Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7530–7534, August 1995
Developmental Biology

In vitro generation of hematopoietic stem cells from an embryonic stem cell line

(hematopoiesis/stromal cells/cytokines)

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ABSTRACT **Hematopoietic stem cells (HSC) are unique in that they give rise both to new stem cells (self-renewal) and to all blood cell types. The cellular and molecular events responsible for the formation of HSC remain unknown mainly because no system exists to study it. Embryonic stem (ES) cells were induced to differentiate by coculture with the stromal cell line RP010 and the combination of interleukin (IL) 3, IL-6, and F (cell-free supernatants from cultures of the FLS4.1 fetal liver stromal cell line). Cell cytometry analysis of the mononuclear cells produced in the cultures was consistent with the presence of PgP-1$^+$ Lin$^-$ early hematopoietic (B-220$^-$ Mac-1$^-$ JORO 75$^-$ TER 119$^-$) cells and of fewer B-220$^+$ IgM$^-$ B-cell progenitors and JORO 75$^+$ T-lymphocyte progenitors. The cell-sorter-purified PgP-1$^+$ Lin$^-$ cells produced by induced ES cells could repopulate the lymphoid, myeloid, and erythroid lineages of irradiated mice. The ES-derived PgP-1$^+$ Lin$^-$ cells must possess extensive self-renewal potential, as they were able to produce hematopoietic repopulation of secondary mice recipients. Indeed, marrow cells from irradiated mice reconstituted (15–18 weeks before) with PgP-1$^+$ Lin$^-$ cell-sorter-purified cells generated by induced ES cells repopulated the lymphoid, myeloid, and erythroid lineages of secondary mouse recipients assessed 16–20 weeks after their transfer into irradiated secondary mice. The results show that the culture conditions described here support differentiation of ES cells into hematopoietic cells with functional properties of HSC. It should now be possible to unravel the molecular events leading to the formation of HSC.**

Blood cells have a limited life-span and therefore must be continuously generated by less differentiated precursor cells throughout life. Hematopoietic stem cells (HSC) are unique in that they give rise both to new stem cells (self-renewal) and to all blood cell types. HSC have extensive self-renewal potential, which is manifested in the repopulation of the hematopoietic system for long periods (>6 mo). Mouse bone marrow and fetal liver cells with properties of HSC were found to express Thy1 and Sca-1 surface markers and c-Kit receptor and to lack most lineage-restricted surface markers (e.g., B-220 for B-lymphocyte lineage, Mac-1 for myeloid lineage, TER 119 for erythroid lineage, and JORO 75 for T-lymphocyte lineage precursors) (1–5). In humans, presumptive HSC were found to be contained in the CD34$^+$ 33$^-$ DR$^-$ and CD34$^+$ 33$^-$ DR$^+$ marrow populations (6–8). HSC can be enriched (1–5), but the numbers obtained are very low. This result has hampered studies on the physiology of HSC and how the self-renewal vs. differentiation choice is made. An unresolved fundamental issue concerning hematopoiesis and HSC in particular is how HSC are formed. The cellular and molecular events responsible for the formation of HSC remain unknown mainly because no system exists to investigate the process.

We report here the generation of hematopoietic precursor cells with properties of HSC—i.e., able to reconstitute the lymphoid-, myeloid-, and erythroid-cell lineages of primary and secondary irradiated mice by inducing embryonic stem (ES) cells to differentiate *in vitro* by coculture with stromal cells and a combination of cytokines.

MATERIALS AND METHODS

Induction of ES Cells. The ESE14.1 cell line (9) was maintained in the undifferentiated state by culture on monolayers of irradiated STO fibroblasts in the presence of exogenous human leukemia inhibitory factor (supernatants from Cos-1 cells transfected with pC10 6R plasmid containing cDNA from human leukemia inhibitory factor) or on monolayers of irradiated SNL 76/7 feeder cells (10). ESE14.1 cells (1–2 × 10^3 cells per well) were cultured on monolayers of mitomycin C-treated (5–10 μg/ml at 37°C for 3–4 hr) or irradiated (2–4 × 10^3 rads of γ rays; 1 rad = 0.01 Gy) RP.0.10 bone marrow stromal cells (11) in Costar six-well plates, in the presence of recombinant interleukin (rIL) 3 (100–300 units/ml) rIL-6 (10–50 units/ml) (12), and F [final concentration 10% (vol/vol)], supernatants from 2-day cultures of confluent FLS4.1 fetal liver stromal cells (13). FLS4.1 supernatant contains FLT3 ligand, steel factor, and a presumably new factor that supports growth of HSC (R.P., C. Bucana, X. Xie, and T. Li, unpublished work) in 2–2.5 ml of culture medium [Iscove's Dulbecco's modified medium/50 μM 2-mercaptoethanol/2 mM L-glutamine/gentamycin at 50 μg/ml/7.5% (vol/vol) fetal calf serum] at 37°C in a 7.5% CO_2/92.5% air atmosphere. Every 5–7 days the cells were harvested and subcultured in new Costar six-well plates containing mitomycin C-treated RP.0.10 stromal cells and freshly prepared cytokine-supplemented culture medium. The cells were harvested at the time indicated in the text and used for cell cytometry analysis, cell-sorter purification, and transfer into severe combined immunodeficiency (SCID) mice, as required.

Purification of ES Cell-Derived-PgP-1$^+$ Lin$^-$ Hematopoietic Cells. ESE14.1 cells were induced to differentiate into hematopoietic precursor cells as detailed above. After 21 days of culture, some cells were harvested and used to document the presence of Thy1$^+$ PgP-1$^+$ c-Kit$^+$ hematopoietic cells. After this procedure, the rest of the cells were harvested and expanded by subculturing in new Costar six-well plates containing mitomycin C-treated RP.0.10 stromal cell monolayers

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Abbreviations: HSC, hematopoietic stem cells; IL, interleukin; rIL, recombinant IL; ES, embryonic stem; FITC, fluorescein isothiocyanate; SCID, severe combined immunodeficiency; PE, phycoerythrin; MHC, major histocompatibility complex.

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and cytokine-supplemented culture medium at 37°C for 6–8 days. The cells were harvested, washed, and depleted of JORO 75^+, JORO 37-5^+, JORO 30-8^+, B-220^+, Mac-1^+, F4/80^+, g/4^+ (granulocytes), and TER 119^+ cells using magnetic beads coupled with sheep anti-rat immunoglobulin (Dynabead, Dynal, Oslo) as described (14). The magnetic particle-free cells were stained with fluorescein isothiocyanate (FITC)-PgP-1 and phycoerythrin (PE)- or biotin-labeled B-220-, 8C5-, TER 119-, Joro 75-, and Mac-1-specific antibodies followed by PE–streptavidin. Cell cytometry analysis indicated that these cell preparations contained <8% Lin^+ cells. We also stained the magnetic particle-free cell preparation with FITC–anti-rat IgG antibody to assess the percentage of cells that bound the lineage-characteristic surface markers used above but which failed to be depleted upon applying the magnetic force. In the two instances tested we found that <2% of the cells were positive; this percentage of cells probably could not be stained with the fluorochrome-labeled antibodies that recognized the same epitopes on the different lineage-characteristic surface markers. Thus, to further purify the PgP-1^+ Lin^- (B-220-, Mac-1-, JORO 75-, TER 119-, 8C5-negative) cells, they were subjected to flow cytometry sorting using either a FACStar plus or an Elite V Coulter as described (14). The cell-sorter-purified cells were >98.0% PgP-1^+ Lin^-, as determined upon reanalysis. The probability levels for the two-color plots used for cell sorting and for re-analysis were 1, 3, 6, 10, 15, and 25. Cells were washed and injected into sublethally irradiated female SCID mice.

Hematopoietic Precursor Potential *in Vivo*. Purified PgP$^-$ 1^+ Lin^- cells (obtained as detailed above) that were generated by induced ESE14.1 cells were injected i.v. ($4–6 \times 10^4$ cells per mouse) into sublethally irradiated (300 rads) CB17 or C3H SCID mice (6- to 12-week-old females with no detectable serum IgM, which were bred and maintained in the animal facilities of the Basel Institute for Immunology or The University of Texas M.D. Anderson Cancer Center). The presence of donor-derived (ESE14.1 cells are of 129sv male origin, H-2b) T-lymphoid-, B-lymphoid-, myeloid-, and erythroid-lineage cells in these mice was determined (*i*) by using FITC- or biotin-labeled H-2b class I major histocompatibility complex (MHC)-specific antibody (hybridoma 28-8-6, Pharmingen); FITC-labeled mouse IgM (Southern Biotechnology Associates); biotin– or PE–CD8; FITC– or PE–CD4 (Becton Dickinson); FITC–T-cell receptor antibody, FITC–CD3, FITC– or biotin–Thy1-specific antibodies (all from PharMingen), and FITC– or PE–streptavidin (Molecular Probes) in two-color cell cytometry analysis and (*ii*) by DNA-based PCR analysis to determine the presence of the *Sry* male locus (present in the genome of donor cells of ES cell origin but not in the genome of cells of the recipient female SCID mice).

Cell Cytometry Analysis. Two-color cell cytometry analysis was done as described (13, 14) using a FACScan or a Profile Coulter. All stainings were done in the presence of purified rat immunoglobulin (250 μg/ml each) and 15% heat-inactivated hamster serum to prevent the constant fragment of the heavy chain (FC)-receptor-mediated binding of labeled antibodies to cells. Bone marrow, spleen, or fetal liver mononuclear cells from normal C57BL/6 mice were used as positive controls. Cells exposed to biotin-conjugated rat IgG and FITC– or PE–steptavidin were negative controls. FITC- or biotin-labeled Thy1 (30H12, PharMingen); FITC- or biotin-labeled PgP-1 (I42/5) (15); biotin-conjugated c-Kit receptor (ACK2, from S. Nishikawa, Kyoto University); biotin-conjugated JORO 75, JORO 37-5, and JORO 30-8 (14); FITC- or PE-labeled B-220 (RA3-6B2) (PharMingen); FITC- or biotin-conjugated Mac-1 (M1/70) (Caltag, South San Francisco, CA); FITC-labeled F4/80 (Serotec); and biotin-conjugated TER 119 (PharMingen)-specific antibodies were used. Fluorescence emitted by single viable cells was measured with logarithmic amplification. Dead cells were excluded from analysis by forward- and side-scatter gating. Data collected from 10^4 cells were analyzed and displayed in the form of contour plots.

DNA PCR Assay. DNA was isolated from the thymus-, bone marrow-, or spleen-derived lymphoid, myeloid, and erythroid cell populations [obtained by using appropiate specific antibodies for mature T lymphocytes (TCR ab, CD3), B lymphocytes (B-220, IgM), myeloid cells (Mac-1, 8C5), or erythroid cells (TER 119) and magnetic beads (beads to cell ratio, 30:1) as described before (14)] from a pool of three to four SCID mice either reconstituted with ES-induced cell-sorter-purified PgP-1^+ Lin^- cells or injected with buffer only 6 mo before analysis. Mouse *Sry* locus sequences (16) on the Y chromosome were amplified by PCR with a specific pair of primers (upstream, 5′-ctgctgtgaacagacactac-3′; downstream, 5′-gactcctctgacttcacttg-3′). DNA samples were heated for 5 min at 94°C and then amplified for 30 cycles (94°C for 15 sec, 62°C for 30 sec, and 72°C for 30 sec) followed by a 10-min extension period at 72°C, using a commercially available kit (Cetus). PCR products were fractionated on agarose gels, blotted to membrane filters, and hybridized with ^{32}P-labeled 25-mer *Sry*-specific oligonucleotide (ctagagatcggagcttggctcaggt) internal to the amplified fragment and processed for autoradiography.

RESULTS AND DISCUSSION

In a previous study we found that ES cells cocultured on monolayers of irradiated RPO10 stromal cells and a mixture of cytokines gave rise to early immature hematopoietic cells including lymphocyte precursors after 21–25 days of culture (17). Our efforts were now directed to search for and document the generation of HSC by induced-ES cells and to optimize the culture conditions that would support this process. We found that mitomycin C-treated RP.0.10 stromal cells performed far better than irradiated RP.0.10 cells. Formation of HSC in the cultures was achieved in the presence of exogenously supplied rIL-3, rIL-6, and F [cell-free supernatant from cultures of the fetal liver stromal cell line FLS4.1 (ref. 13); F contains steel factor, FLT3 ligand, and a presumably new cytokine that supports proliferation of HSC (R.P., C. Bucana, X. Xie, and T. Li, unpublished work)]. ESE14.1 cells were cultured on monolayers of mitomycin C-treated RP.0.10 stromal cells in the presence of rIL-3, rIL-6, and F at 37°C for 5–7 days. The cells were harvested, washed, and subcultured on freshly prepared mitomycin C-treated RP.0.10 stromal cell monolayers and freshly prepared cytokine-supplemented culture medium. This procedure was repeated every 5–7 days as required. ESE14.1 cells underwent progressive differentiation; mononuclear cells with morphological characteristics of hematopoietic cells became apparent by days 15–21 and increased in numbers throughout the 35-day period of observation. Cell cytometry analysis of cells from 25 day-induced ESE14.1 cell cultures showed the presence of significant numbers of early hematopoietic cells bearing PgP-1, Thy1, or c-Kit surface markers and fewer JORO 30-8^+ multipotent and T-lymphocyte progenitors (14), JORO 75^+ and JORO 37-5^+ T-lymphocyte progenitors (14), or B-220^+ IgM^- B^- lymphocyte precursors. Most JORO$^+$ lymphocyte progenitors were $Thy1^+$. Very few Mac-1^+, F4/80^+ myeloid, or TER 119^+ erythroid lineage cells were inconsistently observed in the cultures (Fig. 1). Table 1 summarizes the results obtained in another three experiments. Adding all Lin^+ cells produced in the cultures, we found that the percentages of Lin^- cells (range, 58–72%) were higher than those of Lin^+ cells (TER119^+, Mac-1^+, JORO 75^+, JORO 37-5^+, JORO 30-8^+, B-220^+) (range, 28–42%). Uninduced ESE14.1 cells were negative for all these surface markers (Table 1).

Next we isolated (by a combination of negative- and positive-selection procedures detailed in *Materials and Methods*) the PgP-1^+ Lin^- (JORO$^-$ B-220^- Mac-1^- 8C5$^-$ TER 119^-) cells

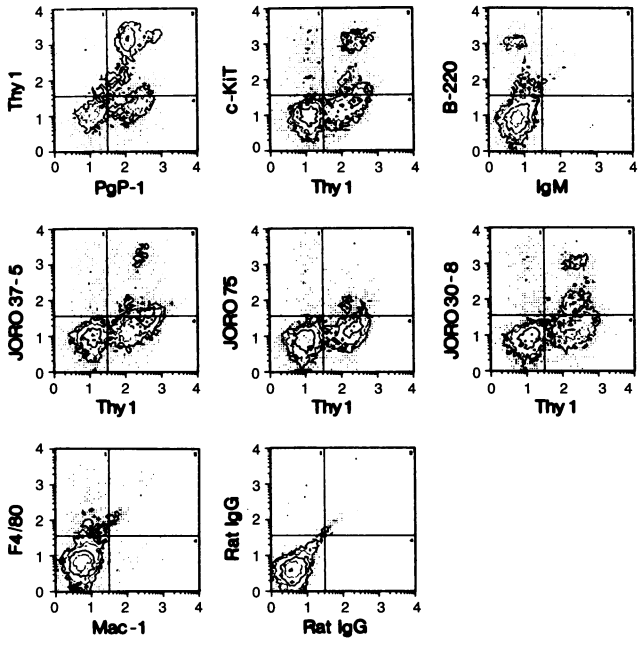

FIG. 1. Production of early hematopoietic cells by induced ES cells *in vitro*. ESE14.1 cells were cocultured with RPO10 stromal cells, IL-3, IL-6, and F, as detailed. Cells were harvested at day 25 of culture, and the presence of early hematopoietic Thy1$^+$, Pgp-1$^+$, c-Kit$^+$ cells, JORO 30-8$^+$ multipotent and lymphocyte progenitors, JORO 75$^+$ T-lymphocyte progenitors, and B-220$^+$ B-lymphocyte precursors was assessed by two-color cell cytometry analysis using surface markers PE–Thy1/FITC–PgP-1, biotin–c-Kit + PE–streptavidin/FITC–Thy1, PE-B220/FITC–IgM, biotin–JORO + PE–streptavidin/FITC–Thy1, biotin–F4/80 + PE–streptavidin/FITC–Mac-1. Uninduced ESE14 cells were negative for all surface markers indicated above. Scale nos. are in $\log_{10}$ units.

developed in the cultures. The purified PgP-1$^+$ Lin$^-$ cells were injected into sublethally irradiated female CB17 or C3H SCID mice to assess their hematopoietic precursor potential. Sublethally irradiated SCID mice are well suited to assess the differentiation of not only lymphocyte precursors but also HSC. The *scid* mutation renders cells much more sensitive to irradiation and hampers their recovery after nonlethal irradiation doses (18). This fact provides us with a competitive assay between host HSC and the HSC population under test without

Table 1. Differentiation of ESE14.1 cells into very early hematopoietic cells

	FACS analysis, % positive cells			
Cell population	Uninduced ESE14	Exp. 2	Exp. 3	Exp. 4
Thy1$^-$ PgP1$^+$	<1	45	51	40
Thy1$^+$ PgP-1$^+$	<1	36	43	31
Thy1$^+$ c-Kit$^+$	<1	17	29	20
Thy1$^+$ JORO 75$^+$	<1	6	5	8
Thy1$^+$ JORO 37-5$^+$	<1	7	8	9
B220$^+$ IgM$^-$	<1	8	10	5
B220$^+$ IgM$^+$	<1	<1	<1	<1
F4/80$^+$	<1	<2	3	<1
Mac-1$^+$	<1	3	3	<2
TER 119$^+$	<1	<2	<1	<2

Two-color cell cytometry analysis using the surface markers indicated of cells generated in culture by ESE14.1 cells induced for 21–25 days. Viable cells were gated by forward and side scatters that also permitted us to exclude large stromal (and nonhematopoietic) cells from analysis. Single-color cell cytometry analysis was used to assess expression of the surface markers on uninduced ESE14.1 cells (viable cells were gated by using propidium iodide exclusion).

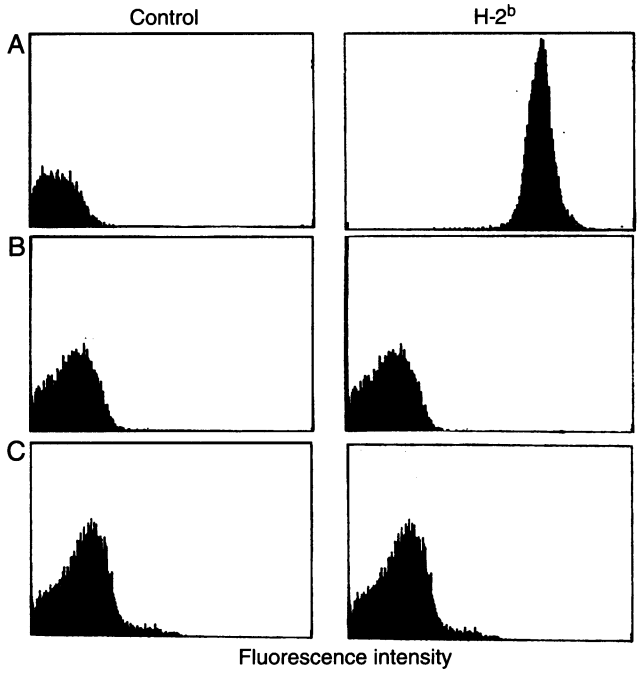

FIG. 2. Specificity of the 28-8-6 antibody for MHC class I H-2b haplotype. The MHC class I H-2b-specific antibody 28-8-6 stains mononuclear marrow cells from C57BL/6 (H-2b) (*A*) but not from CB17 SCID (H-2D) (*B*) or C3H SCID (H-2K) (*C*) mice.

any need to expose recipients to lethal irradiation doses that may severely damage the microenvironments required for survival and differentiation of HSC.

Two markers were used to distinguish the hematopoietic progeny of the donor cells (PgP-1$^+$ Lin$^-$ cells generated by induced ESE14.1 cells) from C3H or CB17 SCID recipient cells. (*i*) A MHC class I H-2b-specific antibody was used along with several hematopoietic cell surface markers and two-color cell cytometry analysis to determine the presence and the nature of donor-derived hematopoietic cells in the bone marrow and spleen of these mice. Cells from CB17 SCID (H-2d), C3H SCID (H-2k), and the RP.0.10 stromal cells (H-2d) did not react with the H-2b-specific antibody (Fig. 2).

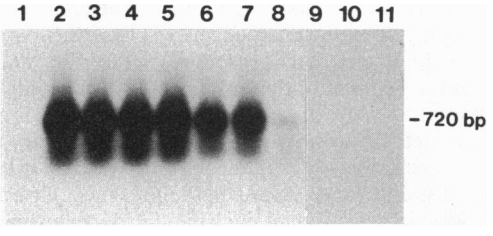

FIG. 3. Detection by *Sry* male-specific DNA-based PCR assay of donor-derived hematopoietic cells in SCID female mice reconstituted with cell-sorter-purified PgP-1$^+$ Lin$^-$ cells generated *in vitro* by induced ESE14.1 cells. DNA was isolated from the different hematopoietic cell populations and subjected to PCR amplification with a pair of primers specific for *Sry* gene in the Y chromosome. Thymocytes (lane 3), TCR ab$^+$-splenic T lymphocytes (lane 4), IgM$^+$ splenic B-lymphocytes (lane 5), bone marrow Mac-1$^+$ myelomonocytic cells (lane 6), TER 119$^+$ erythroid marrow cells (lane 7) from female SCID mice (pool of three mice) injected with purified PgP-1$^+$ Lin$^-$ cells generated by induced ESE14.1 cells, 6 mo before analysis. Controls included thymocytes (lane 8), spleen cells (lane 9), Mac-1$^+$ marrow cells (lane 10), TER 119$^+$ erythroid marrow cells (lane 11), from female SCID mice (pool of four mice) that received no cells. Lane 1 contains DNA from female normal C57BL/6 mice, and lane 2 contains DNA from male normal C57BL/6 mice, which served as negative and positive controls, respectively, for the PCR assay.

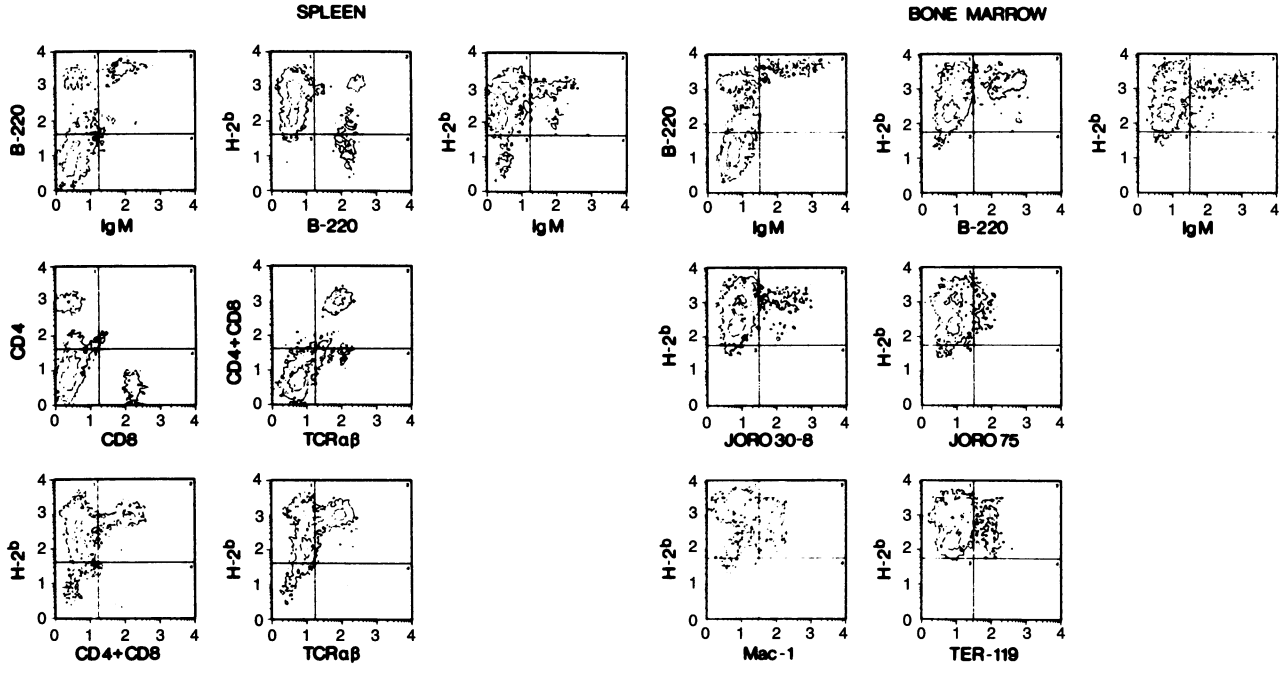

FIG. 4. Cell-sorter-purified PgP-1$^+$ Lin$^-$ cells generated by induced ESE14.1 cells repopulate the lymphoid-, myeloid-, and erythroid-cell compartments of irradiated C3H SCID mice. ESE14.1 cells were induced to differentiate by coculture with RPO10 stromal cells, IL-3, IL-6, and F. The PgP-1$^+$ Lin$^-$ cells developed in the cultures were purified and injected into irradiated C3H SCID mice. Four months later, the presence of hematopoietic cells positive for the cell-surface markers indicated in spleen and bone marrow was determined by cell cytometry analysis. Scale nos. are in $\log_{10}$ units.

(*ii*) The presence of the SRY male gene in the genome of lymphoid, myeloid, and erythroid cells obtained from the thymus, spleen, or bone marrow of reconstituted female SCID hosts was assessed by DNA-based PCR assays (Fig. 3). Both markers are expressed by the donor cells but not by the recipient female SCID mice.

The purified PgP-1$^+$ Lin$^-$ cells generated by ESE14.1 cells in the cultures gave rise to myeloid (H-2b$^+$ Mac-1$^+$), erythroid (H-2b$^+$ TER 119$^+$), B-lymphocyte precursors (H-2b$^+$ B-220$^+$), and T-lymphocyte progenitors (H-2b$^+$ JORO$^+$) in the bone marrow and to mature T lymphocytes (H-2b$^+$ CD4$^+$/CD8$^+$ TCR ab$^+$) and B lymphocytes (H-2b$^+$ B-220$^+$ IgM$^+$) in the spleen assessed 12–18 weeks after their transfer into SCID mice (Fig. 4). Hematopoietic reconstitution of the lymphoid, myeloid, and erythroid lineages by purified PgP-1$^+$ Lin$^-$ ESE14.1-derived progeny occurred in 27 of 30 mice (pool of five independent experiments) tested. Why the hematopoietic system was not repopulated in three cases remains unanswered. In some cases (6 of 27 mice) the PgP-1$^+$ Lin$^-$ cells had even replaced the host bone marrow hematopoietic cells, as

Table 2. Lymphohematopoietic repopulation of secondary SCID mice recipients by marrow cells from primary SCID mice recipients reconstituted by purified PgP-1$^+$ Lin$^-$ cells generated *in vitro* by induced ESE14.1 cells

1er SCID source donor marrow cells	2do SCID recipient	Cell cytometry analysis, % positive cells							
		H-2b$^+$ bone marrow				H-2b$^+$ spleen			
		JORO 75$^+$	B-220$^+$	Mac-1$^+$	TER 119$^+$	CD4$^+$/CD8$^+$	TCR$\alpha\beta^+$	B-220$^+$	IgM$^+$
Mouse 2	1	2.4	16.4	12.6	9.3	22.5	21.9	46.4	43.0
	2	1.8	21.0	9.4	6.5	26.1	27.6	51.2	50.5
	3	3.6	10.7	13.9	10.9	28.0	29.2	50.9	48.7
	4	1.5	13.5	11.7	9.7	20.4	22.4	56.3	52.9
Mouse 5	1	1.8	20.2	15.4	8.8	25.3	27.8	57.2	53.1
	2	2.2	18.8	10.8	9.7	24.7	23.5	53.8	50.7
	3	1.7	14.6	16.3	10.2	20.9	22.0	55.0	51.9
	4	1.1	15.7	13.3	10.0	23.2	23.8	54.3	52.6
Mouse 13	1	0.9	21.5	15.1	7.3	17.4	19.6	40.2	38.1
	2	3.1	26.2	18.2	12.8	28.5	30.3	59.1	57.5
	3	2.3	17.3	10.8	8.5	24.9	25.5	54.8	51.9
	4	1.6	19.8	13.7	7.4	26.1	25.8	56.7	52.8
None		<1	<1	<1	<1	<1	<1	<1	<1
Normal C57BL/6		1.6–2.9	23–29	26–31	22–29	27–32	28–32	68–69	67–68.5

Purified PgP-1$^+$ Lin$^-$ cells generated *in vitro* by ESE14.1 cells were injected into sublethally irradiated SCID mice (1er = primary SCID recipients). Marrow mononuclear cells from such reconstituted SCID mice were obtained 15–18 weeks later and were injected (10^6 cells per mouse) i.v. into sublethally irradiated SCID mice (2do = secondary recipients). After 16–20 weeks, the presence of donor-derived lymphoid (JORO$^+$, B-220$^+$, IgM$^+$, TCR ab$^+$, CD4$^+$/CD8$^+$), myeloid (Mac-1$^+$), and erythroid (TER 119$^+$)-lineage cells in bone marrow and spleen was determined by two-color cell cytometry analysis using an H-2b class I MHC-specific antibody that marks cells of donor origin and the lineage-characteristic surface markers that are indicated. Data from SCID mice that received no cells (negative control; n = 3) and from C57BL/6 normal mice (positive control for cell cytometry; n = 3) are also shown.

illustrated with the example of Fig. 4. The latter perhaps reflects the advantage of the donor cells over the host SCID cells, which because of inherent defects in DNA repair and an increased sensitivity to irradiation (18) may have had further compromises in their capacity for functional recovery and thus became less competitive. Consistent with the cell cytometry data, Fig. 3 shows that the *Sry* male gene was found in thymocytes, splenic lymphocytes, and bone marrow lymphoid, myeloid, and erythroid cells of SCID female mice reconstituted with purified PgP-1$^+$ Lin$^-$ cells generated *in vitro* by induced ESE14.1 cells. No *Sry* male-positive signal was detected in cells from tissues of the control SCID female mice that received no cells (Fig. 3). We have shown before that uninduced ES cells fail to reconstitute the hematopoietic system after injection into SCID mice (17).

The ESE14.1 cell-derived PgP-1$^+$ Lin$^-$ cells must possess extensive self-renewal potential, as they were able to produce hematopoietic repopulation of secondary SCID mice recipients (Table 2). Indeed, marrow cells from SCID mice reconstituted (15–18 weeks before) with PgP-1$^+$ Lin$^-$ cell-sorter-purified cells generated by induced ESE14.1 cells repopulated the lymphoid, myeloid, and erythroid lineages of secondary mouse recipients assessed 16–20 weeks after their transfer into irradiated secondary SCID mice (Table 2).

Taken together, we conclude that PgP-1$^+$ Lin$^-$ cells produced in culture by induced ESE14.1 cells are multipotent hematopoietic precursors with large self-renewal capacity. As these cells possess functional (extensive self-renewal and lymphohematopoietic precursor potential) characteristics ascribed to HSC (1–5), it can also be concluded that the culture system defined here supports the differentiation of ES cells into HSC.

Several groups have reported the generation *in vitro* of myelo/erythroid-cell lineages from ES cells, by culturing embryoid bodies on methylcellulose and IL-1, IL-3, and erythropoietin-supplemented culture medium. However, this system appears inefficient in supporting the generation of lymphocyte-lineage cells, and no evidence for the production of HSC was reported (19–23). Stromal cells play a critical role in systems that support differentiation of ES cells into HSC and lymphoid-precursor cells (this study; refs. 17, 24). Indeed, we find that not all stromal cell lines can support HSC and lymphoid-lineage development from ES cells (refs. 17, 24, and our unpublished results) and that F (a presumably new cytokine secreted by the FLS4.1 stromal cell line) also plays a role in this process.

The establishment of a culture system in which HSC are generated is a key step toward unraveling the molecular events responsible for the formation of HSC.

We are grateful to Dr. A. Smith for human leukemia inhibitory factor cDNA, Dr. S. Nishikawa for c-Kit antibody, K. Ramirez for help with cell cytometry analysis and cell sorter operation, E. Banes for animal care, and P. Love for preparation of the manuscript. Part of this work was supported by National Institutes of Health Grant CA16672-18 and a Sid Richardson Foundation grant. The Basel Institute for Immunology was founded and is supported by F. Hoffmann–La Roche.

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