Clonal analysis of thymus-repopulating cells presents direct evidence for self-renewal division of human hematopoietic stem cells

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To elucidate the in vivo kinetics of human hematopoietic stem cells (HSCs), CD34$^+$CD38$^-$ cells were infected with lentivirus vector and transplanted into immunodeficient mice. We analyzed the multilineage differentiation and self-renewal abilities of individual thymus-repopulating clones in primary recipients, and their descending clones in paired secondary recipients, by tracing lentivirus gene integration sites in each lymphomyeloid progeny using a linear amplification-mediated polymerase chain reaction (PCR) strategy. Our clonal analysis revealed that a single human thymus-repopulating cell had the ability to produce lymphoid and myeloid lineage cells in the primary recipient and each secondary recipient, indicating that individual human HSCs expand clonally by self-renewal division. Furthermore, we found that the proportion of HSC clones present in the CD34$^+$ cell population decreased as HSCs replicated during extensive repopulation and also as the differentiation capacity of the HSC clones became limited. This indicates the restriction of the ability of individual HSCs despite the expansion of total HSC population. We also demonstrated that the extensive self-renewal potential was confined in the relatively small proportion of HSC clones. We conclude that our clonal tracking studies clearly demonstrated that heterogeneity in the self-renewal capacity of HSC clones underlies the differences in clonal longevity in the CD34$^+$ stem cell pool. (Blood. 2006;108:2446-2454)

Introduction

Self-renewal and multilineage differentiation are the 2 fundamental abilities that define hematopoietic stem cells (HSCs) and distinguish them from progenitors. Severe combined immunodeficient mouse (SCID)–repopulating cells (SRCs), originally identified by their ability to reconstitute hematopoiesis in nonobese diabetic (NOD)/SCID mice, are thought to represent human HSCs that are useful clinically to repopulate human recipients.[1-3] Unlike murine HSCs that have been purified and analyzed at the single-cell level,[4] viral gene-marking is the only strategy for the in vivo clonal analysis of human HSCs.[5] Using this approach, several studies documented heterogeneity among SRC clones and implied that some clones have the ability to differentiate into B-lymphoid and myeloid lineages and to self-renew.[6-12] A major shortcoming of using the NOD/SCID mouse model is a lack of reproducible human T-lymphocyte repopulation. Consequently, the multilineage differentiation capacity of SRCs in NOD/SCID recipients has been assessed by reconstitution of only B-lymphoid and myeloid lineages. Because a close relationship between B-lymphocyte and macrophage differentiation has been indicated,[13,14] current analyses cannot clearly distinguish true HSCs from lineage-restricted progenitors such as B-lymphocyte/macrophage progenitors. As a result, the multilineage differentiation and self-renewal of HSCs represented by a single SRC are yet to be proven.

Along with other investigators, we demonstrated that phenotypically normal and polyclonal human T lymphocytes were reproducibly repopulated from human cord blood CD34$^+$ cells in the NOD/SCID/common γ chain (cγ)–null (NOG) mouse.[15-17] Having this unique environment that permits human thymopoiesis, the NOG recipient serves as an excellent model to study self-renewal, as well as multilineage differentiation, of human HSCs. HSCs can be identified as thymus-repopulating cells and distinguished from short-lived oligopotent or monopotent progenitors. Thymopoiesis requires constant recruitment of progenitors into the thymus, which eventually produces mature T lymphocytes in a relatively short period of time.[18,19] Therefore, to maintain thymopoiesis in recipient mice, transplanted HSCs must divide without loss of thymus-repopulating activity. Several classes of SRCs that differ in their proliferative and self-renewal potential have been reported.[20-22]

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Analyzing the thymus-repopulating activity of these cells provides a unique way to distinguish and identify long-term self-renewing stem cells within the SRCs. Self-renewal of HSCs has been assessed by serial transplantation on the basis that HSCs, which are responsible for multilineage hematopoiesis in primary recipients, are also capable of repeating this process in secondary transplant recipients. Confirmation of the persistence of thymus-repopulating cells with multilineage differentiation ability in the secondary recipient would eliminate the possible contribution of some long-lived progenitors and mature cells and at the same time, provide direct evidence for self-renewal of SRCs.

In this study, we established a novel strategy to analyze both self-renewal and multilineage differentiation of a single human thymus-repopulating SRC clone in NOG recipient mice using linear amplification-mediated polymerase chain reaction (LAM-PCR) that verifies individual genomic virus integration sites by direct sequencing.[23] The identification of specific clones in fluorescent-activated cell sorter (FACS)–sorted lymphomyeloid lineage populations by their unique molecular markers allowed us to assess how individual clones contribute to the specific lineages during long-term hematopoiesis in vivo. We focused on CD4/CD8 double-positive (DP) immature thymocyte populations as a starting point of our clonal analysis of the human HSC ability. Our study presented direct clonal evidence that a single human HSC had the ability to produce lymphoid and myeloid lineage cells. Self-renewal division of multilineage clones resulted in expansion of SRCs. However, this clonal expansion of SRCs leads to the clonal exhaustion of SRCs during long-term hematopoiesis in vivo. It was also indicated that, although most of the SRC clones were destined to loose their self-renewal potential, the relatively small proportion of SRC clones retained extensive self-renewal potential.

Materials and methods

Collection and purification of human CB CD34$^+$CD38$^-$ cells

CB samples were obtained from full-term deliveries according to the institutional guidelines approved by the Tokai University Committee on Clinical Investigation. Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Lymphoprep, 1.077 ± 0.001 g/mL; Nycomed, Oslo, Norway) density gradient centrifugation. CD34$^+$ cell fractions were prepared using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's directions. Column-enriched CD34$^+$ cells were cryopreserved in liquid nitrogen until use. For isolation of CD34$^+$CD38$^-$ cells, pooled CD34$^+$-enriched cells from multiple donors were stained with fluorescein isothiocyanate–conjugated anti-CD34 (581; Coulter/Immunotech, Marseille Cedex, France), and phycoerythrin (PE)–conjugated anti-CD38 (HB7; BD Biosciences, San Jose, CA) monoclonal antibodies (mAbs). Cells were sorted using the FACSVantage flow cytometer (BD Biosciences) equipped with HeNe and argon lasers. CD38$^-$ gate was determined in reference to isotype control. CD34$^+$CD38$^-$ cells, which comprise 5% to 8% of the total CD34$^+$ cell population, were isolated with 97% to 99% (n = 16) purity using FACSVantage (BD Biosciences).

Lentivirus infection

Purified CD34$^+$CD38$^-$ cells were plated on fibronectin CH-296 fragment and incubated with highly concentrated viral supernatant at a multiplicity of infection (MOI) of 50 in serum-free StemPro-34 medium (Invitrogen, Carlsbad, CA) containing cytokines (Takara Shuzo, Tokyo, Japan) for 16 hours. Recombinant human thrombopoietin (50 ng/mL; kindly donated by Kirin Brewery, Tokyo, Japan), stem cell factor (50 ng/mL; donated by Kirin Brewery, Tokyo, Japan), and Flk-2/Flt-3 ligand (50 ng/mL; R&D Systems, Minneapolis, MN) were used. The number of virus integration sites per cell was examined in the following experiment. CD34$^+$ cells were infected with enhanced green fluorescent protein (EGFP) at an MOI of 50 and then plated in methylcellulose. Individual colonies expressing EGFP were picked up and analyzed for integration sites by LAM-PCR. Over the 20 colonies examined, none of the colonies demonstrated multiple bands, confirming that the individual colonies contain a single integration site (data not shown).

Estimation of multilineage differentiation potential of SRCs

NOD/Shi-scid, IL-2Rγc^{null} (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained in the animal facility of the Tokai University School of Medicine in microisolator cages; the animals were fed with autoclaved food and water. Nine- to 20-week-old NOG mice were irradiated with 250 cGy X-rays. The following day, transduced CD34$^+$CD38$^-$ cells (1×10^4 cells) were injected intravenously into the NOG mice. All experiments were approved by the animal care committee of Tokai University. Sixteen to 20 weeks after transplantation, the mice were humanly killed, and bone marrow (BM) cells, splenocytes, and thymocytes were analyzed by flow cytometry. Cells were stained with mAbs to human leukocyte differentiation antigens. Human hematopoietic cells were distinguished from mouse cells by the expression of human CD45. APC-conjugated anti–human CD19 mAb (Coulter/Immunotech), ECD-conjugated anti–human CD8 and CD34 mAbs (all Coulter/Immunotech), and PE-conjugated anti–human CD3, CD4, and CD33 mAbs were used. The efficiency of gene transduction was determined by the percentage of cells expressing EGFP. EGFP-expressing CD45$^+$ human hematopoietic cells were further classified into human stem/progenitor (CD34$^+$), myeloid (CD33$^+$), B-lymphoid (CD19$^+$), and T-lymphoid (CD3$^+$ or CD4$^+$/CD8$^+$) subpopulations and were sorted using a FACSvantage Diva option (BD Biosciences). To eliminate the contamination of lineage-committed cells, CD34$^+$ cells were sorted on CD19$^-$ and CD33$^-$ gate. Sorted cells, confirmed to be lineage$^{-/low}$CD34$^+$, were designated as stem/progenitor cells. Double cell-sorting was performed to ensure greater than 99% cell purity. Representative FACS profiles of sorting purity were demonstrated in Figure S1 (available at the *Blood* website; see the Supplemental Materials link at the top of the online article).

Secondary transplantation

BM cells were obtained from mice that received a transplant with CD34$^+$CD38$^-$ cells at 13 to 19 weeks after transplantation. The BM cells of these primary recipients were divided equally and injected intravenously into 2 sublethally irradiated secondary NOG recipients (1.35×10^7–2.1×10^7 cells per recipient). Thirteen to 19 weeks after transplantation, BM cells and thymus were collected from each secondary recipient and used for flow cytometric analysis and lineage cell sorting as described.

Integration site analysis of lentivirally marked SRCs

LAM-PCR was carried out as described previously[24] with some slight modifications. Genomic DNA samples (100 ng), isolated from each sorted subpopulation, were preamplified for a total of 100 cycles by repeated primer extension using 0.25 pmol vector-specific, 5′-biotinylated primer LTR1 (5′-GAACCCACTGCTTAAGCCTCA-3′) using ProofStart DNA polymerase (2.5U; Qiagen, Hilden, Germany). The biotinylated extension products were collected using streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway), and the second strand was synthesized using Klenow polymerase (2 U; Takara Shuzo) and random primer (Takara Shuzo). To prevent virus-vector sequence contamination, samples were first incubated with SacI endonuclease (5 U; Promega, Madison, WI) for 2 hours at 37°C and then digested with Tsp509I endonuclease (5 U; New England BioLabs, Ipswich, MA) for 2 hours at 65°C. After restriction digestion, 100 pmol of a linker cassette (5′-GTACATATTGTCGTTAGAACGCGTAAT-ACGACTCACTATAGGGAGA-3′) was ligated using a DNA ligation kit (Takara Shuzo) at 16°C overnight. Each ligated sample was amplified using a vector-specific primer, LTR2 (5′-AGCTTGCCTTGAGTGCTTCA-3′),

and a linker cassette primer (5′-GTACATATTGTCGTTAGAACGCGTA-ATACGACTCA-3′), using the following conditions: 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute (30 cycles). Each PCR product was subjected to nested PCR with the internal primers, LTR3 (5′-AGTAGTGTGTGCCCGTCTGT-3′) and LC2 (5′-CGTTAGAACGCGTA-ATACGACTCACTATAGGGAGA-3′), under identical conditions. PCR products were sequenced after cloning into the TOPO TA cloning vector (Invitrogen). The proviral integration sites of DP cells were sequenced, and the sequences were examined for alignment to the human genome using NCBI BlastN (http://www.ncbi.nlm.nih.gov/blast). The verified genomic sequence information of these DP cell integration sites was used to design new primers (all primer sequences used in this study are listed in Tables S1-S3). PCR was performed on each LAM-PCR product using the unique genomic flanking primers in combination with the LTR3 primers.

Estimation of clone size by real-time quantitative PCR (RQ-PCR)

Genomic DNA samples from $CD34^+$ cells of secondary recipients were amplified using multiple displacement amplification reagents (REPLI-g; Qiagen) according to the manufacturer's instructions.[25] Briefly, 10 ng template DNA was mixed with the DNA polymerase and incubated for 16 hours at 30°C. Approximately 50 μg amplified DNA was obtained from each sample. For RQ-PCR, each target DNA was amplified on the same plate with β-globin as the reference using the QuantiTect SYBR Green PCR Master Mix (Qiagen) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The relative clone amounts and range were determined in reference to β-globin. Threshold cycles (C_T) were determined as to fit all samples in logarithmic phase. To ensure the efficiency of amplification and the assay precision, calibration curves for each clone sequence were constructed to have the correlations (r^2) of above 0.95 and the efficiency of greater than 98%. A comparative C_T was used to determine the proportion of $CD34^+$ clones in paired secondary recipients that were derived from the parent primary recipient clone. For each sample, the clone C_T value was normalized using the formula $\Delta C_T = \Delta C_T$ clone $- \Delta C_T$ β-globin. To determine relative clone size, the following formula was used: $\Delta\Delta C_T = \Delta C_T$ clone in CD34+ cells of the one secondary recipient $- \Delta C_T$ clone in CD34+ cells of the other secondary recipient, and the value was calculated by the expression $2^{-\Delta\Delta CT}$. Each reaction was performed at least in triplicate. Amplification conditions were as follows: 95°C for 15 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 60 seconds.

The same primer set described in PCR tracking of LAM-PCR procedure was used to amplify each clone. As an internal control, human hematopoietic cell kinase 1 (*HCK1*) gene and ribosomal DNA (*rDNA*) gene were also amplified. Even amplification of each genomic DNA was confirmed by RQ-PCR using all 3 internal control primers (data not shown).[26] The primers for β-globin gene were forward, 5′-GTGCACCTGACTCCTGAG-GAGA-3′, and reverse, 5′-CCTTGATACCAACCTGCCCAG-3′. Primers for *HCK1* gene were forward, 5′-TATTAGCACCATCCATAGGAGGCTT-3′, and reverse, 5′-GTTAGGGAAAGTGGAGCGGAAG-3′. Primers for *rDNA* gene were forward, 5′-CCATCGAACGTCTGCCCTA-3′, and reverse, 5′-TCACCCGTCGTCACCATG-3′.

Statistical analysis

Data are represented as mean ± SD. The 2-sided *P* value was determined by testing the null hypothesis that the 2 population medians are equal. *P* values less than .05 were considered to be significant.

Results

Multilineage differentiation of gene-marked SRCs

To investigate the multilineage differentiation and self-renewal capacity of individual SRC clones, we introduced recombinant lentiviral vector carrying an EGFP-encoding gene to cord blood $CD34^+CD38^-$ cells which can provide long-term engraftment (more than 12 weeks) and multilineage differentiation,[2] and then we transplanted these cells into sublethally irradiated NOG mice. Multilineage differentiation was determined as the proportion of each hematopoietic lineage within the EGFP-expressing human $CD45^+$ cell population using FACS at 16 to 20 weeks after transplantation (Figure 1). Consistent with our previous results,[16] substantial engraftment, including $CD34^+$ primitive cells, $CD33^+$ myeloid, $CD19^+$ B-lymphoid, $CD3^+$ mature, and DP immature T-lymphoid cells, was observed in the BM, spleen, and thymus of the NOG mice (Table 1). Because the proportion of nontransduced $EGFP^-$ cells within the human graft and the percentage of $EGFP^+$ cells within each lineage were not significantly different (data not shown), these data indicated that EGFP transduction did not affect the differentiation and proliferation capacity of the SRCs.

Multilineage differentiation of individual thymus-repopulating SRC clones

To analyze the multilineage differentiation capacity of individually transduced SRCs, we performed in vivo integration site analysis by LAM-PCR that can distinguish the progeny of each transduced cell

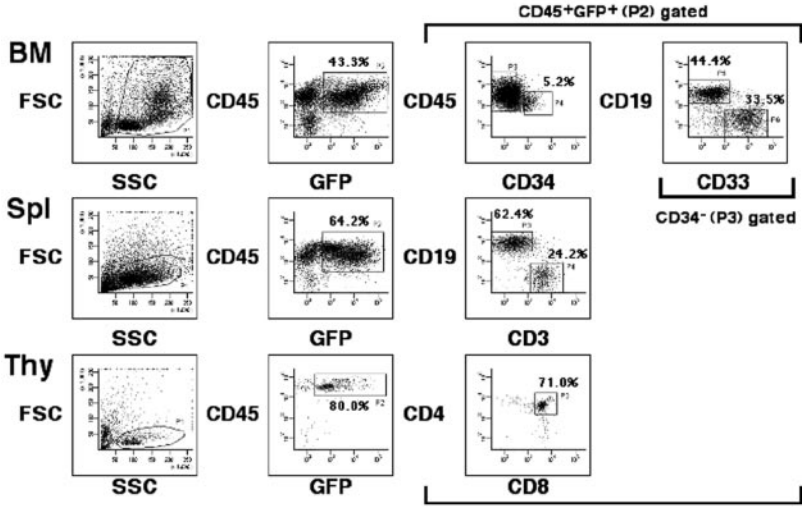

Figure 1. Representative FACS profiles of EGFP-transduced SRCs. Samples were obtained from BM, spleen, and thymus of a NOG mouse, and the proportion of EGFP-transduced human cells was evaluated. The relative frequencies of each cell population are indicated.

Table 1. Proportion of each human cell lineage engrafted in primary NOG mice

		Bone marrow, % engrafted					Spleen, % engrafted				Thymus, % engrafted		
Mouse	Week*	CD45	EGFP	S	M	B	CD45	EGFP	T	B	CD45	EGFP	T
1	17	82.2	84.8	22.1	4.6	ND	81.8	65.3	2.1	88.6	97.8	83.0	82.6
3	17	74.0	58.5	5.2	33.5	44.4	82.1	78.2	24.2	62.4	85.5	80.0	71.0
4	16	88.9	75.4	8.5	17.5	64.4	75.6	77.9	1.6	87.9	95.0	52.9	23.4

The total cellularity of BM and thymus in the primary recipient was $3.56 \times 10^7 \pm 0.53 \times 10^7$ and $1.39 \times 10^5 \pm 1.13 \times 10^5$, respectively. Each mouse listed in the table received a transplant of 10×10^3 $CD34^+CD38^-$ cells.

S indicates $CD34^+$ stem/progenitor cells; M, $CD33^+$ myeloid lineage cells; B, $CD19^+$ B-lymphoid lineage cells; T, $CD3^+$ (spleen) or CD4/CD8 double-positive (thymus) T-lymphoid lineage cells; ND, not done.

*Weeks after transplantation. Bone marrow cells, spleen cells, and thymocytes of NOG mice were stained with an anti–human CD45 mAb and analyzed. The proportion of EGFP-expressing cells within the $CD45^+$ cells and cells positive for each lineage marker in the $CD45^+/EGFP^+$ was calculated.

by its unique proviral-genomic fusion sequence (Figure 2A). Direct sequencing of PCR products derived from $EGFP^+CD45^+$ DP cells verified that each product with a unique band length represented individual and different clones. LAM-PCR analysis of $EGFP^+CD45^+$ FACS-purified lineage populations detected multiple integration sites in each cell lineage (Figure 2B). It is reported that the number of vector copies per cell can be controlled by adjusting the MOI, without reducing the transgene expression levels.[27] Because we optimized the experimental conditions to have each cell carrying one insertion per cell, confirmed by both colony-forming assay (see "Materials and methods") and transplantation assay,[24] multiple integration sites detected by LAM-PCR indicated polyclonal repopulation in the NOG mice.

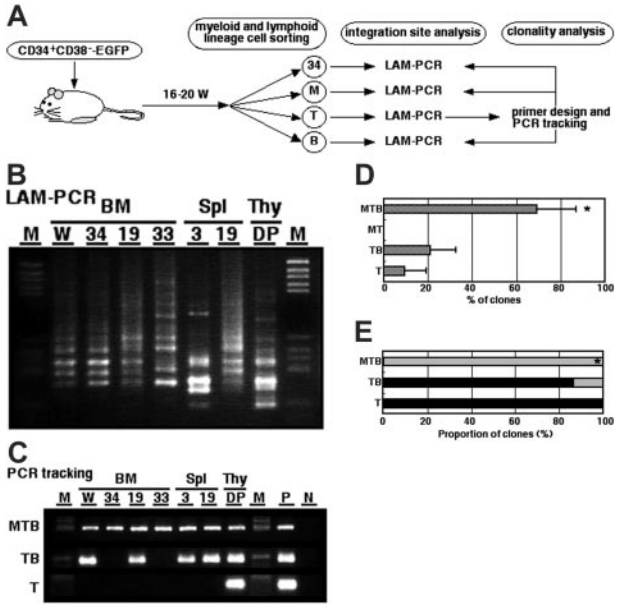

Figure 2. Clonal analysis of primary transplanted SRCs. (A) Study design for clonal analysis of primary grafts. 34 indicates $CD34^+$ stem/progenitor cells; M, $CD33^+$ myeloid lineage cells; B, $CD19^+$ B-lymphoid lineage cells; T, $CD3^+$ (spleen) or CD4/CD8 double-positive (thymus) T-lymphoid lineage cells. (B) Representative LAM-PCR profiles of SRCs. Each band represents a different insertion locus in the assayed material. W indicates unseparated whole BM MNCs; M, size marker. (C) DP-derived T-lymphoid insertion sites were traced by PCR. The clones detected in all lymphomyeloid lineage cells were designated as multipotent type (MTB). TB indicates clones restricted in T-lymphoid and B-lymphoid cells; T, clones detected in T-lymphoid cells; W, unseparated whole BM MNCs; M, size marker; P, TA-cloned LAM-PCR product was used as a positive control; N, DW. (D) Relative frequencies of each clone type detected in primary SRCs. Data represent mean ± SD of 3 independent experiments. $*P < .01$ relative to other type of clones. (E) The proportion of clones detected in the $CD34^+$ cell population. A total of 27 clones in 3 independent experiments were analyzed. Gray bars represent the clones detected in $CD34^+$ cells. Black bars represent the clones not detected in $CD34^+$ cells. $*P < .01$ relative to other type of clones.

A total of 27 clones were identified in 3 independent experiments based on the genomic sequence information of the LAM-PCR products from the DP cells (Table S1 summarizes the results of the integration site analysis of DP cells). Using primers designed to correspond to individual integration sites, and therefore unique clones, we were able to track the individual clones and their progenies, including $CD34^+$ stem/progenitor, myeloid, and B-lymphoid cells (Figure 2A). Three different clone types were observed in this experiment (Figure 2C): a multipotent type (MTB), in which insertion sites originally detected in the DP cells were also detected in the highly purified myeloid and B-lymphoid cell populations; a unipotent progenitor containing exclusively T cells; and a bipotent T/B progenitor. However, a bipotent progenitor containing myeloid and T lymphocytes was not detected. As expected, analysis of thymus-repopulating cells revealed that the majority of SRC clones (70.4%) found in the recipient mice were of the MTB multilineage type (Figure 2D; Table 2). We also used CD14 and CD66b, a more mature myeloid marker, for clonal analysis and detected the equivalent proportion of the 3 clone types (data not shown). Interestingly, all MTB clones were found in the $CD34^+$ cell population (Figure 2E), suggesting that the transduced SRC clones self-replicated within the $CD34^+$ stem cell pool without losing their ability to contribute to both lymphoid and myeloid lineages during long-term hematopoiesis. However, as the differentiation capacity of clones became limited to bipotency or unipotency, the proportion of clones that were also found in the $CD34^+$ cell population decreased (Figure 2E), indicating that some SRC clones had been exhausted from the stem cell pool during lineage commitment.

In vivo expansion of individual thymus-repopulating SRC clones

To directly demonstrate the self-renewal capacity of the SRCs, we injected BM cells from each primary mouse into 2 secondary mice

Table 2. Differentiation potential of thymus-repopulating SRC clones

		Clone type, no. (%)		
Mouse	No. of clones	MTB	TB	T
1	5	3 (60)	1 (20)	1 (20)
3	12	7 (58.3)	4 (33.3)	1 (8.3)
4	10	9 (90)	1 (10)	0 (0)
Total	27	19 (70.4)	6 (22.2)	2 (7.4)

Lineage contribution of individual thymus-repopulating SRC clones was evaluated by PCR tracking based on integration site analysis of DP cells. No clones were differentiated into M and T lineages.

MTB indicates clones that gave rise to myeloid (M), T-lymphoid (T), and B-lymphoid (B) lineages; TB, clones differentiated into T and B lineages; T, clones differentiated into T lineage only.

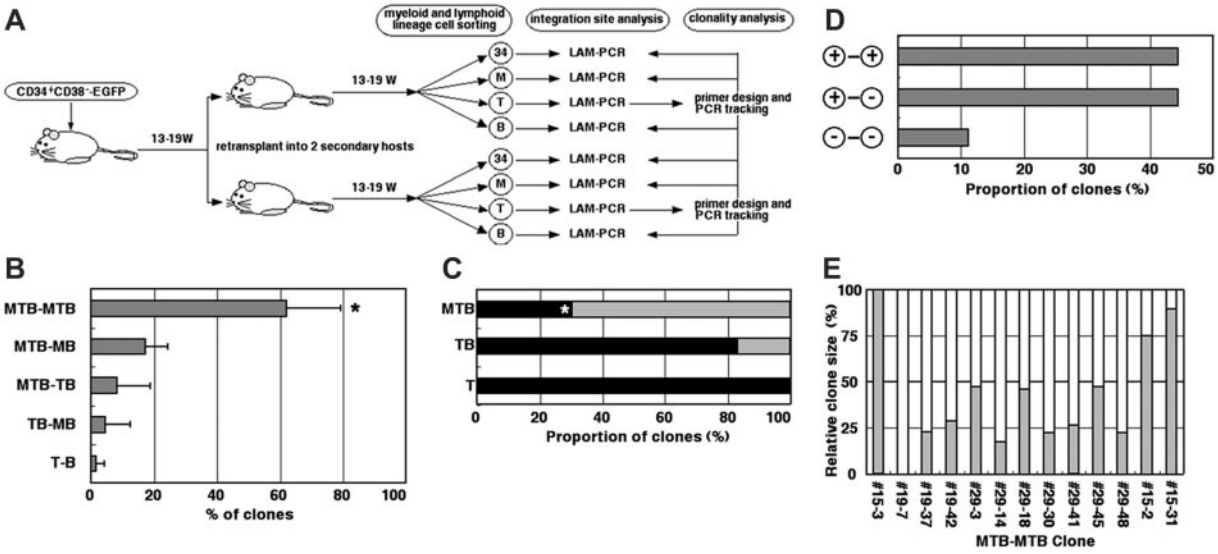

Figure 3. Clonal analysis of secondary transplanted SRCs. (A) Study design for clonal analysis of secondary grafts. 34 indicates $CD34^+$ stem/progenitor cells; M, $CD33^+$ myeloid lineage cells; B, $CD19^+$ B-lymphoid lineage cells; T, $CD3^+$ (spleen) or CD4/CD8 double-positive (thymus) T-lymphoid lineage cells. (B) Relative frequencies of each clone type detected in paired secondary transplanted recipients. Data represent mean ± SD of 3 independent experiments. *$P < .01$ relative to other type of clones. (C) The proportion of clones detected in $CD34^+$ cells is shown. A total of 43 clones in 3 independent experiments were analyzed. Gray bars represent the clones detected in $CD34^+$ cells. Black bars represent the clones not detected in $CD34^+$ cells. *$P < .01$ relative to MTB clones found in primary recipients (shown in Figure 1E). (D) The proportion of MTB clones found in $CD34^+$ cells of paired secondary recipients. A total of 27 clones in 3 independent experiments were analyzed. Notation of the left vertical axis: +/+, MTB-MTB clone pairs were detected in $CD34^+$ cells of both secondary recipient pairs; +/−, MTB-MTB clone pairs were detected in the $CD34^+$ cells of 1 of the 2 secondary recipient pairs; and −/−, MTB-MTB clone pairs were not detected in the $CD34^+$ cells of either secondary recipient pairs. (E) Relative clone size of individual clones in each MTB-MTB clone pairs in $CD34^+$ stem cell pool found in paired secondary recipient. The relative clone size of individual clones in 11 MTB-MTB clone pairs detected in $CD34^+$ cells of both secondary recipients was examined by RQ-PCR. The relative clone size of individual clones in each MTB-MTB pair is expressed as the proportion of one clone relative to the other clone. The MTB-MTB clone pairs no.15-3 and no. 19-7 that was detected in the $CD34^+$ cells of only 1 of the 2 secondary recipient pairs were used as experimental control and demonstrated complete skewing to either one recipient.

(Figure 3A). Although each primary BM cell population was divided into 2 recipients, substantial engraftment was observed in these secondary-recipient NOG mice (Table 3). Clone tracking analysis was then performed to examine the fate of individual SRC clones in paired secondary mice. Integration site analysis by LAM-PCR of FACS-sorted cells showed polyclonal reconstitution in each secondary host. A total of 43 clones were identified by integration site analysis of DP T lymphocytes found in 3 secondary recipient pairs (results are summarized in Table S2). All clones detected in paired secondary recipients were also detected in the primary donor. Strikingly, all 43 clones were found as a pair; clones detected in one of the secondary recipient pairs were always observed in the other pair. In greater than 90% of these clone pairs (39 of 43), at least 1 of the daughter clones inherited MTB differentiation potential from its parent clone. Moreover, in 69.2% of these 39 clone pairs, both daughter clones remained multipotent (MTB-MTB type), whereas the other daughter clone in the remaining 30.8% of clone pairs became committed to specific cell lineages (MTB-MB or MTB-TB type) (Figure 3B; Table 4). The existence of MTB-MTB type clones in secondary recipients indicated that a single SRC clone self-replicated in the primary recipients and produced 2 daughter clones that retained SRC potential, thereby resulting in the in vivo expansion of multipotent SRC clones.

Table 3. Proportion of the primary and the secondary human graft

Mouse	Cell dose*	Week†	Bone marrow, %					Thymus, %		
			CD45	EGFP	S	M	B	CD45	EGFP	T
Primary recipient										
101	17	13	80.3	72.7	ND	ND	ND	ND	ND	ND
109	6	19	66.2	71.0	ND	ND	ND	ND	ND	ND
113	10	14	43.2	71.8	ND	ND	ND	ND	ND	ND
Secondary recipient										
101-1	21	13	63.0	93.3	3.4	7.4	63.7	98.2	80.1	83.6
101-2	21	13	62.5	88.1	4.2	14.6	54.9	96.5	78.6	71.2
109-1	13.5	17	29.4	69.4	1.3	49.5	29.9	91.3	78.2	90.5
109-2	13.5	17	33.3	75.1	1.9	27.6	45.9	93.0	70.1	81.1
113-1	19.3	19	72.3	68.7	3.4	17.3	38.7	95.5	88.1	88.0
113-2	19.3	19	86.3	50.0	0.6	17.2	17.3	92.9	72.3	70.3

The total cellularity of BM in the primary and the secondary recipient was $3.86 \times 10^7 \pm 0.83 \times 10^7$ and $3.72 \times 10^7 \pm 0.91 \times 10^7$, respectively. The total cellularity of thymus in the primary and the secondary recipient was $3.36 \times 10^5 \pm 3.28 \times 10^5$ and $2.43 \times 10^5 \pm 1.59 \times 10^5$, respectively.

ND indicates not done.

*Number of $CD34^+CD38^-$ cells transplanted (primary recipient, $\times 10^3$; secondary recipient, $\times 10^6$).

†Number of weeks after transplantation.

Table 4. Differentiation potential of paired secondary clones

Mouse	No. of clones	Clone type, no. (%)				
		MTB-MTB	MTB-MB	MTB-TB	MB-TB	T-B
101	22	12 (54.5)	5 (22.7)	1 (4.5)	3 (13.6)	1 (4.4)
109	11	9 (81.8)	2 (18.2)	0 (0)	0 (0)	0 (0)
113	10	6 (60)	2 (20)	2 (20)	0 (0)	0 (0)
Total	43	27 (62.8)	9 (20.9)	3 (7)	3 (7)	1 (2.3)

Differentiation potential of paired secondary clones was determined by PCR tracking strategy based on integration site analysis of DP cells developed in secondary recipients.

Individual thymus-repopulating SRC clones have different self-renewal capacities

Consistent with our earlier observations in the primary recipient mice, the proportion of MTB clones present in the $CD34^+$ cell population decreased as the differentiation capacity of the clones became limited (Figure 3C). In addition, note that 30.3% of MTB clones in the secondary recipient mice were no longer found in the $CD34^+$ cell population, contrasting with the initial finding that all MTB clones in the primary mice were found in the $CD34^+$ cell population (Figure 2E). Therefore, phenotypic and possibly functional differences exist between MTB clones in the primary and secondary recipient mice, although both were capable of repopulating and giving rise to multilineage progenitors in the host. To assess how the MTB clone cell division affected the status of daughter MTB clones, we examined how many of in vivo–expanded MTB-MTB clone pairs were also found in the $CD34^+$ cell population and classified into 3 groups (Figure 3D). First, in 44.4% of the MTB-MTB clone pairs, both daughter clones were found in the $CD34^+$ cell population. Second, in another 44.4% of MTB-MTB clone pairs, only one of the daughter clones was found in the $CD34^+$ cell population. These results indicated that the former type clones have relatively higher SRC activity and that, as a result of unequal distribution of SRC activity in 2 daughter clones after cell division, 1 of the daughter clones exited from the $CD34^+$ stem cell pool. Finally, in 11.1% of MTB-MTB clone pairs, neither daughter clone was detected in the $CD34^+$ stem cell pool, which may reflect the extensive replication required to repopulate both primary and secondary recipients that eventually leads to exhaustion of the stem cells. These results demonstrate the heterogeneity among clones that repopulate both the primary and secondary recipient mice.

To further confirm our findings of heterogeneity of SRC clones, we quantitatively examined what was the relative clone size ratio of each MTB-MTB clone pair in the $CD34^+$ stem cell pool by RQ-PCR. In the majority of MTB-MTB clone pairs, the proportion of individual clone in each clone pair varied widely (Figure 3E). Interestingly, only a small proportion of MTB-MTB clone pairs (no. 29-3, no. 29-18, and no. 29-45) were found to be equally distributed to each paired recipient. Those clone pairs that were able to repopulate equally in a paired secondary recipient may have more extensive self-renewal capacity. The results indicated that individual thymus-repopulating SRC clones have different self-renewal capacities, which is a basis for a hierarchically organized stem cell pool.

We also performed tertiary transplantation and analyzed SRC clones found in the recipients. The level of engraftment in tertiary recipients was less than 1% in BM and 0.1% in the thymus ($n = 4$). We performed integration site analysis on unseparated BM MNCs of tertiary recipients and obtained 17 clones from 4 mice. The status of individual tertiary SRC clones in the secondary recipients was examined by clone tracking analysis using the LAM-PCR products from tertiary graft as a starting point of clonal analysis. We found that all SRC clones in the tertiary mice were detected in the $CD34^+$ stem cell pool of secondary recipient (Table S3 summarizes the results of the integration site analysis of SRCs). The results of tertiary transplantation experiment confirmed that only HSC clones that continuously replicate themselves in the $CD34^+$ stem cell pool could produce descendents to maintain long-term hematopoiesis.

Discussion

This study provides the first direct evidence for the multilineage differentiation and self-renewal of human HSCs at the single-cell level in vivo using PCR tracing analysis of individual thymus-repopulating clones. We demonstrated that polyclonal thymus-repopulating clones with multilineage differentiation and self-renewal abilities were able to maintain long-term human hematopoiesis. This study revealed several features of human HSCs that are important biologically and clinically. First, self-renewal division of individual thymus-repopulating clones resulted in clonal expansion of cells with multilineage differentiation in vivo. Second, a single thymus-repopulating clone produced progeny that were heterogeneous in SRC activity. Third, as a result of continuous division and/or advancement of lineage commitment, some of these multipotent thymus-repopulating clones were destined to limit their capability for multilineage differentiation and self-renewal. Our study indicated that, although the majority of thymus-repopulating clones loose their self-renewal potential, a relatively small proportion of thymus-repopulating clones retain extensive self-renewal potential. Therefore, the self-renewal capacity distinctly different in individual thymus-repopulating clones may cause a hierarchically organized stem cell pool.

Controlling the copy number of the virus vector in each transduced cell is important for our clonality analysis. We recently reported the direct evidence for single virus integration per cell in a transplantation study.[24] When EGFP-transduced $CD34^+$ cells expanded in vitro were divided and transplanted into multiple recipient mice, the unique integration site representing individual clones were detected in multiple mice; in other words, multiple mice were engrafted with the same clone. If a cell contains more than one integration site and assessed as "different" clones, these different clones should be detected in the same recipients. However, none of the clones demonstrated the identical engraftment pattern. In addition, clonal tracking analyses in this study clearly demonstrated that the individual SRC clones were both qualitatively and quantitatively heterogenous. Everything being considered, our infection condition achieves one copy per cell. Even if there is a slight possibility that the number of copies per cell is more than one, the clonality is not denied. Because a virus gene integrates into the host genome at a random site, progenies having a common integration site are developed from a single clone. It could affect the numeric calculation of the number of clones, but it would not influence our interpretation of the result.

Although the concept of HSCs was proposed decades ago and is well accepted, little experimental data regarding multipotency of human HSCs is available. Using a genetic marking strategy in both experimental[6-12] and clinical studies,[28-33] it has been suggested that hematopoietic reconstitution after transplantation is attributed to oligoclonal or polyclonal HSC activity and that the repopulation capacity of individual HSCs is substantially heterogeneous. However, the results of these studies, such as the presence of transgene expression in B lymphocytes and myeloid cells and the detection of similar genomic bands in multiple hematopoietic recipient organs, were not sufficient to unequivocally determine the multipotency of human HSCs at the single-cell level. Recently, Schmidt et al[34] reported clonal evidence for multilineage

human hematopoietic differentiation from IL-2Rcγ gene-transduced CD34$^+$ cells transplanted into patients with X-linked SCID. Considering that the IL-2Rcγ has been known to play a critical role in lymphoid development,[35] the observations based on the IL-2Rcγ gene–expressing clone may not reflect authentic hematopoietic development because of possible lineage commitment redirection mediated by cytokines that act on IL-2Rcγ. Although the HSC's potential was elaborately assessed clonally in vitro,[36,37] there exists some concerns; the lineage commitment could be easily fluctuated by culture conditions; homing of HSCs to thymus was neglected. The field has thus far lacked experimental evidence showing that purified human HSCs possess the potential for multilineage differentiation at the single-cell level. In this study, we succeeded in demonstrating that a single HSC gave rise to myeloid, T-, and B-lymphoid lineage cells by using LAM-PCR–based clonal tracing analysis in highly purified lymphomyeloid cell populations. Our results now provide data supporting the multipotency of a single human HSC.

Transplantation of prospectively isolated donor cells in mice has demonstrated that only HSCs can sustain thymopoiesis for a long period.[38,39] A lack of reliable in vivo experimental models for human thymocyte reconstitution and clonal stem cell assays has precluded determining whether the same applies to human HSCs. In this study, we used the thymus-repopulating potential of individual SRC clones as a means to analyze human HSCs at a single-cell level in vivo. Previously, we have demonstrated that human T lymphocytes derived from CD34$^+$ cells and developed in NOG recipient mice bore polyclonal Vβ TCR and responded not only to mitogenic stimuli but also to allogeneic human cells, which reflects normal human T-lymphoid cell development.[16-19] By analyzing individual thymus-repopulating clones in the NOG recipient, we successfully demonstrated that human thymus-repopulating cells were derived from a single multipotent-type HSC clone and were capable of maintaining long-term thymopoiesis in vivo. This is the first clonal evidence that multipotent HSCs contribute to long-term human thymopoiesis.

Self-renewal is essential for HSCs to maintain homeostasis in the blood system by the sequential generation of mature blood cells throughout a lifetime. To maintain the total pool of HSCs, this ability must be passed on to at least one of the daughter cells in each division. If both daughter cells undergo terminal differentiation, HSCs will eventually be lost. On the other hand, if both daughter cells retain stem cell properties, the total number of HSCs will increase, resulting in expansion of HSCs.[40] In fact, an increase in the number of murine[41,42] (and human[43,44]) repopulating cells was reported in serial transplantation studies. Limiting dilution analysis showed that secondary recipients contained larger numbers of HSCs than was originally injected. These results suggest that HSCs can replicate vigorously under certain conditions. However, it has been shown that HSCs intrinsically limit their potential for self-renew.[45-47] Recently, Ema et al[48] observed the self-renewal ability of HSCs by single murine HSC serial transplantation experiments and statistically determined that the number of HSCs increased in the BM of recipients, although the mean activity of individual HSCs was reduced. Taken together, this finding suggests that, although HSCs replicate themselves, which results in expansion of HSCs, they sequentially loose their potential as a HSC. Our clonal analysis of thymus-repopulating cells in paired secondary recipients provides direct evidence to address this innate property of HSCs in vivo.

Our finding that the same multipotent HSC clone was detected in paired secondary recipients (MTB-MTB type) indicates that a single SRC clone can self-replicate to produce 2 daughter cells with multilineage differentiation and self-renewal potential, leading to the in vivo expansion of SRCs. It has been considered that SRCs that can engraft and give rise to multilineage cells in secondary recipients are self-renewed HSCs. Thus, these MTB-MTB clones in this study could be defined as HSCs. However, when the MTB-MTB clone pairs were further examined whether they remained in the stem cell pool, one of the daughter clones in the pair was no longer found in the CD34$^+$ cell population in approximately half of MTB-MTB clone pairs. Furthermore, the stem cell phenotype was not retained in 11.1% of MTB-MTB clones. Considering that 100% of MTB clones in primary recipients possess the stem cell phenotype, these results indicate that SRCs with the stem cell phenotype progressively decrease during serial transplantation, leading to exhaustion of SRCs. This is consistent with our finding that the proportion of clones with the stem cell phenotype decreased as the clone committed to specific lineages. By assessing the phenotype of self-replicated multilineage clone pairs, determined by the presence of common integration site in CD34$^+$ cell population, we were able to reveal the status of HSCs during aging. The loss of stem cell phenotype may be caused by extensive replication required for hematopoietic reconstitution in recipient. Although the total SRC population appears to expand, our data indicate that the ability of individual SRCs, in more than half of the clones, may become restricted during long-term hematopoiesis in vivo.

Our clonal tracking analysis clearly demonstrated that heterogeneity in the self-renewal capacity of individual multipotent SRC clones underlies the differences of clonal longevity in the stem cell pool (Figure 4). Although most of the SRC clones loose their self-renewal potential, a relatively small proportion of SRC clones

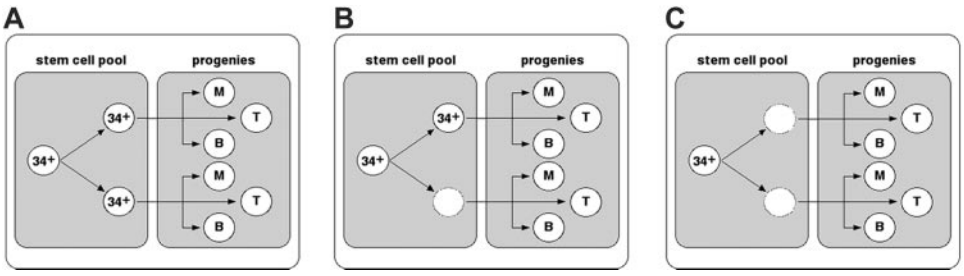

Figure 4. Schema of in vivo expansion. (A) A HSC replicates and produces 2 daughter cells, both of which retain the HSC phenotype. The paired daughter HSCs in the stem cell pool contribute to hematopoiesis; even so, the self-renewal activity of the parent HSC may be equally distributed to both daughters or may be skewed to either daughter cell. (B) As a result of heterogeneous HSC replication, one of the daughter HSCs loses the stem cell potential and therefore exits from the stem cell pool, but still remains in the progenitor pool. (C) Both paired daughter cells have lost their HSC potential, leading to exhaustion from the stem cell pool. 34 indicates CD34$^+$ stem/progenitor cells; M, CD33$^+$ myeloid lineage cells; B, CD19$^+$ B-lymphoid lineage cells; T, CD3$^+$ (spleen) or CD4/CD8 double-positive (thymus) T-lymphoid lineage cells.

(3 of 43 total secondary descending clones) are able to continuously self-renew. Our strategy which combined lineage-cell sorting and LAM-PCR enabled identification of the MTB clone that continuously self-renews in the stem cell pool and represents the long-term HSC. Our study provides a method that can accurately evaluate in vivo properties of human HSCs, and further studies may lead to elucidation of the mechanisms of self-renewal of the long-term human HSC at the single-cell level. It has been demonstrated that long-term leukemic stem cells have extensive self-renewal potential, and their hierarchic organization of stem cell pool was notably similar to the normal HSC compartment.[49,50] These findings propose the idea that some forms of leukemia imitate a system of the normal long-term HSC and retain or acquire the extensive self-renewal capacity. The clonal analysis for properties of long-term HSCs in vivo will be a powerful tool to understand the mechanisms for tumor initiation, progression, and relapses and will lead to efficient use of HSCs in clinical transplantation medicine.

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