

# Efficient marking of human cells with rapid but transient repopulating activity in autografted recipients

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**Short-term hematopoietic reconstituting cells have been identified in mice, nonhuman primates, and among human cells that engraft xenogeneic hosts. We now present clonal marking data demonstrating a rapid but unsustainable contribution of cultured human autografts to the initial phase of hematologic recovery in myeloablated patients. Three patients received transplants of granulocyte colony-stimulating factor–mobilized autologous peripheral blood (PB) cells, of which a portion (8%-25% of the CD34<sup>+</sup> cells) had**

**been incubated in vitro with growth factors (5 days) and clinical grade LN retrovirus (3-5 days). More than 9% of the clonogenic and long-term culture-initiating cells harvested were transduced. Semiquantitative and linear amplification-mediated polymerase chain reaction analyses of serial PB samples showed that marked white blood cells appeared in all 3 patients within 11 days and transiently constituted up to 0.1% to 1% of those produced in the first month. However, within another 2 to 9 months, marked cells had**

**permanently decreased to very low levels. Analysis of more than 50 vector insertion sites showed none of the clones detected in the first month were active later. Eighty percent of inserts were located within or near genes, 2 near *CXCR4*. These findings provide direct evidence of cells with rapid but transient repopulating activity in patients and demonstrate their efficient transduction in vitro. (Blood. 2005;106:893-898)**

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## Introduction

The existence of human hematopoietic stem cells able to permanently repopulate the hematopoietic system has been well established from clinical studies of allogeneic transplants,<sup>1</sup> clonality analyses,<sup>2</sup> and gene marking.<sup>3</sup> Evidence of short-term repopulating human cells (STRCs) has come from comparative studies of the pattern of reconstitution obtained from different prospectively isolated subsets of human cells transplanted into preimmune fetal sheep<sup>4</sup> or genetically immunodeficient mice.<sup>5-7</sup> Results obtained using these xenograft models have identified 4 types of human cells with different phenotypes and biologically distinct differentiation and self-renewal properties: CD34<sup>+</sup>CD38<sup>+</sup> myeloid-restricted short-term (3-week) repopulating cells (STRC-Ms), CD34<sup>+</sup>CD38<sup>-</sup> lympho-myeloid short term (6- to 12-week) repopulating cells (STRC-MLs), CD34<sup>+</sup>CD38<sup>-</sup> lympho-myeloid cells with long-term repopulating activities (CD34<sup>+</sup> LTRCs) and CD34<sup>-</sup>CD38<sup>-</sup> lympho-myeloid cells with long-term repopulating activities (CD34<sup>-</sup> LTRCs). However, as yet there have been no studies to determine whether the human STRCs identified in these experimental models play a similar role in patients. To address this question we examined the appearance, persistence, clonality, and sites of proviral integration in retrovirally marked peripheral blood (PB)

cells that were regenerated in 3 patients who received transplants with partially transduced autografts of mobilized PB cells.

## Patients, materials, and methods

### Clinical study

Between March 1998 and July 1999, 3 patients with chronic myelogenous leukemia (CML; no. 1 and no. 3 in chronic phase and no. 2 in early blast crisis) received transplants of partially transduced autologous PB cells at the University Hospital in Freiburg, Germany, according to a protocol approved by the Institutional Review Board, State and National Review Commission. Informed consent was provided according to the Declaration of Helsinki. All 3 patients were given 24 mg/m<sup>2</sup> idarubicin, 450 mg/m<sup>2</sup> etoposide phosphate, and 1600 mg/m<sup>2</sup> cytosine-arabioside followed by subcutaneous administration of granulocyte colony-stimulating factor (G-CSF). PB cells were then collected when the white blood cells (WBCs) reached  $0.5 \times 10^9/L$  (500/ $\mu$ L) or the CD34<sup>+</sup> cells exceeded 1% of the mononuclear cells. From  $2.9 \times 10^6$  to  $6.2 \times 10^6$  CD34<sup>+</sup> cells/kg body weight were isolated from a portion of the collection using the CellPro device (CellPro, Seattle, WA), and remaining cells were frozen. Patients were conditioned with 16 mg/kg body weight busulfan and received

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subcutaneous injections of 300  $\mu\text{g}/\text{d}$  G-CSF starting 7, 9, or 13 days after transplantation until the neutrophils were greater than  $1.5 \times 10^9/\text{L}$  or the WBCs were greater than  $5 \times 10^9/\text{L}$  for 2 consecutive days.

### Retroviral transduction

Supernatants of LN retrovirus-producing PG13 cells (A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) containing  $1.6 \times 10^5$  infectious units/mL were collected under good manufacturing practice (GMP) guidelines in a GMP facility (Magenta, Stirling, Great Britain) using a clinical grade serum-free medium (CellGro SCGM; Cell Genix, Freiburg, Germany). Transductions were also carried out in a GMP clean room facility as described<sup>8</sup> with minor modifications. Briefly, cells from patients no. 1 and no. 2 were prestimulated for 48 hours in 850  $\text{cm}^2$  roller bottles (Costar, Cambridge, MA) at  $2 \times 10^5$  cells/mL in serum-free medium (CellGro) containing 100 ng/mL flt3-ligand (FL), 100 ng/mL stem cell factor (SCF), and 20 ng/mL each of interleukin (IL)-3, IL-6, and G-CSF (all from Genzyme, Cambridge, MA). The cells were then harvested, resuspended in virus-containing medium (VCM) plus  $10^{-6}$  M hydrocortisone, 4  $\mu\text{g}/\text{mL}$  protamine, and the same growth factors and transferred into new roller bottles. Cells from patient no. 3 were suspended directly in VCM with  $10^{-6}$  M hydrocortisone, 4  $\mu\text{g}/\text{mL}$  protamine, 300 ng/mL FL, 100 ng/mL SCF, and 30 ng/mL IL-3. Half of the VCM was replaced with fresh VCM each day. After a total of 5 days in culture, an aliquot ( $\sim 5\%$  of the cells) was removed to perform helper virus assays ( $S^+/L^-$ ), sterility testing, and other safety and gene transfer efficiency tests. The remaining cells were frozen. The fraction of colony-forming cells (CFCs) and 5-week long-term culture-initiating cells (LTC-ICs)<sup>9</sup> transduced was inferred from colony yields in the presence and absence of 1.5 mg/mL G418 (Sigma Chemicals, St Louis, MO).

### Quantification of retrovirally marked hematopoietic cells

Semiquantitative polymerase chain reaction (PCR) was performed to measure the contribution of gene-marked cells in samples of PB cells obtained from all patients at regular intervals after the transplantation was performed. DNA was isolated using the blood and cell culture DNA kit (Qiagen, Valencia, CA). A 589-base pair (bp) fragment of the 5' vector sequence was amplified from 1  $\mu\text{g}$  of each sample and compared with dilutions of DNA isolated from HeLa cells containing a single copy of LN provirus. Taq polymerase (2.5 U; Qiagen) was used in 35 PCR cycles (denaturation at 95°C for 60 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 60 seconds) after initial denaturation for 5 minutes and before final extension for 10 minutes with primers LTR1 (5'-GTGGTCTCGCTGTTCCCTT-3'; Roth, Karlsruhe, Germany) and MISC (5'-CGCTCGACATCTTTCCAGT-3'; Roth). PCR products were separated on a 2% agarose gel and quantified by comparing the intensity of the test signal to a limited dilution set of HeLa cell controls.

### 5'-Long terminal repeat integration site analysis

Genomic proviral junction sequences were detected by linear amplification-mediated PCR (LAM-PCR).<sup>10,11</sup> In brief, 1  $\mu\text{g}$  DNA from PB leukocytes was used as template for 2 rounds of a 50-cycle linear PCR with a biotinylated vector-specific primer followed by immobilization on streptavidin-coupled paramagnetic beads and separation using a magnet. This step was followed by hexanucleotide random priming, restriction digestion with *Sse9I* or *isoschizomers*, ligation of the linker, and exponential PCR. Two percent of the first PCR product served as template (either directly or after

an additional magnetic capture purification step) for a second (semi-) nested PCR that enabled the visualization of the LAM-PCR amplicons (Figure 1B). LAM-PCR amplicons derived from DNA isolated from patients 1 and 2 were separated on a high-resolution Spreadex gel (Elchrom Scientific, Cham, Switzerland). For patient 3, PCR products were separated on a 2% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham, Piscataway, NJ) by pressure blot (Stratagene, La Jolla, CA), probed with a digoxigenin-labeled LTR/extended packaging signal probe, and documented by chemiluminescence exposure to x-ray film (Roche Diagnostics, Mannheim, Germany). LAM amplicons were either isolated, purified, and directly sequenced or shotgun cloned into the TOPO TA vector (Invitrogen, Carlsbad, CA) and sequenced. The length of 37 bp in the unprimed LTR was taken to indicate the specificity and correctness of the insertion sites sequenced. A representative analysis of 96 wells showed 14 contained no bacterial colonies, and in 9 the genomic-LTR junction could not be sequenced. As a result, from 96 wells, a total of 73 specific inserts were isolated.

## Results

### Retrovirally marked mobilized PB cells display transient repopulating activity in autografted patients

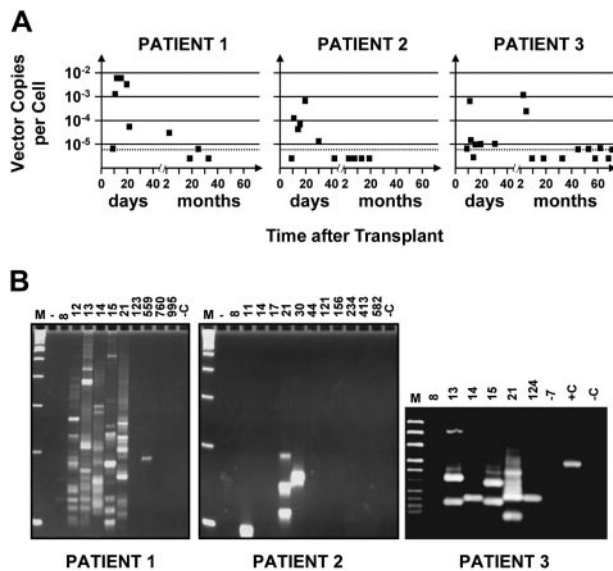
G-CSF-mobilized PB cells were harvested from 3 patients with CML (2 in chronic phase and 1 in early blast crisis), and a portion of the total  $\text{CD}34^+$  cells present (8%, 25%, and 25%, respectively; Table 1) were then isolated and cultured for 5 days in the presence of a potent growth factor cocktail (FL, SCF, and IL-3  $\pm$  G-CSF and IL-6). During the same 5 days, or only during the last 3 days, the cells were also exposed to a clinical grade preparation of a neo-encoding retrovirus, as described in "Materials and methods." These cultured cells were then harvested and transplanted together with the unmanipulated cells into their respective autologous hosts who, in the interim, had been treated with a myeloablative dose of busulfan. The proportion of transduced (G418-resistant) CFCs was at least 13%, and the total number of transduced CFCs infused varied from 2.9 to  $3.8 \times 10^4$  per kg body weight (Table 1). Corresponding values for the LTC-ICs harvested from the same cultures were greater than 9% marked LTC-ICs and a calculated 2 to  $11 \times 10^4$  transduced LTC-ICs per kg body weight transplanted (Table 1). Hematologic recovery was timely in all 3 patients, with achievement of a sustained WBC count of greater than  $10^9/\text{L}$  and a platelet count of greater than  $2 \times 10^{10}/\text{L}$  within 13 to 18 days.

Figure 1 shows the results of semiquantitative PCR and highly sensitive LAM-PCR analyses of sequential PB samples obtained from all 3 patients from 10 days after transplantation until follow-up was terminated (18, 33, and 72 months later). Marked cells were first detected in all 3 patients on day 11 (Figure 1A). In one case (patient no. 2), such cells were detected only at that time among several samples taken before day 21 and then again on days 21 and 30 but not thereafter. In the other 2 patients (no. 1 and no. 3), evidence of a first peak of marked WBC production persisted throughout the period between days 11 and 21. Quantification of

**Table 1. Numbers of transduced cells transplanted**

Patient no.	No. $\text{CD}34^+$ cells infected $\times 10^6/\text{kg}$ bwt (% of total)	Total no. cells harvested from transduction cultures and transplanted $\times 10^6/\text{kg}$	No. transduced CFCs transplanted/kg	No. transduced LTC-ICs transplanted/kg
1	1.0 (25)	4.0	29 000	4300
2	1.1 (25)	8.8	38 000	1100
3	0.5 (8)	1.1	35 000	2100

The fraction of CFCs and 5-week LTC-ICs transduced was inferred from a comparison of the yields of colonies obtained in methyl cellulose cultures containing 1.5 mg/mL G418 or not. Bwt indicates body weight.



**Figure 1. Kinetics of appearance and disappearance of marked cells in recipients of retrovirally transduced mobilized PB cell autografts.** (A) Time course studies revealing an early contribution of marked cells to the WBCs produced in the first 3 weeks in all 3 patients studied with variable patterns of contribution at later times. DNA was extracted from sequentially obtained samples of PB cells from each patient, and proviral sequences were detected using primers designed to amplify a 589-bp 5' vector sequence. Quantification of the proportion of WBCs that were marked was achieved by comparing the intensity of the signal obtained from each sample to control DNA from HeLa cells that contained a single copy of the provirus that was then serially diluted in DNA extracted from nontransduced HeLa cells. Analysis of 1  $\mu$ g template DNA ( $\sim 1.5 \times 10^5$  test cells) at each time point allowed the limit of gene-marked cells detectable to be reduced to close to 0.001% (assuming 1 vector copy per cell, dashed horizontal line). (B) Detection of different proviral integration sites in PB cells using LAM-PCR. The data indicate that multiple clones contributed to the early output of retrovirally marked WBCs in all 3 patients. M indicates a 100-bp DNA ladder; +C, positive control (0.02 ng DNA from monoclonal HeLa cells transduced with the same vector); and -C (1  $\mu$ g nontransduced HeLa DNA), as well as -7 (DNA extracted from the PB of this patient taken 7 days before the transplantation was performed), negative controls.

the amount of proviral DNA within the WBCs sampled during the first month showed that marked cells contributed approximately 0.1% to 1% of the total, assuming 1 copy of proviral DNA per transduced cell. Only approximately 10% of the CD34<sup>+</sup> cells transplanted were exposed to the original retroviral preparation, and it is unlikely that the number of cells with STRC activity would have increased during the 5-day transduction culture.<sup>7,12</sup> Thus, from the level of marking detected in these patients, a transduction efficiency of approximately 1% to 10% can be inferred for the cells infused that were able to generate an initial burst of WBC production. This would be consistent with the assumption of 1 copy of proviral DNA per transduced cell.

In patients no. 1 and no. 3, evidence of a second transient output of marked cells was seen 4 to 5 months after transplantation. However, thereafter, marked cells permanently decreased to undetectable or barely detectable levels in both.

#### Multiple transient clones contribute to the early phase of hematologic recovery in autografted patients

We also used LAM-PCR and sequence analysis of proviral integration sites to investigate the clonal composition of the marked PB cells identified at different times after transplantation (Figure 1B; Table 2 and Table S1, which is available on the *Blood* website; see the Supplemental Table link at the top of the online article). None of the insertion sites were repeated in sequential samples taken from patient no. 1 on days 12, 13, 14, 15, or 21 after

transplantation or from patient no. 3 on days 13, 14, and 15 after transplantation. Of note, the clone identified in the second wave of marked WBC production that occurred 4 to 5 months after transplantation in patient no. 3 was different from those seen earlier or detected later. However, a repeat analysis performed on an early sample from patient no. 2 demonstrated some bands of identical size and one with a different size. Since we can estimate that the template DNA analyzed for LAM-PCR from these samples was derived from fewer than 150 marked cells, this finding supports the inference that low levels of marked cells reflect small numbers of transduced cells in the transplant. Taken together, these results confirm the transient nature of the populations of marked cells detected in this initial phase of hematologic recovery and, thus, support the concept of their origin from STRCs.

The numbers of unique integration sites detected in all 3 patients during the first month after transplantation (59 inserts detected in cells from patient no. 1, 3 in cells from patient no. 2, and 5 cells from patient no. 3) also demonstrates the multiplicity of clones active during this time, assuming 1 clone per insert. The greater number of clones ( $\sim 10$ -fold) of gene-marked cells identified in the WBCs produced within the first 3 weeks in patient no. 1 by comparison to patients 2 and 3 is consistent with the correspondingly greater representation of marked cells in the PB of patient no. 1 during this period (Figure 1A).

Sequencing of shotgun-cloned LAM-PCR products revealed 69 unique integration sites (Table 2 and Table S1). Fifty-one of these integration sites could be mapped unequivocally to previously identified sequences in the human genome (44 from patient no. 1, 1 from patient no. 2, and 6 from patient no. 3). Forty-one of the 51 mapped inserts (80%) were found near ( $\pm 100$  kb) or within genes defined in RefSeq, and all but 2 of these were located within introns. Interestingly, 2 of the integration sites detected in WBCs produced early after transplantation in 2 different patients (no. 1 and no. 3) were 19 kb and 23 kb upstream of *CXC* chemokine receptor 4 (*CXCR4*), the gene encoding the receptor for stromal-derived factor-1 (SDF-1).

## Discussion

In this study, we analyzed serial PB blood cell samples from 3 recipients of partially marked autografts. The results provide new evidence that human hematopoietic cells with predetermined short-term repopulating activity contribute to the initial but not later phases of hematopoietic reconstitution seen in such patients. This conclusion is based on the following findings. First, in all 3 patients, there was a well-defined early wave of marked WBCs that reached a peak between the 11th and 20th days after transplantation. Second, the kinetics and ultimate level of early and late repopulation in individual patients was found to be highly dissociated. Third, multiple (differently marked) clones were found to be uniquely active during the first 4 weeks after transplantation with different clones showing activity at later times. These studies thus extend to human autografts the concept of a hierarchy of cells with in vivo repopulating ability that vary in their durability of mature cell output.

It is noteworthy that the substantial, but transient, early burst of marked WBCs seen in the patients followed in this study mirrors closely in time and content the output of cells produced during the first 3 weeks after transplantation in immunodeficient nonobese diabetic (NOD)/SCID- $\beta$ 2microglobulin<sup>-/-</sup> mice that received transplants of CD34<sup>+</sup>CD38<sup>+</sup> STRC-Ms.<sup>5-7</sup> These results suggest that

Table 2. LAM-PCR analysis of provirus integrants in identified sites

Patient no. and Seq no.	Time after transplantation, d	Genomic length	LAM amplicon length, bp	Integrand-host-junction sequence	Identity, %	Chromosome	Sequence orientation	Integration locus	Genomic locus/RefSeq gene
80852 H02	12	54	147	TCCATTTAATACAGTtgaagacc	100.0	4	Plus	48114519	1777 bp upstream of <i>TEC</i>
78933 B06	12	177	270	GACCAGATGTGGAAtgaagacc	98.4	5	Plus	94448967	6025 bp upstream of <i>MCTP1</i>
78933 C06	12	139	232	TGCTATAGTTACCGCtgaagacc	100.0	10	Minus	121145584	187767 bp in intron 3 of <i>GRK5</i>
80852 F01	12	26	119	ATAAAAGGGAAGCCTtgaagacc	100.0	11	Plus	128037827	31372 bp upstream of <i>FLI1</i>
78933 B07	13	38	131	CACACACACTCATAAAtgaagacc	100.0	1	Minus	155874598	14878 bp upstream of <i>AIM2</i>
78933 F07	13	77	170	GACCTCAGGTGATCCtgaagacc	100.0	1	Plus	164251126	3588 bp in intron1 of <i>CREG1</i>
78933 D07	13	52	145	TTGCTTACCCACTGtgaagacc	100.0	2	Minus	136728494	19044 bp upstream of <i>CXCR4</i>
79697 C08	13	130	223	ATGTCACAGACAAAGtgaagacc	100.0	2	Plus	17225602	41799 bp upstream of <i>VSNL1</i>
78933 H06	13	28	121	AACAAATGAGGTAGtgaagacc	100.0	6	Plus	2935514	1582 bp in exon 1 of <i>DKFZp686115217</i>
78933 E07	13	125	218	CTGAAGGGTGGGTGtgaagacc	100.0	7	Minus	94707759	1187 bp in intron 1 of <i>PON2</i>
80852 G03	13	289	382	CTCCCTATATGATGtgaagacc	100.0	7	Plus	43647712	27533 bp downstream of <i>BLVRA</i>
80852 C04	13	79	172	CCAATAGCTACCTTtgaagacc	100.0	8	Plus	62757554	32002 bp in intron 2 of <i>ASPH</i>
80852 G04	13	80	173	ATCGCTTGAACCCGGtgaagacc	98.8	9	Minus	129009529	8983 bp downstream of <i>IER5L</i>
78933 F06	13	27	120	TCTGTCTTCAAGTTCtgaagacc	100.0	12	Minus	107639068	11307 bp upstream of <i>CORO1C</i>
80852 E03	13	278	371	TCCAGTAAGAGCTATtgaagacc	99.7	12	Minus	92744212	170593 bp in intron 2 of <i>CRADD</i>
80852 A04	13	26	119	GCCGTGCAATACCTTtgaagacc	100.0	12	Plus	15927381	768 bp in intron 1 of <i>STRAP</i>
79697 C09	14	26	119	GGAACTACTTTTTCCtgaagacc	100.0	4	Plus	185981905	35854 bp in exon 9 of <i>FLJ33167</i>
80852 A06	14	60	153	CATAGTTTCAAATAAAtgaagacc	100.0	5	Minus	59965457	66256 bp in intron 7 of <i>DEPDC1B</i>
79697 E09	14	93	186	GTGGGAGGATCGCTTtgaagacc	100.0	6	Minus	109393783	117465 bp in intron 17 of <i>ARMC2</i>
78933 A09	14	49	142	GAAAGTTATTTAGTTtgaagacc	100.0	8	Plus	124166005	11904 bp in intron 4 of <i>MGC21654</i>
80852 G07	14	24	117	GAACGGTCTCTGACTgaaagacc	100.0	15	Plus	48215414	16703 bp upstream of <i>ATP8B4</i>
79697 F09	14	23	116	CAGGATAGGATGCAGtgaagacc	100.0	18	Minus	40593847	58380 bp in intron 1 of <i>SETBP1</i>
78933 H07	14	119	212	TTAAGAGCTTGAGGCTgaaagacc	99.2	20	Minus	30763159	31877 bp in intron 3 of <i>COMMD7</i>
78933 A08	14	207	300	ATACAGGTTCCAGGGtgaagacc	99.1	20	Plus	237848	8887 bp downstream of <i>ZCCHC3</i>
80852 A08	15	76	169	GTGTGGCCTAATAAtgaagacc	98.7	2	Plus	36543852	48804 bp in intron 2 of <i>CRIM1</i>
80852 H08	15	159	252	ATCTTAACAAACAGGtgaagacc	100.0	3	Plus	15815709	2372 bp upstream of <i>ANKRD28</i>
80852 A09	15	89	182	GTTAAGATATTTTCATgaaagacc	100.0	3	Minus	159553012	242418 bp in intron 5 of <i>MGC12197</i>
80852 G08	15	21	114	TTTCATTCAGAATCtgaagacc	100.0	13	Plus	77171831	41659 bp upstream of <i>FLJ30046</i>
79697 H10	21	137	230	AATATGACTGACATCtgaagacc	100.0	5	Minus	10558333	40196 bp downstream of <i>ROPN1L</i>
78933 E11	21	167	260	TGCTTTGTGTCGCAGtgaagacc	98.3	7	Minus	23111987	193 bp upstream of <i>C7orf30</i>



**Table 2. LAM-PCR analysis of provirus integrants in identified sites (continued)**

Patient no. and Seq no.	Time after transplantation, d	Genomic length	LAM amplicon length, bp	Integrand-host-junction sequence	Identity, %	Chromosome	Sequence orientation	Integration locus	Genomic locus/RefSeq gene
78933 E02	21	22	115	AGGTGCCATTCCCTTTtgaagaccc	100.0	12	Minus	121193424	16278 bp in Intron 1 of <i>MGC35140</i>
78933 H10	21	176	269	CCCCACCCACCTACTgaagaccc	99.5	17	Minus	7181218	612 bp in intron 1/exon 1 of <i>CENTB1</i>
78933 A11	21	86	179	TGCAAAGATTGTTTCtgaagaccc	100.0	18	Plus	27468862	49764 bp in intron 5 of <i>B4GALT6</i>
78933 D10	21	115	208	GTCTGGGGTTCAAACtgaagaccc	97.4	19	Plus	44428610	1159 bp upstream of <i>IL28B</i>
80852 F12	21	101	194	TCCCCTCAGTCTCAtgaagaccc	100.0	20	Plus	5573746	34074 bp upstream of <i>KIAA1434</i>
<b>2</b>									
78933 A02	21	54	147	CTGAATACTTACTATtgaagaccc	100.0	16	Minus	28531941	3791 bp upstream of <i>SULT1A1</i>
<b>3</b>									
9084	13	79	172	GTCTTTGTTTCATAGtgaagaccc	100.0	2	Minus	136732882	23434 bp upstream of <i>CXCR4</i>
9086	14	110	203	GAGCTGAGATCGCGTgaagaccc	99.1	12	Minus	120560220	10060 bp in intron 1 of <i>LOC283385</i>
9088	15	89	182	ACATGGGCACATCGctgaagaccc	100.0	12	Plus	18867724	84336 bp upstream of <i>CAPZA3</i>
4065	152	212	305	GGGGAAGGAAAAAActgaagaccc	99.6	1	Plus	181568775	106526 bp in intron 5 of <i>C1orf24</i>
9706*	335	93	186	CAAGGAGAGTTTGATtgaagaccc	94.6	2	Plus	128781865	10617 bp in intron 1 of <i>HS6ST1</i>

All patient samples analyzed were derived from cloned and sequenced PB LAM products as shown in Figure 1. Each sequenced LAM-PCR amplicon was aligned to the human genome database, and the insertion site was identified using University of California, Santa Cruz BLAT search tools (<http://genome.ucsc.edu/>). Integrand-host-junction sequence present in the first 15 nucleotides of the genomic DNA flanking the 5'-LTR (10 nucleotides). Genomic length denotes the size of the LAM-PCR amplicon without linker and LTR sequences. A complete list of all integrants analyzed is provided in Table S1. All cells analyzed were WBCs, except as shown by the asterisk (\*), where purified granulocytes were analyzed.

STRCs defined by their repopulating activities in such xenogeneic recipients display similar kinetics of growth and differentiation when transplanted into myeloablated human hosts, thus underscoring the promise of such xenotransplantation assays for predicting WBC recoveries in patients receiving transplants.<sup>13</sup>

Transplantation experiments to examine the properties of individual repopulating cells have further suggested that hematopoietic recovery in mice can be carried out by a very small number of clones when grafts of freshly isolated murine cells are transplanted.<sup>14-17</sup> In humans, it seems much less likely that the neutrophils and platelets produced immediately after transplantation would represent clonal populations for a number of reasons. First, the cell numbers required to meet the daily needs for new mature PB cells in adult humans are much larger (~ 100 times those needed in mice). Second, human cells also have longer cell cycle times (~ 2 times longer than murine cells). Thus, it would not be anticipated that single human cells could achieve clonal dominance within 2 to 3 weeks in patients with a normalized WBC count by that time. Assuming an 80-kg adult human normally contains approximately  $10^{10}$  WBCs, a single repopulating cell would have to produce a minimum of  $10^8$  progeny within 11 days to regenerate just 1% of these. This, in turn, would require a minimum of 26 divisions in the same time frame, assuming no lag time and no cell loss during this phase of cell expansion in vivo. The present evidence of oligo/polyclonal reconstitution of human recipients of marked autologous cells is consistent with these calculations. In fact, clonal repopulation of humans with transplanted cells has rarely been documented and then only after a minimum of 28 days.<sup>2,11</sup> Similarly in nonhuman

primates, early repopulation by marked cells has also been found to be highly polyclonal.<sup>10</sup>

We have recently shown that the leukemic clone in patients with chronic-phase CML contains cells with features of STRCs<sup>18</sup> as well as more primitive leukemic cells with a more prolonged repopulating activity detectable in immunodeficient mice.<sup>19-21</sup> It is, therefore, conceivable that some of the repopulating activity detected in the present study might have been attributable to BCR-ABL<sup>+</sup> cells. Although the data available cannot completely exclude this possibility, we believe such cells did not play a significant role in the repopulation obtained in the autografting studies reported here for several reasons. First, in 2 cases (patients no. 2 and no. 3), fluorescence in situ hybridization analyses were performed on the CD34<sup>+</sup> cells isolated from the freshly harvested leukapheresis products subsequently used as autografts, and these analyses showed the frequency of BCR-ABL<sup>+</sup> cells to be either low (7% and 30% in the 2 collections obtained from patient no. 2) or undetectable (< 6% in the case of patient no. 3). In the case of patient no. 2, who received a transplant after showing symptoms of incipient blast crisis and who relapsed with terminal blast-phase disease 18 months after transplantation, no evidence of gene marking was detectable in the relapse PB or marrow cells using a sensitive nested (LTR) PCR procedure (data not shown). Finally, the chemotherapy used in this study has been demonstrated to mobilize predominantly normal hematopoietic progenitors in patients with chronic-phase CML, and we and others have previously shown that the number of primitive BCR-ABL<sup>+</sup> cells (LTC-ICs) declines rapidly in vitro under the type of culture conditions used in our 5-day transduction protocol.<sup>22</sup>

Our results also add significantly to a growing body of data documenting a preferential integration of Moloney virus-based oncoretroviral vectors, and lentiviral vectors near or within genes.<sup>23-26</sup> In this study we were able to identify a total of 51 integration sites by sequence alignment and found that 80% of these inserts were also located within or near genes ( $\pm 100$  kb). The concept that such inserts occur preferentially in genes whose expression is relevant to the function of the cells transduced was reinforced here by the finding that 2 inserts in cells from different patients were sited within 25 kb upstream of the *CXCR4* gene. *CXCR4* is the receptor for SDF-1 and is known to be expressed on the surface of primitive human hematopoietic cells where it participates in the regulation of both their cell cycle arrest<sup>27,28</sup> and chemotactic/homing responses to SDF-1.<sup>29</sup> No untoward consequences of retroviral insertional mutagenesis were noted in any of

the 3 recipients of marked autografts in the present study. Nevertheless, our findings point to the need for continuing vigilance of this possibility in future trials using transduced grafts, particularly given the demonstrated efficiency ( $\sim 10\%$ ) with which STRCs can be retrovirally transduced, even in the virtual absence of transduction of cells with long-term repopulating activity.

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## References

- Gratwohl A, Hermans J, Niederwieser D, et al. Bone marrow transplantation for chronic myeloid leukemia: long-term results. *Bone Marrow Transplant*. 1993;12:509-516.
- Turhan AG, Humphries RK, Phillips GL, Eaves AC, Eaves CJ. Clonal hematopoiesis demonstrated by X-linked DNA polymorphisms after allogeneic bone marrow transplantation. *N Engl J Med*. 1989;320:1655-1661.
- Brenner MK, Rill DR, Holladay MS, et al. Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet*. 1993;342:1134-1137.
- Civin CI, Almeida-Porada G, Lee M-J, Olweus J, Terstappen LWMM, Zanjani ED. Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. *Blood*. 1996;88:4102-4109.
- Kerre TC, De Smet G, De Smedt M, et al. Both CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>+</sup>38<sup>-</sup> cells home specifically to the bone marrow of NOD/LISZ scid/scid mice but show different kinetics in expansion. *J Immunol*. 2001;167:3692-3698.
- Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34<sup>+</sup> cord blood cells transplanted into NOD/SCID mice. *Proc Natl Acad Sci U S A*. 2002;99:413-418.
- Glimm H, Eisterer W, Lee K, et al. Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID- $\beta_2$  microglobulin-null mice. *J Clin Invest*. 2001;107:199-206.
- Glimm H, Flugge K, Möbest D, et al. Efficient serum-free retroviral gene transfer into primitive human hematopoietic progenitor cells by a defined, high-titer, nonconcentrated vector-containing medium. *Hum Gene Ther*. 1998;9:771-778.
- Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ. Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci U S A*. 1990;87:3584-3588.
- Schmidt M, Zickler P, Hoffmann G, et al. Polyclonal long-term repopulating stem cell clones in a primate model. *Blood*. 2002;100:2737-2743.
- Schmidt M, Carbonaro DA, Speckmann C, et al. Clonality analysis after retroviral-mediated gene transfer to CD34<sup>+</sup> cells from the cord blood of ADA-deficient SCID neonates. *Nat Med*. 2003;9:463-468.
- Glimm H, Schmidt M, Fischer M, et al. Evidence of similar effects of short term culture on the initial repopulating activity of mobilized peripheral blood transplants assessed in NOD/SCID- $\beta_2$  microglobulin null mice and in autografted patients. *Exp Hematol*. 2005;33:20-25.
- Shier LR, Schultz KR, Imren S, et al. Differential effects of granulocyte colony-stimulating factor on marrow- and blood-derived hematopoietic and immune cell populations in healthy human donors. *Biol Blood Marrow Transplant*. 2004;10:624-634.
- Lemischka IR, Raulet DH, Mulligan RC. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell*. 1986;45:917-927.
- Dick JE, Magli MC, Huszar D, Phillips RA, Bernstein A. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W<sup>v</sup> mice. *Cell*. 1985;42:71-79.
- Benveniste P, Cantin C, Hyam D, Iscove NN. Hematopoietic stem cells engraft in mice with absolute efficiency. *Nat Immunol*. 2003;4:708-713.
- Uchida N, Dykstra B, Lyons KJ, Leung FYK, Eaves CJ. Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Exp Hematol*. 2003;31:1338-1347.
- Eisterer W, Jiang X, Christ O, et al. Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. *Leukemia*. 2005;19:435-441.
- Sirard C, Lapidot T, Vormoor J, et al. Normal and leukemic SCID-repopulating cells (SRC) co-exist in the bone marrow and peripheral blood from CML patients in chronic phase while leukemic SRC are detected in blast crisis. *Blood*. 1996;87:1539-1548.
- Dazzi F, Capelli D, Hasserjian R, et al. The kinetics and extent of engraftment of chronic myelogenous leukemia cells in non-obese diabetic/severe combined immunodeficiency mice reflect the phase of the donor's disease: an in vivo model of chronic myelogenous leukemia biology. *Blood*. 1998;92:1390-1396.
- Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood*. 1999;94:2056-2064.
- Petzer AL, Eaves CJ, Barnett MJ, Eaves AC. Selective expansion of primitive normal hematopoietic cells in cytokine-supplemented cultures of purified cells from patients with chronic myeloid leukemia. *Blood*. 1997;90:64-69.
- Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 2003;300:1749-1751.
- Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*. 2002;110:521-529.
- Baum C, von Kalle C, Staal FJ, et al. Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. *Mol Ther*. 2004;9:5-13.
- Imren S, Fabry ME, Westerman KA, et al. High-level  $\beta$ -globin expression and preferred intragenic integration after lentiviral transduction of human cord blood stem cells. *J Clin Invest*. 2004;114:953-962.
- Cashman J, Clark-Lewis I, Eaves A, Eaves C. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. *Blood*. 2002;99:792-799.
- Cashman J, Dykstra B, Clark-Lewis I, Eaves A, Eaves C. Changes in the proliferative activity of human hematopoietic stem cells in NOD/SCID mice and enhancement of their transplantability after in vivo treatment with cell cycle inhibitors. *J Exp Med*. 2002;196:1141-1149.
- Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*. 1999;283:845-848.