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Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice

(gene therapy/retroviral vectors)

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ABSTRACT **Gene transduction of pluripotent human hematopoietic stem cells (HSCs) is necessary for successful gene therapy of genetic disorders involving hematolymphoid cells. Evidence for transduction of pluripotent HSCs can be deduced from the demonstration of a retroviral vector integrated into the same cellular chromosomal DNA site in myeloid and lymphoid cells descended from a common HSC precursor. $CD34^+$ progenitors from human bone marrow and mobilized peripheral blood were transduced by retroviral vectors and used for long-term engraftment in immune-deficient (beige/nude/XID) mice. Human lymphoid and myeloid populations were recovered from the marrow of the mice after 7–11 months, and individual human granulocyte–macrophage and T-cell clones were isolated and expanded *ex vivo*. Inverse PCR from the retroviral long terminal repeat into the flanking genomic DNA was performed on each sorted cell population. The recovered cellular DNA segments that flanked proviral integrants were sequenced to confirm identity. Three mice were found (of 24 informative mice) to contain human lymphoid and myeloid populations with identical proviral integration sites, confirming that pluripotent human HSCs had been transduced.**

Pluripotent hematopoietic stem cells (HSCs) give rise to all lineages of mature blood cells. Gene transduction of human HSCs is a requisite for successful gene therapy of a variety of genetic disorders of hematolymphoid cells. In contrast to the widely demonstrated ability to transduce high percentages of pluripotent murine stem cells (1–4), large animal models and preliminary human trials have shown much lower extents of gene transfer into stem cells (5–9). Thus, a key basic research goal must be to derive techniques to study gene transduction of human stem cells. Numerous studies performed with murine bone marrow have used integrated retroviral vectors as clonal tags. Because retroviral DNA integrates at random sites in the target cell chromosomes, the site of integration creates a unique restriction fragment length polymorphism containing vector sequences. The presence of the same vector integrant in cells of multiple lineages, including myeloid and lymphoid, has been used to verify that pluripotent murine stem cells could be transduced and would retain the capacity for multilineage differentiation (1–4). Similar analyses with human hematopoietic stem cells have not previously been possible due to the lack of *in vitro* systems for pluripotent differentiation of human cells and low-level gene transfer in initial trials of human stem cell marking (8).

We have developed a unique model system for the growth of adult human bone marrow cells *in vivo* in immune-deficient mice (beige/nude/XID, bnx). Sustained hematopoiesis from human $CD34^+$ progenitors occurs after a single tail vein injection, by cotransplantation with human bone marrow stromal cells engineered to secrete human interleukin (IL) 3 (10). When this model of human hematopoiesis was used to study gene transfer into human hematopoietic cells by using retroviral vectors, the presence and function of the inserted gene was consistently demonstrated in human colony-forming progenitor cells isolated from the mice after 7–11 months (10, 11). We now report use of the bnx/human (hu) cotransplantation model to study gene transduction of pluripotent human HSCs. $CD34^+$ progenitors from human bone marrow or granulocyte colony-stimulating factor-mobilized peripheral blood were transduced *in vitro* by the LN vector (12) and transplanted into bnx mice as described (11). After engraftment periods of 7–11 months, human $CD3^+$ lymphoid and $CD33^+$ myeloid populations were recovered from the marrow of the mice. Individual human granulocyte–macrophage and T-cell clones were isolated by automated cell deposition (ACDU), expanded *ex vivo*, and screened for the presence of the LN provirus. Inverse PCR from the retroviral long terminal repeat (LTR) into the flanking DNA was then performed on each sorted cell population and vector-marked clone. The recovered cellular DNA segments that flanked proviral integrants were sequenced to confirm identity.* Three mice were found (of the 24 mice studied) to contain both human lymphoid and myeloid clones with identical proviral integration sites, confirming that pluripotent human HSCs had been transduced *in vitro* by the LN vector prior to transplantation. To our knowledge, these data provide the first direct evidence of retroviral-mediated transduction of human HSCs that retained the potential to differentiate into both lymphoid and myeloid progeny.

MATERIALS AND METHODS

Human Marrow Transduction. $CD34^+$ progenitors were isolated by immunomagnetic selection from normal human bone marrow and granulocyte colony-stimulating factor-mobilized peripheral blood as described (11). Use of these samples was approved by the Committee on Clinical Investigations at Childrens Hospital, Los Angeles. Cells were transduced by cell-free supernatant from the LN vector (12) with stromal support as described (11). Producer cell lines were determined to be free of both ecotropic and amphotropic recombinant helper virus by marker rescue assay and PCR for amphotropic *env*. After the 72-h *in vitro* transduction period,

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Abbreviations: HSC, hematopoietic stem cell; bnx, beige/nude/XID; LTR, long terminal repeat; IL, interleukin; ACDU, automated cell deposition; neo, neomycin resistance.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X95499 and X95500).

portions of the transduced cells were plated in methylcellulose-based colony-forming unit assay with and without the selective agent G418 to assess the extent of gene transfer into 14-day colony-forming progenitors as described (11). The remainders of each sample were transplanted into immune-deficient mice to assess transduction of more primitive progenitors.

Mice. All studies used 6-week-old bnx homozygous mice bred at Childrens Hospital Los Angeles. Cotransplantation of transduced human progenitors and stromal cells producing IL-3 was performed as described (10). Strict isolation precautions were used in the bnx colony. Mice were sacrificed by 90% CO_2/10% O_2 narcosis, 7–11 months after transplantation with human cells. Bone marrow was flushed from the tibiae and femurs of each mouse into 1× phosphate-buffered saline (PBS), dispersed with a fine needle, and used for flow cytometry analysis, cell sorting, and myeloid- and T-cell cloning. The remainder of each bone marrow sample was viably cryopreserved for later analyses.

Human T-Lymphoid- and Myeloid-Cell Separation. A portion of the marrow cells from each mouse was incubated with an anti-CD3 monoclonal antibody specific for human T lymphocytes (Amac, Westbrook, ME). A second sample was incubated with the monoclonal antibody My9 (Coulter), specific for human cells of the granulocyte and macrophage lineages. Both antibodies were assayed for species specificity at the time of each cell separation by parallel analyses using non-transplanted bnx mouse bone marrow and human peripheral blood. Immunoreactive cells were isolated from each fraction by goat anti-mouse-linked immunomagnetic beads (Dynal) and a magnetic field. Next, cryopreserved samples of marrow from mice that showed common vector integrants in bulk-sorted human $CD3^+$ and $CD33^+$ populations were thawed and subjected to single cell cloning to verify the lineages of marked cells. To generate T-cell clones, samples were blocked with unlabeled mouse immunoglobulin (MsIgG, Coulter) and then incubated with fluorescein-conjugated HLE-1 [anti-human CD45, Becton Dickinson (BD)] and phycoerythrin-conjugated anti-human CD3. After gating on the double-positive human $CD45^+/CD3^+$ population, human T cells were deposited individually into each well of 96-well plates (Linbro Titertek, Flow Laboratories) by ACDU (Becton Dickinson). Human peripheral blood mononuclear cells [1×10^5 irradiated (5400 cGy) cells] were added to each well with 0.1 μg of phytohemagglutinin and 1.0 unit of IL-2 in RPMI 1640 medium with 10% (vol/vol) fetal calf serum. After 6 days of incubation, wells were refed IL-2 and phytohemagglutinin without disturbing the cell pellet. Fourteen days later, each resulting human T-cell clone of 500–1000 cells was individually recovered, washed, and lysed for DNA isolation. To obtain human granulocyte–macrophage clones, marrow samples were blocked and then incubated with fluorescein-conjugated HLE-1 (anti-human CD45) and phycoerythrin-conjugated My9. Acquisition gates were set on human $CD45^+$/human $CD33^+$ cells with small size and low side scatter, to exclude mature cells. Human myeloid progenitors were deposited individually by ACDU into 96-well plates that contained human-specific methylcellulose-based medium (10) with G418 (GIBCO/BRL; 0.9 mg/ml). Individual wells with colonies containing at least 500 cells were harvested for clonal analysis.

DNA Purification and Inverse PCR. Samples containing 500–1000 cells were lysed in 200 μl of proteinase K buffer (0.01 M Tris·HCl, pH 7.40/0.15 M NaCl/0.01 M EDTA, pH 8.0/0.01% SDS) with 30 μg of proteinase K (Sigma) for 2 h at 56°C. One extraction with 200 μl of buffered phenol/chloroform/isoamyl alcohol, (25:24:1) (vol/vol), was then performed. The aqueous phase was precipitated by addition of 2 μg of glycogen (Boehringer Mannheim), 18 μl of 10 M NH_4Ac, 500 μl of absolute ethanol and incubation at −20°C for at least 2 h. DNA was centrifuged in a tabletop microcentrifuge in a Dupont H1394 rotor at 10,000 rpm for 10 min, rinsed in 70% ethanol, and dried on the benchtop for 30 min. DNA pellets were resuspended in 82 μl of 1× TE buffer [10 mM Tris·HCl, pH 7.6/1 mM EDTA, pH 8.0/3 μl of spermidine (0.1 M, Sigma)/10 μl of React 2 buffer (GIBCO/BRL)/1 μl (10 units) of *Taq* I restriction enzyme (GIBCO/BRL)] for 2 h at 65°C, with readdition of *Taq* I after the first hour of incubation. An 8-μl sample was then ligated by addition of 2 μl of 5× T4 ligase buffer and 1 μl of T4 ligase (Gibco/BRL) at 12–15°C for 2 h. The first round of amplification of 2 μl of the circularized DNA used the primers INVa (5′-AGGAACTGCTTACCACA) and INVb (5′-CTGTTCCTTGGGAGGGT) in Perkin–Elmer PCR buffer with a final Mg^{2+} concentration of 1.25 mM. The first cycle was 95°C denaturation for 5 min, 50°C annealing for 2 min, and 72°C extension for 4 min. The subsequent 29 cycles were identical, except that the denaturation time was reduced to 1 min. Nested PCR was then performed on 2 μl of the amplified product with primers INVc (5′-TCCTGACCTTGATCTGA) and INVd (5′-CTGAGTGATTGACTACC) by using the same reaction conditions and cycles. The resulting PCR products were electrophoresed on a 2% gel (1% Seakem LE agarose/1% Nu-Sieve; FMC), transferred to nylon membrane (Biotrace, Pall Biodyne, East Hills, NY), and hybridized with an oligonucleotide probe (5′-GGCAAGCTAGCTTAAGT) specific for LTR sequences, end-labeled with [γ-^{32}P]ATP by T4 kinase (GIBCO/BRL). To verify identity of clonal patterns by sequencing, the corresponding PCR product bands were first excised from a 1% agarose gel. DNA was recovered from the agarose by centrifugation through a Spin-X column (Costar) at 10,000 rpm for 10 min in a Dupont H1394 rotor and then precipitated by addition of 0.1 vol of 10 M NH_4Ac with 2.5 vol of ethanol. DNA pellets were washed in 70% ethanol, dried, resuspended in 8 μl of 1× TE buffer, and then sequenced with the Circum *Vent* thermal cycle kit (New England Biolabs) and the primer INVc. Sequencing reactions were done as described (13).

RESULTS

Transduction and Transplantation of Human $CD34^+$ Progenitors. Human bone marrow and granulocyte colony-stimulating factor-mobilized peripheral blood cells were enriched for $CD34^+$ progenitors by immunomagnetic separation. Enriched populations were 90–95% $CD34^+$, as demonstrated by flow cytometry analysis (14). Cells were transduced by cell-free retroviral supernatant with the support of an irradiated allogeneic stromal layer as described (11). The LN vector, which carries the bacterial neomycin-resistance (neo) gene (12) packaged by the amphotropic producer PA317, was used for all transductions. The duration of transduction was 72 h in all cases. After transduction, a portion of each cell sample was plated in the methylcellulose colony-forming assay. The average extent of transduction prior to transplantation was 35.6% colonies resistant to the selective agent G418, as reported (11). After the *in vitro* transduction, each sample was cotransplanted with primary human stromal cells engineered to produce human IL-3 in 6-week-old bnx mice as described (10). Mice were sacrificed 7–11 months later. Bone marrow was recovered from the mice, analyzed for the percentage of human $CD45^+$ cell engraftment by flow cytometry analysis, and screened for the presence of LN provirus by PCR for the neo gene as described (10). A total of 24 bnx mice had significant levels of human hematopoietic cell engraftment (average, 4.8% human $CD45^+$; range, 1.8–9.9%) and had proviral neo sequences in their bone marrow (Table 1). Human-specific colony-forming assays were plated from a portion of the recovered marrow. The average extent of G418-resistant human colonies recovered from the long-term engrafted animals was 23.1%.

Clonal Integration Analysis of Bulk-Sorted Myeloid and T-Cell Populations. Human cells recovered from the marrow of the 24 mice engrafted with neo-marked progenitors were

Table 1. Inverse PCR analysis of human myeloid and lymphoid populations recovered from bnx/hu mice

bnx mouse	BMT		% human CD45$^+$ in bnx BM	No. of integrants		No. common integrants
	Source	Months		T cell	Myeloid	
25B1	BM	7.0	7.2	2	3	0
25B2	BM	7.0	3.5	3	2	0
25B3	BM	10.5	2.1	1	2	0
25B4	BM	10.5	5.2	2	10	0
25D3	PB	7.5	2.7	0	0	0
25D4	PB	7.5	6.4	1	5	1
26B2	BM	10.5	5.8	0	0	0
26B3	BM	11.0	5.9	0	4	0
26C2	BM	10.5	9.9	1	3	0
26C3	BM	11.0	8.6	0	4	0
27A1	PB	8.5	2.0	3	1	0
27A2	PB	8.5	4.8	2	3	0
27B1	PB	9.5	3.9	1	4	0
27B2	PB	10.5	3.8	1	3	1
28A1	PB	10.0	5.3	0	4	0
28A2	PB	10.0	4.2	4	8	0
28B2	PB	10.0	3.8	2	4	0
28E1	PB	10.0	2.0	0	2	0
28E2	PB	10.0	8.6	2	5	0
28F2	PB	10.0	2.3	0	2	0
29C1	PB	8.0	3.3	1	2	0
30B1	BM	7.0	7.4	2	6	1
30C1	BM	7.0	4.6	1	2	1
39C4	BM	7.0	1.8	2	7	0

BM, bone marrow; PB, peripheral blood; Int, proviral integrants; BMT, bone marrow transplant.

sorted by immunomagnetic selection into two populations: human CD3$^+$ T-lymphocyte pools and human CD33$^+$ myeloid-cell pools (Fig. 1). Both populations were then subjected to clonal integration analysis using an inverse PCR method adapted from Rill *et al.* (15) (Fig. 2). Briefly, genomic DNA from each human cell population was digested with the restriction enzyme *Taq* I (GIBCO/BRL). Each LTR was then on a restriction fragment of unique size, determined by the distance to the nearest *Taq* I recognition site in the genomic DNA flanking the provirus. The LTR-containing fragments were then self-ligated to form closed circles. PCR amplification using primer pairs complementary to the LTR directed in opposite directions around the closed circle produced a PCR product of characteristic size for each integration site. Each Moloney-based proviral vector integrant yields two PCR products, one from each LTR. The progeny of each transduced stem cell will contain the same clonotypic vector integrant and will show the same pattern of PCR products.

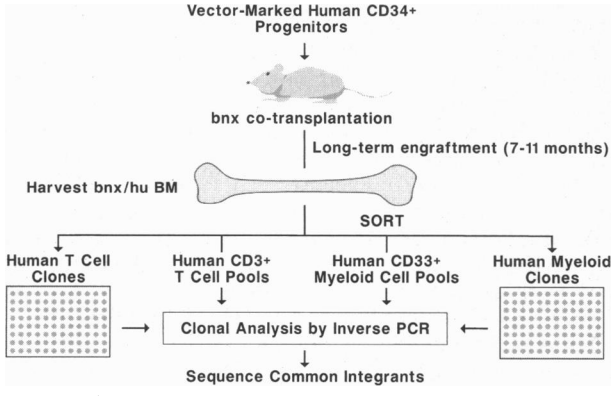

FIG. 1. Experimental schema. Human CD34$^+$ progenitors were transduced by the retroviral vector LN and cotransplanted into bnx mice with human stroma engineered to produce human IL-3. After a period of 7–11 months, hematopoietic cells were recovered from the marrow of the mice and bulk-sorted into human CD3$^+$ lymphoid and human CD33$^+$ myeloid populations. Individual human T-lymphoid and myeloid clones were obtained by ACDU and expanded. Each sample was then subjected to clonal integration analysis by inverse PCR to determine the number of proviral vector integrants in each population and then sequenced to confirm identity of common bands.

The results of the clonal analyses of the human myeloid and T-lymphoid populations recovered from bnx/hu mice are shown in Table 1 and Fig. 3. Oligoclonal marking of both populations was seen in most cases. Two mice were not informative. The number of individual integrants in T lymphocytes ranged from one to four separately marked populations in each informative animal. In five mice, only the myeloid cells were marked. The number of proviral integrants in the myeloid cells ranged from 1 to 10 per animal. In the event that PCR product bands representing the integrant from both human myeloid and lymphoid cells isolated from an individual mouse appeared identical in size, DNA from each was sequenced to confirm identity (Fig. 3). Of 24 mice tested, 4 were confirmed to harbor engrafted human myeloid and T-lymphoid cells with identical sites of proviral integration (Table 1). These results indicate that some cells in both marked populations were derived from the same pluripotent stem cell that differentiated into the two disparate lineages after retroviral integration.

Clonal Integration Analysis of Myeloid and T-Cell Clones. We next analyzed clones expanded from individual human CD3$^+$ and CD33$^+$ cells recovered from the long-term engrafted mice. Cryopreserved marrow from the four mice with common bands in T- and myeloid-cell pools was thawed and single CD3$^+$ T-lymphoid cells or single CD33$^+$ myeloid cells were sorted by ACDU into individual wells for clonal expansion. Myeloid cells were grown in the presence of G418 and thus all isolated clones contained proviral sequences. The T-lymphoid clones were expanded without selection and then screened for the presence of the LN vector by PCR for the neo gene. All clones bearing LN provirus were then subjected to inverse PCR to define vector integrant patterns.

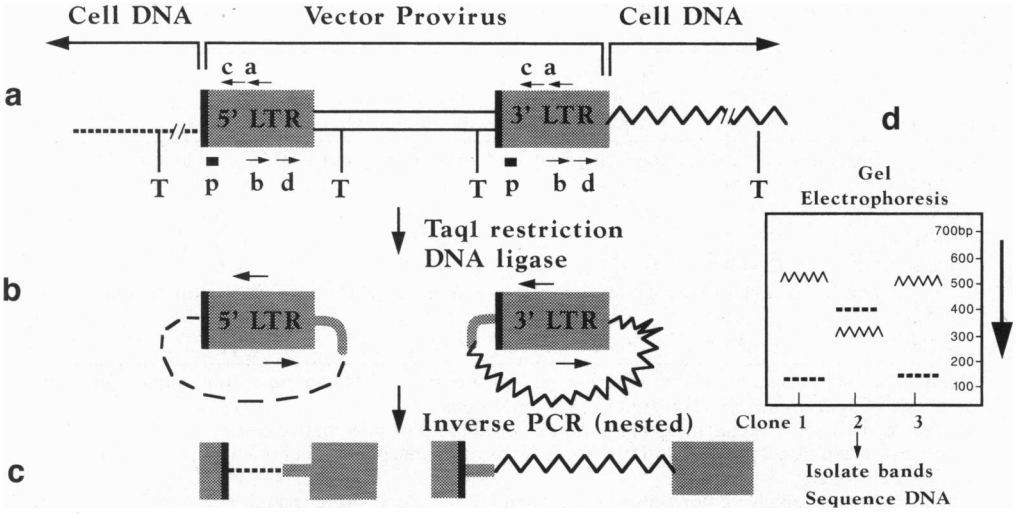

FIG. 2. Inverse PCR to assess proviral clonal integration sites. (*a*) Genomic DNA was digested with the restriction enzyme *Taq* I (T), which cuts twice within the integrated provirus and again in the flanking cellular DNA. The resulting fragments of DNA had variable size, dependent on integration site, and, therefore, provided unique fragment lengths. (*b*) The fragments containing the 3′ and 5′ LTR with flanking DNA were self-ligated. (*c*) The first round of inverse PCR was done by using the circular ligated DNA as a template and subsequent cycles amplified the linear products. Nested PCR was then performed to further amplify the fragments. (*d*) PCR products were size-fractionated by gel electrophoresis, and DNA bands containing the amplified PCR products were recovered from agarose and sequenced.

Three mice were found to harbor individual human T-cell and myeloid clones with proviral integrants at the same site (Table 2). From mouse 27B2, engrafted for 10.5 months, four individual human T-cell clones were isolated with clonal vector integration sites identical to those in five myeloid clones. An inverse PCR band of 290 bp, amplified from all nine clones, had also been amplified from the sorted human $CD3^+$ and $CD33^+$ pools from that mouse. The sequences of the portions of the corresponding 290-bp bands, which represented flanking cellular DNA, confirmed that the proviral integration sites were identical (Table 3). Mouse 30B1, engrafted for 7.0 months, had six individual patterns of vector integrants among the human myeloid clones, and two among the T-cell clones. One inverse PCR band, of 520 bp, was common to clones of both lineages (one T and two myeloid), and was also present in the bulk-sorted human myeloid and lymphoid populations.

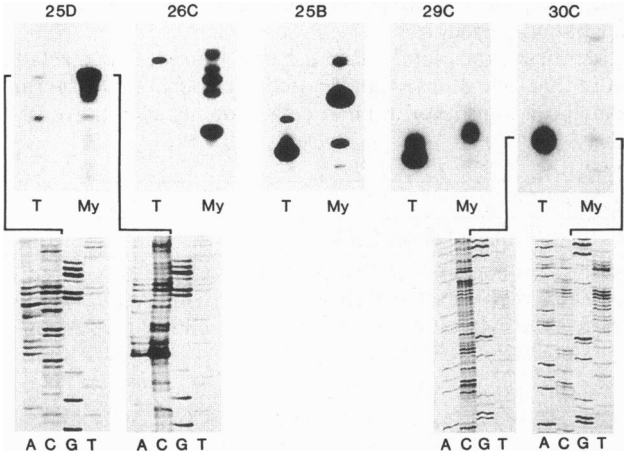

FIG. 3. Inverse PCR to assess proviral clonality in marked human T and myeloid cells isolated from bnx/hu mice 7–11 months after transplantation. Bulk populations of human $CD3^+$ T-lymphoid and $CD33^+$ myeloid cells were recovered after long-term engraftment in bnx mice 25D4, 26C2, 25B3, 29C1, and 30C1 (Table 1). Clonal integration analysis by inverse PCR was performed on each population. DNA was recovered from bands of the same molecular weight and sequenced to confirm that the cellular sequences flanking the integrated proviral DNA were identical.

Again, sequencing confirmed the identity of the bands (Fig. 4). One mouse, 25D4, had a common integrant band in the $CD3^+$ and $CD33^+$ populations, which was also detected in individual myeloid clones. However, only three marked T-cell clones were expanded from that animal, and they lacked the common integrant band. It is possible that the T-cell pattern observed in the bulk $CD3^+$ population was derived from mature vector-marked T lymphocytes that were no longer clonogenic at the time of harvest and expansion. In the bulk sorted populations from mouse 30C1, also engrafted for 7 months, only two major integrants were seen in the bulk-sorted myeloid population and one in the T-cell population (Fig. 3). Additional patterns were uncovered by the single cell cloning. There were 11 proviral integration patterns in myeloid clones, and 4 individual patterns in T-lymphoid clones. Again, one band matched in two lymphoid and four myeloid clones, as well as in both bulk-sorted populations.

In the human DNA sequences (Table 3) flanking the integrated proviral LTRs, there are two instances in which the virus had integrated into unlisted human DNA and a third in which the third provirus had integrated into the gene encoding human atrial natriuretic factor, in the stem cell producing both T-lymphocyte and myeloid progeny in bnx/hu mouse 30B1. Apparently alteration of this gene did not hamper hematopoietic differentiation. The inverse PCR method, modified for analysis of small cell numbers, provides a vital technique for assessing sites of random retroviral integration.

DISCUSSION

Transduction of pluripotent human stem cells will be necessary for successful gene therapy of a variety of genetic disorders of hematopoietic cells. An essential research goal must be to derive techniques to study the transfer of genes into human stem cells. *In vitro* systems are inadequate for analyses of the biology and transduction of pluripotent human stem cells. Committed progenitor cells expand in the cultures, obscuring the differentiation of progeny from the rare pluripotent stem cells that may be present. Another limitation is the inability to support the simultaneous growth and differentiation of both lymphoid and myeloid lineages from one human HSC in any culture system developed to date, in contrast to success in murine systems (16). All cells analyzed *in vitro* may, therefore,

Table 2. Integration patterns in individual human myeloid and T-cell clones recovered from bnx/hu mice

bnx mouse	No. $G418^R$ GM clones*	No. myeloid patterns†	No. neo-positive T-cell clones‡	No. T-cell patterns§	No. common integrants
bnx/hu mice with common integrants in human $CD3^+$ and $CD33^+$ populations					
25D4	17/480	14/16	3/480	2/2	0
27B2	12/576	2/12	6/768	2/6	1
30B1	9/522	6/9	2/288	2/2	1
30C1	22/480	11/17	13/884	4/6	1
bnx/hu mice with no common integrants in human $CD3^+$ and $CD33^+$ populations					
25B2	6/384	4/4	2/336	2/2	0
39C4	5/480	5/5	6/864	4/5	0

*Number of G418-resistant ($G418^R$) granulocyte–macrophage (GM) colonies containing >500 cells/number of $CD33^+$ human cells plated in individual wells.
†Number of individual vector integrant patterns per number of informative clones.
‡Number of T-cell clones positive for LN by PCR for the neo gene per number of human $CD3^+$ cells plated in individual wells.
§Granulocyte–macrophage colony-forming units and T-cell clones were plated from bnx/hu 39C4 and 25B2 mice to provide controls that had no apparent common integration patterns in the bulk-sorted $CD3^+$ and $CD33^+$ populations.

be the progeny of committed progenitors rather than pluripotent stem cells. Due to the lack of *in vivo* transplant systems that achieved significant levels of transduced human cells, transduction of human hematopoietic progenitors has traditionally been studied *in vitro*. Therefore, proof of human stem cell transduction has not previously been possible.

We used an immune-deficient mouse xenograft system to maintain transduced human $CD34^+$ progenitor cells for periods of 7–11 months. The durability of engraftment and the concurrent development of transduced progeny of both lymphoid and myeloid lineages allowed examination of the issue of pluripotent human stem cell transduction. Definition of the number of marked stem cells capable of producing progeny of both lineages was accomplished by single cell cloning and clonal analysis by inverse PCR. The progeny of each marked stem cell were identified by clonal integration analysis using an inverse PCR method adapted from Rill *et al.* (15). Inverse PCR products were examined for identical clonal integration patterns in pools of human lymphoid and myeloid populations recovered from each mouse, as well as in individual human T-cell and myeloid clones. We identified 3 mice, out of 24 analyzed, that contained human T-cell and myeloid clones with identical junctions between the proviral LTR and flanking cellular DNA (Table 3), confirming that human HSCs capable of differentiating into progeny of both lineages had been transduced by the LN vector. Since the T-cell lineage is thought to diverge from the myeloid lineage early in development (17), our data provide evidence that very primitive human hematopoietic cells can be transduced by retroviral vectors while retaining multilineage differentiation potential.

Mice that did not have common integrants in both lineages may have had vector-marked lineage-committed progenitors contributing separately to each lineage. For instance, mouse 25B4 had 10 individual proviral integrants in human cells of the myeloid lineage and 2 in the T-lymphoid lineage (Table 1). None of the myeloid integrants were found in restriction fragments of the same size as those obtained from human T-lymphoid cells recovered from the same mouse. These data indicate that the LN-transduced progenitors engrafted in mouse 25B4 lacked the potential for pluripotent differentiation. The human progenitors may have been lineage-restricted at the time of transduction. Alternatively, pluripotent stem cells may have been transduced, but then lost the capacity for multipotent differentiation during the 72-h *in vitro* transduction period or during engraftment in the mice. Further definition of these possibilities will aid in developing transduction protocols for human gene therapy trials, to ensure that the pluripotentility of stem cells is not compromised by the *in vitro* incubation period.

Identical clonal integration patterns were never detected in more than one mouse transplanted with portions of the same sample of transduced human cells. For instance, mice 25B1

Table 3. Sequences of inverse PCR products

Sample	Sequence: MoMuLV	Sequence: Flanking human DNA
27B2/GM5	aaatcttttattttat	CGAAGCTCAGAGAGGTATGGAACACCCTTAGGGCACACCGTAGCAAGTGGCGGG
27B2/T2	aaatcttttatttxat	CGAAGCTCAGAGAGGTATGXAACACCCTTAGGGCACACCGTAGCAAGTGGCGNG
27B2/$CD3^+$	aaatcttttattttat	CGAAGCTCAGAGAGGTATGGAACACCCTTAGGGCACACCGTAGCAAGTGGCGGG
27B2/$CD33^+$	aaatcttttattttat	CGAAGCTCAGAGAGGTATGGAACACCCTTAGGNCACACCGTAGCAAGTGNCGGG
30C1/GM6	ggtggcgtctttcatt	GAAAGACCNCCCTCCACGGGTGAGTACAACCACCCANNCCACCGCCACCCACA
30C1/T1	ggtxgggtctxtcatt	GAAAGACCCCXCTCCACGGGTGAGTACAACCACCCATTCCACCGCCACCCACA
30C1/$CD3^+$	ggtggcgtcttncatt	GAANGACCNCCCTCCACGGGTGAGTXCAACCACCCATTCCACCGCCACCCXCA
30C1/$CD33^+$	ggtggcgtctttcatt	GAAAXACCNCCCTCCACGGNTGAGTACAACCACCCATXCCACCGCCACCNACA
30B1/GM3	aaatcttttattttat	CGAGACACANCCTGGCCAACATGGTGAAACCCTGTCTGTACGNAAATTAGCTGXG
30B1/T1	aaatcttttattntat	CGAGACACAACCTGGCCAACATGGTGAAACCCTGTCTGTACGAAAATTAGCTGGG
30B1/$CD3^+$	aaatcttttattttat	CGAGACACAACCTGGCCAACATGGTGAAACCCTGTCTGTACGAAAATTAGCTGGN
30B1/$CD33^+$	aaatcttttaxtttat	CGAGACACAACCTGGCCAACATGGTGNAACCCTGTCTGTACGAAXATTAGCTGGG

N, base mismatch; X, base missing. A portion of the sequence from a representative human T-lymphoid (T) clone and a myeloid (GM) clone that matched the sequence of a band from the bulk-sorted human T-lymphoid ($CD3^+$) and myeloid ($CD33^+$) cells from the same mouse is shown. The Moloney murine leukemia virus (MoMuLV) sequences are shown in lowercase type, and the flanking cellular DNA is in uppercase type.

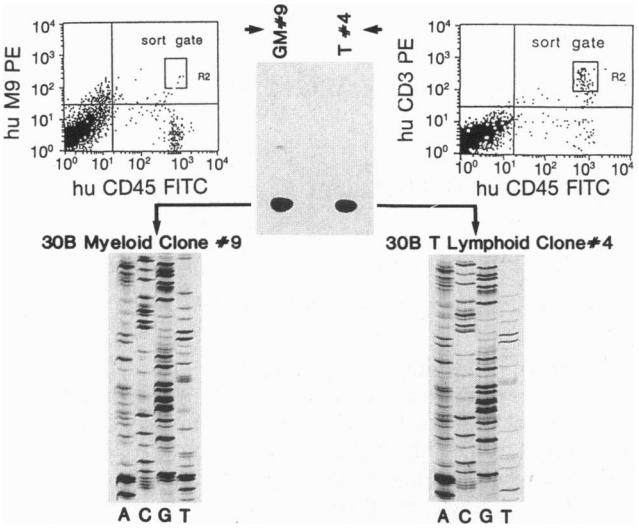

FIG. 4. Proviral clonal integration analysis of individual human T and myeloid clones. Bone marrow from bnx/hu mice was sorted into single-cell cultures by an ACDU. All wells that expanded to at least 500 cells were harvested. Genomic DNA was prepared from each clone and screened for the presence of provirus by PCR for neo sequences. Clonal analysis by inverse PCR was then performed on all neo-marked clones. Identity of bands with the same apparent molecular weight from T-cell and myeloid clones was verified by sequencing. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

through 25B4 (Table 1) were all transplanted with human $CD34^+$ progenitors from the same transduction flask. However, none of these mice had clonal integration bands in human T-lymphoid or myeloid pools that matched bands from the other mice in the same set. Finding two mice with shared vector integrant patterns among human T or myeloid populations would provide evidence that primitive progenitor cells had expanded during the 72-h *in vitro* transduction period, as has been shown in murine bone marrow transplant systems (18). Among the possible explanations for lack of *in vitro* expansion of marked human stem cells are (*i*) stem cell division did not occur during the 72-h *in vitro* incubation, but only after the cells had been transplanted, (*ii*) asymmetric division of the transduced stem cell occurred, with vector integrating into only one of the two daughter cells, or (*iii*) asymmetric division of the transduced stem cell occurred, resulting in one pluripotent stem cell and one lineage-restricted progenitor. Further studies using human stem cell marking and long-term engraftment in xenograft systems or human recipients followed by clonal analysis (19) may further define the parameters of stem cell division.

The unique system we report here combines sustained growth and multilineage differentiation of transduced human hematopoietic stem cells with the capacity to determine clonal relationships among transduced cells by inverse PCR. This model will allow analysis of conditions to increase the frequency of gene transduction of pluripotent human HSCs, including the use of purified human stem cell populations, stimulation with novel cytokines, such as flt-3 ligand, or other gene delivery systems, such as AAV-based vectors.

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