells. In this regard, the challenge will be to identify environmental conditions that induce the same effects, to recapitulate \textit{in vitro} the environment that the primitive HSC experiences in the embryo at the time of its definitive transition. This may involve the use of signaling molecules normally involved in these processes, as well as synthetic constructs such as transducible proteins, small molecule agonists or inhibitors, and transiently expressed nucleic acids. In any case, we envision that therapeutic differentiation protocols will ultimately combine elements of both instruction of lineage choice and selection and expansion of target cells.