Expression of the LIM-homeobox gene *LH2* generates immortalized Steel factor-dependent multipotent hematopoietic precursors

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The genes controlling self-renewal and differentiation in the hematopoietic system are largely unknown. The LIM-homeobox genes are known to be important for asymmetric cell divisions and differentiation of specific cell types and organs. One member of this family, *LH2*, is expressed in fetal liver at the time of active hematopoiesis. Therefore, we have assessed the function of *LH2* during the formation and initial expansion of the hematopoietic system by differentiating *LH2*-transduced embryonic stem (ES) cells *in vitro*. This procedure generated multipotent hematopoietic precursor cell (HPC) lines that required Steel factor for growth. HPC lines have been maintained in an undifferentiated state in culture for >7 months. Other growth factors tested efficiently induce terminal differentiation of HPCs into various mature myeloid lineages. Steel factor is also required and acts synergistically with the other growth factors to generate multilineage colonies from the HPCs. These HPC lines express transcription factors that are consistent with an immature progenitor, and the pattern of cell surface marker expression is similar to that of early fetal multipotent hematopoietic progenitors. Collectively, these data suggest that the HPC lines represent an early fetal multipotent hematopoietic progenitor, and suggest a role for *LH2* in the control of cell fate decision and/or proliferation in the hematopoietic system.
Keywords: ES cells/LIM-homeobox gene/multipotent hematopoietic precursor/self-renewal versus differentiation/Steel factor

Introduction

The hematopoietic system is sustained throughout life by a small number of hematopoietic stem cells (HSCs). HSCs are distinct from other hematopoietic precursors in that they have the unique capacity to generate progeny of all hematopoietic lineages as well as the potential to generate more stem cells, a characteristic often referred to as self-renewal (Harrison *et al*., 1988; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990). While these characteristics of HSCs are central to the maintenance of a normal hematopoietic system, the underlying mechanisms which control differentiation and self-renewal are poorly understood because stem cells are extremely rare and difficult to access experimentally in normal hematopoietic tissues.

In contrast to the adult marrow, a substantial increase in the number of HSCs occurs during embryonic development (Ikuta and Weissman, 1992; Sánchez *et al*., 1996). Therefore, elucidation of the mechanisms responsible for the expansion of the hematopoietic system during embryonic development might offer insights into the mechanisms of self-renewal in the adult system. In the mouse, the first sign of hematopoiesis is the appearance of blood islands on the yolk sac at embryonic day 7.5 (e7.5) (Metcalf and Moore, 1971; Russel, 1979). The yolk sac is the major hematopoietic organ until e11–12 when the fetal liver supersedes the yolk sac as the major hematopoietic organ. After birth hematopoiesis declines in the liver and the bone marrow becomes the prevalent site for hematopoiesis (Metcalf and Moore, 1971; Russel, 1979). HSCs have different properties depending on the anatomical site and developmental stage of the mouse. For instance, early HSCs generate primitive erythroid cells in the yolk sac and as hematopoiesis shifts to the fetal liver this lineage disappears and definitive erythroid cells emerge (Barker, 1968; Brotherton *et al*., 1979). Similarly, HSCs able to long-term repopulate newborn mice are detected in the yolk sac at e9 and HSCs able to long-term repopulate adult mice are detected within the embryo at e10–11 (Müller *et al*., 1994; Yoder *et al*., 1997b). A substantial increase in the number of HSCs occurs subsequently in the fetal liver (Ikuta and Weissman, 1992; Sánchez *et al*., 1996), indicating that the microenvironment in the developing embryo promotes self-renewal of HSCs.

Mice carrying null-mutations in cell-surface receptors and their respective ligands have been informative in deducing cellular interactions and signalling molecules which control early hematopoietic development. Mice deficient in the receptor tyrosine kinase Flk-1 are unable to generate both hematopoietic cells and endothelial cells and mice deficient for its ligand, vascular endothelial growth factor (VEGF), display a similar but less severe phenotype (Carmeliet *et al*., 1996; Ferrara *et al*., 1996; Shalaby *et al*., 1997). Steel factor (also called stem cell factor, mast cell growth factor or c-kit ligand) and its receptor c-kit are essential for hematopoietic development in fetal liver and mice lacking functional c-kit (*W* mutants) or Steel factor (*Sl* mutants) die of a severe anemia *in utero* (Russel, 1979; Nocka *et al*., 1990). In contrast to the Flk-1/VEGF interaction, the c-kit/Steel factor interaction is not required for the formation of the system in the yolk sac but plays a critical role in the establishment or expansion of hematopoietic cells in the fetal liver (Nocka *et al*., 1989; Ogawa *et al*., 1993). Although the c-kit receptor is expressed on HSCs at all developmental stages, e.g. on HSCs in yolk sac, AGM (aorta, gonad, mesonephros) region, fetal liver and adult bone marrow

(Ogawa *et al.*, 1991; Ikuta and Weissman, 1992; Sánchez *et al.*, 1996; Yoder *et al.*, 1997a), the precise role(s) of Steel factor with respect to self-renewal and differentiation is not clear.

Developmental decisions during hematopoiesis are largely controlled by transcription factors (Orkin, 1996). This is exemplified by the absence of blood formation in mice lacking the basic helix–loop–helix transcription factor SCL, and the inability to initiate fetal liver hematopoiesis in mice lacking the runt family transcription factor AML1 (Shivdasani *et al.*, 1995; Okuda *et al.*, 1996). Another class of transcription factors, homeodomain proteins, have been found to be expressed in hematopoietic cells (Kongsuwan *et al.*, 1988; Sauvageau *et al.*, 1994). Due to their importance in cellular differentiation and patterning during embryogenesis (McGinnis and Krumlauf, 1992), a role for homeodomain proteins in the regulation of the hematopoietic system has been suggested (Kongsuwan *et al.*, 1988). Moreover, deregulated homeobox gene expression in hematopoietic cells is believed to contribute to the development of leukemias (Lawrence and Largman, 1992), immortalization of different precursor populations (Perkins and Cory, 1993; Hawley *et al.*, 1994a) and selective expansion of primitive hematopoietic progenitors (Sauvageau *et al.*, 1995; Helgason *et al.*, 1996). The physiological relevance of these findings is unclear, since most mouse strains with the respective homeobox gene inactivated show no, or subtle, hematopoietic-specific phenotypes (Brandon *et al.*, 1995a,b,c; Stein *et al.*, 1996). One candidate homeobox gene, *LH2* (*Lhx2*) may be particularly important for hematopoietic development since it is expressed in the fetal liver during active hematopoiesis (Xu *et al.*, 1993 and our unpublished observation), and *LH2*-homozygous mutant mice die *in utero* at e15–17 due to a severe anemia (Porter *et al.*, 1997). Similar to the *W* and *Sl* mutant mice, the anemia is the result of impaired definitive erythropoiesis in the fetal liver, suggesting that *LH2* plays a role in the initiation or expansion of the hematopoietic system in the fetal liver. *LH2* is a member of the LIM class of homeobox genes (also Lhx genes) (Way and Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Dawid *et al.*, 1995). Given the role of this class of homeobox genes in the control of asymmetric cell division, tissue specification and differentiation of specific cell types (Way and Chalfie, 1988; Freyd *et al.*, 1990; Cohen *et al.*, 1992; Taira *et al.*, 1992; Shawlot and Behringer, 1995; Pfaff *et al.*, 1996), *LH2* might play an important role in developmental decisions in the hematopoietic system.

Analyses of the developing hematopoietic system during embryonic development are hampered by limited access to tissues derived from the early embryo. An alternative system for analysing the formation and initial expansion of the hematopoietic system *in vitro* is provided by embryonic stem (ES) cells. When ES cells are induced to differentiate *in vitro* they form three dimensional structures referred to as embryoid bodies (EBs) (Keller, 1995). EBs contain hematopoietic precursor cells which respond to specific growth factors in clonal assays, and the development of precursors follows closely the *in vivo* process (Keller *et al.*, 1993). In addition, the ES cells can also be genetically manipulated without concern for *in utero*
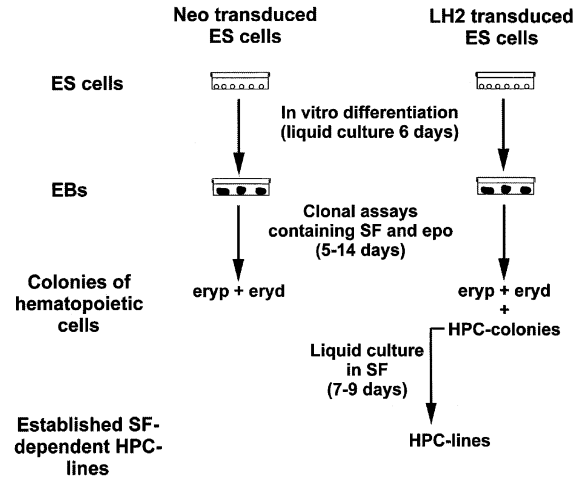
Generation of HPC-lines

Fig. 1. Schematic representation of the generation of HPC lines from *LH2*-transduced ES cells. SF, Steel factor; epo, erythropoietin.

lethality, making it possible to study a wide range of loss or gain-of-function mutations.

To examine a putative role for *LH2* during the formation and the initial expansion of the hematopoietic system, we expressed the mouse *LH2* cDNA in ES cells and subjected them to the differentiation assay *in vitro*. *LH2* expression in hematopoietic precursors leads to the generation of multipotent hematopoietic precursor cell (HPC) lines which express high levels of vector-derived *LH2* and require Steel factor for growth. The HPCs respond quantitatively to a variety of hematopoietic growth factors by terminal differentiation into different types of mature blood cells. These properties of HPC lines resemble both the self-renewal and the differentiation process occurring in the hematopoietic system *in vivo*. *LH2*-dependent generation of HPCs during *in vitro* differentiation of ES cells is consistent with an important role for this transcription factor in hematopoietic self-renewal or differentiation or both. Moreover, the ability to manipulate self-renewal and differentiation of HPCs in culture should provide a useful model for understanding how these processes are regulated *in vivo*.

Results

Retroviral infection of ES cells and LH2-expression analysis within EBs

To examine the role of *LH2* in the developing hematopoietic system, the mouse *LH2* cDNA was inserted into the murine stem cell virus (MSCV) retroviral vector and virions containing either the MSCV (Neo control) or the MSCV-*LH2* (*LH2*) construct were used to infect ES cells. Sublines and polyclonal populations from the control and the *LH2*-transduced ES cells were expanded in media containing leukemia inhibitory factor (LIF). EBs were generated by *in vitro* differentiation of the respective ES cell populations (Figure 1). No obvious differences in morphology or growth were observed between the various undifferentiated ES cells or EBs. *LH2* expression was not detected in EBs generated from control-transduced ES cells as determined by *in situ* hybridization (Figure 2A).
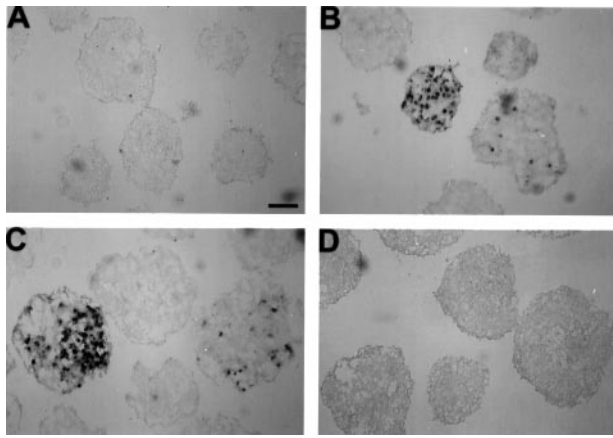
Fig. 2. Analysis of *LH2* expression by *in situ* hybridizations of day 6 EBs using an antisense (**A–C**) and sense control (**D**) probes for the *LH2* gene. EBs were generated from control ES cells (A), polyclonal MSCV-*LH2* ES cells (B), and MSCV-*LH2* subline #7 ES cells (C and D). Scale bar indicates 100 μm.

Approximately 5% of EBs generated from the polyclonal *LH2*-transduced ES cells and 0–30% of EBs generated from the ES cell sublines contained *LH2*$^+$ cells (data not shown). In individual EBs where *LH2* expression was detected, 1–50% of the cells were *LH2*$^+$ and they appeared to be randomly distributed within the EBs, independent of whether the EBs were generated from the polyclonal population or different sublines of *LH2*-transduced ES cells (Figure 2B and C).

EBs containing LH2$^+$ cells form unique hematopoietic progenitors

Considering the similarity between the *LH2*$^{-/-}$, *W* and *Sl* mutant phenotypes, we analysed the EBs for the presence of precursors that respond to Steel factor and erythropoietin (Epo) in progenitor (clonal) assays (Figure 1). The precursors which respond to Steel factor/Epo generate colonies containing either primitive erythroid (eryp) cells (Figure 3A and C) or definitive erythroid (eryd) cells (Figure 3A and B). The frequency of eryp precursors in EBs generated from control and *LH2*-transduced ES cells ranged between 1225–4419 per 10^5 EB cells (Table I). The frequency of eryd precursors in EBs generated from control or *LH2*-transduced ES cells ranged between 4–26 per 10^5 EB cells (Table I). In addition, a distinct type of colony containing mostly non-red cells appeared upon replating of *LH2*-expressing EBs (Figure 3D). The majority of cells within these colonies showed an immature blast-like morphology with large nuclei and a small rim of cytoplasm (Figure 3E), and will hereafter be referred to as HPC colonies (see below and Figure 1). The frequency of the precursor giving rise to HPC colonies was 0.5–1 per 10^5 EB cells in the polyclonal population of *LH2*-transduced ES cells, and 10- to 20-fold higher in some of the sublines (Table I). HPC colonies could not be generated from EBs derived from one *LH2*-transduced ES subline (#6) which did not contain *LH2*$^+$ cells (Table I, data not shown). There is no obvious correlation between the frequency of HPC colonies versus the frequency of eryp or eryd colonies, respectively (Table I). The HPC colonies continued to increase in size after 2–3 weeks of
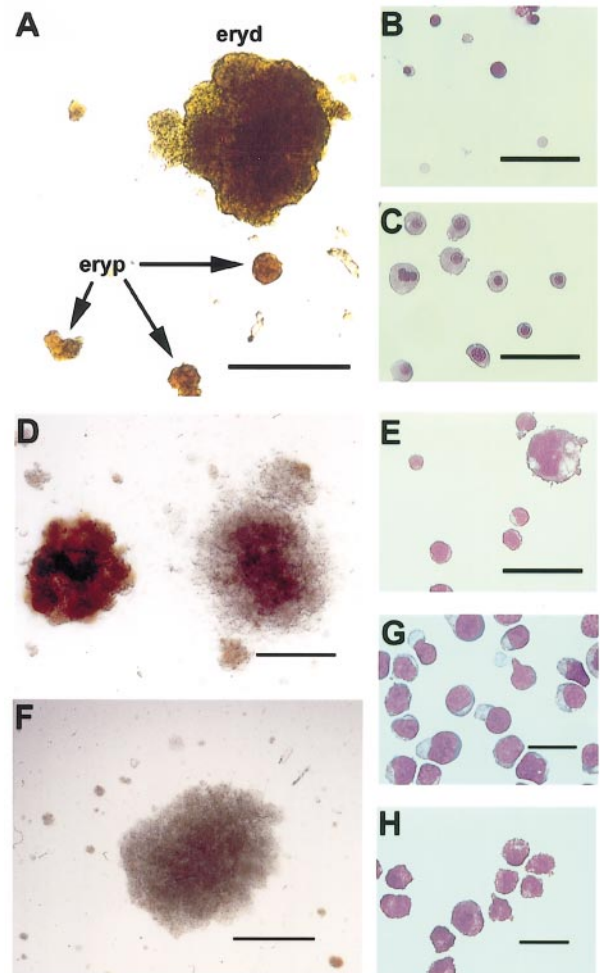

Fig. 3. *LH2*-expressing EBs give rise to unique HPC colonies. Two types of colonies derived from control EB cells replated in Steel factor and erythropoietin (**A**). The small tight colonies consist of primitive erythroid (eryp) cells and the large irregular shaped colony consists of definitive erythroid (eryd) cells. (**B**) and (**C**) show the cell morphology by May-Grünwald Giemsa staining of cells within eryd colonies and eryp colonies, respectively. A distinct type of colony termed HPC colony formed when *LH2*-expressing EBs were replated in Steel factor and erythropoietin (**D** and **F**). (D) shows a HPC colony (right) which is 12 days old and is compared to an eryd colony (left), and (F) shows a 14 days old HPC colony. Note that (D) is a higher magnification as compared with (F). May-Grünwald Giemsa staining show the immature 'blast-like' morphology of cells within the HPC colony (**E**) and of an HPC line that has been expanded in liquid culture in the presence of Steel factor (**G**). May-Grünwald Giemsa staining of cells showing mast cell morphology derived from wild-type EBs after two weeks of culture in liquid culture in the presence of Steel factor (**H**). Scale bars: (A and D) 0.25 mm, (F) 0.5 mm, (B, C and E) 50 μm, (G and H) 20 μm.

culture and the fraction of red cells remained low (Figure 3F). A low number of megakaryocytic cells was also observed in the HPC colonies (Figure 3E). When cells from the HPC colonies were replated into secondary clonal assays with Steel factor/Epo, both HPC colonies and eryd colonies reappeared. Based on colony and cell morphology we were unable to detect eryp cells under these culture conditions. This procedure could be repeated several times, indicating that a subpopulation of hematopoietic precursor cells that had apparently lost the ability to generate

Table I. Frequency of precursors in day 6 EBs per 10^5 cells

ES cells used to generate EBs	Eryp[a]	Eryd[b]	HPC colony[c]
Polyclonal control	2636 ± 83	15 ± 2	0
Subline #2 control	1499 ± 99	4 ± 2	0
Subline #3 control	1989 ± 234	18 ± 6	0
Polyclonal MSCV-*LH2*	1225 ± 35	26 ± 1	1 ± 0.6
Subline #3 MSCV-*LH2*	1396 ± 174	4 ± 2	12 ± 2
Subline #6[d] MSCV-*LH2*	4419 ± 116	9 ± 2	0
Subline #7 MSCV-*LH2*	2656 ± 107	13 ± 5	7 ± 2
Subline #9 MSCV-*LH2*	3927 ± 149	7 ± 2	6 ± 2
Subline #19 MSCV-*LH2*	2310 ± 31	7 ± 1	10 ± 1

[a]Primitive erythroid colony.
[b]Definitive erythroid colony.
[c]The distinct type of colony described in Figure 3D and F.
[d]*LH2* expression could not be detected in EBs derived from this subline.

eryp cells was regenerating the HPC colonies. No cell proliferation was observed in semisolid cultures lacking growth factors.

Cells within the HPC colonies can be expanded and maintained as immature cells in Steel factor

To characterize further the growth requirements of the cells within the HPC colonies, cells from individual HPC colonies were transferred into liquid media containing Epo, Steel factor or IL-3. We tested IL-3 since it is an early acting factor that stimulates growth of many immortalized hematopoietic precursor cell lines (Ymer *et al.*, 1985; Spooncer *et al.*, 1986; Perkins *et al.*, 1990; Perkins and Cory, 1993; Hawley *et al.*, 1994a). Without factors all cells died within 3 weeks of culture. In the presence of Epo alone, the cells divided a few times and then differentiated into erythrocytes. With IL-3 no significant increase in cell number was observed over a 5-week period, but a few clusters of large adherent macrophage-like cells appeared. Since the adhesive cells did not proliferate for an additional 3-week period they were not studied further. In contrast, in the culture containing Steel factor an increase in the number of non-adherent cells was apparent after 7–9 days of culture. These cells expanded further and cells from single colonies have been maintained in culture for >7 months retaining their immature blast cell-like morphology (Figure 3G). Thus, of the growth factors tested, only Steel factor was sufficient to allow the establishment of cell lines. These Steel factor-dependent cell lines are referred to as HPC lines (see Figure 1). HPC lines have been established from all different sublines of *LH2*-transduced ES cells that generate HPC colonies upon differentiation. Therefore, the generation of HPC lines is due to *LH2* expression and not due to fortuitous activation (or inactivation) of endogenous genes by retroviral DNA integration. We have not been able to establish HPC lines from precursors present in Neo control EBs or *LH2*-transduced EBs which do not contain *LH2*+ cells, since these precursors consistently generate terminally differentiated mast cells within 2 weeks of culture in the presence of Steel factor (Figure 3H).

Next we wanted to determine whether any factor known to have an effect on early hematopoietic precursors could replace Steel factor in the maintenance of an established HPC line. We compared the growth of an HPC line when cultured in Steel factor, IL-3, Tpo or VEGF. We tested Tpo since it is an early acting factor for normal stem cells (Sitnicka *et al.*, 1996; Ramsfjell *et al.*, 1997), and VEGF since it promotes growth of a common precursor for primitive and definitive hematopoiesis (Kennedy *et al.*, 1997). Each culture was monitored regularly with respect to cell growth and types of cells present and one representative experiment is shown in Figure 4. An increase in the number of undifferentiated cells was only observed in the culture with Steel factor with a doubling-time of ~40–48 h (Figure 4A and B). A transient increase in the number of cells was evident in the cultures containing Tpo but this proliferation was accompanied by differentiation into megakaryocytes (Figure 4A and C). A slight and transient increase in the number of cells also occurred in the cultures containing IL-3 but this proliferation was accompanied by differentiation into neutrophils and macrophages (Figure 4A and D). The HPCs cultured with VEGF or without growth factors showed a >60% decrease of viable cells after 24 h. A lack of response to VEGF was confirmed in clonal assays where no VEGF-responsive cells could be detected per 10^6 HPCs (data not shown). In conclusion, the *LH2*-induced immortalized HPC lines depend on Steel factor for both growth and maintenance in an immature state, e.g. for self-renewal. Furthermore, the lack of response to VEGF distinguishes HPCs from the common precursor of primitive and definitive hematopoiesis defined recently (Kennedy *et al.*, 1997).

HPCs exhibit broad hematopoietic potential

To assess, qualitatively and quantitatively, the potential of individual HPCs to generate different hematopoietic lineages, one of the HPC lines was chosen for prolonged growth. This line was continuously analysed in progenitor assays containing a broad spectrum of factors [IL-3, thrombopoietin, granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF) and Epo] with or without Steel factor. Three different types of colonies appeared in the clonal assays of the HPC line. The first type was large and compact and contained hemoglobinized cells (Figure 5A and B). These colonies were multilineage colonies since they contained cells of several lineages, e.g. erythrocytes, megakaryocytes, macrophages and neutrophils (Figure 5C). A second type of colony resembled the first type but lacked hemoglobinized cells. This type of colony contained megakaryocytes in combination with another lineage, the most common being macrophages (Figure 5D). The third type of colony was considerably smaller, diffuse and contained a single lineage, usually macrophages (Figure 5E). A summary of independent clonal assays carried out over a 2-month period of the HPC line is displayed in Table II. Thirty-one percent of the colonies generated in the presence of Steel factor were multilineage colonies and the remaining 69% were bi- or single-lineage colonies (Table II). This ability to generate multilineage colonies persisted for at least 7 months of continuous culture. The relationship between the number of HPCs plated and the total number of colonies which developed is linear (Figure 5G), suggesting that all colonies are of a single cell origin. Under these experimental conditions we have not been
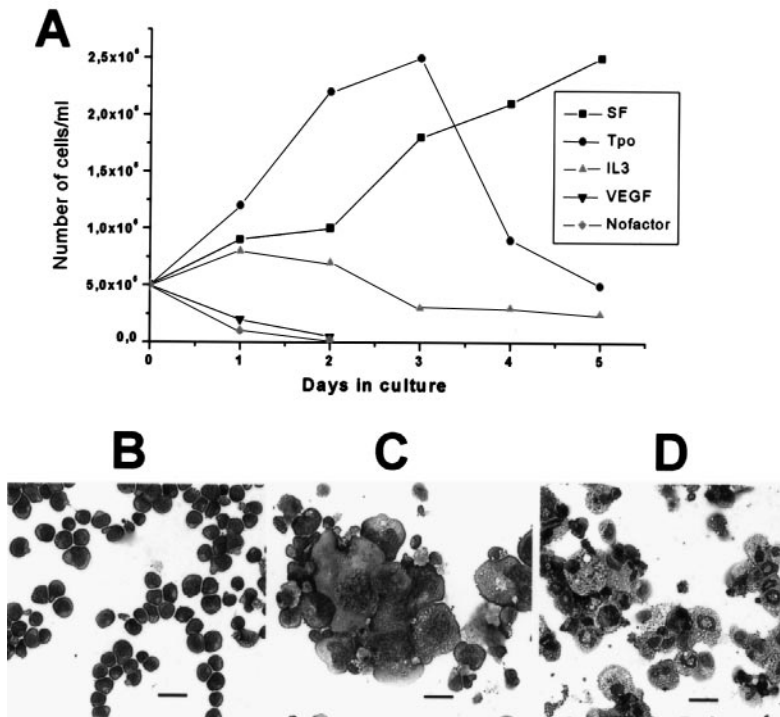
Fig. 4. Steel factor dependence for proliferation and maintenance of established HPC lines. Growth curves of an HPC line cultured in the indicated factors (**A**). After six days of culture the HPCs cultured in Steel factor (**B**), Tpo (**C**), and IL-3 (**D**) were stained by May-Grünwald Giemsa stain. Scale bars indicate 20 μm.

able to detect eryp cells within the multilineage colonies as determined by the expression of βH1-globin (data not shown). In clonal assays lacking Steel factor the plating efficiency decreased by 65% and only small bi-lineage and single-lineage colonies developed (Table II). Thus, Steel factor was required for optimal colony formation and for efficient generation of multilineage colonies.

When multilineage colonies generated from HPCs were replated into secondary clonal assays supplemented with Steel factor and factor mix, a majority of the multilineage colonies (23 of 30) generated small colonies containing mainly mast cells (Figure 5F). The remaining colonies (7 of 30) did not generate any secondary colonies. Thus, the HPCs can also differentiate into mast cells.

Under the culture conditions described, no additional myeloid cell types could be detected. In addition, when co-cultured with the S-17 stromal cell line and IL-7, conditions known to promote B-cell differentiation, no B-cell development could be detected based on B220 expression (data not shown). Thus, the HPC line appears to represent a myeloid restricted progenitor. It is not clear whether the lack of certain lineages reflects the true *in vitro* potential of the HPCs or if we have not yet been able to define the appropriate culture conditions.

LH2 is preferentially expressed in HPC colonies, HPC lines and their differentiated progeny

To compare the level of *LH2* expression between different stages during the generation of HPC lines, we analysed *LH2* expression in ES cells, EBs, HPC colonies, HPC lines and in the differentiated progeny of the HPC lines.

Initially, *LH2* expression in two independent HPC lines, the ES cell sublines they were originally isolated from and the corresponding EBs were compared by Northern blot analysis (Figure 6A, left panel). Both of the HPC lines expressed high levels of *LH2*. Furthermore, the size of transcript corresponds to an MSCV-derived transcript of 4.2 kb. The endogenous *LH2*-transcript is ~2.3 kb (Xu *et al.*, 1993; our unpublished observation) and is hardly, if at all, detected in any cell population analysed. This is consistent with the lack of *LH2* expression in control EBs as determined by *in situ* hybridization (Figure 2A). One of the ES cell sublines (Subline #3) did express detectable levels of *LH2* whereas *LH2* expression was not detected in the EBs. The latter result is in agreement with the observation that very few *LH2*$^+$ cells were detected by *in situ* hybridization analysis of these EBs (Figure 2C). Expression of the 4.2 kb transcript starting from the long terminal repeat (LTR) in the retroviral vector was confirmed by hybridizing the filters to a Neo-probe (Figure 6A, right panel). All populations analysed contained the MSCV-derived 1.3 kb Neo-transcript. These results confirm the silencing of LTR-driven expression in ES cells upon differentiation (Laker *et al.*, 1998). Semi-quantitative RT–PCR assays revealed that both cells within HPC colonies and the HPC line expressed similar levels of *LH2* (Figure 6B, HPC col., HPC LQ respectively). Cells within multilineage-containing colonies derived from the HPCs also expressed *LH2* (Figure 6B, HPC mix), as did mast cells derived from secondary replatings of the latter colonies (Figure 6B, HPC mast). No *LH2* mRNA was detected in cells derived from control eryd colonies (Figure
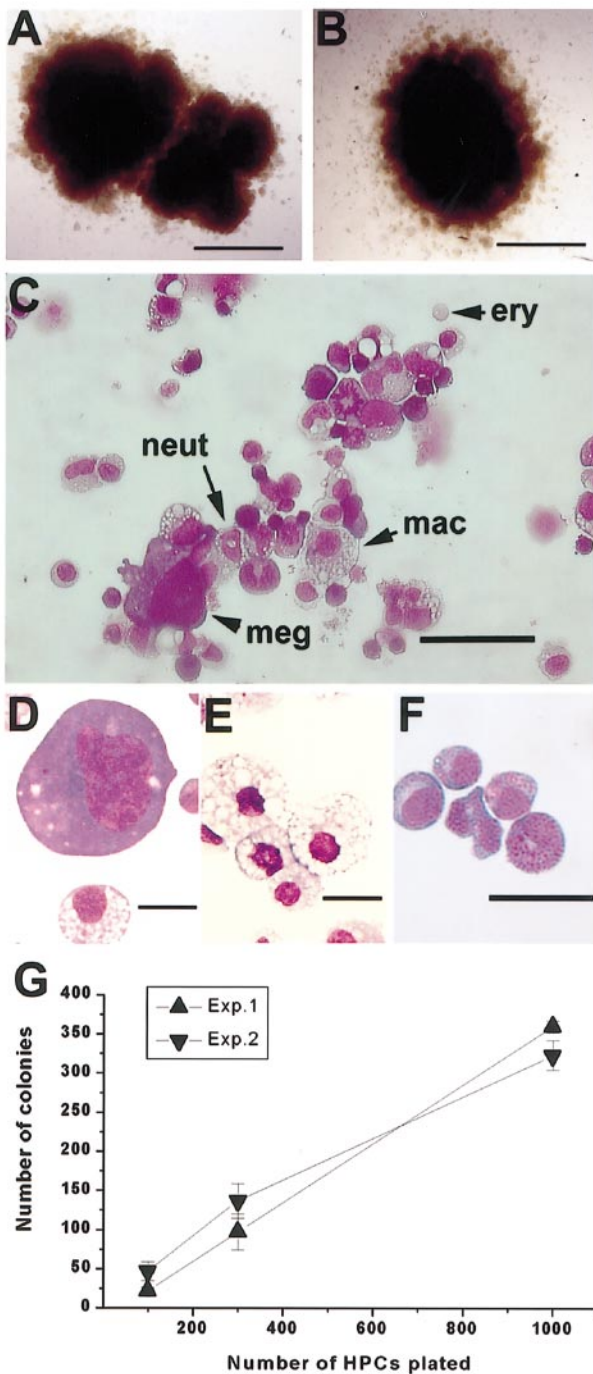
Fig. 5. Multilineage colonies generated from HPCs in precursor assays containing Steel factor + factor mix (IL-3/Tpo/GM-CSF/G-CSF/M-CSF/Epo) (**A** and **B**). May-Grünwald Giemsa staining of a representative multilineage colony is shown in (**C**). May-Grünwald Giemsa staining of the cells within representative bi-lineage colony (**D**), single-lineage (**E**) colony, and the mast cells generated when cells within multilineage colonies were replated in secondary progenitor assays containing Steel factor + factor mix (**F**). Relationship between the number of HPCs plated and the total number of colonies that develop in two independent experiments carried out at a three months interval (**G**). ery, anucleated definitive erythroid cell; neut, neutrophilic granulocyte; mac, macrophage; and meg, megakaryocyte. Scale bars: (A and B) 0.5 mm, (C) 50 µm, (D, E and F) 20 µm.

Table II. Colony formation per 100 HPCs

Factor combination[a]	Multilineage colonies[b]	Bi-lineage colonies[c]	Single-lineage colonies[d]	Total
+ SF	16 ± 7	25 ± 20	8 ± 4	51 ± 21
− SF	0	4 ± 3	14 ± 5	18 ± 4

SF, Steel factor; +SF represents the average of five independent, and − SF represents the average of three independent experiments.
[a]The factor combination is IL-3, Tpo, GM-CSF, M-CSF, G-CSF and Epo.
[b]Colonies containing erythroid cells plus at least two more lineages.
[c]Colonies containing two different lineages, usually megakaryocytes together with macrophages.
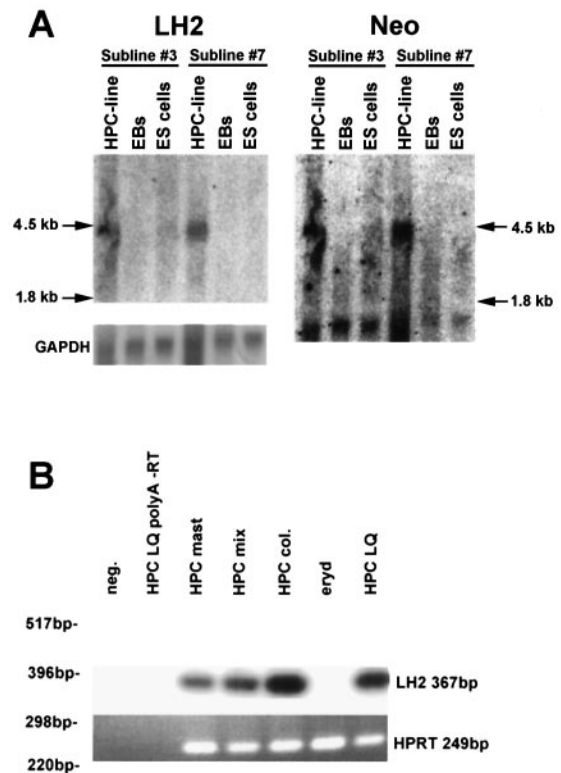[d]Small colonies containing one lineage, usually macrophages.

Fig. 6. Analysis of MSCV-derived transcripts in different cell populations by Northern blot and RT–PCR. (**A**) *LH2* expression was analysed by Northern blot analysis of total RNA (10 µg) from two independent sublines (#3 and #7) of *LH2*-transduced ES cells, the EBs after *in vitro* differentiation, and one established HPC line derived from each ES cell subline (LH2). Sizes indicate locations of the 28 and 18S rRNA. The same blot was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe followed by a second stripping and rehybridization to a Neo probe (Neo). (**B**) RT–PCR analysis was performed on cDNA generated from eryd colonies derived from control EB cells (eryd), HPC colonies derived from EBs generated from *LH2*-transduced ES cells (HPC col.), and HPCs that were continuously cultured in liquid culture (HPC LQ). *LH2* expression analyses on the differentiated progeny of HPCs was performed on either multilineage colonies (HPC mix) or mast cells (HPC mast). Negative controls are PCR carried out on a mock RNA preparation (neg.), and on poly(A)+ RNA preparation of continuously cultured HPCs where no cDNA synthesis have been carried out [HPC LQ poly(A)–RT].

6B, eryd). These results show that *LH2* was expressed during the establishment and the maintenance of the HPC lines, giving further support to the notion that the

generation and the maintenance of HPC lines is mainly due to *LH2* expression. *LH2* was also expressed in the differentiated progeny of the HPCs, indicating that *LH2*
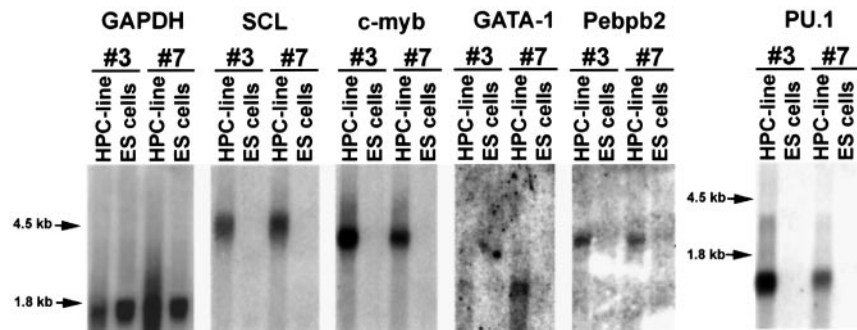
Fig. 7. Northern blot analysis of hematopoietic progenitor/stem cell associated transcription factors. Total RNA (10 μg) from two independent HPC lines and the ES cell sublines they were generated from was loaded onto gels and subsequently blotted and hybridized to probes of the indicated genes. Equal loading and integrity of the RNA was confirmed by hybridization to a GAPDH probe. Sizes indicate locations of the 28 and 18S rRNA.

expression *per se* does not restrict the ability of the HPCs to differentiate.

Transcription factors expressed by the HPCs are consistent with an immature hematopoietic progenitor

Expression of transcription factors functionally associated with early hematopoietic progenitor/stem cells was analysed in the HPCs by Northern blot analysis. The expression of five transcription factors in two independent HPC lines and the ES cell subline they were originally derived from is shown in Figure 7. Both HPC lines expressed *SCL*, *c-myb*, *GATA-1*, *Pebpb2/CBFb* (gene encoding a non-DNA binding transcriptional activator which interacts with the AML1 protein), and *PU.1*, whereas none of these transcription factors could be detected in ES cells. Thus, the HPCs express transcription factors associated with normal hematopoietic progenitor/stem cells. The respective homozygous mutant mice of each of these transcription factors have severe defects at the level of hematopoietic progenitor/stem cells (Mucenski *et al.*, 1991; Scott *et al.*, 1994; Weiss *et al.*, 1994; Shivdasani *et al.*, 1995; Sasaki *et al.*, 1996; Niki *et al.*, 1997), suggesting that they play an important role in these cell types.

Cell-surface markers expressed by the HPCs are consistent with early fetal hematopoietic progenitors

HPCs were examined for cell-surface markers using immunofluorescence flow cytometry. Similar results were obtained for two independent HPC lines (Figure 8). Approximately 50–80% of the HPCs expressed CD34 throughout the culture period. All HPCs expressed c-kit, CD44, CD29 (β1-integrin), and low to medium levels of major histocompatibility complex (MHC) class 1 antigen. A small but consistent fraction of the HPCs expresses AA4.1 (1–4%) whereas no significant expression of Sca-1 (Ly6A/E) could be detected. The HPCs did not express significant levels of Thy1.2 (T cell and adult precursor marker), CD16/32 (low affinity Fcγ receptors type III and II), TER119 (erythroid marker), Gr-1 (neutrophil marker), Mac-1 (monocyte/macrophage marker), CD4 (T-cell marker) CD3 (T-cell marker) and B220 (B-cell marker). The expression of c-kit and CD34 together with the lack of Sca-1 and Thy1.2 expression supports the notion that the HPCs are multipotent precursors of early fetal origin.
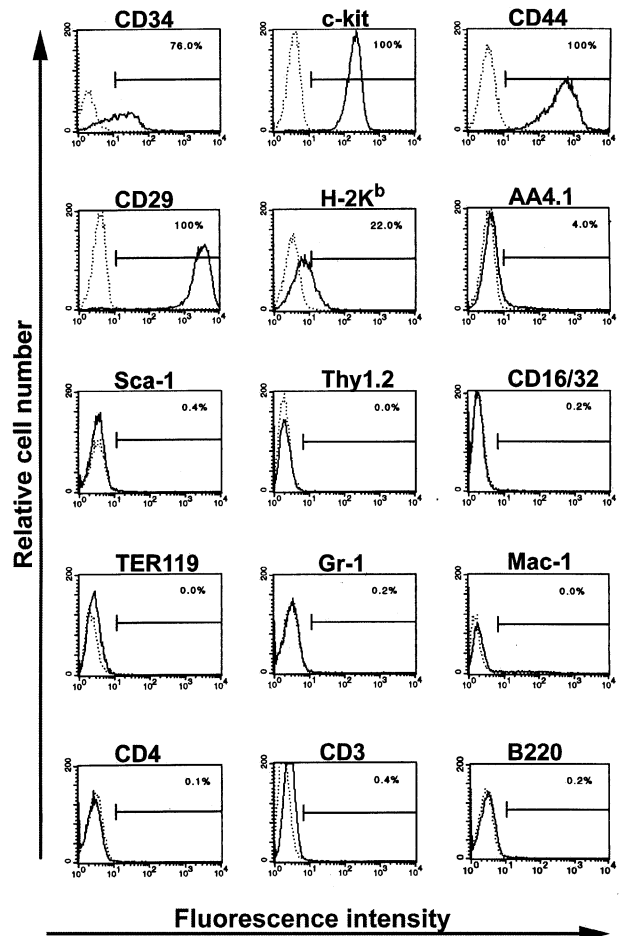

Fig. 8. FACS analysis of a representative HPC line cultured in Steel factor. Histograms displaying cell surface marker expression on the HPCs. Percentage of positive cells is indicated in each histogram. Stippled line histograms represent unstained cells and solid line histograms represent cells labelled with antibodies specific for the indicated cell surface markers.

HPCs induced to differentiate down-regulate stem-cell markers and up-regulate markers for committed cells

Next we determined if HPCs induced to differentiate into myeloid and erythroid cells also showed a corresponding change in the pattern of cell-surface marker expression. HPCs were cultured in liquid culture in the presence of Steel factor and factor mix. After 10 days of culture cell-
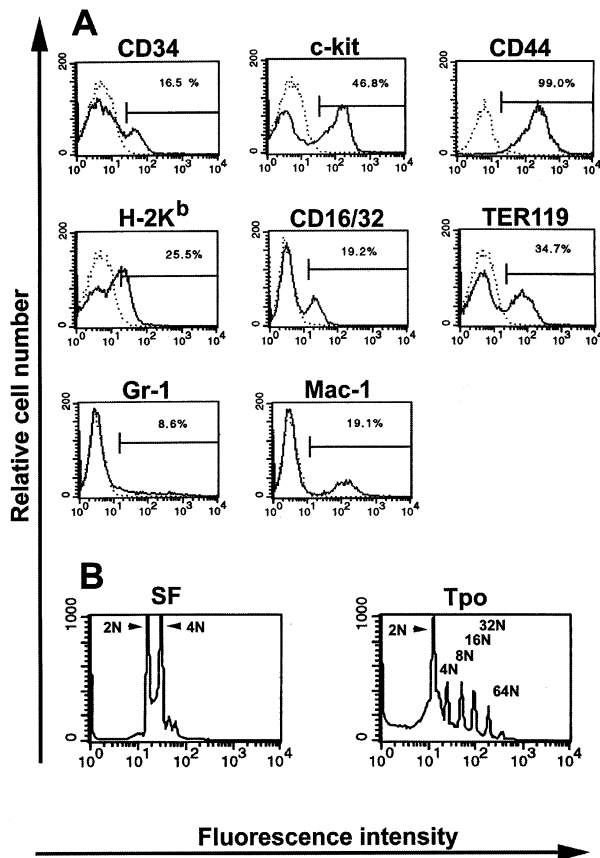
Fig. 9. FACS analysis of an HPC line induced to differentiate for 10 days in Steel factor and factor mix (**A**). See legend to Figure 8 for further details. Analysis of ploidy by propidium iodide staining of HPCs cultured in Steel factor (SF) and thrombopoietin (Tpo) for 6 days (**B**).

cells. A significant proportion of the cells cultured in Tpo for 4 days had a ploidy of 8, 16, 32 and 64N (Figure 9B, Tpo), whereas the undifferentiated HPCs showed the normal distribution of 2 and 4N ploidy (Figure 9B, SF). In conclusion, similar to normal progenitors, megakaryocyte endoreduplication does occur when the HPCs are cultured in Tpo.

Discussion

Expression of the LIM-homeobox gene *LH2* in immature hematopoietic precursors generated by *in vitro* differentiation of ES cells allowed the isolation of Steel factor-dependent multipotent HPC lines. *LH2* was expressed during the establishment and the maintenance of the HPC lines, e.g. in the HPC colonies and the continuously cultured HPC lines. Furthermore, HPC lines express high levels of vector-derived *LH2* and could be established from independent differentiation assays of the same and several different *LH2*-transduced ES sublines, strongly suggesting that the generation of HPC lines is directly due to *LH2* expression. When exposed to a broad spectrum of cytokines a high proportion of individual HPCs form multilineage colonies containing erythrocytes, megakaryocytes, macrophages, neutrophilic granulocytes and mast cells. The HPCs apparently represent a definitive fetal multipotent precursor distinct from the early embryonic primitive hematopoietic precursor recently described in the ES system (Kennedy *et al.*, 1997), since the HPCs neither respond to VEGF nor are capable of generating eryp cells. The level of *LH2* expression is similar in the HPC lines and their differentiated progeny, indicating that *LH2* expression *per se* does not block differentiation. HPC lines have been maintained in culture for >7 months without significant loss or change of their immature phenotype. Steel factor is important for the establishment and self-renewal of the HPCs, and it also acts as a synergistic factor for the terminal differentiation of the HPCs.

All HPC lines examined are c-kit$^+$ and express CD34 to a large extent (50–80%). Interestingly, c-kit and CD34 are thought to be markers for HSCs in both mice and humans at different ontogenetic stages (Berenson *et al.*, 1991; Ikuta and Weissman, 1992; Lu *et al.*, 1993; Krause *et al.*, 1994; Morel *et al.*, 1996; Sánchez *et al.*, 1996; Yoder *et al.*, 1997a). HPCs are also CD44$^+$, CD29$^+$ and a small but significant fraction expresses AA4.1 whereas they do not express detectable levels of Sca-1. The HPCs did not express significant levels of CD16/32 or lineage specific cell surface markers. This pattern of expression of cell surface markers (e.g. c-kit$^+$, CD34$^+$, CD44$^+$, Sca-1$^-$, Thy1.2$^-$, AA4.1$^{neg/low}$, Lin$^-$) resembles to a high degree that of multipotent hematopoietic precursors detected in yolk sac at e9 (Yoder *et al.*, 1997a), and in fetal liver at e11 (Sánchez *et al.*, 1996). Furthermore, the HPCs express relatively low levels of MHC class I as compared to adult bone marrow derived precursors, which is also similar to HSCs isolated from early mouse fetuses (Huang and Auerbach, 1993; Yoder *et al.*, 1997a). The cell surface phenotype of the HPCs resembles to a high degree that of early fetal-derived multipotent hematopoietic precursors, and transcription factors expressed by the HPCs are consistent with an immature hematopoietic progenitor.

surface marker expression was analysed by immuno-fluorescence flow cytometry. The result of a representative experiment is shown in Figure 9A. Compared with the undifferentiated HPCs (see Figure 8), a significant down-regulation of CD34 (16.5% versus 76%) and c-kit (46.8% versus 100%) has occurred. Furthermore, a concomitant up-regulation of the lineage markers TER119 (34.7% versus 0%), Gr-1 (8.6% versus 0.2%), and Mac-1 (19.1% versus 0%) was evident. In addition, the expression of CD16/32 was also up-regulated (19.2% versus 0%), whereas no significant difference in the expression of CD44 was observed (Figure 9). Two different populations with respect to the level of MHC class 1 expression appeared after differentiation: one with a slight but significant increase in the level of MHC class 1 molecules and one that was negative for expression. The latter population corresponds to late erythroid precursors and mature erythrocytes, based on low forward versus side scatter and TER119 expression (data not shown). Thus, HPCs induced to differentiate show a co-ordinated down-regulation of stem-cell markers and an up-regulation of relevant lineage markers.

During megakaryopoiesis the cells become polyploid (>4N). Megakaryocytic cells develop when HPCs are cultured in the presence of Tpo (Figure 4C). To confirm that megakaryocyte endoreduplication does occur in the presence of Tpo we analysed the DNA content in these

Similar to normal progenitors, HPCs induced to differentiate down-regulate stem cell markers (c-kit and CD34), up-regulate lineage specific markers (TER119, Mac-1 and Gr-1), and show megakaryocyte endoreduplication in response to a physiological stimuli, e.g. Tpo.

In recent years a number of multipotent hematopoietic cell lines have been established. The HPC lines differ from the previously described cell lines in numerous ways. In contrast to most previous cell lines which were derived from adult bone marrow (Greenberger *et al.*, 1983; Palacios and Steinmetz, 1985; Spooncer *et al.*, 1986; Tsai *et al.*, 1994; Wong *et al.*, 1994), the HPC lines are established from an embryonic source and the pattern of cell surface marker expression is compatible with a fetal multipotent precursor. In addition, the HPC lines are homogenous with respect to cell surface marker expression as compared to previously analysed cell lines (Palacios and Steinmetz, 1985; Ford *et al.*, 1992; Tsai *et al.*, 1994; Itoh *et al.*, 1996). Both the generation and maintenance of the HPC lines correlates to *LH2* expression whereas the mechanisms for the generation of most of the previously described cell lines are largely unknown (Greenberger *et al.*, 1983; Palacios and Steinmetz, 1985; Wyke *et al.*, 1986; Wong *et al.*, 1994; Itoh *et al.*, 1996; Palacios *et al.*, 1996). A majority of the previously established multipotent cell lines are dependent on IL-3 for continuous growth (Greenberger *et al.*, 1983; Palacios and Steinmetz, 1985; Spooncer *et al.*, 1986; Lee *et al.*, 1991), whereas the HPCs can neither be established nor maintained in IL-3. The dual role of Steel factor with respect to self-renewal and differentiation of HPCs distinguish them from most previously described cell lines. One of the previously described multipotent cell line is Steel factor-dependent (Tsai *et al.*, 1994). This line was established by expressing a dominant-negative mutant of retinoic acid receptor (RAR) in hematopoietic precursors. However, in contrast to the HPCs, the cell line described by Tsai *et al.* appears to have a specific block in the differentiation of myeloid cells in response to GM-CSF and IL-3.

Expression of *LH2* in immature hematopoietic precursors also reveals several distinct phenotypic properties in comparison with previous descriptions of homeobox gene expression in immature hematopoietic precursors. Cell lines could not be established in several of these studies (Sauvageau *et al.*, 1995, 1997; Helgason *et al.*, 1996; Thorsteinsdottir *et al.*, 1997). When cell lines could be established they were IL-3-dependent and appear to have a more mature myelo-monocytic precursor phenotype as compared to the HPCs (Perkins and Cory, 1993; Hawley *et al.*, 1994a). Deregulated expression of homeobox genes in the hematopoietic system correlates to the development of leukemia (Lawrence and Largman, 1992). A possible involvement of *LH2* in development of leukemia has not yet been proven. Although *LH2* is expressed in human chronic myelogenous leukemia (CML) cells its expression appears to be secondary to the BCR-ABL translocation (chromosomal translocation that creates a fusion gene between the Brakepoint Cluster Region gene and the c-Abl gene) and not directly involved in disease progression (Wu, 1995; Wu *et al.*, 1996; Wu and Minden, 1997). Finally, what also distinguishes *LH2* from the other homeobox genes discussed so far, is the lack of genetic evidence that any of the latter genes are involved in the development of the normal hematopoietic system (Brandon *et al.*, 1995a,b,c; Stein *et al.*, 1996).

The finding that Steel factor is required for the immortalization of the HPC lines is intriguing for several reasons. First, *LH2*, *Steel factor,* and *c-kit* are expressed in the fetal liver (Matsui *et al.*, 1990; Keshet *et al.*, 1991; Xu *et al.*, 1993 and our own unpublished results), at a developmental stage when HSCs are expanding (Ikuta and Weissman, 1992; Sánchez *et al.*, 1996). Secondly, the hematopoietic phenotypes of the corresponding mouse mutants ($LH2^{-/-}$, *Sl* and *W*) are almost identical. Primitive hematopoiesis is apparently normal but definitive hematopoiesis is severely impaired, resulting in a lethal anemia at e15–17 (Russel, 1979; Ogawa *et al.*, 1993; Porter *et al.*, 1997). Precursor assays of fetal liver cells from the respective mutant mice reveal a slight or no reduction of early erythroid precursors and a drastic reduction of late erythroid precursors, whereas the precursor of granulocytes/macrophages appears unaffected (Chui *et al.*, 1978; Russel, 1979; Nocka *et al.*, 1989; Porter *et al.*, 1997). In addition to these apparently specific erythroid defects in early fetal liver hematopoiesis, the entire precursor pool is reduced in adult bone marrow in the viable *W* and *Sl* mutant mice (McCulloch *et al.*, 1964; Russel, 1979), indicating a more general precursor defect. The relationship between *LH2*, *c-kit* and *Steel factor* presented herein, might indicate a similar precursor defect in the $LH2^{-/-}$ mice. However, the early embryonic lethality precludes such an analysis in adult bone marrow in the $LH2^{-/-}$ mice. Thirdly, most experiments suggest that Steel factor, at least *in vitro*, acts synergistically with other growth factors to induce terminal differentiation of immature hematopoietic precursors (Broxmeyer *et al.*, 1991; Metcalf and Nicola, 1991; Migliaccio *et al.*, 1991). This is similar to the observed synergistic effect of Steel factor on HPCs in the presence of factor mix in progenitor assays. Furthermore, the c-kit receptor is expressed on HSCs (Ogawa *et al.*, 1991; Ikuta and Weissman, 1992; Sánchez *et al.*, 1996; Yoder *et al.*, 1997a), and some studies have indicated that the c-kit–Steel factor interaction is required for long-term hematopoiesis following transplantation (Harrison and Astle, 1991; Fleischman, 1996; Miller *et al.*, 1997). These data suggest that the Steel factor–c-kit-signalling pathway might play a role in both differentiation and self-renewal of HSCs and expression of *LH2* in multipotent hematopoietic precursors might modulate these activities depending on the cellular environment. In addition to the hematopoietic phenotype the $LH2^{-/-}$ mice also have severe neuronal defects. *LH2* encodes a putative transcription factor and the hematopoietic-specific phenotype in $LH2^{-/-}$ mice is cell non-autonomous whereas the neuronal defect appear to be cell autonomous (Roberson *et al.*, 1994; Porter *et al.*, 1997). The identification of its target genes is essential to elucidate whether the *LH2*-induced immortalization described here is due to a cell autonomous or non-autonomous mechanism and this in turn might give clues for the role of *LH2* in the hematopoietic system *in vivo*. In this regard, HPCs offer a valuable tool to unravel the mechanisms of self-renewal versus differentiation in the normal hematopoietic system at the molecular, cellular and biochemical level.

Materials and methods

Cells
The 129/Sv derived, feeder-independent ES cell line CCE (Robertson *et al.*, 1986) (kindly provided by Dr G.Keller) was maintained in an undifferentiated state on gelatinized tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM), supplemented with leukemia inhibitory factor (LIF) (R&D Systems), 15% fetal calf serum (FCS) (Boehringer) and 1.5×10^{-4} M monothiolglycerol (MTG) (Sigma). Two days prior to the differentiation assay, the ES cells were passaged into Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL) supplemented as the DMEM. All cultures were maintained in a humidified environment with 5% CO_2 in air at 37°C.

Growth factors
Recombinant growth factors utilized were mouse Steel factor, GM-CSF, M-CSF, G-CSF, thrombopoietin, VEGF (R&D Systems), and human erythropoietin (Eprex, Cilag). IL-3 was obtained from medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector containing murine IL-3 (Karasuyama and Melchers, 1988). For all applications the growth factors were used at the following predetermined optimal concentrations; erythropoietin 4 U/ml, Steel factor 100 ng/ml, M-CSF 20 ng/ml, G-CSF 20 ng/ml, GM-CSF, 10 ng/ml, thrombopoietin 20 ng/ml, VEGF 5 µg/ml and IL-3 conditioned media was used at 1% final concentration.

Retrovirus production and infection of ES cells
The mouse *LH2* cDNA (gift from Dr R.Maurer) was cloned into the *Eco*RI site upstream of the pgk-neo cassette in the MSCV 2.1 vector (Hawley *et al.*, 1994b) (a gift from Dr R.Hawley). Virus was produced by transfecting BOSC-23 cells with the respective vector construction (Pear *et al.*, 1993). Briefly, just prior to transfection the media was supplemented with 25 µM chloroquinone (Sigma) to increase the virus titer. The MSCV vector and the MSCV-*LH2* vector were transiently transfected into BOSC-23 cells. Twenty-four h after transfection the medium was changed to 3 ml of ES medium. Supernatant containing virus was collected 48 h after transfection, filtered through a 0.45 µm filter and used directly or frozen in aliquots at –70°C. For infection of ES cells, 10^5 ES cells were plated on 10 cm Petri dishes (Falcon 3003). The following day the ES cells were incubated for 4–5 h with 4 ml ES medium supplemented with the virus containing supernatant (100–200 µl) and 4 µg/ml Polybrene (Hexadimethrine bromide, Sigma). Forty-eight h after infection the ES cells were passaged into ES medium supplemented with 0.5 mg/ml G418 (Gibco-BRL). The G418 selection was carried out until no live adherent cells were left in the mock-infected (Polybrene only) ES cells. ES cell sublines were isolated by gently scraping individual colonies with a pipette tip from Petri dishes. The ES cell colonies were transferred to 200 µl Trypsin–EDTA and incubated at room temperature for 5–10 min. After vigorous pipetting the cells were transferred to a well in a 24 well plate (Falcon) containing ES cell medium. All cells were expanded and frozen.

Differentiation of ES cells
ES cells were trypsinized and a single cell suspension was prepared and cultured in liquid culture in IMDM supplemented with 15% FCS (Integro Inc, the Netherlands), 4.5×10^{-4} M MTG, and 25 µg/ml ascorbic acid (Sigma). Generally 10^3 ES cells/ml were plated in a final volume of 5 ml in 60-mm suspension culture Petri dishes (Corning 25060-60). After six days of differentiation EBs were collected and resuspended in Trypsin–EDTA solution and incubated for 3 min. Two ml of FCS was added and the cells were gently passaged 2–3 times through a syringe with a 20-gauge needle. Ten ml of IMDM medium was added, the cells were spun down, resuspended in IMDM medium and 10^4 –2×10^5 cells/ dish were plated in the progenitor assays.

Progenitor (clonal) assays on EB cells
The progenitor assays were carried out in IMDM containing 1% methyl-cellulose (Fluka) and supplemented with L-glutamine, 300 µg/ml iron saturated transferrin (Boehringer), 5% protein-free hybridoma medium II (PFHM II, Gibco), 10% plasma-derived serum (PDS, Antech Inc. Texas), and Steel factor/Epo. The cells were plated in a final volume of 1.25 ml in 35-mm Petri dishes (Falcon 1008) in triplicates. Primitive erythroid colonies were scored at days 4–6 of incubation and definitive erythroid colonies were scored at days 8–11 of incubation. HPC colonies were scored at days 12–14. Colony identities were verified by May-Grünwald Giemsa (Merck) staining of cytospin preparation of individual colonies.

In situ hybridization
For analysis of sections, whole day 6 EBs were fixed in 4% para-formaldehyde in 0.1 M phosphate buffer (PB) at 4°C overnight. The EBs were subsequently transferred to 30% sucrose solution in PB and incubated for at least 6 h at 4°C. The EBs were mounted in O.C.T. Tissue Tek and sectioned at 10 µm and collected on superfrost slides (Fischer). A linearized cDNA clone of 1.3 kb containing the whole mouse *LH2* coding region was transcribed with T3 and T7 RNA polymerase and digoxigenin labelling mix to generate both the antisense and the sense strand of *LH2* (Boehringer). *In situ* hybridization was performed on sections as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Detection of labelled cells was carried out with anti-digoxigenin antibodies according to the DIG Nucleic Acid Detection Kit (Boehringer). EBs containing >5 brightly stained cells were scored as positive for *LH2* expression.

Northern blot analysis and RT–PCR assays
For all Northern blot analyses RNA was prepared using the RNAgents system for isolating total RNA (Promega). Ten µg of total RNA was separated on a 1% formaldehyde agarose gel and blotted onto a Zeta-Probe GT blotting membrane (Bio-Rad), and hybridized to radioactively labelled probes of the indicated genes according to standard procedures (Sambrook *et al.*, 1989). All cDNA probes used were of mouse origin. All membranes were analysed in a PhosphorImager (Molecular Dynamics). For RT–PCR analysis were 10–20 individual colonies derived from the different methylcellulose cultures pooled. Poly(A)$^+$ RNA was prepared from the pooled colonies by the Quick Prep Micro mRNA Purification Kit (Pharmacia) and first strand cDNA was synthesized by the First strand cDNA Synthesis Kit (Pharmacia). HPCs expanded in liquid culture were harvested and mRNA preparation and cDNA synthesis were carried out in a similar fashion. The amount of cDNA was estimated by PCR for the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) for each individual preparation. The amount of cDNA was adjusted so that an equal amount of the amplified HPRT product was obtained from all cDNA reactions from the different samples. The *LH2*-specific PCR was subsequently carried out with the same number of cycles on the same amount of cDNA as determined by the HPRT-specific PCR, and an equal volume of the HPRT and *LH2*-specific PCRs were loaded into the same lane on a 1.5% agarose gel. Since the genomic configuration of the expressed *LH2* gene is the same as for the cDNA, the poly(A)$^+$ RNA was analysed for genomic DNA contamination by carrying out a *LH2*-specific PCR with 35 cycles on five times the amount of poly(A)$^+$ RNA as used in the whole cDNA reaction. In order to confirm the *LH2*-specific PCR product, the gel was blotted onto a Nylon filter (Amersham) and hybridized to a radioactively labelled *LH2* gene. The following PCR primers were used; HPRT 5′ primer CACAGGACTAGAACACCTGC and 3′ primer GCTGGTG-AAAAGGACCTCT which generates a 249 bp PCR product. LH2 5′ primer AAAAGACAAAGCGCATGCGGG and 3′ primer CAGGCACA-GAAGTTAAGACTG which generates a 367 bp PCR product.

Establishment, maintenance and differentiation of the HPC lines
Individual colonies identified as HPC colonies were split into four different wells in a 24 well plate. Each well contained 1 ml of IMDM medium supplemented with 10% PDS or FCS, 1.5×10^{-4} M MTG and either no growth factor or Epo, IL-3, or Steel factor. Proliferating cell populations which exhibited an undifferentiated morphology by May-Grünwald Giemsa staining were expanded. HPC lines were routinely maintained at cell densities between 5×10^5 and 2×10^6 cells/ml in IMDM supplemented with 10% PDS or FCS, 1.5×10^{-4} M MTG, and Steel factor. Progenitor assays of the HPCs were carried out as described above but with the following cytokines: Tpo, IL-3, GM-CSF, G-CSF, M-CSF, and Epo with or without Steel factor. The different types of colonies were scored at day 12–16 of incubation. Colony identity was confirmed by May-Grünwald Giemsa staining of cytospin preparation of individual colonies. To ensure single cell origin of the colonies we examined the dishes for the presence of individual cells and cell doublets directly after plating. After counting >500 cells, we could detect ~1 doublet per 100 cells ($\pm$ 1) which is much lower than the frequency of multilineage colonies. Together with the linear relationship between cells plated and number of colonies obtained, and that the multilineage colonies develop when the cells are plated at such low density as 100 cells/dish (e.g. 100 cells/1.25 ml), strongly argues for a single cell origin of the multilineage colonies. HPCs induced to differentiate and analysed by flow cytometry were cultured as normal HPC lines but the media was supplemented with the factor mix.

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Flow cytometry

The monoclonal antibodies used in this study were direct conjugates with phycoerythrin (PE), fluorescein isothiocyanate (FITC), or biotin. The following antibodies were purchased from Pharmingen (San Diego, CA): PE-anti-CD4 (GK1.5), PE-anti-CD5 (Ly-1), PE-TER119, PE-anti-CD44 (IM7), FITC-Thy1.2 (53–2.1), FITC-anti-Mac-1 (M1/70), FITC-anti-CD3 (145–2C11), FITC-2.4G2 (low affinity Fcγ receptors type II and III), biotinylated and FITC-anti-Gr-1 (RB6-8C5), biotinylated anti-Sca-1 (E13-161.7), biotinylated anti-CD29 (anti-integrin β1 chain, clone 9EG7), biotinylated anti-H-2K^b (AF-88.5), biotinylated anti-CD34 (RAM34), biotinylated anti-B220 (RA3-6B2), and biotinylated anti-c-kit (2B6). The AA4.1 antibody (McKearn et al., 1985) was purified by protein G-coupled Sepharose (Pharmacia) from the hybridoma supernatant and subsequently biotinylated. The HPCs were incubated in a supernatant from the 2.4G2 hybridoma on ice for 15 min prior to all antibody labelling, except when the cells were labelled with FITC-2.4G2. The cells were incubated with specific antibodies on ice for 20 min, washed twice and subsequently incubated with PE-conjugated streptavidin (Southern Biotechnology). Labelled cells were washed twice before analysed in a FACScan (Becton Dickinson, San Jose, CA). All labelled antibodies were titrated on normal mouse bone marrow or spleen cells. For analysis of ploidy the cultured cells were fixed in 70% ethanol overnight, and subsequently washed and incubated for 30 min on ice in hypotonic citrate solution consisting of 0.1% sodium citrate, 50 µg/ml propidium iodide, 30 µg/ml DNase-free RNase and 0.1% Triton X-100. Stained cells were washed and analysed in the FL3 channel of a FACScan.

Acknowledgements

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