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Long-term culture of lymphohematopoietic stem cells

(stem cells/hematopoiesis/gene expression/transplantation/cell growth/differentiation)

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ABSTRACT **Pluripotent hematopoietic stem cells (PHSCs) show self-renewal and give rise to all blood cell types. The extremely low number of these cells in primary hematopoietic organs and the lack of culture systems that support proliferation of undifferentiated PHSCs have precluded the study of both the biology of these cells and their clinical application. We describe here cell lines and clones derived from PHSCs that were established from hematopoietic cells from the fetal liver or bone marrow of normal and p53-deficient mice with a combination of four growth factors. Most cell lines were Sca-1$^+$, c-Kit$^+$, PgP-1$^+$, HSA$^+$, and Lin$^-$ (B-220-, Joro 75-, 8C5-, F4/80-, CD4-, CD8-, CD3-, IgM-, and TER 119-negative) and expressed three new surface markers: Joro 177, Joro 184, and Joro 96. They did not synthesize RNA transcripts for several genes expressed at early stages of lymphocyte and myeloid/erythroid cell development. The clones were able to generate lymphoid, myeloid, and erythroid hematopoietic cells and to reconstitute the hematopoietic system of irradiated mice for a long time. The availability of lymphohematopoietic stem cell lines should facilitate the analysis of the molecular mechanisms that control self-renewal and differentiation and the development of efficient protocols for somatic gene therapy.**

Pluripotent hematopoietic stem cells (PHSCs) are unique in that they give rise to both new stem cells [self-renewal, manifested in the repopulation of the hematopoietic system for long time (>5 months)] and all blood cell types (1, 2). Mouse bone marrow (BM) and fetal liver (FL) cells with properties of PHSCs were found to express Sca-1 surface marker and c-Kit receptor and to lack most lineage-restricted hematopoietic surface markers (3, 4). In humans, putative PHSCs were found to be contained in the CD34$^+$33$^-$38$^-$DR$^-$ or DR$^+$ marrow populations (5, 6). The extremely low number of these cells in primary hematopoietic organs and the lack of culture systems that support proliferation of undifferentiated PHSCs have precluded the study of both the biology of these cells and their clinical application. PHSCs can be enriched (reviewed in refs. 7 and 8) but the numbers obtained are very low. Purified PHSCs appear to still be heterogeneous in terms of size, cell cycle status, and rhodamine 123 staining (2, 9, 10). The only way to generate homogeneous PHSCs in large numbers is to establish culture conditions that support proliferation without differentiation. We describe here clones that were established from hematopoietic cells from the FL or BM of normal and p53-deficient mice with a combination of four growth factors. The clones share the phenotypic characteristics and the two functions of PHSC with the freshly isolated populations enriched for PHSCs; namely, these two functions are the ability to provide long-term repopulation of the hematopoietic system and the capacity of giving rise to lymphoid, myeloid, and erythroid cell lineages.

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MATERIALS AND METHODS

Isolation of Lin$^-$ Mononuclear Cells. BM mononuclear cells from 3- to 6-week old mice and day 12 to 13 FL mononuclear cells free of erythrocytes from p53-deficient homozygous (ref. 11; purchased from GenPharm, Mountain View, CA) or normal C57BL/6 mice (bred and maintained in the animal barrier facility of our department) were prepared as described (12, 13). Lin$^-$ BM and FL cells were obtained by removing CD4$^+$, CD8$^+$, CD3$^+$, IgM$^+$, Joro 75$^+$, B-220$^+$, F4/80$^+$, 8C5$^+$, and TER 119$^+$ cells by a combination of negative selection using magnetic beads and positive selection by fluorescence-activated cell sorter (FACS) as described in detail (12, 14). A proportion of the cell sorter-selected cells were used for reanalysis and showed that >99.5 of the cells were negative for all the surface markers indicated above and they will be referred to as Lin$^-$ cells. The FL or BM Lin$^-$ cells were washed and resuspended in culture medium [Iscove's modified Dulbecco's medium supplemented with 7.5–10% heat-inactivated defined fetal bovine serum (cat. no. A-1111, HyClone), 2-mercaptoethanol (5×10^5 M), 2 mM L-glutamine, and gentamycin (50 μg/ml)].

Cytokines. Recombinant interleukin (IL)-1β was a gift from Hoffmann-LaRoche (Basel, Switzerland). Supernatants were from X63Ag8 or J558/L myeloma cells transfected with cDNAs coding for mouse (m)IL-2, mIL-3, mIL-4, mIL-5, human (h)IL-6, (15) mIL-7 (16), or hIL-10 [plasmid pHIL-10-550p, gift of P. Dellabona (H. S. Raffaele, Milan)]. Cos-1 cells were transfected with cDNA coding for steel factor (13) and cDNA coding for human leukemia-inhibiting factor (hLIF) (plasmid pC10–6R, gift from A. Smith, Centre for Animal Genome Research, Edinburgh). cDNA coding for basic fibroblast growth factor (plasmid pbFGF) was a gift of G. Neufeld (Israel Institute of Technology, Haifa). mIL-9 was a gift of J. Van Snicke (University of Brussels, Belgium); B9-transfected cells producing IL-11 were provided by R. G. Hawley (University of Toronto). J558/L/FLT3-lig/5 transfectant cells producing recombinant mouse FLT3-lig were developed by subcloning FLT3/FLK2-mouse cDNA into the *Eco*RI–*Xho*I site of the pCDNA3 mammalian expression vector (X.X. and R.P., unpublished results), erythropoietin, macrophage colony-stimulating factor (CSF), granulocyte CSF, tumor necrosis factor α, transforming growth factor β, and IL-12 were purchased from R & D Systems, and mouse granulocyte/macrophage CSF was a gift from Biogen. The cell-free supernatants collected and filtered from 2- to 3-day confluent cultures of the FLS4.1 stromal cell line are referred to as F (13).

Establishment of Lin$^-$ FL and BM Cell Lines. Several cytokine mixtures (list is available upon request) were tested within a period of 17 months until the cytokine mixtures that support growth with little or no differentiation of Lin$^-$ cells

Abbreviations: PHSC, pluripotent hematopoetic stem cell; BM, bone marrow; FL, fetal liver; IL, interleukin; CSF, colony-stimulating factor; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex, TCR, T-cell receptor; SCID, severe combined immunodeficient; FACS, fluorescense-activated cell sorter; LIF, leukemia-inhibiting factor.

were found. The mixtures of LIF, steel factor, F, and either IL-3 or FLT3-lig were found to best support proliferation with no or little differentiation. These mixtures were then used to try to establish Lin^- cell lines. To this end, Lin^- cells (1–10 cells) were placed in round-bottomed microculture wells containing 100 μl of either 3LSF medium [culture medium supplemented with IL-3 (50–100 units), hLIF (500–1000 units), steel factor (100–300 units), and 10% F (final concentration, vol/vol)], or FLT3-ligLSF medium [culture medium supplemented with FLT3-lig (100–500 units), LIF, steel factor, and F at the concentrations indicated above]. The cultures were incubated at 37°C in a 7.5% CO_2/92.5% air atmosphere. Every 5–7 days the cultures were supplemented with 50–100 μl of freshly prepared 3LSF-medium (with prior removal of an approximately equal volume of old medium). Similar bulk cultures at higher cell densities (3×10^4 Lin^- cells per 0.5 ml of 3LSF medium) were also grown in 24-well Linbro tissue culture plates. Between 13% and 26% of the microculture wells were positive for cell growth (assessed by visual inspection with an inverted microscope) after 2–5 weeks of culture in the three separate experiments we carried out. A total of 60 of these cultures were transferred to 24-well Linbro plates containing a final volume of 0.5 ml of 3LSF medium. As the cultures reached confluence, each well was split into three new Linbro wells in 0.5–1 ml of 3LSF-medium. Forty-nine of the original 60 lines showed continued growth in culture, could be transferred to tissue culture flasks, and were thus considered established cell lines. They were propagated in 3LSF medium at a density of $1–2 \times 10^5$ cells per ml by transferring them into fresh medium every 2–3 days. Aliquots of each line were frozen [14% (vol/vol) DMSO and 14% FCS in Iscove's modified Dulbecco's medium] by standard procedures at this time. The cell lines, called FL or BM (according to tissue of origin) $p53^-$, were phenotyped by FACS analysis using antibodies to several surface markers on different hematopoietic cell lineages. Twelve out of 49 cell lines studied comprised some (<25%) F4/80-, 8C5-, or TER 119-positive cells, and these cell lines were therefore discarded. A total of six $p53^-$ cell lines that exhibited an stable Lin^- phenotype, three from FL and three from BM, were first cloned by micromanipulation (12), and selected Lin^- clones were recloned by limiting dilution (0.1 cell per well) in 3LSF medium. The expanded clones were phenotyped and aliquots were frozen by standard procedures (12). Essentially the same protocol was used to establish Lin^- cell lines from FL of C57BL/6 normal mice. The Lin^- $p53^-$ cell lines were established in October–November 1993, and the Lin^- cell lines from normal C57BL/6 mice were established in April–May 1994. In general, these as well as other previously described hematopoietic cell lines established in our laboratory are maintained in continuous culture for up to 4–5 months. After this, we usually thaw a new vial of the cells to conduct experiments and propagate them in culture for another 4–5 month period.

FACS Analysis. This was carried out as described before (12–14). Single- and two-color FACS analyses were performed using Coulter Profile and ELITE V instruments. The following antibodies were used: biotin-, phycoerythrin-, or fluorescein isothiocyanate (FITC)-conjugated antibodies against Thy-1, LyT2 (CD8), L3T4 (CD5), CD3 (hybridoma 145–2c11), B-220 (hybridoma 6B2), major histocompatibility complex (MHC) class I of the $H\text{-}2^b$ haplotype (hybridoma AF6-88.5), MHC class I of the $H\text{-}2^k$ haplotype (hybridoma AF3-12.1), Mac-1 (hybridoma m1/70), TER 119, 8C5, T-cell receptor (TCR) ab (hybridoma H57-597), TCR gd (hybridoma GL3), VLA-4, VLA-5, ICAM-1, ICAM-2, heat-stable antigen (HSA), VCAM-1, and Sca-1 were purchased from PharMingen; FITC- and phycoerythrin-conjugated F4/80 antibody was from Serotec; purified EA1 antibody against VLA-6 was a gift from B. A. Imhof (Basel Institute For Immunology, Switzerland); FITC-conjugated anti-mouse IgM, κ chain, λ chain, and IgG, phycoerythrin–anti-rat IgG, and phycoerythrin–streptavidin were from Southern Biotechnology Associates; FITC–streptavidin was from Vector Laboratories; FITC-conjugated anti-rat IgG, mouse Ig, and rat Ig were from Jackson ImmunoResearch; and FITC-anti-rat IgM was from The Binding Site (Birmingham, U.K.). The antibodies against the following cell surface markers were prepared in our laboratory: Joro 37-5, 75, 96, 184, 177; PgP-1 (I/45), c-Kit (ACK2), LFA-1 (FD441.8), and HSA (J11D) (12–14).

Northern Blot and Reverse Transcriptase-PCR assays. Northern blot assays using total RNA (20 μg per lane) were performed as described (12, 13) with ^{32}P-labeled specific probes for the genes indicated. The reports describing the origin and characteristics of these probes are listed elsewhere (12, 13). The generation of cDNAs, the primer combinations, and cycle protocols used in reverse transcriptase-PCR assays for the CD34, Tal-1, E12, Gata-2, Id-1, and CD19 genes will be described in detail elsewhere (R.P. and B. A. Imhof, unpublished data).

Hematopoietic Reconstitution *in Vivo*. For repopulation of sublethally irradiated [300 rad (1 rad = 0.01 Gy) of gamma rays] C3H-SCID-SCID mice [female 8- to 12-week-old mice with no detectable serum Ig, termed severe combined immunodeficient (SCID)], the Lin^- clones from normal mice (FLSC 8 and FLSC 14) or from p53-deficient mice ($Bmp53^-$ A11 and $FLp53^-$ B4), freshly isolated FL mononuclear cells from C57BL/6 embryos (10^6 cells in 0.4 ml of PBS), or PBS alone were injected IV into the recipient animal 2–4 hr after irradiation. All mice were housed in sterile isolators with sterile food in a laminar flow hood. Hematopoietic reconstitution in BM and spleen of SCID mice was assessed by single- and two-color FACS analysis 5 to 6 months later.

Radioprotection Assay. The Lin^- clones from p53-deficient mice (BMp53A11 and FLp53B4) or from normal mice (FLSC 8 and FLSC 14) alone (10^6, 5×10^6, or 10^7 cells in 0.4 ml of PBS) or the Lin^- clones (10^6 cells) together with freshly isolated BM cells from Rag-2-deficient mice (10^5 cells) were injected i.v. into lethally irradiated (1000 rad of gamma rays provided in two equal exposures given 4 hr apart) Rag-2-deficient mice (ref. 17; 6- to 8-week-old, male and female). Control groups included lethally irradiated Rag-2-deficient mice that received no cells and mice that received 1.1×10^6 freshly isolated BM cells from syngeneic Rag-2-deficient mice. All mice were housed in sterile isolators with sterile food and antibiotics in the drinking water. Radioprotection was assessed by following the survival of recipient mice. The presence of donor derived mature T cells ($TCRab^+$) and mature B lymphocytes (IgM^+) in peripheral blood of reconstituted Rag-2-deficient mice was assessed by FACS analysis with TCRab and mouse IgM-specific antibodies at the time indicated in the text.

RESULTS AND DISCUSSION

To find culture conditions that support long-term growth of undifferentiated PHSCs, we thought that it would be advantageous to use PHSCs from mutant mice that lack the p53 gene (11, 18, 19). PHSCs from p53-deficient mice might survive longer in culture and might have a lower threshold to enter into mitosis. p53 controls the G_1 to S phase transition of the cell cycle without affecting hematopoiesis (11, 18, 19). Enriched populations of PHSCs from day 12–13 FL cells or BM of young adult p53-deficient mice were obtained by a combination of negative selection using magnetic beads and positive selection by FACS (12, 14). Two cytokine combinations out of several tested were found to support proliferation with no or little differentiation, namely, LIF, steel factor, F (supernatants from the FLS4.1 FL stromal cell line), and either IL-3 or FLT3-lig. These mixtures will be referred to as 3LSF or FLT3-ligLSF, respectively. If F is removed from the cytokine mixture, the cells will continue to proliferate but will also differentiate,

usually into F4/80, 8C5, and TER 119-positive myeloid/erythroid cells. Both 3LSF and FLT3-ligLSF-conditioned medium supported the initiation and long-term (>6 months) maintenance of PHSC lines. In cultures that received FLT3-ligLSF medium, the frequency of positive wells (12–19%) and the time required for the cultures to reach confluency (3–5 weeks) were lower and longer, respectively, when compared to the frequency of growing wells (up to 26%) and the time required to reach confluency (2–3 weeks) observed if the same Lin^- FL cells were cultured in 3LSF medium. Periodic monitoring by FACS for signs of differentiation revealed few myeloid or erythroid lineage cells in some cell lines, mostly in cultures containing 3LSF. The differentiated cells were depleted by magnetic bead selection and 12 of 49 lines were discarded because of persistent and significant (10–20% F4/80-, 8C5-, TER 119-positive cells) differentiation. A total of six $p53^-$ cell lines with stable Lin^- phenotype, three from FL (called $FLp53^-$ A, B. . .) and three from BM (called $Bmp53^-$ A, B. . .), were cloned first by micromanipulation, and selected clones were recloned twice by limiting dilution. We then used the same protocol to establish Lin^- cell lines from day 12–13 FL of normal C57BL/6 mice. Two out of several cell lines with stable Lin^- phenotype established from C57BL/6 normal mice were cloned as indicated above. In the following we describe the details of the characterization of two clones derived from FL of normal C57BL/6 mice (called FLSC 8 and FLSC 14) and two clones, one derived from BM ($Bmp53^-$A11) and one from FL ($FLp53^-$ B4), of $p53^-$-deficient mice. All studies were performed 3–5 months after the establishment of the cell lines in culture. The $p53^-$ clones and the clones from normal mice divide approximately every 10–12 and 16–20 hr, respectively. The clones from wild-type mice die within 36–48 hr, whereas the clones from $p53^-$ mice can survive up to 5–6 days in the absence of their exogenous growth factors.

All four clones are ≈5–8 μm in diameter, round, and exhibit scanty cytoplasm and a prominent dense nucleus, as determined by Giemsa staining. Scanning electron microscopy shows that they have a homogeneous morphology and display microvilli uniformly on their cell surface (data not shown).

Table 1 summarizes the phenotypic characteristics of all clones as determined by FACS analysis using a panel of antibodies against several hematopoietic surface markers. These results can be summarized as follows. First, the clones are negative for several hematopoietic lineage-restricted surface markers normally present on myeloid cells (8C5 and F4/80), erythroid cells (TER 119), immature and mature B-lymphocyte lineage cells (B-220 and IgM), and immature and mature T-lymphocyte lineage cells (Joro 75, CD4, CD8, CD3). Intriguingly, the FL-derived but not the BM-derived clones expressed low levels of Mac-1 antigen. Second, the cells are positive for Sca-1, PgP-1, c-Kit receptor, and express no or very low levels of Thy-1 surface antigen. Third, they stained with the Joro 177, Joro 184, and Joro 96 monoclonal antibodies, indicating that besides reacting with early lymphoid progenitors and myeloid precursors, these surface proteins are also expressed by PHSCs (this study and ref. 20). Fourth, the cells are positive for the VLA-5, VLA-6, ICAM-1, ICAM-2, HSA, and PgP-1, very weakly positive for VLA-4 adhesion molecules, and negative for VLA-2 and VCAM-1 (Table 1). Expression was somewhat variable for c-Kit, Thy 1, Joro 184, Joro 96, Mac-1, VLA 4 and 6, Icam-1 and Icam-2.

Table 1. Phenotypic and genotypic characteristics of the Lin^- clones from C57BL/6 normal and p53-deficient mice

Characteristics	Presence in clone			
	$Bmp53^-$A11	$FLp53^-$B4	FLSC 8	FLSC 14
Surface markers* FACS analysis				
Sca-1	+	+	+	+
c-Kit	+	+	+	+
B-220	−	−	−	−
IgM	−	−	−	−
CD3	−	−	−	−
CD4	−	−	−	−
CD8	−	−	−	−
Joro 75	−	−	−	−
8C5	−	−	−	−
F4/80	−	−	−	−
Mac-1	−	+/−	+/−	+/−
TER 119	−	−	−	−
Joro 96	+	+	+	+
Joro 177	+	+	+	+
HSA	+	+	+	+
VLA-2	−	−	−	−
VLA-4	+/−	+/−	+	+
VLA-5	+	+	+	+
VLA-6	+	+	+	+
VCAM-1	−	−	−	−
PgP-1	+	+	+	+
Gene expression†				
Rag-1, Rag-2	−	−	−	−
CD3γ, CD3ζ	−	−	−	−
MB-1, vPre-B, λ 5	−	−	−	−
TCF-1, LEF-1	−	−	−	−
Gata-1, Gata-3	−	−	−	−
CD34	+	+	+	+
CD19	−	−	−	−
Tal-1/SCL, Gata-2, E12, Id-1	+	+	+	+

*FACS analysis.
†Northern blot/reverse transcriptase-PCR analysis.

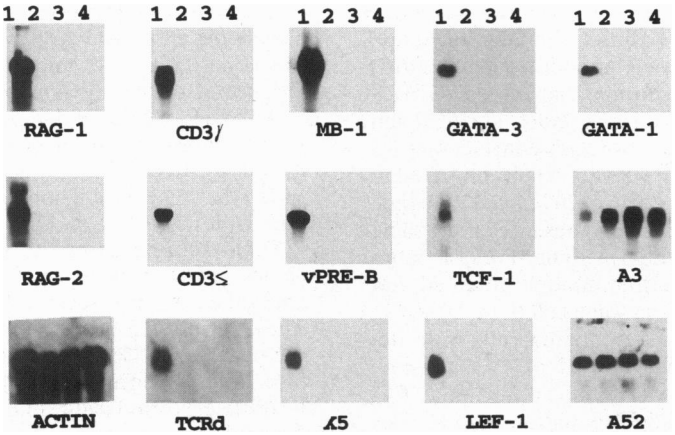

FIG. 1. mRNA synthesis of genes expressed early during lymphohematopoietic development. mRNA expression for the genes indicated by the clones BMp53$^-$ A11 (lane 2), FLp53$^-$B4 (lane 3), and FLSC 8 (lane 4), and positive controls (lane 1 thymus, FL, BM, or spleen as required) was determined by Northern blot analysis of total RNA (20 μg per lane). Films were exposed for 8 hr (Actin and A52), 24 hr (A3), and 48 hr (the rest of the genes indicated).

Northern blot analysis revealed the absence of RNA transcripts for genes expressed at very early stages of lymphocyte development (CD3γ, CD3ζ, Rag-1, Rag-2, TCF-1, Gata-3, LEF-1, MB-1, vPRE-B, λ5, μ Ig heavy chain, and TCRδ), and of Gata-1 mRNA expressed at early stages of erythroid/myeloid differentiation (Fig. 1 and Table 1). All clones synthesized mRNA for the A3 nuclear transport gene and for the A52 gene encoding ribosomal protein L13 (unpublished results; Fig. 1). Reverse transcriptase-PCR assays revealed that all four clones synthesize RNA for CD34 and for Tal-1, Gata-2, E12, and Id-1 transcriptional regulator genes and were negative for CD19 B-cell lineage specific gene (Table 1). These results indicate that the Lin$^-$ p53$^-$ and the Lin$^-$ p53$^+$ clones are at an earlier stage of differentiation than lymphoid-, erythroid-, and myeloid-committed progenitors. In addition, the phenotypic and genotypic characteristics readily distinguish these clones from previously described multipotent, pro-B cell, pro-T cell, B-cell/myelocytic, and myeloid progenitor cell lines (12, 13, 21).

The clones were tested for their capacity to provide long-term reconstitution of the hematopoietic system of x-irradiated mice. We and others (14, 22) have shown before that sublethally irradiated SCID mice are well-suited to assess the differentiation of not only lymphocyte precursors but also lymphohematopoietic stem cells. The SCID mutation renders cells more sensitive to x-irradiation and hampers their hematopoietic recovery after nonlethal doses of radiation (22). This provides with a competitive assay between host PHSCs and the PHSC population under test without the need to expose the recipients to lethal doses of irradiation, which may cause severe damage of the microenvironments required for survival and differentiation of PHSCs.

Two markers were used to distinguish the hematopoietic progeny of the donor cells (Lin$^-$ clones) from C3H-SCID recipient cells, namely, H-2b assessed using an MHC class I H-2b specific antibody and FACS analysis (12, 14) and the Neo^r gene [present in the genome of the p53-deficient mice (11)] assessed by DNA-based PCR (12, 14)]. We have shown

Table 2. *In vivo* repopulation of the hematopoietic system by Lin$^-$ clones from normal and p53-deficient mice

Clone injected	SCID mouse recipient	Time of analysis, months	Percentage positive donor-derived cells (FACS Analysis)					
			Bone marrow				Spleen	
			H-2b$^+$B-220$^+$	H-2b$^+$8C5$^+$	H-2b$^+$Mac-1$^+$	H-2b$^+$TER119$^+$	H-2b$^+$TCR/CD3$^+$	H-2b$^+$IgM$^+$
BMp53$^-$A11	1	6	22.4	27.4	13.8	7.7	36.4	51.2
	2	6	27.3	19.5	10.2	8.1	31.0	48.6
	3	6	20.8	23.1	14.4	6.9	33.5	46.9
	4	5	31.7	25.0	15.9	5.5	38.1	57.3
FLp53$^-$B4	1	5	19.3	18.2	10.7	5.8	26.5	41.5
	2	5	22.1	20.7	13.1	7.3	32.3	47.2
	3	6	14.8	13.6	15.8	4.4	29.8	40.9
	4	6	18.6	15.2	12.6	6.2	30.3	43.3
FLSC 8	1	5	20.1	14.4	18.1	5.2	22.8	43.3
	2	5	18.3	12.7	14.7	6.1	31.3	40.9
	3	6	12.8	16.2	13.3	5.0	26.5	39.8
	4	6	17.5	10.8	15.4	4.7	29.7	44.6
FLSC 14	1	6	13.4	15.5	12.8	5.1	30.0	38.7
	2	6	18.6	11.1	10.9	4.5	32.6	37.3
	3	5	16.2	13.4	14.1	5.3	34.2	41.8
None	1	6	<1	<1	<1	<1	<1	<1
	2	6	<1	<1	<1	<1	<1	<1
	3	5	<1	<1	<1	<1	<1	<1
	4	5	<1	<1	<1	<1	<1	<1

The clones (10^6 cells per mouse) or control buffer without cells were injected into sublethally irradiated C3H SCID mice (H-2K). Five to 6 months later, the presence of donor-derived (H-2b$^+$) cells in the BM and spleen was determined by two-color FACS analysis.

before that the H-2b MHC class I antibody used here does not react with cells bearing H-2d or H-2k (C3H-SCID mouse MHC antigens (14). Thus, both markers are expressed by the donor cells but not by the recipient mice. Five to 6 months after transfer of the Lin^- clones, the BM and/or spleens of C3H-SCID mice contained donor cells, which included granulocytes/macrophages ($H\text{-}2^{b+}$ Mac-1/F4/80/8C5$^+$), erythroid cells ($H\text{-}2^{b+}$ TER 119$^+$), B cells ($H\text{-}2^{b+}$ B-220$^+$, IgM$^+$), and T cells ($H\text{-}2^{b+}$ CD4/CD8/TCR/CD3$^+$) (Table 2). No $H\text{-}2^{b+}$ cells were found in C3H-SCID mice that received no cells. As shown in Fig. 2, the Neo^r gene was found in thymocytes, splenic lymphocytes, and myeloid cells and BM lymphoid, myeloid, and erythroid cells of SCID mice reconstituted with the Lin^- clones from $p53^-$ Neo^r-positive mice. No Neo^r-positive cells were detected in cells from tissues of the control SCID mice that received FL mononuclear cells from $p53^+$ Neo^r-negative normal C57BL/6 mice. These findings indicate that the Lin^- clones have the capacity to provide long-term reconstitution of the lymphohematopoietic system.

In another set of experiments, we studied whether the Lin^- clones could rescue mice from a lethal dose of radiation. Thus, the Lin^- $p53^-$ and the Lin^- $p53^+$ clones from normal mice were injected i.v. either alone or together with BM cells from Rag-2-deficient mice into lethally irradiated syngeneic Rag-2-deficient mice. Control groups included irradiated mice that received either no cells or marrow cells from Rag-2-deficient mice only. Survival of the mice was monitored daily during the first 20 days and twice weekly aftewards. The presence of donor-derived mature T and B lymphocytes in the mice that survived was monitored by FACS analysis of peripheral blood for the presence of TCRab$^+$ mature T lymphocytes and IgM$^+$ mature B lymphocytes. Rag-2-deficient mice cannot rearrange their antigen receptor-encoding genes and thereby do not have mature T and B lymphocytes (17). Thus, the presence of mature T and/or B lymphocytes in the Rag-2-deficient mice will indicate that these mature lymphocytes are the progeny of the Lin^- clones injected. Table 3 shows that lethally irradiated Rag-2-deficient mice that received the $p53^-$ Lin^- clones or the $p53^+$ Lin^- clones alone all died between 5 and 14 days after exposure to a lethal dose of irradiation, as did the control group of mice that received no cells. The Lin^- clones failed by themselves to rescue lethally irradiated mice at all cell concentrations tested (10^6, 5×10^6, and 10^7 cells per mouse). The latter also precluded to assess their potential to give rise to CFU-S, a property of less immature progenitor cells than PHSCs (2, 23). In contrast, all lethally irradiated Rag-2-deficient mice that received the Lin^- $p53^-$ clones or the Lin^- $p53^+$ clones together with few marrow cells from Rag-2-deficient mice survived during the period of observation (6 months). These mice had mature T- and B-lymphocytes (range of percentages of TCRab$^+$ plus IgM$^+$ mature lymphocytes detected was 37% to 63% 6 months after transfer of the Lin^- clones) generated by the Lin^- lymphohematopoietic precursor clones injected (Table 3). The latter results ruled out the possibility that the Lin^- clones were unable to survive, engraft, and differentiate in these lethally irradiated mice. Eight out of 10 lethally irradiated Rag-2-deficient mice that received only BM cells from Rag-2-deficient mice survived, but none of these mice had detectable mature T or B lymphocytes (Table 3). The finding that the Lin^- $p53^-$ and the Lin^- $p53^+$ lymphohematopoietic precursor clones could not by themselves rescue mice from a lethal dose of radiation agree with some (23, 24) but not with other (2, 25) previous studies in which the ability of freshly isolated enriched PHSC populations to rescue mice from lethal irradiation was also investigated. There are several potential explanations for these contrasting results. For instance, the PHSC population might be heterogenous in terms not only of cell cycle status, rhodamine 123 staining, and size (2, 9, 10), but also in their ability to rescue mice from a lethal dose of irradiation. The clones described here would represent the PHSC subset that lacks this property. Another explanation is that a few more differentiated precursor cells contained in a given preparation of freshly isolated enriched PHSC cells could account for survival of lethally irradiated mice. The latter implies that PHSCs would not possess the capacity of rescuing mice from a lethal dose of irradiation (23, 24) and would be consistent with the results obtained here with the Lin^- clones. Although this possibility is virtually impossible to rule out in experiments using freshly isolated enriched PHSC populations, a recent study reported that as few as 25 cells from a purified BM PHSC cell population were able to protect one-third of irradiated recipient mice, and 100 cells protected all recipients (25), findings that argue against this explanation. A third possibility is that the Lin^- $p53^-$ and the Lin^- $p53^+$ clones, while retaining the functions of extensive self-renewal and lymphohematopoietic precursor potential, might have lost, after prolonged culture, the ability to rescue mice from a lethal dose of radiation. Further work is necessary to distinguish among these possibilities.

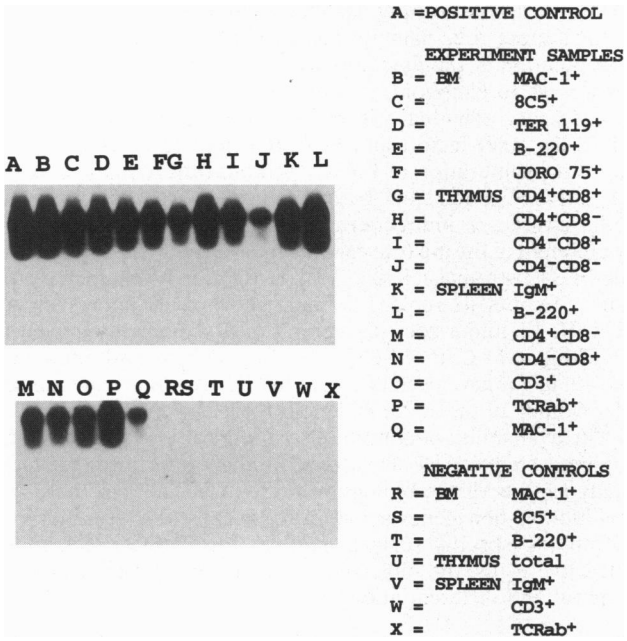

FIG. 2. Neo^r-positive cells in the thymus, spleen, and BM of SCID mice reconstituted with the BMp53$^-$A11 clone. The hematopoietic cell populations indicated were isolated from SCID mice reconstituted with either BMp53$^-$A11 cells (experiment samples). [The p53-deficient mice from which the BMp53A11 cells were isolated carry in their genome the Neo^r gene (ref. 11)] or FL mononuclear cells from p53$^+$Neo^r-negative normal C57BL/6 embryos (negative controls). Thymuses, spleens, and BMs of three SCID mice injected with the same $P53^-$ clone were pooled and used to isolate thymocyte subsets, splenic T and B lymphocytes, BM B-cell precursors and Ig$^+$ B lymphocytes, myeloid cells, and erythroid cells by using magnetic beads coupled with sheep anti-mouse IgG and/or goat anti-rat IgG or by cell sorter, as required, using appropriate antibodies. DNA was isolated from these various populations to detect the presence of the Neo^r gene by PCR assays. The presence of cells carrying the Neo^r gene in their genome among these cell populations was determined by DNA-based PCR assays as described (12, 14). The primers 5′-NeoTTCGGCTATGACTGGGCACAAC and 3′-NeoTCAGTGA-CAACGTCGAGCACA and the Neo-specific probe (the 1.4-kb *Hin*dIII–*Sma*I fragment of the PSV-2 Neo plasmid) were used. The PCR products were fractionated on agarose gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled Neo-specific probe, followed by autoradiography. Exposure times were usually between 2 and 6 hr. The presence of Neo^r-positive cells unambigously documents that these cells were the progeny of the BMp53$^-$A11 clone, which carries the Neo^r gene in its genome and distinguishes it from hematopoietic cells of SCID host or FL cells from C57BL/6 normal mice origin, which do not.

Table 3. Rescue of mice from a lethal dose of irradiation

Cells injected	Mice, alive/ total injected	Presence of donor-derived T and B lymphocytes
None	0/5	NP
BMP53$^-$A11	0/10	NP
FLP53$^-$B4	0/10	NP
FLSC 8	0/10	NP
FLSC 14	0/9	NP
BMP53$^-$A11 + BM	10/10	10/10
FLP53$^-$B4 + BM	9/10	9/9
FLSC 8 + BM	9/10	9/9
FLSC 14 + BM	8/9	8/9
BM	8/10	0/8

NP = not possible.

In summary, we show here that lymphohematopoietic stem cells can grow in long-term cultures without differentiating. The clones from normal and p53-deficient mice described here share phenotypic characteristics and the two functions of PHSC with the freshly isolated populations enriched for PHSC; these are, namely, the ability to provide long-term repopulation of the hematopoietic system and the capacity of giving rise to lymphoid, myeloid, and erythroid cell lineages. Among the individual cytokines in the mixtures (IL-3, LIF, FLT3-lig, steel factor, and F) found here to be required to support proliferation of PHSCs without differentiation, IL-3, LIF, FLT3-lig, and steel factor have previously been reported to have direct or indirect effects on PHSCs (26–29). The new component in the mixture called F [cell-free supernatants from the FLS4.1 stromal cell line (13)], remains to be characterized. Our attempts to replace F with known cytokines such as IL-1–IL-12, tumor necrosis factor, transforming growth factor, FLT3-lig, GM-CSF, M-CSF, G-CSF, activin, and fibroblast growth factor have been unsuccessful. Efforts toward the molecular cloning of the active moiety in F are currently underway.

The availability of continuously proliferating lymphohematopoietic stem cell clones offers a unique opportunity to study many aspects of the biology of these cells—for instance, the isolation of new genes active in these cells, the possibility of identifying specific surface markers for PHSCs, and, most attractive to us, the molecular mechanisms that control self-renewal and differentiation.

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