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Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution

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Objective. **Ex vivo expansion of human hemopoietic stem cells (HSC) is an important issue in transplantation and gene therapy. Encouraging results have been obtained with cord blood, where extensive amplification of primitive progenitors was observed. So far, this goal has been elusive with adult cells, in which amplification of committed and mature cells, but not of long-term repopulating cells, has been described.**

Methods. **Adult normal bone marrow (BM) and mobilized peripheral blood (MPB) $CD34^+$ cells were cultured in a stroma-free liquid culture in the presence of Flt-3 ligand (FL), thrombopoietin (TPO), stem cell factor (SCF), interleukin-6 (IL-6), or interleukin-3 (IL-3). Suitable aliquots of cells were used to monitor cell production, clonogenic activity, LTC-IC output, and in vivo repopulating capacity.**

Results. **Here we report that BM and MPB HSC can be cultured in the presence of FL, TPO, SCF, and IL-6 for up to 10 weeks, during which time they proliferate and produce large numbers of committed progenitors (up to 3000-fold). Primitive NOD/SCID mouse repopulating stem cells (SRC) are expanded sixfold after 3 weeks (by limiting dilution studies) and retain the ability to repopulate secondary NOD/SCID mice after serial transplants. Substitution of IL-6 with IL-3 leads to a similarly high production of committed and differentiated cells but only to a transient (1 week) expansion of SRC_s, which do not possess secondary repopulation capacity.**

Conclusion. **We report evidence to show that under appropriate culture conditions, adult human SRC can also be induced to expand with limited differentiation.** **© 2003 International Society for Experimental Hematology Published by Elsevier Science Inc.**

Hematopoiesis is a dynamic system, originating from primitive, multipotent stem cells that, through proliferation and differentiation, produce many classes of committed progenitors and precursors with increasingly lineage-restricted potential. The hallmark of stem cells is self-renewal, the ability to divide without significant alteration of their proliferation and differentiation potential. Self-renewal can be triggered in vivo and is thought to be regulated, at least in part, by interactions between cytokines and their receptors. Thus, several combinations of cytokines known to act on primitive hemopoietic stem cells (HSC) have been employed in vitro in an attempt to produce culture conditions suitable for HSC expansion. The ligand for c-kit (stem cell factor; SCF) and flt3/flk2 ligand (FL), known to transduce signals crucial for HSC proliferation, and c-mpl ligand, thrombopoietin (TPO), shown to stimulate primitive HSC expansion, have all been regarded as key factors for triggering self-renewal [1–12]. In addition, interleukin-6 (IL-6), IL6/IL6-receptor chimera, interleukin-3 (IL-3), and granulocyte colony-stimulating factor (G-CSF) have reported to play different, although controversial, roles in HSC expansion [13–18].

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Until recently, most HSC expansion studies employed in vitro assays for $CD34^+$ cells, colony-forming cells (CFCs) in clonal culture, cobblestone area-forming cells (CAFCs), and long-term culture-initiating cells (LTC-ICs) to optimize culture conditions [15,19,20], but these surrogate assays may not accurately reflect stem cell activity [21,22]. The transplantation assay available in mouse has been instrumental in defining and characterizing the most primitive cells of the hemopoietic system. Development of a similar assay also for human cells has been recently reported. This assay measures the ability of human stem cells to completely and durably reconstitute human hematopoiesis in sublethally irradiated nonobese diabetic (NOD)/ severe combined immunodeficient (SCID) mice [23–27].

We and others demonstrated that human long-term NOD/ SCID repopulating stem cells (SRC) of cord blood (CB) or fetal tissues can be expanded several-fold after up to 10 weeks of culture in suitable culture conditions [12]. Expansion, however, has proved much more difficult to obtain with adult HSC as these are hard to manipulate ex vivo and a stromal cell–based system may be necessary for optimal adult $CD34^+$ cell expansion and LTC-IC or week-5 CAFC amplification [28]. Indeed, several findings highlight that CB and adult bone marrow (BM) and mobilized peripheral blood (MPB) HSC are quite different: the former possess greater proliferation potential, different cytokine requirements, and responsiveness to a number of growth regulators and are more easily transduced by retroviral vectors [29].

The aim of this work was to investigate whether adult BM and MPB primitive, in vivo repopulating cells could be cultured in vitro for long periods of time in the presence of combinations of growth factors (FL, SCF, TPO, IL-6, IL-3) previously shown to induce a larger expansion of CB progenitors and more primitive cells; whether in these culture conditions, transplantable SRC could be maintained or even expanded and still retain their proliferation and multilineage differentiation capacity.

Materials and methods

Human cells

Human BM was obtained by aspiration from the posterior iliac crest of (fully informed) hematological normal donors. MPB was collected from leftovers of leukapheresis procedures from normal volunteers donating stem cells for allogeneic transplants, who had received G-CSF subcutaneously for five consecutive days prior to the apheresis procedure. In both cases, approved institutional procedures involving written informed consent from each patient were followed.

$CD34^+$ cell purification

Mononuclear cells (MNC) were isolated from BM and MPB using Ficoll Hypaque (density 1077; Nyegaard, Oslo, Norway) density centrifugation. Only for MPB, MNC were subjected to plastic adherence (60 minutes) in tissue culture flasks, then the nonadherent fraction collected. The $CD34^+$ MNC fraction was directly isolated with superparamagnetic microbead selection using high-gradient magnetic field and miniMACS column (Miltenyi Biotech, Gladbach, Germany). The efficiency of the purification was verified by flow cytometry counterstaining with α-CD34-phycoerythrin (PE; HPCA-2; Becton-Dickinson, San Jose, CA, USA) antibody. In the

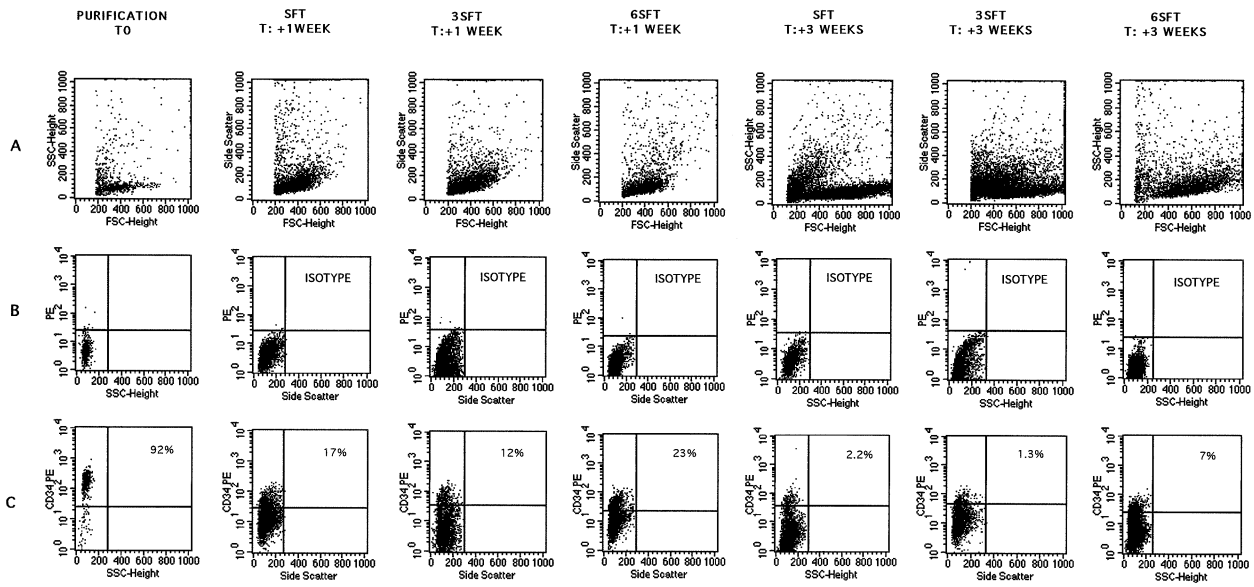

Figure 1. Representative FACS profile of CD34 antigen expression before and during ex vivo expansion of BM CD34-enriched cells. Analysis of CD34 antigen expression on bone marrow $CD34^+$ cells at start of cultures (T_0) and at weeks 1 and 3 of ex vivo expansion in stroma-free cultures supplemented with different growth factor combinations as described in Materials and methods. **(A)** FACS analyses of the population at the start of the culture and at weeks 1 and 3 based upon forward and side scatter. **(B)** Isotype of cultured cells. **(C)** FACS analyses of CD34 expression; the numbers in the upper right quadrants show the percentage of $CD34^+$ cells after the purification and during the expansion cultures.

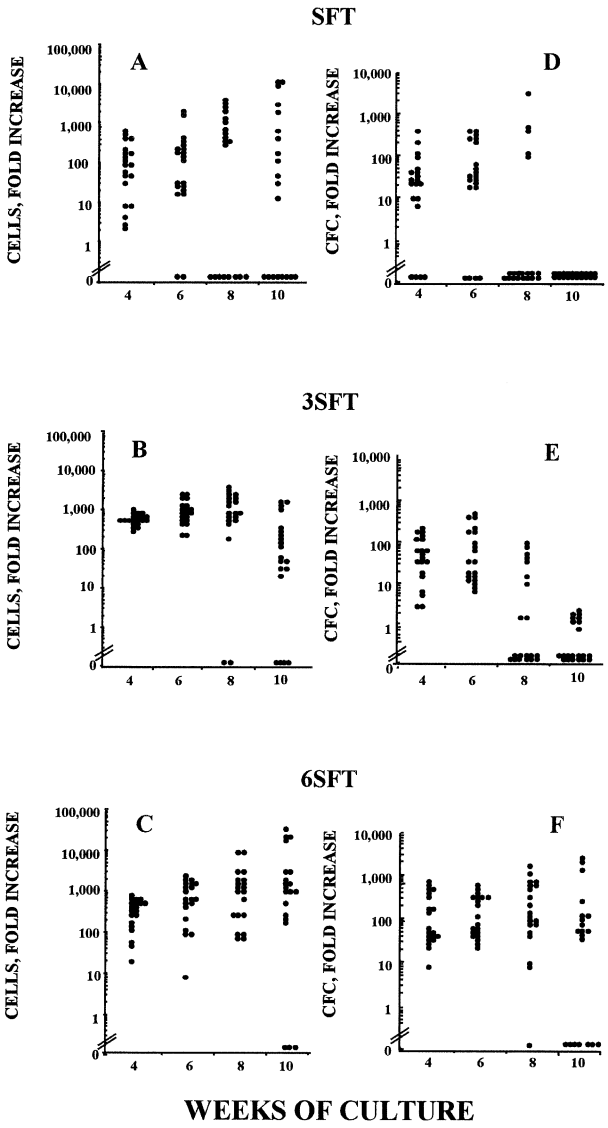

Figure 2. Hematopoietic cell production and clonogenic progenitor output during stroma-free long-term cultures of BM $CD34^+$ cells. Expansion of hematopoietic cells **(A,B,C)** and of hematopoietic progenitors **(D,E,F)** in cultures stimulated by SCF, FL, and TPO (SFT); IL-3, SCF, FL, and TPO (3SFT); and IL-6, SCF, FL, and TPO (6SFT). The starting population was represented by 2×10^4 BM $CD34^+$ cells. Each dot (•) represents one individual sample. Results are expressed as fold increase (compared with the starting T_0 population). Cells and CFC were assessed weekly from triplicate wells. To calculate the fold increase, the cumulative number of cells and of CFC (calculated as described in Materials and methods) was divided by the input cell and CFC number.

cell fraction containing purified cells, $CD34^+$ cells ranged from 87 to 92%.

Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human (rh) SCF was a generous gift from Amgen (Thousand Oaks, CA, USA); rh granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and rhIL3 were from Sandoz (Basel, Switzerland); rhIL6 from PeproTech (Rocky Hill, NJ, USA); rh erythropoietin (rhEPO; EPREX) was from Cilag (Milan, Italy); rhFL was kindly provided by S.D. Lyman (Immunex Corp, Seattle, WA, USA), rhTPO was a generous gift from Kirin (Kirin Brewery, Tokyo, Japan).

Stroma-free liquid cultures

Stroma-free expansion cultures were performed as follows. 1) Twenty thousand $CD34^+$ BM or MPB cells were cultured at 37°C in flat-bottomed 24-well plates in 1 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT, USA) with different growth factors: SCF (50 ng/mL), FL (50 ng/mL), TPO (10 ng/mL), IL-6 (10 ng/mL), and IL-3 (10 ng/mL), as a 3-factor (SCF + FL + TPO = SFT) or a 4-factor (IL-6 + SCF + FL + TPO = 6SFT; IL-3 + SCF + FL + TPO = 3SFT) combination, were added to each series of microwells twice a week [12,26]. At initiation of the culture, the number of CFC present in 1 mL of a single well was determined by semisolid assays. Every week all the wells were demi-depopulated, after vigorous pipetting and resuspension of the cells, by removing one half of the culture volume, which was replaced with fresh medium and growth factors. Cells of the harvested media were counted and suitable aliquots of the cell suspension were assayed for colony assays. 2) To obtain enough cells to perform in vivo transplantation experiments, 5×10^4 $CD34^+$ BM or MPB cells/mL in the presence of SFT, 3SFT, and 6SFT (added twice a week) and 10% FCS were deposited on the bottom of tissue culture T_{25} or T_{75} flasks in duplicate or triplicate. Every week, instead of dividing in half, the cell suspension volume was increased by adding fresh medium and growth factors in order to maintain the cell density at 5×10^5/mL [26].

Animals

NOD/LtSz scid/scid (NOD/SCID) mice were supplied by Jackson Laboratories (Bar Harbor, ME, USA) and maintained at the animal facilities of C.I.O.S. (Torino) and at the Weizmann Institute for Science (Rehovot, Israel). All animals were handled, according to Institutional regulations, under sterile conditions and maintained in microisolator cages. Mice to be transplanted were given total-body irradiation at 6 to 8 weeks of age with 350 to 375 cGy from a ^{137}Cs source, and then after 24 hours were given a single intravenous injection of: 1) human BM or MPB $CD34^+$ cells; 2) cells harvested from expansion cultures as described. Mice were sacrificed at 6 to 8 weeks posttransplant to assess the number and types of human cells detectable in murine BM harvested from femurs and tibias.

Flow cytometric detection of human cells in murine BM

Bone marrow cells were flushed from the femurs and tibias of each mouse to be assessed using a syringe and a 26-gauge needle. Staining was performed as previously described [26]. Flow cytometric analysis was performed using a FACSVantage SE (Becton-Dickinson). At least 20,000 events were acquired for each analysis. Successful engraftment by human hemopoietic cells was defined by the presence of at least 0.1% of human $CD45^+$, $CD71^+$, and GpA^+ cells in the BM of NOD/SCID mice 6 to 8 weeks after transplantation.

DNA extraction and analysis of human cell engraftment

High-molecular-weight DNA was extracted from the BM of transplanted mice by phenol-chloroform extraction using standard protocols. Ten μg of DNA was digested with EcoRI and separated by

Table 1. Bone marrow and mobilized peripheral blood LTC-IC expansion in liquid cultures containing different growth factor combinations*

	Growth factors added	Week 1	Week 2	Week 3	Week 4	Week 5
BM	FL+SCF+TPO	2.9 ± 2.1‡ (6/9)†)	3.57 ± 3.6 (6/9)	1.7 ± 1.5 (6/9)	1 ± 0.3 (6/9)	0.6 ± 0.1 (6/9)
	IL3+SCF+FL+TPO	3.5 ± 1.5‖ (9/9)	4.9 ± 5.8 (7/9)	2.4 ± 1.3‡ (7/9)	0.5 ± 0.2 (7/9)	0‖
	IL6+SCF+FL+TPO	3.9 ± 1.1‖ (9/9)	3.6 ± 1.9‡ (9/9)	3.9 ± 1.3‖ (9/9)	7.4 ± 3.1‖ (9/9)	11.3 ± 5.6‖ (8/9)
MPB	FL+SCF+TPO	5.5 ± 3.6‡	1.3 ± 0.4 (4/7)	2.6 ± 1.7 (3/7)	3.3 ± 2.5‡ (3/7)	0.6 ± 0.3 (3/7)
	IL3+SCF+FL+TPO	4.4 ± 3.7‡ (7/7)	6.6 ± 2.7‖ (6/7)	2.9 ± 1.5‡ (7/7)	3.1 ± 1.8‡ (6/7)	0.6 ± 0.5 (2/7)
	IL6+SCF+FL+TPO	4.1 ± 1.9‡ (7/7)	5.1 ± 2.4‖ (7/7)	6.2 ± 2.4‖ (7/7)	8.9 ± 4.3‖ (7/7)	8 ± 3.6‖ (6/6)

*Mean ± SD of fold increase of LTC-IC numbers (LTC-IC generated by culturing limiting dilutions of week-1-to-week-5-expanded cells/LTC-ICs generated by culturing limiting dilutions of input $CD34^+$ cells on preirradiated stroma layers for 5 weeks).

†number of cases in which LTC-IC were observed/total cases evaluated for LTC-IC generation.

‡$p < 0.05$ as compared to the value on day 0; ‖$p < 0.001$ as compared to the value on day 0.

agarose gel electrophoresis, transferred onto a positively charged nylon membrane, and probed with a labeled human chromosome 17-specific α-satellite probe (p17H8) (limit of detection, approximately 0.05% human DNA). To quantify the level of human cell engraftment, the intensity of the characteristic 2.7-kb band in samples was compared with those of human: mouse DNA control mixture (0%, 1%, 5%, 10%, 20%, 50% human DNA) as previously described [23,26]. The level of human engraftment was quantified by densitometric analysis with the use of Phoretix 1D Standard software (Phoretix Inc., Newcastle-Upon-Tyne, UK) by comparing the characteristic 2.7-kb band with human to mouse DNA mixture controls (limit of detection, approximately 0.1% human DNA).

Hematopoietic cell cultures

Assays for granulopoietic, erythroid, megakaryocytic, and multilineage granulocyte-erythroid-macrophage-megakaryocyte colony-forming units (CFU-GM, BFU-E, CFU-Mk, and CFU-GEMM respectively) were performed as previously described [12].

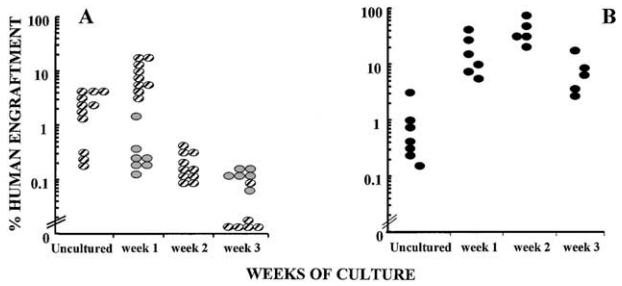

Figure 3. Engraftment in the BM of NOD/SCID mice of human BM cells at start of cultures and of their progeny after 1 to 4 weeks of expansion in the presence of SFT and 3SFT **(A)** or 6SFT **(B)**. The level of human engraftment was evaluated by flow cytometry as the percent of human $CD45^+$, $CD71^+$, and GpA^+ cells within the total, unseparated BM cells in individual NOD/SCID mice. **(A)** Each animal was injected with $\geq 3 \times 10^5$ uncultured $CD34^+$ cells or their equivalents expanded with SFT (gray circles) or 3SFT (striped circles) harvested at the indicated time points. **(B)** Each animal was injected with $\geq 3 \times 10^5$ (black circles) uncultured $CD34^+$ cells or their equivalents expanded with 6SFT harvested at the indicated time points. Each circle represents an individual mouse.

LTC-IC

The LTC-IC content of cell suspension was determined by limiting dilution assays as previously described [12]. Briefly, different initial cell concentrations (from 10 to 10^3) or suitable aliquots of expansion cultures were seeded onto preirradiated stroma layers in 24-well plates for 6 weeks and then the numbers of CFC generated by methylcellulose cultures were enumerated as described 14 to 21 days later.

Statistical analysis

The frequency of SRC in a population of cells was determined by injecting cohorts of mice with different dilutions of cells. After 6 to 8 weeks the BM was analyzed and a mouse was scored as positive if both myeloid and lymphoid lineages were detectable by FACS (>0.1% $CD45^+$, $CD71^+$, and GpA^+ cells). The frequency of SRC was then calculated from the proportions of negative mice in each cohort, using the L-Calc software program (StemCell Technologies, Vancouver, BC, Canada), which uses Poisson statistics and the method of maximum likelihood.

Differences between CFC outputs elicited by the different growth factor combinations in LTC were calculated by the paired *t*-test.

Results

Normal bone marrow cells

Twenty normal BM samples from different donors were studied. Three-factor combination SFT and four-factor combinations, 6SFT and 3SFT, were tested (Fig. 1).

In vitro expansion potential. At week 8, BM samples cultured with SFT and 3SFT showed a good proliferative response in respectively 8/20 (fold increase range: 300–5000) and 18/20 cases (fold increase range: 200–4000) (Fig. 2A and B). 6SFT was able to maintain a high degree of cell proliferation in all cases (range: 90–10,000) (Fig. 2C).

After six weeks of culture all samples cultured with 3SFT and 6SFT were still capable of a significant CFC production (Fig. 2E and F), whereas 4/20 of the SFT-stimulated samples no longer contained detectable CFC (Fig. 2D). At week 10 the vast majority of samples grown with 6SFT only,

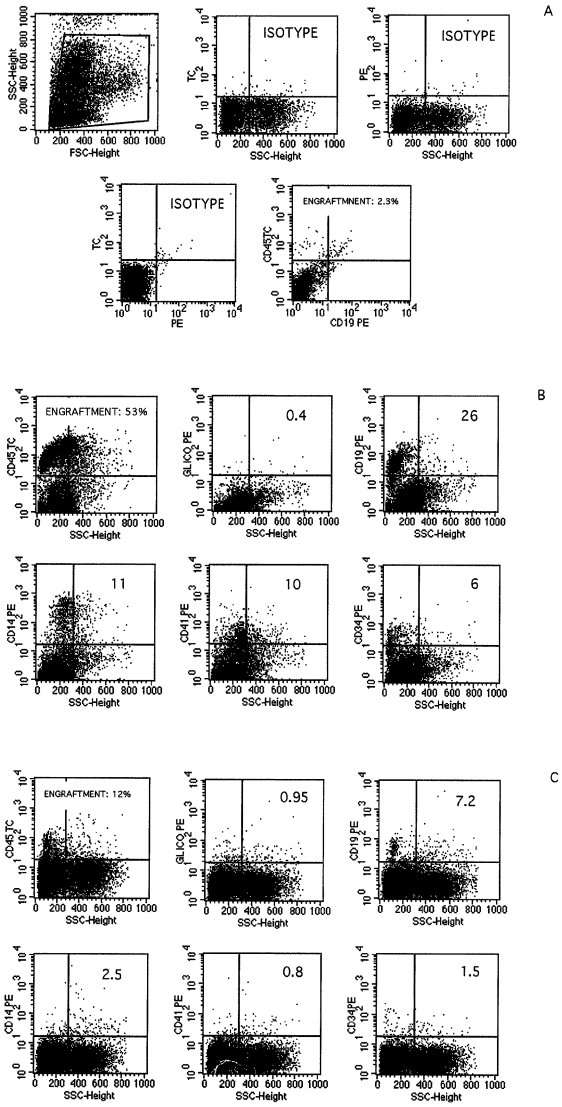

Figure 4. Human BM cells cultured for 3 weeks in 6SFT-expansion cultures produce multilineage engraftment in primary and secondary NOD/SCID mice. **(A)** Isoptype controls and example of a NOD/SCID mouse transplanted 8 weeks previously with 5×10^5 unmanipulated BM $CD34^+$ cells. CD45/CD19 analysis was performed on total BM cells. **(B)** Representative FACS profile of marrow cells from a primary NOD/SCID mouse transplanted 8 weeks previously with the progeny of 5×10^5 $CD34^+$ BM cells expanded for 3 weeks with 6SFT. Total engraftment ($CD45^+$ cells and GpA^+ cells) and lineage marker analysis were performed on total marrow cells. **(C)** Secondary engraftment was evaluated in the BM of a NOD/SCID mouse injected 8 weeks previously with 20×10^6 unseparated BM cells of primary mouse.

still contained variable but significant numbers of CFC (Fig. 2D–F).

Expansion cultures of BM cells contained detectable LTC-IC for up to 5 weeks (Table 1). SFT-expanded BM cells generated an increased number of LTC-IC in some, but not all, cases (fold increase of 2.9 and 3.6 at weeks 1 and 2 respectively), but from then on, LTC-IC number decreased. LTC-IC expansion was detected in 3SFT-expanded BM cells: it peaked at week 2 (4.9 ± 5.8-fold), but from then on, it decreased. Kinetics of LTC-IC expansion in 6SFT-expanded BM cells were different: the LTC-IC number increased at week 1 and kept increasing with time (7.4-fold at week 4 and 11.3-fold at week 5).

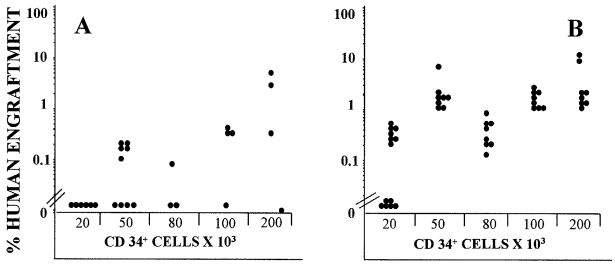

Figure 5. Human engraftment evaluation. Summary of the level of human engraftment in the BM of 66 mice transplanted 6–8 weeks previously with reducing doses of unmanipulated BM $CD34^-$ cells (**A**) or their 1 to 3 week expansion equivalents (**B**) cultured with 6SFT. The level of human engraftment in the mouse BM was evaluated by FACS analysis (a positive mouse was defined by the presence of $\geq 0.1\%$ human $CD45^+$, $CD47^+$ and GpA^+ cells on total BM cells), confirmed by DNA analysis as described in materials and methods.

Long-term in vivo repopulation capacity: primary transplants. Unmanipulated $CD34^+$ BM cells were inoculated into NOD/SCID mice. Human engraftment was observed in all animals that had received more than 300,000 $CD34^+$ cells (Figs. 3 and 4A). Mice receiving less than 300,000 $CD34^+$ cells showed no or very little human engraftment (data not shown). Transplantation of week-1 to week-3 expansion equivalents obtained by culturing same numbers of initial cells with 6SFT led to the detection of higher levels of engraftment in all transplanted animals (Fig. 3B). Human cells that repopulated the murine marrow belonged to all hemopoietic lineages (Fig. 4B). Human colonies were generated by $CD34^+$ cells present in the BM of engrafted mice (not shown). Cells cultured with 3SFT rapidly lost their long-term in vivo repopulation ability: while week-1-expanded cells were capable of high levels of engraftment, week-2- and week-3-expanded cells showed little or no engraftment (Fig. 3A).

By contrast, low levels of engraftment were detected upon injection of cells expanded for 1 week with SFT and very little engraftment could be detected upon injection of week-3 expanded cells (Fig. 3A).

Limiting dilution assays (LDA) on BM $CD34^+$ cells, at start of cultures and after 1 to 3 weeks of 6SFT-induced expansion, were performed to quantitate the extent of SRC expansion (Fig. 5).

Human engraftment was not detectable in all mice transplanted with 20,000 uncultured $CD34^+$ cells (Fig. 5A), while the expansion equivalent of 20,000 $CD34^+$ cells engrafted 7 of 13 mice (Fig. 5B). Also, the expansion equivalent of 50,000 $CD34^+$ cells engrafted all of the mice (Fig. 5B). The

Table 2. Primary and secondary NOD/SCID mouse repopulation ability of week-3-expanded BM and MPB cells

CD34$^+$/SOURCE	Starting CD34$^+$ cells	Primary engraftment %*	Multilineage engraftment†	Secondary engraftment %‡	Multilineage engraftment†	CFC5 $^\parallel$
MPB	0.5×10^6	16.3	+	11	+	39.7 ± 5.1
		22.7	+	6.3	+	18.3 ± 6.7
	0.6×10^6	36.7	+	2.6	+	3 ± 2.6
		23.4	+	7.9	+	24.3 ± 5.7
BM	3.5×10^5	21.6	+	6.25	+	5.3 ± 3.5
		27.4	+	9.15	+	43 ± 13.4

CD34$^+$cells were cultured at 5×10^4/mL in T flasks as described for 3 weeks in the presence of 6SFT in duplicate. At the end of cultures all cells were harvested, washed, and injected in 6- to 8-week-old NOD/SCID mice.

*Each value represents the percentage of human CD45$^+$, CD71$^+$, and GpA$^+$ cells detected in the BM of each individual mouse.

†Multilineage engraftment was defined by the presence of CD19$^+$, CD34$^+$, CD33$^+$, CD14$^+$, and CD41$^+$ cells within the CD45 gate.

‡15 to 20 $\times 10^6$ unseparated BM cells of a primary mouse were injected into sublethally irradiated NOD/SCID recipients, which were sacrificed 6 weeks later. Engraftment was evaluated on BM cells of the sacrificed animals with the same parameters used for primary engraftment.

$^\parallel$Mean ± SD of the CFC number/1 $\times 10^6$ total BM cells of the secondary transplanted mice seeded in triplicate dishes for semisolid assays. Recombinant human IL-3, GM-CSF, SCF, and EPO were added at the start of cultures. Dishes were scored after 14 days of incubation, after staining with human specific MoAb. Only fluorescent colonies were considered of human origin.

frequency of SRC in unmanipulated and in 6SFT-expanded cells was found to be 1 in 126,588 (95% confidence limits [CL] 1 in 67,361 to 1 in 237,588) and 1 in 19,005 (95% CL 1 in 11,521 to 1 in 31,350) respectively; expansion was over sixfold. The value of χ^2 in all cases was not statistically significant ($p > 0.05$), demonstrating internal consistency in our assay and allowing pooling of the data.

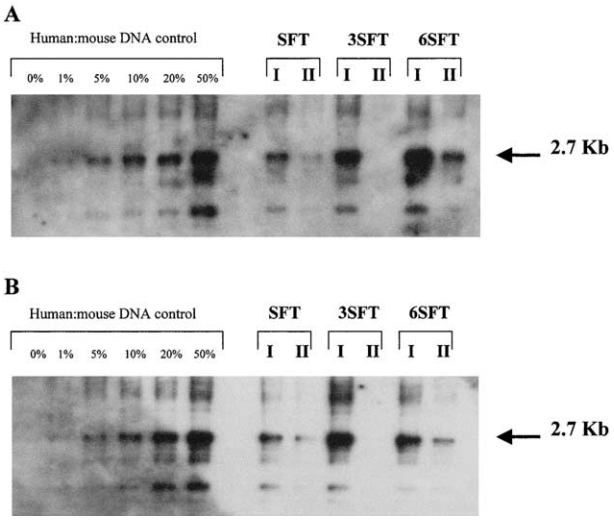

Figure 6. Southern blot analysis. Representative Southern blot analysis of a primary NOD/SCID mouse transplanted with 5×10^5 1-week SFT- or 3STF-expanded BM or MPB CD34$^+$ cells and a NOD/SCID mouse transplanted with 5×10^5 3-week 6SFT-expanded BM or MPB CD34$^+$ cells. The BM from the primary (I) mice was injected in distinct secondary (II) sublethally irradiated NOD/SCID mice. BM of primary and secondary NOD/SCID mice injected with 6SFT-expanded CD34$^+$ cells were the same utilized for FACS analysis reported in Figures 4 and 9. DNA was extracted from the murine BM at week 8 after transplant and hybridized with a human chromosome 17-specific α-satellite probe. Human:mouse controls are given as percent human DNA. r = 0.96 and 0.95 respectively for panel A and for panel B standard.

Long-term in vivo repopulation capacity: secondary transplants. To assess whether expanded cells retained long-term repopulation ability, unseparated BM cells of the week-6 or week-8 engrafted animals, transplanted with the progeny of more than 300,000 CD34$^+$ cells ex vivo expanded, were injected into secondary sublethally irradiated recipients (Figs. 4 and 6). Human engraftment (either by fluorescein-activated cell sorting [FACS] or by human chromosome 17 α-satellite quantitative DNA analysis) was consistently and reproducibly detected in all mice injected with the BM of primary mice previously transplanted with week-3 6SFT-expanded cells, but not with week-1 SFT and 3SFT-expanded cells. In secondary engrafted mice erythroid, myeloid, and lymphoid lineages were represented (Table 2, Fig. 4C).

Mobilized peripheral blood cells

MPB samples from 12 different normal individuals who received G-CSF for stem cell donation were studied. Culture conditions and growth factor combinations were the same as described for BM expansion (data not shown).

In vitro expansion potential. After 6 weeks of culture, cell output induced by SFT, 3SFT, and 6SFT showed respective fold-increase range of 2–1800, 80–1900, and 110–2214 (Fig. 7A–C). At week 8, SFT sustained cell expansion in 10 of 12 cases, 3SFT in 11 of 12 cases, and 6SFT in all 12 cases (Fig. 7A–C). After 10 weeks of culture, the combinations SFT and 3SFT no longer supported the growth in four cases (Fig. 7A and B), whereas 6SFT-stimulated suspension cultures continued proliferating for a few additional weeks (not shown).

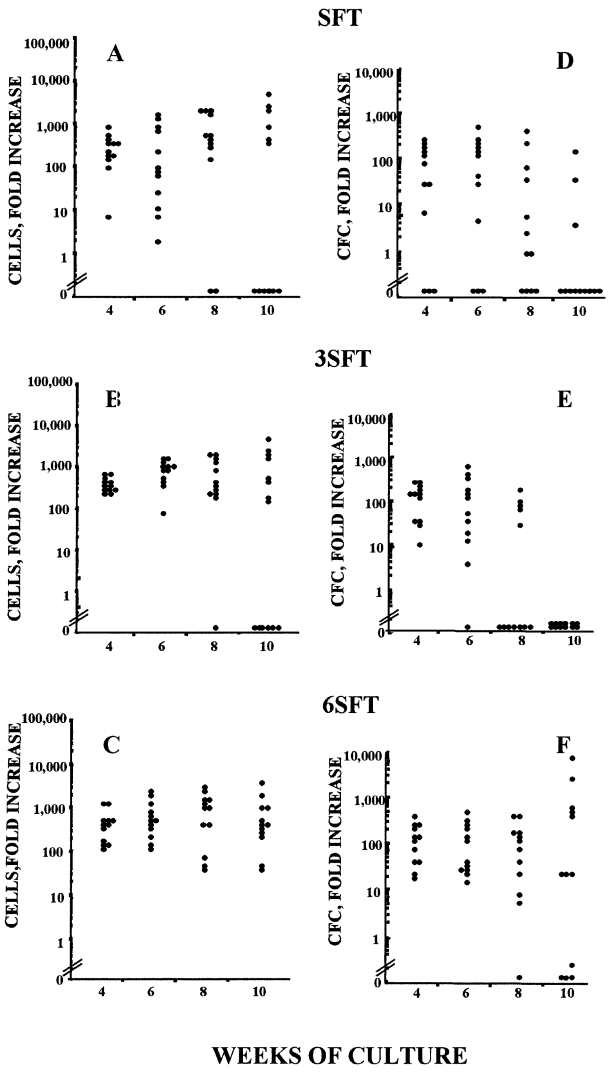

Figure 7. Hematopoietic cell production and clonogenic progenitor output during stroma-free long-term cultures of MPB CD34$^+$ cells. Expansion of hematopoietic cells **(A,B,C)** and of hematopoietic progenitors **(D,E,F)** in cultures stimulated by SCF, FL, and TPO (SFT); IL-3, SCF, FL, and TPO (3SFT); and IL-6, SCF, FL, and TPO (6SFT). The starting population was represented by 2×10^4 MPB CD34$^+$ cells. Each dot (•) represents one individual sample. Results are expressed as fold increase (compared with the starting population). Cells and CFC were assessed weekly from triplicate wells. To calculate the fold increase, the cumulative number of cells and of CFC (calculated as described in Materials and methods) was divided by the input cell and CFC number.

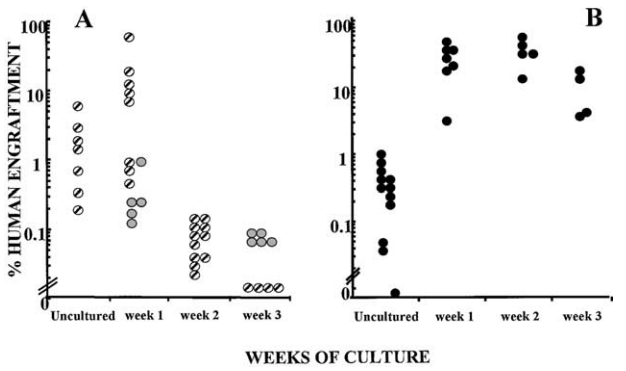

Figure 8. Engraftment in the BM of NOD/SCID mice of human MPB cells at start of cultures and of their progeny after 1 to 3 weeks of expansion in the presence of 3SFT **(A)** or 6SFT **(B)**. The level of human engraftment was evaluated by flow cytometry as the percent of human CD45$^+$, CD71$^+$, and GpA$^+$ cells within the total, unseparated BM cells in individual NOD/SCID mice. **(A)** Each animal was injected with $\geq 3 \times 10^5$ uncultured CD34$^+$ cells or their equivalents expanded with SFT (gray circles) or 3SFT (striped circles) harvested at the indicated time points. **(B)** Each animal was injected with $\geq 3 \times 10^5$ (black circles) uncultured CD34$^+$ cells or their progeny cultured with 6SFT harvested at the indicated time points. Engraftment was assessed as explained in Figure 3.

At weeks 8 and 10 an increasing number of cases did not contain assessable CFC, if cultured in the presence of SFT and 3SFT (Fig. 7D and E). By contrast, 6SFT still sustained a prolonged generation of CFC in 11 of 12 cases tested at week 8 and in 9 of 12 cases at week 10 (Fig. 7F).

Kinetics and pattern of LTC-IC expansion of MPB cells were very similar to those of BM (Table 1). SFT-expanded cells contained assessable LTC-IC in 4 of 7 cases after 1-week culture (5.5-fold increase). Conversely, LTC-IC expansion was detected in all samples expanded with 3SFT and 6SFT. In the former culture condition, LTC-IC number peaked at week 1 and then slowly decreased; in the latter culture condition LTC-IC number increased from 4.1-fold at week 1 to eightfold at week 5.

Long-term in vivo repopulation capacity: primary transplants. Unmanipulated CD34$^+$ MPB cells were injected into sublethally irradiated NOD/SCID mice. Undetectable or low levels of human engraftment were achieved upon injection of less than 300,000 unmanipulated CD34$^+$ cells (data not shown). Injection of higher numbers of CD34$^+$ cells led to the detection of higher levels of human engraftment (Figs. 8 and 9A). Transplantation of week-1 to week-3 expansion equivalents obtained by culturing same numbers of initial cells with 6SFT led to the detection of higher levels of engraftment in all transplanted animals (Fig. 8B). Once again, human cells that repopulated the murine marrow belonged to all hemopoietic lineages (Fig. 9B). Human colonies were generated by CD34$^+$ cells present in the BM of engrafted mice (not shown). By contrast, cells cultured ex vivo with 3SFT rapidly lost their long-term in vivo repopulation ability: high levels of engraftment were detected upon injection of cells expanded for 1 week, but no or very little engraftment could be detected upon injection of week-2- and week-3-expanded cells (Fig. 8A).

Week-1 SFT-cultured cells could sustain only a low level of engraftment that was even lower with week-3-expanded cells (Fig. 8A).

Limiting dilution assays were performed to quantitate the extent of SRC expansion (Fig. 10). All mice inoculated with 20,000 unmanipulated MPB CD34$^+$ cells did not show human engraftment (Fig. 10A). By contrast, 7 of 8 mice trans-

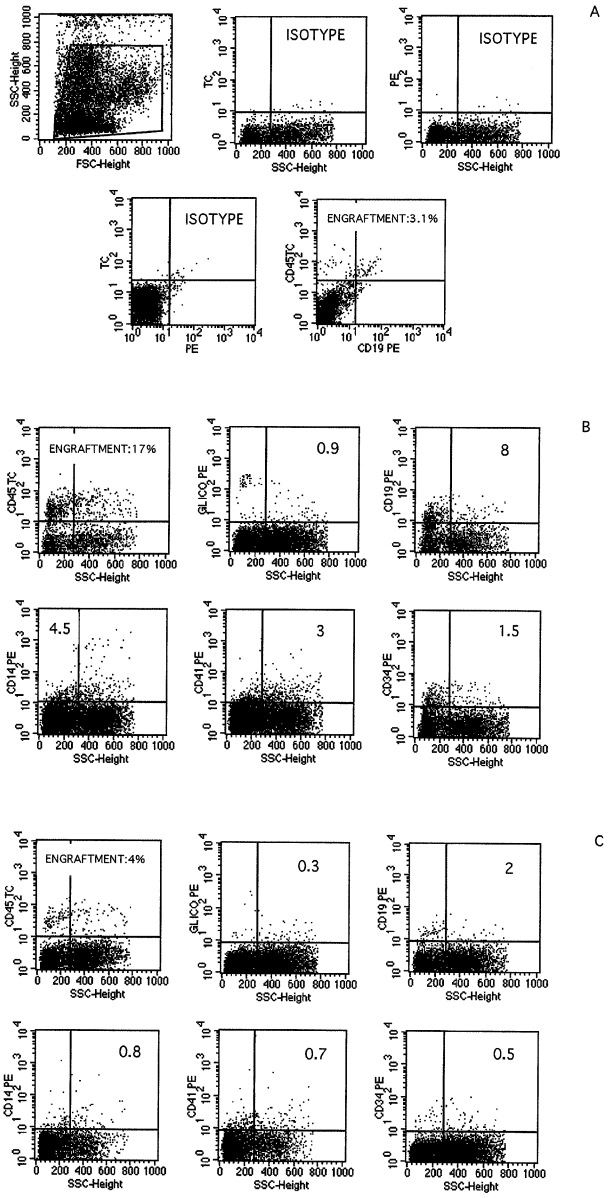

Figure 9. Human MPB cells cultured for 3 weeks in 6SFT expansion cultures produce multilineage engraftment in primary and secondary NOD/SCID mice. **(A)** Isotype controls and example of a mouse transplanted 8 weeks previously with 5×10^5 MPB $CD34^+$ cells. CD45/CD19 analysis was performed on total BM cells. Multilineage engraftment ability of primary **(B)** and secondary **(C)** NOD/SCID mice transplanted with week-3 6SFT-expanded MPB cells. Analysis of lineage markers was evaluated as in Figure 4.

planted with the expansion equivalent of 20,000 6SFT-stimulated $CD34^+$ cells possessed detectable human cells in their BM (Fig. 10B). The frequency of SRC in unmanipulated MPB $CD34^+$ cells was calculated to be 1 in 93,193 (95% CL 1 in 50,212 to 1 in 172,963), while that of 1- to 3-week-expanded MPB cells was 1 in 15,664 (95% CL 1 in 8,116 to 1 in 30,232); expansion was about sixfold. The value of χ^2 in all cases was not statistically significant ($p > 0.05$).

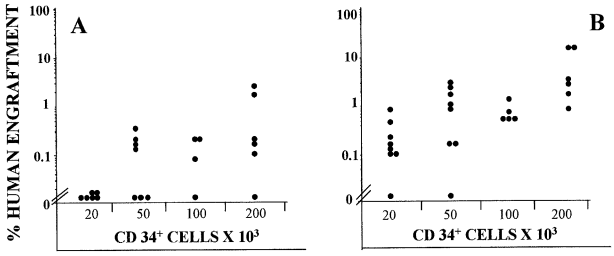

Figure 10. Human engraftment evaluation. Summary of the level of human engraftment in the MPB of 48 mice transplanted 6–8 weeks previously with reducing doses of unmanipulated MPB $CD34^+$ cells **(A)** or their 1- to 3-week expansion equivalents **(B)** cultured with 6SFT. Engraftment was evaluated as explained in Figure 5.

Long-term in vivo repopulation capacity: secondary transplants. The ability of expanded cells to also repopulate secondary recipients was evaluated by injecting secondary NOD/SCID mice with unseparated cells of BM primary recipients, transplanted with the progeny of more than 300,000 MPB $CD34^+$ cells ex vivo expanded. As represented in Figures 6 and 9C and Table 2, injection of 15 to 20×10^6 unseparated BM cells of primary mice transplanted with human MPB cells expanded with 6SFT, resulted in the secondary engraftment of human cells in all of the injected mice. Mice transplanted with the BM of primary mice that had been inoculated with SFT and 3SFT week-1-expanded cells failed to repopulate secondary mice (Fig. 6).

Discussion

In this work clonal cultures, LTC-IC, and SRC assays were employed in an attempt to ascertain whether human primitive BM and MPB cells of adult normal individuals can be cultured ex vivo for long periods of time, generate high numbers of committed progenitors, and maintain or, if possible, improve their long-term in vivo repopulating ability as already demonstrated for CB in the NOD/SCID mouse in vivo assay [12,15,26,30–32]. Indeed, human primitive long-term in vivo repopulating cells can be more reliably detected by a functional in vivo assay based on their ability to completely and durably repopulate the BM of sublethally irradiated NOD/SCID mice [10,21–26,33–37].

The current studies show that the SRC activity is retained by BM and MPB cells that have been cultured for up to three weeks with 6SFT, demonstrating that this combination promotes proliferation and potential expansion of human long-term in vivo repopulating cells. By contrast, the in vivo repopulating ability is maintained for only one week when the same cells are cultured with 3SFT and for 1 and 3 weeks in the presence of SFT, however without improving the level of engraftment compared to the unmanipulated cells.

Expanded cells showed enhanced long-term reconstituting ability when compared with unmanipulated $CD34^+$ cells.

Because expanded BM and MPB cells contain both $CD34^+$ cells and more differentiated $CD34^-$ progeny, we cannot rule out the possibility that the enhanced BM reconstituting ability of expanded cells might be due to the presence of known or unknown accessory cells generated during the expansion procedure. It has been shown that, whereas CB $CD34^+CD38^-$ cells are capable of long-lasting marrow reconstitution, and $CD34^+CD38^+$ of transient engraftment, the simultaneous presence of both populations leads to a much higher and long-lasting BM repopulation [38,39].

The present in vivo studies were performed on cells cultured ex vivo for 1 to 3 weeks, demonstrating that adult $CD34^+$ cells maintain and actually gain an even greater NOD/SCID mouse repopulation ability after 3 weeks of in vitro cultures.

The possibility that SRC were indeed expanded in ex vivo cultures was tested: quantitation of SRC in initial and in 1- to 3-week-expanded cells was obtained by limiting dilution transplantation assays. Our data suggest that a sixfold expansion of SRC occurs in 1- to 3-week-expansion cultures of both BM and MPB grown in the presence of 6SFT.

The long-term repopulation ability of stem cells can be determined by their ability to also repopulate secondary transplanted recipients with both myeloid and lymphoid cells. Successful secondary engraftment was observed with 6SFT-espanded cells; however, negligible or no secondary engraftment was obtained respectively with SFT- or 3SFT-cultured cells, particularly demonstrating the transient nature of the latter expansion.

The finding that $CD34^+$ cells expanded for 3 weeks can repopulate the BM of primary and secondary mice, never reported so far, shows that human BM and MPB $CD34^+$ cells can be cultured in vitro for up to 3 weeks, maintain or even increase their full, long-term in vivo repopulating ability, and preserve high proliferative and multilineage differentiation potential.

Variable and contrasting effects of IL-3 on HSC expansion and on their durable in vivo engraftment have been reported: while IL-3 did not seem to negatively affect the long-term repopulating ability of human HSCs [16], this cytokine was recently demonstrated to exhibit an inhibitory effect on the expansion of human long-term repopulating cells in a NOD/SCID mouse model [31,32]. As IL-3 could stimulate the increase of mature blood cells and HPCs, it was concluded that IL-3 might consume human HSCs by speeding up their differentiation. The culture conditions reported, in particular growth factor combination, were similar to ours, suggesting that IL-3 may be detrimental to stem cell self-renewal when this is triggered by some specific growth factors or growth factor combinations.

In conclusion, 6SFT-expanded cells not only fully retain their ability to completely and long-term repopulate the BM of myeloablated recipients, but are also capable of secondary hemopoietic reconstitution. One-week 3SFT-expanded cells are capable to completely engraft the BM of NOD/SCID mice; however, they cannot repopulate secondary recipients; also, week-2-expanded cells do not even retain primary reconstitution ability. These observations could be regarded as an indication that these cells spent all their proliferation potential differentiating, rather than self-renewing.

Although the "expansion potential" [the ability to generate progressively higher numbers of cells and CFCs for prolonged periods of time (up to 10 weeks for BM and MPB, compared to more than 30 weeks for CB] and the magnitude of expansion (a few thousand-fold in adults compared to million-fold for CB) of adult stem cells is lower than that of CB stem cells, both in vitro and in vivo data support the conception that also these two adult stem cell sources can be cultured in vitro and generate progeny of cells at different maturation levels, including more primitive long-term repopulating cells.

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