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Clonal analysis of differentiating embryonic stem cells reveals a hematopoietic progenitor with primitive erythroid and adult lymphoid-myeloid potential

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SUMMARY

Embryonic stem (ES) cells differentiate into multiple hematopoietic lineages during embryoid body formation in vitro, but to date, an ES-derived hematopoietic stem cell has not been identified and subjected to clonal analysis in a manner comparable with hematopoietic stem cells from adult bone marrow. As the chronic myeloid leukemia-associated BCR/ABL oncogene endows the adult hematopoietic stem cell with clonal dominance without inhibiting pluripotent lymphoid and myeloid differentiation, we have used BCR/ABL as a tool to enable engraftment and clonal analysis. We show that embryoid body-derived hematopoietic progenitors expressing BCR/ABL maintain a primitive hematopoietic blast stage of differentiation and generate only primitive erythroid cell types in vitro. These cells can be cloned, and when injected into irradiated adult mice, they differentiate into multiple myeloid cell types as well as T and B lymphocytes. While the injected cells express embryonic (β-H1) globin, donor-derived erythroid cells in the recipient express only adult (β-major) globin, suggesting that these cells undergo globin gene switching and developmental maturation in vivo. These data demonstrate that an embryonic hematopoietic stem cell arises in vitro during ES cell differentiation that constitutes a common progenitor for embryonic erythroid and definitive lymphoid-myeloid hematopoiesis.

Key words: Embryonic stem cells, Hematopoiesis, Adult reconstitution, BCR/ABL

INTRODUCTION

Hematopoiesis during embryoid body (EB) differentiation follows a well-defined temporal program of development, and is thought to recapitulate the yolk sac stage of hematopoiesis (Keller et al., 1993; Schmitt et al., 1991; Wiles and Keller, 1991). The earliest hematopoietic population consists of primitive yolk-sac type erythrocytes, which are nucleated and express embryonic forms of β-globin (Barker, 1968; Brotherton et al., 1979). As EB differentiation continues, there is a switch to the adult program, characterized by emergence of definitive erythroid, myeloid and multipotent precursors. Recently, a common precursor for the primitive erythroid and definitive erythroid-myeloid hematopoietic programs, designated the blast colony-forming cell (BL-CFC), was identified during EB differentiation (Kennedy et al., 1997). However, it has not been demonstrated that this cell can generate lymphoid cell types or reconstitute hematopoiesis in irradiated adult mice, a requirement if ES cells are ever to be exploited as a source for hematopoietic cellular therapies. In this regard, previous reports using different methods of ES differentiation have shown that ES-derived hematopoietic cells have only a limited capacity to engraft adults under normal circumstances (Gutierrez-Ramos and Palacios, 1992; Hole et al., 1996; Müller and Dzierzak, 1993; Palacios et al., 1995; Potocnik et al., 1997). To date an ES-derived hematopoietic stem cell (HSC) has not been identified through a rigorous clonal analysis in a manner comparable with HSCs from adult bone marrow (BM).

The clonal analyses that proved the existence of the bone marrow-derived HSC involved lineage marking by unique radiation-induced karyotypes (Wu et al., 1968) or retroviral integration sites (Dick et al., 1985; Keller et al., 1987; Lemischka et al., 1986) followed by engraftment in irradiated adult mice. In humans, the first direct evidence for the hematopoietic stem cell was the presence of a unique chromosomal rearrangement, the Philadelphia chromosome, in lymphoid and myeloid cells of individuals with the disease chronic myeloid leukemia (CML) (Fialkow et al., 1977). Subsequent studies have shown that retroviral transduction into murine bone marrow of BCR/ABL, the oncoprotein encoded by the Philadelphia chromosome, transforms the hematopoietic stem cell and generates a CML-like myeloproliferative disorder in transplanted mice that manifests clonal repopulation of lymphoid and myeloid lineages (Daley et al., 1990; Li et al., 1999). The CML-associated BCR/ABL

oncogene endows the adult HSC with clonal dominance while allowing lymphoid and myeloid differentiation. BCR/ABL augments the proportions of myeloid populations derived from infected HSCs, but does not alter the fundamental potential of the HSC to differentiate along lymphoid, myeloid or erythroid lineages. To date, clonal analysis of embryoid body hematopoietic progenitors has been precluded by the rarity of these progenitors and their inefficiency in engraftment of adults (Medvinsky and Dzierzak, 1996; Müller et al., 1994). Thus, we have used BCR/ABL as a tool to enable engraftment and clonal analysis of hematopoietic precursors from EBs. Our data demonstrate that a common progenitor of the primitive erythroid and definitive lymphoid-myeloid hematopoietic programs arises during the differentiation of ES cells into EBs in vitro.

MATERIALS AND METHODS

Growth and differentiation of ES cells

The 129/Sv-derived ES cell line CCE (Robertson, 1987) was maintained on gelatinized flasks in DMEM (Sigma) supplemented with 1000 U/ml LIF (leukemia inhibitory factor; Amgen), 15% knockout serum replacement (Gibco), 0.1 mM non-essential amino acids (Sigma), and 0.1 mM of β-mercaptoethanol (Sigma) in the absence of feeders for up to 15 passages. For differentiation cultures, the cells were dissociated with trypsin (0.25%; Gibco)/EDTA (1 mM; Sigma) to form a single-cell suspension. Cells were washed three times with phosphate-buffered saline (PBS) and EBs were generated by plating 10^4 cells per ml in IMDM (Sigma) with 15% fetal calf serum (FCS; Sigma), 50 μg/ml ascorbic acid (Sigma), 200 μg/ml iron-saturated transferrin (Sigma), 4.5×10^{-4} M monothioglycerol (MTG; Sigma), and 0.9% methylcellulose (MCM-3120, StemCell Technologies) in 35 mm Petri dishes (StemCell).

Retroviral infection of EB-derived cells and co-culture on OP9 stroma

MSCV-BCR/ABLiresGFP and MSCViresGFP (control vector) retroviruses were transfected into 293T cells using the Ca^{2+} phosphate precipitation method (Grignani et al., 1998). Virus-containing supernatant was collected 48 hours after transfection and filtered through a 0.45 μm filter before use. During the EB growth period, OP9 stromal cells (generously provided by Dr Toru Nakano) were expanded in MEMα (Sigma) supplemented with 20% FCS and plated in six-well dishes 1 day before harvesting EBs. EBs were collected at 5 days post differentiation, washed in PBS and treated with 0.25% collagenase (Sigma) for 60 minutes at 37°C. EBs were disrupted to single cells by repeated passage through a 23 G needle. Approximately 3×10^5 cells were suspended in 10 ml of viral supernatant containing 5 ng/ml mouse IL3 (interleukin 3; Peprotech), 500 ng/ml human IL6 (interleukin 6; Peprotech), 500 ng/ml human SCF (stem cell factor; Peprotech), 50 μM β-mercaptoethanol and 4 μg/ml polybrene (Sigma). Cells were transferred to six well dishes (Corning) pre-plated with semi-confluent OP9 stromal cells and centrifuged at 1300 ***g*** for 90 minutes on a Beckman centrifuge (GH-3.8 rotor). After an overnight incubation at 37°C with 5% CO_2 in air, suspension cells were removed, centrifuged, suspended in fresh viral supernatant and replated onto the same OP9 stromal cells. Suspension cells were removed 18 hours post second infection, centrifuged, suspended in IMDM, 10% FCS, 50 ng/ml human IL6, 0.5 ng/ml mouse IL3, 50 ng/ml human SCF, 50 μM β-mercaptoethanol and replated onto the same OP9 cells.

Culture of infected cells

After 8 days, the non-adherent cells were harvested, plated in methylcellulose medium (MCM) for primitive blast (media containing 5 ng/ml of vascular endothelial growth factor VEGF, 100 ng/ml of SCF and 25% of conditioned medium from the D4T cell line) (Kennedy et al., 1997), myeloid (3434; StemCell) and cytokine-independent colonies (MCM without growth factors; 3231; StemCell). Cells were also expanded in the absence of OP9 cells, vigorously growing cultures were passaged to new dishes as necessary to maintain appropriate cell densities. Clones were generated by plating cells in blast MCM (as described above). Five days later, individual primitive blast colonies were transferred to microtiter wells containing IMDM with human IL6, mouse IL3, human SCF and human FL (FLT3 ligand; Peprotech). Rapidly growing populations were passaged to larger cultures as required.

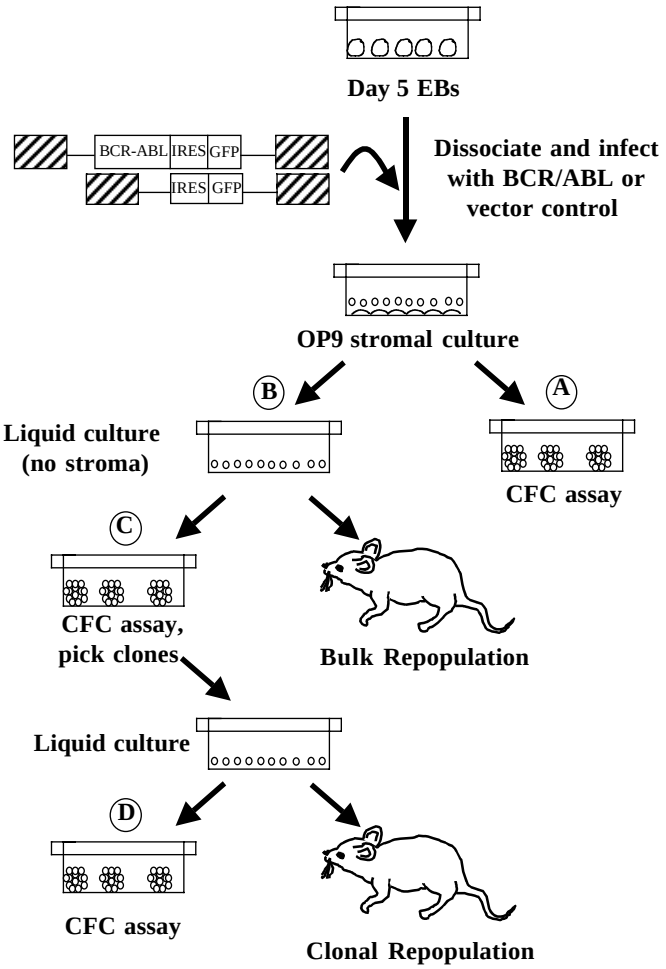

Fig. 1. Strategy for retroviral expression of BCR/ABL in differentiated ES cells to target and expand hematopoietic progenitors. A-D refer to panels in Fig. 2.

Reconstitution of irradiated recipient mice

Cells were injected intravenously (4×10^6 cells in 0.4 ml PBS) into 4 to 8 week old 129Sv/Ev (Taconic) or NOD/SCID (Jackson) mice, preconditioned with sublethal irradiation (500 and 350 rads, respectively). Mice were monitored daily for symptoms, including reduced body temperature, decreased activity and hunched posture. Peripheral blood was collected by retro-orbital venous sinus sampling. Moribund animals were sacrificed and analyzed by necropsy. Leukocytes were purified using red blood cell lysis buffer (Sigma). Spleens were dissected, weighed and minced to prepare single-cell suspensions. Femur and tibia were dissected and bone marrow cells were harvested in PBS 1% FCS by flushing with a 25 or 27 gauge syringe.

Fluorescent-activated cell sorting (FACS) analysis

We used R-phycoerythrin (PE)-conjugated antibodies to AA4 (provided by Dr Ihor Lemischka), Sca1, Kit, CD45, Thy1, Ter119, B220, CD4, CD8, CD19, Gr1, Mac1 and Flk1, and biotinylated antibodies to CD34 (Pharmingen). Cells were washed once in blocking buffer (PBS 1% FBS), suspended at 10^7 cells/ml in the same buffer containing 0.25 μg/10^6 cells of Fc block (Pharmingen) and placed on ice for 5 minutes. Antibody was added at 1 μg/10^6 cells and incubated at 4°C for 30 minutes before washing with blocking buffer. Biotinylated antibody (CD34) was then counterstained with PE-conjugated streptavidin for 30 minutes at 4°C, followed by washing with blocking buffer. Stained cells were analyzed on a FACScan cytometer (Becton-Dickinson) after addition of propidium iodide (Pharmingen) to exclude dead cells.

RT-PCR for globin analysis

Globin expression patterns in the cell lines and peripheral blood from reconstituted mice were determined using the global amplification strategy of Brady et al. (Brady et al., 1990). Total RNA was isolated using RNA STAT-60 reagent (Tel-Test 'B') as recommended by the manufacturer. First strand cDNA was produced using Superscript II reverse transcriptase (Gibco). Total RNA (1 μg) was hybridized with 140 ng random hexamers in first strand reverse transcriptase buffer (50mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μm dNTPs) followed by addition of 200 U Superscript II reverse transcriptase and incubation at 42°C for 50 minutes. Five percent of the first strand reaction was used for each ensuing PCR reaction. Primer sequences were as follows: β-major forward, CTGACAGATGCTCTCTTGGG; β-major reverse, CACAACCCCAGAAACAGACA; β-H1 forward, AGTCCCCATGGAGTCAAAGA; β-H1 reverse, CTCAAGGAGACCTTTGCTCA; β-actin forward, GTGGGGCGCCCCAGGCACCA; and β-actin reverse, CTCCTTAATGTCACGCACGATTTC.

RESULTS

To determine whether BCR/ABL transformation would enable adult engraftment of primitive hematopoietic precursors derived from embryonic stem cells differentiated in vitro, we infected cells after 5 days of EB differentiation with a BCR/ABL-ires-GFP retrovirus, and cultured the cells on OP9 stroma in the presence of IL3, IL6 and SCF (Fig. 1). Cells infected with the empty vector (control) failed to proliferate under these conditions, but BCR/ABL-infected cultures generated abundant semi-adherent cells. After 8 days, these cells were plated in media previously defined to support growth of the most primitive hematopoietic element from EBs, the blast-colony-forming cell (BL-CFC; methylcellulose with VEGF, SCF and conditioned medium from the D4T cell line) (Kennedy et al., 1997). Primitive hematopoietic blast colonies resulted (Fig. 2A, left), which when replated gave rise to secondary primitive and definitive erythroid colonies as well as CFU-GEMM and macrophage/mast cell colonies (data not

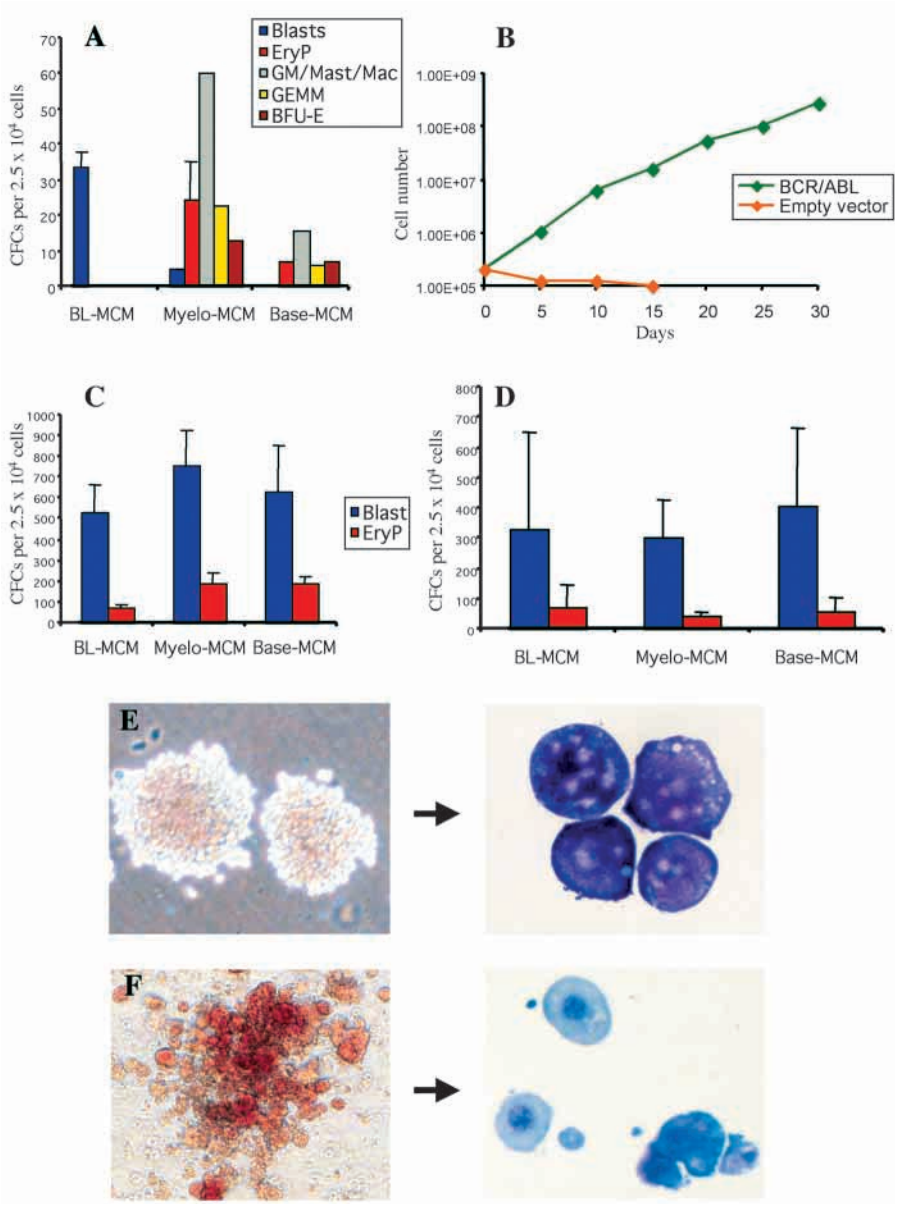

Fig. 2. Growth and hematopoietic potential of BCR/ABL-infected EB cells.
(A) Methylcellulose colony formation of BCR/ABL-infected EB cells after 8 days on OP9 cells under several culture conditions. BL-MCM represents MCM containing SCF, VEGF and D4T-conditioned medium; Myelo-MCM represents MCM containing IL3, IL6, SCF and Epo; Base-MCM represents MCM without growth factors. (B) Growth of a representative liquid culture of BCR/ABL (bulk) and empty vector-infected EB cells in the presence of IL3, IL6 and SCF after 8 days on OP9 cells.
(C) Colony formation of bulk cells after 30 days in liquid culture (without stroma). (D) Colony formation of clonal cells derived from BCR/ABL bulk cells. (E) Morphology of blast colonies (BL-CFC) (left) and corresponding cell populations (right). (F) Morphology of a primitive erythroid colony (EryP) (left) and corresponding nucleated primitive erythroid cells (right). Cytospins were stained with May-Grunwald-Giemsa. Experiments A, C and D were performed two, four and five times, respectively.

shown), demonstrating that this culture system maintains the earliest committed hematopoietic BL-CFCs (Fig. 2E). When plated in an alternative medium supplemented by myeloid cytokines, primitive erythroid and multi-lineage myeloid colonies were observed as well as a smaller number of blast colonies (Fig. 2A, middle), indicating that these cultures also contain large numbers of committed myeloid precursors. We also observed some multilineage colony formation in the absence of growth factors (Fig. 2A, right), presumably owing to complementation of cytokine signaling by BCR/ABL (Gishizky and Witte, 1992). Interestingly, this complementation was not observed for blast colony formation. Concomitantly, the cells were expanded in bulk liquid culture in the presence of IL3, IL6 and SCF (Fig. 2B) to obtain sufficient material for transplantation studies. In this regard, we injected mice only with non-adherent cells in order to exclude any residual non-hematopoietic cells that may have been present in the day 5 EBs.

Before injection of mice at day 30, cells were again assayed for CFC activity. In contrast to the first analyses, definitive erythroid and myeloid precursors were no longer present, being replaced by much larger numbers of blast-type and primitive erythroid colonies (Fig. 2C). These blast colonies are of a different character than the BL-CFC described above, as BCR/ABL complemented their growth in the absence of growth factors (Fig. 2C, right) and upon serial replating, only more blast and primitive erythroid colonies were formed (not shown). These results suggest that while BCR/ABL initially expands a variety of committed progenitors in bulk cultures of infected EB cells, a more primitive cell type with extensive self-renewal potential eventually dominates in liquid culture. These cells were examined for their differentiation potential following transplantation into mice.

Cells from bulk cultures engrafted substantially in hematopoietic organs of transplanted mice (Fig. 3A). Engraftment was evaluated between 5 and 9 weeks after transplantation. After this time, recipient mice succumbed to a myeloproliferative disorder characterized by splenomegaly and high white cell counts in the peripheral blood, with a particular expansion in the erythroid compartment. Flow cytometric analyses showed that GFP-positive cells had differentiated into Gr1/Mac1-positive myeloid cells, Ter119-positive erythroid cells and, to a lesser extent, B220-positive and CD4/CD8-positive lymphoid cells (Fig. 3C).

Rigorous proof that a culture system maintains hematopoietic stem cells requires clonal analyses to exclude the possibility that the cells that repopulate lymphoid and myeloid lineages derive from lineage-committed precursors that arose separately in culture. In order to determine whether the myeloid-lymphoid reconstitution we observed in recipient mice was derived from a single HSC, perhaps the blast colony-forming cell in our system, a vial of early passage frozen cells was thawed, plated in BL-MCM, and individual blast colonies were picked. These were then expanded briefly in liquid culture in the presence of IL3, SCF, IL6 and FL. The limited extent of lymphoid differentiation of the bulk cells and our previous failures to demonstrate lymphoid differentiation of BCR/ABL-infected EB cells in vivo (Peters et al., 2001) prompted us to include FL in an attempt to increase lymphoid potential.

When plated in MCM, clonal cells generated only blast-type and primitive erythroid colonies (Fig. 2D). Flow cytometric analysis revealed that the clones were predominantly positive for Kit, and negative for lineage-specific antigens (Fig. 4A), including the endothelial marker Flk1 (data not shown).

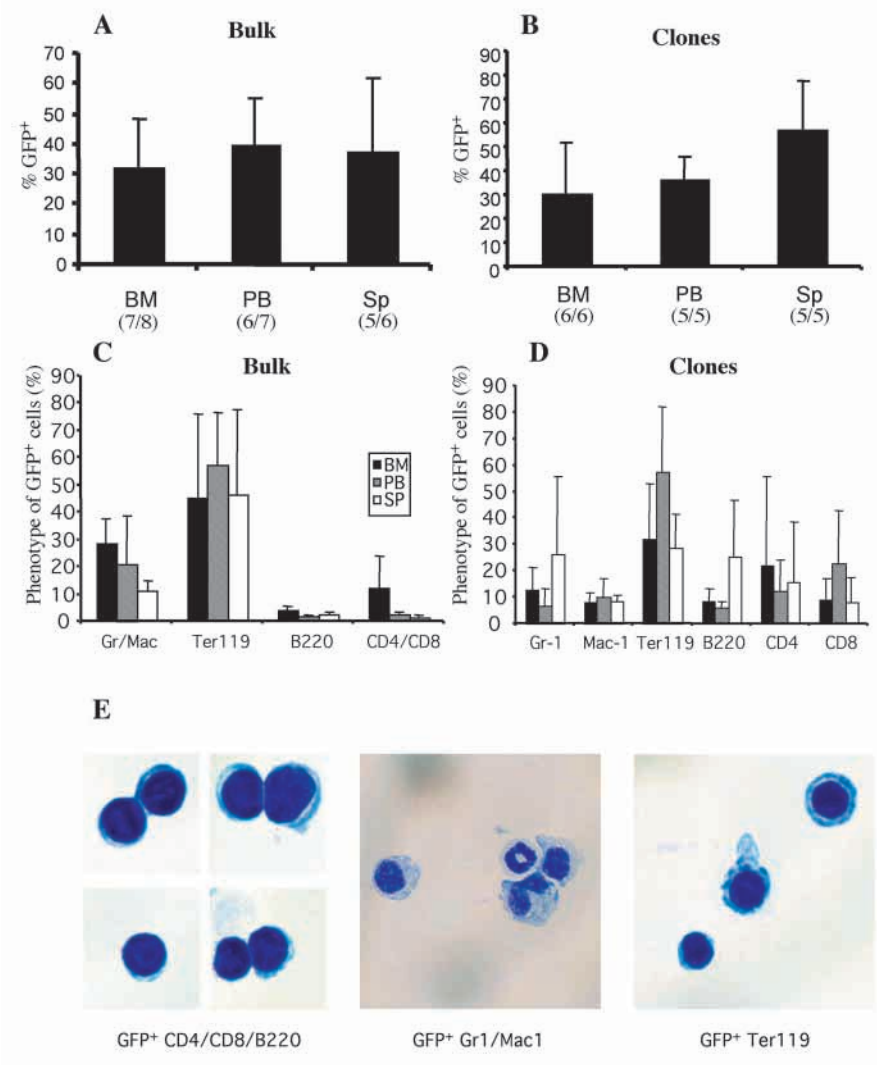

Fig. 3. Engraftment of adult mice. Frequency of GFP-positive cells in bone marrow (BM), peripheral blood (PB) and spleen (Sp) of mice injected with bulk cells (A) or clonal cells (B). (C) Antibody staining profile of GFP-positive cells for Gr1/Mac1, Ter119, B220 and CD4/CD8 in BM, PB and Sp of mice injected with bulk cells. (D) Antibody staining profile of GFP-positive cells for Gr1/Mac1, Ter119, B220 and CD4/CD8 in BM, PB and Sp of mice injected with clonal cells. The number of mice engrafted and the total number of mice injected are indicated, respectively, in parentheses. (E) GFP-positive cells from peripheral blood were stained with lineage-specific antibodies, sorted, centrifuged onto glass slides and stained with May-Grunwald-Giemsa.

Southern analyses of bulk cells as well as all derived subclones demonstrated that the bulk population was already substantially clonal by day 16 of expansion and that all the subclones derived from the same original infected cell (data not shown). This observation of clonal dominance is typical of our studies with cultures of BCR/ABL-infected EB cells (Peters et al., 2001), and our earlier experience with retroviral infections of murine bone marrow, which has been attributed to the infrequency of the HSC target as opposed to secondary mutational events (Daley et al., 1990; Li et al., 1999).

In vivo, all mice injected with clones demonstrated GFP-positive engraftment of bone marrow, peripheral blood and spleen (Fig. 3B), which was characterized by significant contributions to T and B lymphoid, myeloid and erythroid lineages (Fig. 3D). The superior lymphoid engraftment observed in this second round of transplantation seems to confirm the key role attributed to FL during lymphoid development (Borge et al., 1999; McKenna et al., 2000).

Given the embryonic origin of these cells, we examined three clones for expression of adult (β-major) and embryonic (β-H1) forms of β-globin. As expected, all the clones expressed both β-major and β-H1 globins (Fig. 4B). However, sorted cells (GFP-positive Ter119-positive) from spleen and bone marrow of two different mice expressed only adult β-major (Fig. 4B), suggesting that the clones can undergo the globin gene switching associated with definitive adult erythropoiesis.

GFP-positive cells were FACS purified from bone marrow of engrafted mice and assayed for colony formation in methylcellulose. Multi-lineage myeloid colonies were observed (Fig. 5), in addition to large numbers of BL-CFCs. This confirms that the ES-derived cells undergo differentiation into myeloid colony-forming cells in vivo. Furthermore, bone marrow from primary mice could be transplanted into secondary animals, generating a more aggressive secondary disease which also contained lymphoid and myeloid components (not shown). These data demonstrate that the cell we have targeted with BCR/ABL is capable of primitive erythropoiesis as well as definitive lymphoid-myeloid-erythroid hematopoiesis and of self-renewal in primary and secondary animals.

DISCUSSION

It is generally accepted that there are two sites of de novo generation of hematopoietic cells in the mammalian embryo, the yolk sac for the generation of primitive erythroblasts (Barker, 1968; Brotherton et al., 1979; Moore and Metcalf, 1970) and an intra-embryonic site, the floor of the dorsal aorta, that gives rise to definitive hematopoietic progenitors (Godin et al., 1995; Medvinsky and Dzierzak, 1996). However, the relative contributions of these two sites to definitive hematopoiesis in the adult remains controversial. Given that the yolk sac is the first place where blood cells are detectable during development, past authors have postulated that the definitive HSCs originate in this site, migrate to the fetal liver and initiate definitive hematopoiesis there (Moore and Metcalf, 1970). When transplanted into secondary animals via the placental circulation, yolk sac progenitors have the potential to contribute to hematopoiesis in the adult (Toles et al., 1989; Weissman et al., 1978). More recently, Palis and colleagues have shown the presence of a second wave of definitive erythroid and myeloid progenitors that arise in the yolk sac and are found soon thereafter in the bloodstream of the embryo proper, arguing that the yolk sac serves as the first source of definitive hematopoietic progenitors during embryonic development (Palis et al., 1999). The same authors demonstrate that definitive erythroid and macrophage progenitors arise from high proliferative potential colony-forming cells (HPP-CFC) in the yolk sac, which emerge at early somite stages (E8.25) (Palis et al., 2001). According to this hypothesis, a hematopoietic stem cell should exist at some point in development that has both embryonic erythroid potential and adult lymphoid-myeloid-erythroid potential. The alternate hypothesis, that two distinct types of hematopoietic stem cells arise independently, one with embryonic erythroid potential and the other with adult lymphoid-myeloid-erythroid potential is supported by reconstitution experiments using irradiated adult mice, which demonstrated that long-term repopulating HSCs (LTR-HSCs) are not present in the yolk sac before circulation and are first seen in the aorta-gonad-mesonephros (AGM) region followed by amplification in the fetal liver (Müller et al., 1994; Medvinsky and Dzierzak,

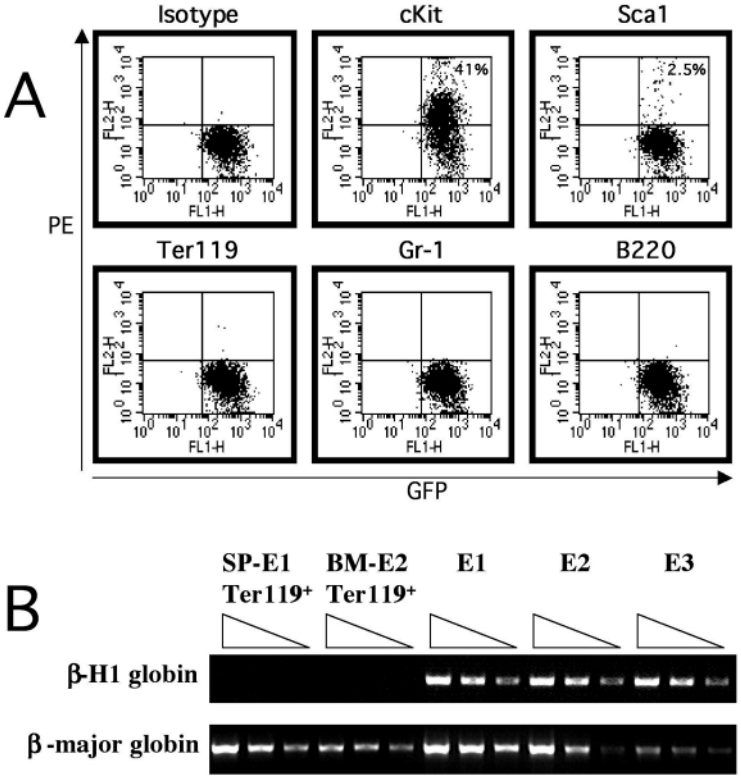

Fig. 4. Flow cytometric analysis and globin gene expression of clones. (A) FACS-staining profile of clonal cells maintained in liquid culture in vitro. (B) RT-PCR analysis for adult (β-major) and embryonic (β-H1) globin mRNA from clones derived from BCR/ABL bulk cells (E1, E2 and E3) and sorted cells (GFP-positive Ter119-positive) from spleen (Sp) and bone marrow (BM) of mice injected with clones E1 and E2, respectively. A threefold dilution series for each sample confirmed that the PCR was in the linear range.

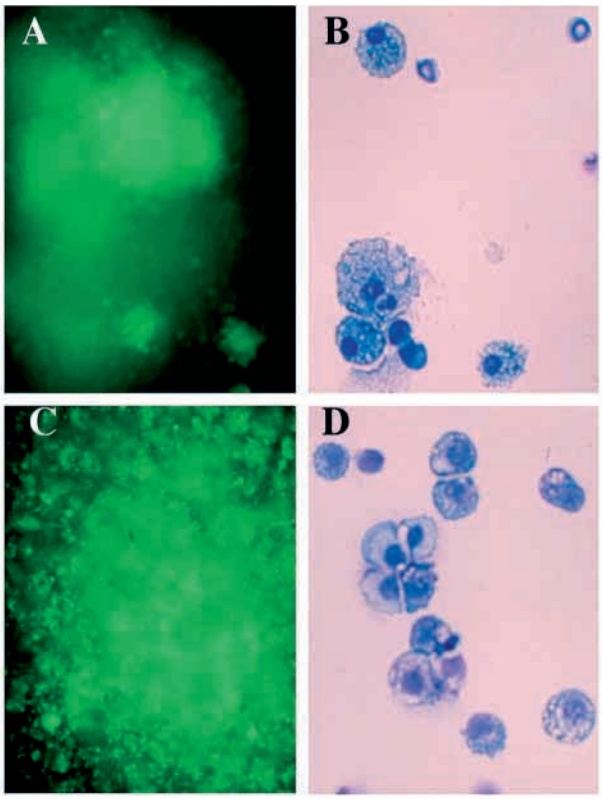

Fig. 5. Hematopoietic colonies derived from the bone marrow (BM) of engrafted mice. Bone marrow from a primary engrafted animal was harvested and plated in MCM supplemented with growth factors (IL3, IL6, SCF and Epo). After 7-10 days, GFP-positive colonies were observed by fluorescence microscopy (A,C). Colonies were plucked, disrupted, centrifuged onto a glass slide, and stained to reveal hematopoietic morphology (B,D). (A,B) CFU-GEMM; (C,D) CFU-GM.

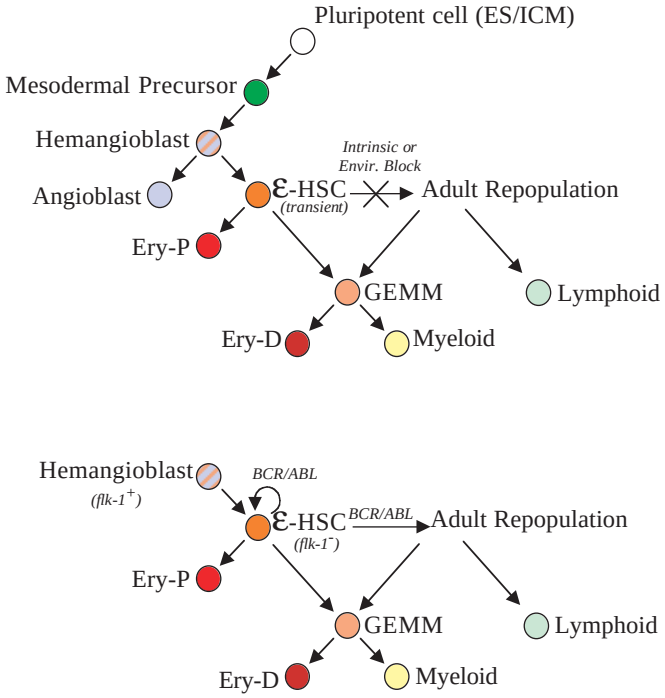

Fig. 6. Schematic representation of hematopoietic differentiation in embryos and embryoid bodies. Pluripotent cells (ES cells or cells from the inner cell mass, ICM) undergo mesodermal commitment and differentiation into the common precursor of hematopoietic and endothelial lineages (hemangioblast). We have demonstrated a cell with both primitive erythroid and definitive lymphoid-myeloid potential, properties predicted for the earliest embryonic hematopoietic stem cell: ε-HSC. The ε-HSC is deficient at repopulation of adult hosts owing to some combination of its transient nature, its homing properties and its response to the adult environment (top panel). BCR/ABL (lower panel) enables repopulation by altering one or more of these deficiencies, allowing both lymphoid and myeloid potential to be observed in engrafted animals.

1996). Our clonal analysis shows that during in vitro differentiation of ES cells as EBs, a cell fulfilling the requirements of the first hypothesis is generated.

EB-derived hematopoiesis mirrors that of the yolk sac in the types of CFC produced, the temporal sequence of their production and the lack of efficient lymphoid-myeloid repopulating activity when assayed in irradiated adult mice (Keller et al., 1993; Wiles and Keller, 1991). It has proven exceedingly difficult to demonstrate that EB-derived hematopoietic cells can efficiently repopulate adults under normal circumstances (Gutierrez-Ramos and Palacios, 1992; Hole et al., 1996; Müller and Dzierzak, 1993; Palacios et al., 1995; Potocnik et al., 1997). Moreover, rigorous proof that EB differentiation produces hematopoietic stem cells is lacking, because this requires clonal analyses to exclude the possibility that lymphoid and myeloid lineages derive from lineage-committed precursors that arose separately in culture. Without a marker to trace clonal relationships, engraftment alone does not prove that ES cells transit through a pluripotent hematopoietic stem cell stage during differentiation within EBs. The limitation in demonstrating extensive multilineage repopulation with cells of ES origin, under normal circumstances, has generated uncertainty as to whether a definitive HSC develops during EB differentiation. By enabling both clonal expansion and multi-lineage engraftment, BCR/ABL has allowed an HSC to be identified.

Recent studies have shown that CD34-positive/Kit-positive cells isolated from E9.0 yolk sacs are able to provide long-term repopulation when conditioned newborn mice instead of adults are used as recipients for transplantation (Yoder et al., 1997). Although these studies were carried out following the onset of circulation, which starts at E8.5, the fact that 37-fold more CD34-positive/Kit-positive cells were found in the yolk sac than in the embryo proper suggests that these LTR cells are of extra-embryonic origin. An AGM-derived cell line has recently been shown to induce adult-repopulating ability in cells derived from pre-circulation yolk sac, provided that the cells are co-cultured in vitro for a minimum of 4 days before transplantation (Matsuoka et al., 2001). In the absence of clonal analysis, the relationship of the adult-repopulating yolk sac cells demonstrated in these studies and the yolk sac primitive erythroid progenitors was unclear. However, taken together with our results, these data suggest that a yolk sac embryonic HSC exists, which has the potential to undergo both embryonic erythropoiesis as well as definitive lymphoid-myeloid hematopoiesis, and, furthermore, that repopulating

activity is dependent on the environment, with that of the irradiated adult host being unsuitable for the embryonic HSC. It may be that the embryonic HSC does not home to the bone marrow, or alternatively that the adult microenvironment fails to provide the appropriate signals required to maintain the embryonic HSC. BCR/ABL overcomes this deficiency perhaps by some combination of altering the homing properties of the donor cell, complementing a missing cytokine signal or blocking apoptosis of the donor cell, providing it with time to acclimatize to the adult environment, allowing us to assay the lymphoid-myeloid potential of this cell (Fig. 6). It may also be the case that the embryonic HSC is by nature a transient cell type, and BCR/ABL endows it with enhanced self-renewal properties. We are currently investigating these hypotheses.

An assumption implicit in our study is that BCR/ABL does not induce a novel or atypical mode of differentiation of EB-derived hematopoietic progenitors. While the relative proportions of myeloid cells are augmented by BCR/ABL in individuals with CML, there is no direct evidence that the multilineage differentiation potential of the HSC is fundamentally altered (Daley et al., 1990; Li et al., 1999). Indeed detection of the Philadelphia chromosome in all blood lineages of individuals with CML is widely regarded as direct evidence for the existence of the HSC in humans (Fialkow et al., 1977). We therefore believe that BCR/ABL is exposing the intrinsic developmental potential of the embryonic HSC. In support of this contention, we have preliminary evidence that conditional expression of STAT5, a downstream mediator of BCR/ABL signaling, also enables multi-lineage hematopoietic engraftment (unpublished).

Where does the cell we have demonstrated fit into the proposed hierarchy of embryonic hematopoiesis? Based on its primitive and definitive erythroid potential and the absence of Flk1 expression by flow cytometry, we place it below the hemangioblast, which has been characterized as Flk1 positive (Choi et al., 1998; Nishikawa et al., 1998) and at or above the primitive-definitive bipotent erythroid precursor (Kennedy et al., 1997) (Fig. 6). Its preference for the production of primitive erythroid CFC in addition to self-renewal in vitro reflects its similarity to the yolk sac HSC, whose primary function is the production of embryonic erythrocytes. Its ability to generate the lineages characteristic of adult hematopoiesis suggests that its developmental potential is fundamentally similar to the adult HSC.

The results of this clonal analysis demonstrate the existence of an ES-cell derived hematopoietic progenitor with primitive erythroid potential in vitro and lymphoid-myeloid potential in engrafted mice, and suggests that an embryonic cognate of the adult repopulating hematopoietic stem cell arises during EB differentiation. Harnessing the potential of such a cell to reconstitute hematopoiesis in adult animals is a crucial step to modeling hematopoietic cell transplantation using ES cell sources.

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REFERENCES

Barker, J. E. (1968). Development of the mouse hematopoietic system. *Dev. Biol.* **18**, 14-29.

Borge, O. J., Adolfsson, J. and Jacobsen, A. M. (1999). Lymphoid-restricted development from multipotent candidate murine stem cells: distinct and complimentary functions of the c-kit and flt3-ligands. *Blood* **94**, 3781-3790.

Brady, G., Barbara, M. and Iscove, N. (1990). Representative in vitro cDNA amplification from individual hemopoietic cells and colonies. *Methods Mol. Cell. Biol.* **2**, 17-25.

Brotherton, T., Chui, D., Gauldie, J. and Patterson, M. (1979). Hemoglobin ontogeny during normal mouse fetal development. *Proc. Natl. Acad. Sci. USA* **76**, 2853-2855.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

Daley, G. Q., Etten, R. A. V. and Baltimore, D. (1990). Induction of chronic myelogenous leukemia in mice by the P210$^{bcr/abl}$ gene of the Philadelphia chromosome. *Science* **247**, 824-830.

Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. and Bernstein, A. (1985). Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hematopoietic system of W/Wv mice. *Cell* **42**, 71-79.

Fialkow, P. J., Jacobsen, J. R. and Papayannopoulou, T. (1977). Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am. J. Med.* **63**, 125-130.

Gishizky, M. L. and Witte, O. N. (1992). Initiation of deregulated growth of multipotent progenitor cells by *bcr-abl* in vitro. *Science* **256**, 836-839.

Godin, I., Dieterlen-Lievre, F. and Cumano, A. (1995). Emergence of multipotent hematopoietic cells in the yolk sac and para-aortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc. Natl. Acad. Sci. USA* **92**, 773-777.

Grignani, F., Kinsella, T., Mencarelli, A., Valtieri, M., Riganelli, D., Grignani, F., Lanfrancone, L., Peschle, C., Nolan, G. P. and Pelicci, P. G. (1998). High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res.* **58**, 14-19.

Gutierrez-Ramos, J. C. and Palacios, R. (1992). *In vitro* differentiation of embryonic stem cells into lymphocyte precursors able to generate T and B lymphocytes *in vivo*. *Proc. Natl. Acad. Sci. USA* **89**, 9171-9175.

Hole, N., Graham, G. J., Menzel, U. and Ansell, J. D. (1996). A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonal stem cell differentiation *in vitro*. *Blood* **88**, 1266-1276.

Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol.* **13**, 473-486.

Keller, G., Paige, C., Gilboa, E. and Wagner, E. F. (1987). Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature* **318**, 149-154.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N. and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive hematopoiesis. *Nature* **386**, 488-493.

Lemischka, I. R., Raulet, D. H. and Mulligan, R. C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45**, 917-927.

Li, S., Ilaria, R. L., Jr, Million, R. P., Daley, G. Q. and Van Etten, R. A. (1999). The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J. Exp. Med.* **189**, 1399-1412.

Matsuoka, S., Tsuji, K., Hisakawa, H., Xu, M-j., Ebihara, Y., Ishii, T., Sugiyama, D., Manabe, A., Tanaka, R., Ikeda, Y. et al. (2001). Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells. *Blood* **98**, 6-12.

McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., Smedt, T. D., Maraskovsky, E., Maliszewski, C. R., Lynch, D. H., Smith, J., Pulendram, B. et al. (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95**, 3489-3497.

Medvinsky, A. and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.

Moore, M. A. S. and Metcalf, D. (1970). Ontogeny of the haematopoietic

system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. *Br. J. Haematol.* **18**, 279-296.

Müller, A. M. and Dzierzak, E. A. (1993). ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. *Development* **118**, 1343-1351.

Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301.

Nishikawa, S.-I., Nishikawa, S., Hirashima, M. and Matsuyoshi, N. (1998). Progressive lineage analysis by cell sorting and culture identifies $FLK1^{+}VE\text{-}cadherin^{+}$ cells at a diverging point of endothelial and hematopoietic lineages. *Development* **125**, 1747-1757.

Palacios, R., Golunski, E. and Samaridis, J. (1995). *In vitro* generation of hematopoietic stem cells from an embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* **92**, 7530-7534.

Palis, J., Chan, R. J., Koniski, A., Patel, R., Starr, M. and Yoder, M. C. (2001). Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proc. Natl. Acad. Sci. USA* **98**, 4528-4533.

Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-5084.

Peters, D. G., Klucher, K. M., Perlingeiro, R. C. R., Dessain, S. K., Koh, E. Y. and Daley, G. Q. (2001). Autocrine and paracrine effects of an ES-cell derived, BCR/ABL-transformed hematopoietic cell line that induces leukemia in mice. *Oncogene* **20**, 2636-2646.

Potocnik, A. J., Kohler, H. and Eichmann, K. (1997). Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94**, 10295-10300.

Robertson, E. J. (1987). Embryo-derived stem cell lines. In *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. E.J. Robertson), pp. 71-112. Oxford: IRL Press.

Schmitt, R. M., Bruyns, E. and Snodgrass, H. R. (1991). Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. *Genes & Development* **5**, 728-740.

Toles, J. F., Chui, D. H. K., Belbeck, L. W., Starr, E. and Barker, J. E. (1989). Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood. *Proc. Natl. Acad. Sci. USA* **86**, 7456-7459.

Weissman, I., Papaioannou, V. and Gardner, R. (1978). In *Differentiation of Normal and Deoplastic Hematopoietic Cells*, pp. 33-47. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Wiles, M. V. and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111**, 259-267.

Wu, A. M., Till, J. E., Siminovitch, L. and McCulloch, E. A. (1968). Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. *J. Exp. Med.* **127**, 455-464.

Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D. (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-344.