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INHERITED DISEASE ***RESEARCH ARTICLE***

An in vitro system for efficiently evaluating gene therapy approaches to hemoglobinopathies

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A variety of gene therapy strategies are under development for the treatment of sickle cell anemia and other hemoglobinopathies. A number of alternative vectors have been developed to transfer and express the β-globin gene and other therapeutic molecules, but none has resulted in efficient transduction and stable long-term expression in primary hematopoietic cells. One reason for this problem is that most vectors are initially evaluated in immortalized cell lines which may not faithfully recapitulate the biology of primary erythroid cells. In order to provide a more relevant system for efficiently evaluating alternative vector constructs for β-globin disorders, we have developed (1) a simple method for generating primary human red blood cell (RBC) precursors in liquid culture established with mononuclear cells obtained from normal donors as well as patients with Hb SC disease; (2) a high titer retroviral vector which can be easily modified to optimize gene transfer and transgene expression; and (3) methods for transducing the RBC precursors at high efficiency. The development of simple and efficient methods and reagents for generating and transducing primary human RBC precursors provides a facile and effective means for screening alternative gene therapy strategies. Gene Therapy (2000) **7,** 215–223.

Keywords: *gene therapy; retrovirus; erythrocytes; sickle cell anemia*

Introduction

Sickle cell anemia and other hemoglobinopathies are among the most common inherited blood disorders and have been well characterized at the molecular level.[1,2] Conventional medical therapy can treat some of the symptoms associated with these diseases, however bone marrow transplantation is the only available curative treatment.[3,4] Unfortunately, most patients lack an HLA-matched sibling, and the use of alternative sources of stem cells for transplantation is associated with a high risk of morbidity and mortality.[5,6] In addition, the conventional treatment of sickle cell disease and other hemoglobinopathies is associated with significant toxicities including transfusion-related iron overload.[7] The lack of an effective form of therapy for many of these patients has sparked an interest in developing gene therapy based strategies as possible alternatives or adjuncts to conventional medical approaches. Examples of such gene therapy approaches include gene replacement strategies where wild type β-globin or γ-globin transgenes are delivered to erythroid lineage cells and genetic repair strategies where molecules such as trans-splicing ribozymes are employed to amend mutant globin transcripts.[8–11]

In order to develop effective gene therapy approaches to hemoglobinopathies using any of these strategies, a variety of vector constructs will have to be evaluated in order to optimize stability, toxicity and transgene expression. Many investigators have employed the murine erythroleukemia (MEL) cell line as well as other immortalized cell lines in order to evaluate alternative vectors.[12–15] These cell models have proven to be very useful for dissecting some of the factors required for effective β-globin transgene expression. Unfortunately, lessons learned from these artificial cell systems do not necessarily predict how transgenes will behave in primary human erythroid lineage cells.[16–20] A more relevant system for evaluating alternative vector constructs would be to transduce primary human erythroid cells. Fibach *et al*[21] described a method for generating mature human red blood cells (RBCs) from peripheral blood mononuclear cells using liquid cultures. In this two step procedure, cells were first grown for a week in conditioned media taken from bladder carcinoma cell cultures and then transferred to media containing erythropoietin. While this method is effective, it relies on conditioned media, which has undefined components, and the two step procedure is relatively complex. Moreover, no information is available regarding the ability to modify erythroid cells grown under these conditions genetically. More recently, Malik *et al*[22] developed a one step culture system for generating erythroid cells from $CD34^+$ selected progenitors and demonstrated that these cells could be readily transduced with retroviral vectors. While this system appears useful for studying a variety of gene transfer related issues, it requires the collection of large number of hema-

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topoietic cells in order to perform the $CD34^+$ cell selection procedure. This limits the utility of this approach for efficiently evaluating alternative vectors, particularly in erythroid cells generated from patients with hemoglobinopathies since it is difficult to obtain large numbers of hematopoietic cells from these individuals.

In the current study, we sought to develop simpler and more efficient procedures for evaluating alternative gene transfer vectors in primary erythroid precursors generated from healthy donors and from persons with hemoglobinopathies. A single-step serum-free culture system was developed for generating RBC precursors from mononuclear cells obtained from the umbilical cord blood of healthy neonates and from the peripheral blood of adults with Hb SC disease. In addition, retroviral vectors and techniques were developed which resulted in efficient gene transfer into the erythroid precursors generated in these culture conditions. The retroviral vector developed in the course of these studies was designed in a modular fashion so that future modifications designed to optimize the expression and stability of therapeutic transgenes could be readily performed. These methods and reagents for generating and efficiently transducing erythrocyte precursors from a widely available source provide a simple, quick, and effective means for evaluating vector constructs in clinically relevant cells.

Results

Generation of erythrocyte precursors in serum-free liquid cultures from unfractionated UCB mononuclear cells and from peripheral blood mononuclear cells obtained from patients with hemoglobin SC disease

To facilitate evaluation of therapeutic genes in primary human RBCs, we established culture conditions for generating erythroid lineage cells directly from mononuclear cells. Fresh umbilical cord blood mononuclear cells were isolated and grown in liquid culture for 3 weeks in the presence of Flt-3-ligand, IL-3, and erythropoietin (Epo). This cytokine combination and culture duration was determined by comparing a variety of alternative culture conditions and incubation times geared towards generating erythroid lineage cells (data not shown). Morphologic analysis revealed that the majority of erythroid lineage cells present on day 1 of incubation were mature red blood cells (RBCs) (Figure 1a). After 8 days in culture however, mature RBCs accounted for less then 20% of the total erythroid lineage, being replaced by more immature nucleated erythrocyte precursors (Figure 1a). FACS analysis of cultured cells stained with the erythroid specific antibody E6[23] revealed that the percentage of erythroid lineage cells increased from a mean of 40% on day 1 to a mean of 90% on day 15 in culture, with a slight decline thereafter (Figure 1b). Overall, total cells expanded a mean of 25-fold (range 1.9–55-fold) and erythroid lineage cells expanded a mean of 78-fold (range 3–127-fold) (Figure 1c). During the culture period, the hemoglobin content of the red cells shifted from primarily hemoglobin F at the start of incubation to primarily hemoglobin A at the end of the culture period (Figure 1d).

These same culture conditions were employed to generate RBC precursors from peripheral blood mononuclear cells (PBMCs) obtained from patients with hemoglobin SC disease undergoing therapeutic phlebotomy. Cells from two of five samples (both from the same individual) failed to expand during the culture period. In the other samples obtained from three different patients however, total cells expanded 4.8-fold (range 2.3–9.5) and erythroid cells expanded 11-fold (range 4.6–24) (Figure 2b). The purity of erythroid lineage cells was similar to that seen with umbilical cord blood, with the majority of erythroid cells on day 1 being mature red blood cells and those at later time points being nucleated red cells (Figure 2a). Although considerable inter-individual variation exists, these data indicate that it is feasible to expand erythroid lineage cells using simple culture conditions directly from most UCB mononuclear cells and from many PBMCs obtained from patients with Hb SC disease.

Retroviral vector mediated gene transfer into RBC precursors generated from mononuclear cells

Next we sought to develop procedures for efficiently transducing RBC precursors generated in the mononuclear cell cultures. In order to facilitate these studies, a novel retroviral vector was developed, termed Luv (Figure 3). Important components of the Luv vector included: (1) utilization of an extended packaging signal which includes a portion of the gag coding sequence to enhance packaging of the viral genomic RNA and increase titer;[24] (2) utilization of the wild-type MoMLV splice signals and the viral envelope start codon (ATG) for initiation of translation of vector encoded transgenes to facilitate viral RNA processing and transgene expression;[15] (3) disruption of the Pr65 *gag* open reading frame with a stop codon to minimize the possibility of translating gag peptides which could contribute to vector immunogenicity and toxicity; (4) elimination of any MoMLV envelope sequences which could contribute to vector immunogenicity and toxicity; and (5) inclusion of a multiple cloning site within the 3′ LTR U3 region for development of double copy vectors.[25] In order to identify cells transduced with Luv-derived vectors, a truncated version of the nerve growth factor receptor (NGFR) gene as well as the optimized green fluorescence protein (GFP) were cloned into the Luv vector to yield LuvNM and LuvGM, respectively.[26–28] These marker genes permit rapid determination of retroviral titer and evaluation of transduced cell populations using multiparameter FACS analysis. In addition, Luv was constructed in a modular manner so that important components of the vector could be easily exchanged in future studies with alternative sequences designed to enhance titer, stability and transgene expression. When transduced into NIH3T3 cells, both LuvNM and LuvGM demonstrated high level expression of the marker gene and were produced at titers of $1–3 \times 10^6$ IU/ml.

Initial screening experiments showed the LuvNM vector to be slightly more efficient than the LuvGM vector in transducing erythroid lineage cells (data not shown). Using the LuvNM vector, a variety of gene transfer procedures were evaluated in erythroid cells generated from UCB mononuclear cells. These studies demonstrated that the most efficient method was to transfer mononuclear cells on to culture plates previously coated with fibronectin fragments and then add retroviral supernatant daily for 3 days (data not shown). Transduced erythroid cells could be easily recognized by immunofluorescent microscopy and FACS analysis (Figures 4 and 5). These

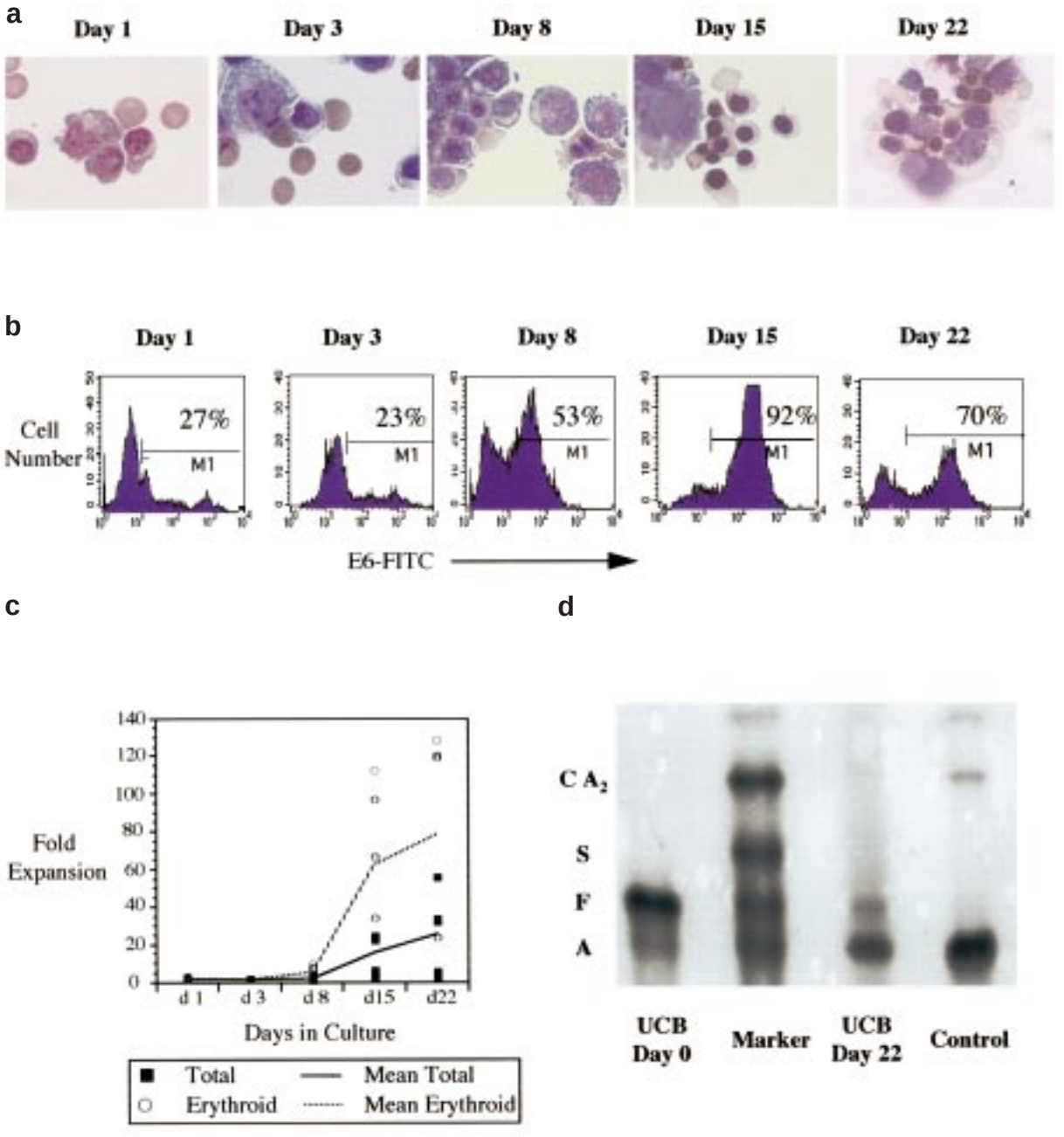

Figure 1 *Erythroid lineage cells derived from umbilical cord blood mononuclear cells. (a) Representative morphology of erythroid cells generated from UCB. Mononuclear cells were obtained and cultured as described in Materials and methods. Wright–Giemsa staining was then performed on days 1, 3, 8, 15 and 22. (b) Representative FACS analysis of erythroid cells generated from UCB. Cells were stained at progressive time points with the erythroid cell specific antibody E6. The percentage of E6 expressing cells is presented above the M1 cursor. (The data in panels a and b is representative of five experiments). (c) Fold expansion of total cells and erythroid lineage cells generated from UCB. The data is the mean of five experiments. (d) Cellulose acetate electrophoresis analysis of erythroid cells generated from UCB. Y axis indicates migration pattern for various hemoglobin types. The data are representative of three experiments.*

techniques resulted in a mean transduction efficiency of the erythroid cells of 51% (range 36.4–66%) using the LuvNM vector (Figure 6a). Expression of the marker gene remained consistent throughout the duration of the cultures indicating that stable gene transfer and transgene expression had occurred. In order to determine whether the vector or the transduction process altered the biology of the RBC precursors, UCB-derived erythroid cells were transduced with the LuvNM vector and analyzed for total cell and erythroid cell growth. No difference was noted in the total fold expansion or erythroid lineage expansion of transduced cells relative to non-transduced cells (Figure 6b and c).

Next, these gene transfer procedures were evaluated in erythroid cells generated from PBMCs obtained from patients with Hb SC disease. While the gene transfer

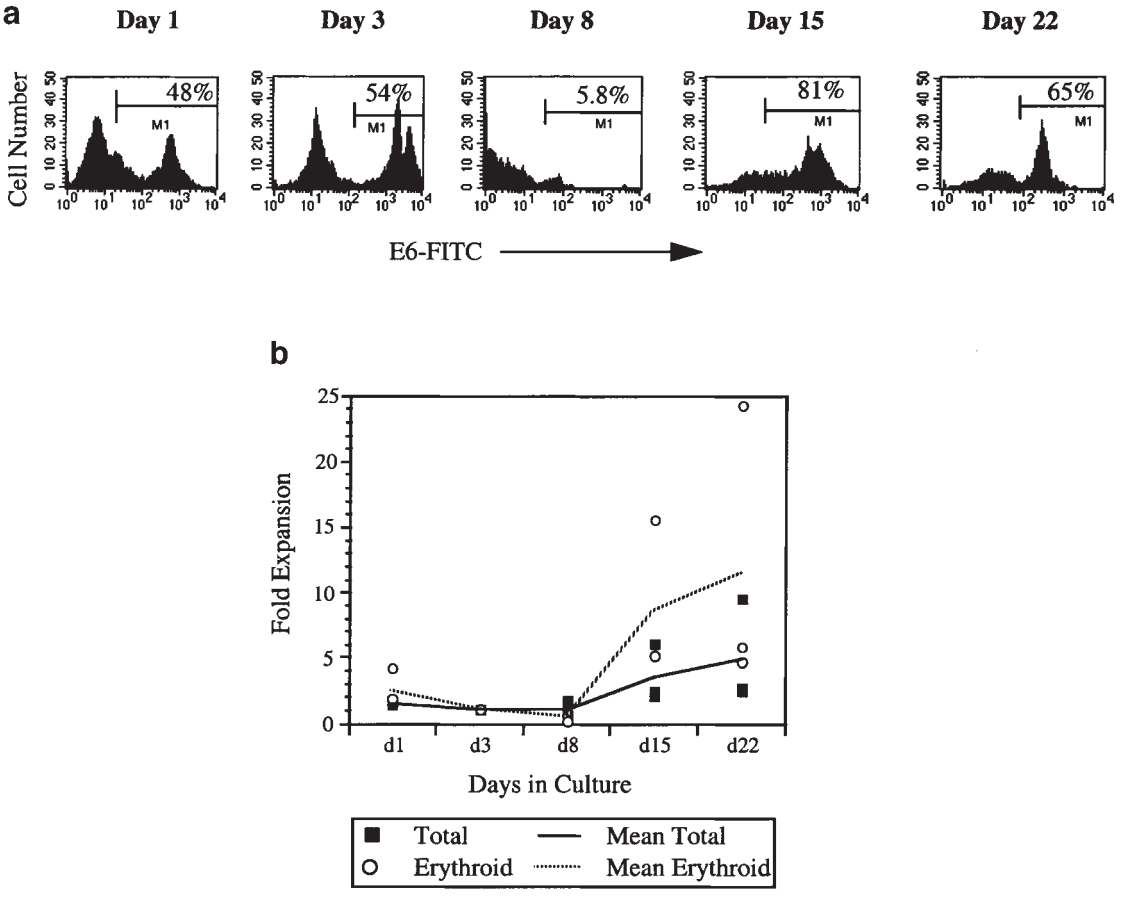

Figure 2 *Peripheral Hb SC mononuclear cell cultures. (a) FACS analysis of erythroid cells generated from Hb SC mononuclear cells stained at progressive time points with the erythroid cell specific antibody E6. The percentage of E6 expressing cells is presented above the M1 cursor. Data is representative of three experiments. (b) Fold expansion of total cells and erythroid lineage cells generated from Hb SC mononuclear cells. The data are the mean of three experiments.*

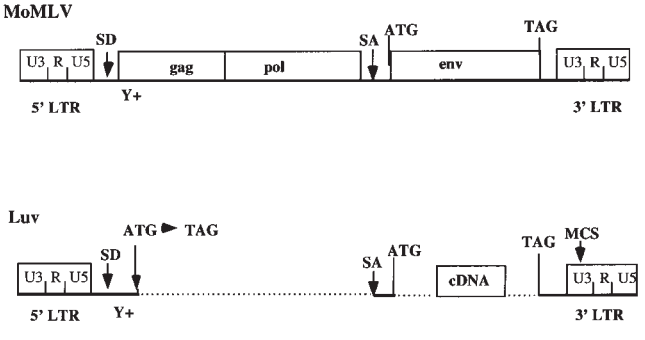

Figure 3 *The Luv vector backbone compared to the Moloney murine leukemia virus (MoMLV) genome. LTR, long terminal repeat; SD, splice donor; Y, the packaging sequence; ATG → TAG, mutation in the gag open reading frame to eliminate translation of gag peptides; SA, splice acceptor; ATG, envelope start codon cloned in frame with the marker or therapeutic transgene (cDNA); TAG, envelope stop codon; MCS, the multiple cloning site in the 3′ LTR.*

efficiency in the Hb SC erythroid cells was slightly lower and showed greater variability than in UCB erythroid cells, a mean of 50% of the Hb SC erythroid cells were stably transduced (Figure 7a). Again, the growth pattern of total cells and Hb SC erythroid lineage cells was unaffected by transduction with the LuvNM vector (Figure 7b and c). These observations indicate that erythroid cells generated from mononuclear cells obtained from both UCB and peripheral blood of patients with Hb SC disease can be efficiently and stably transduced with the Luv vector without obvious effects on erythroid biology.

Discussion

In this report, simple techniques were described for culturing and transducing human erythroid cells at high efficiencies. These procedures are based on generating erythroid lineage cells from mononuclear cells obtained from UCB and peripheral blood of patients with hemoglobin SC disease, both of which are readily available. This is a considerable advantage for comparing alternative vectors in primary cells relative to previously described techniques which rely on multi-step culture techniques and selecting $CD34^+$ cells. In addition, the Luv vector developed during the course of these studies may further facilitate gene therapy studies since it is easy to modify with alternative transgenes and elements which may enhance transgene expression and titer. An additional advantage of the Luv vectors is that the gfp and NGFR marker genes permit easy determination of gene transfer efficiencies, as well as rapid isolation of transduced cells using flow cytometric or immunoadhesion techniques.[26–28]

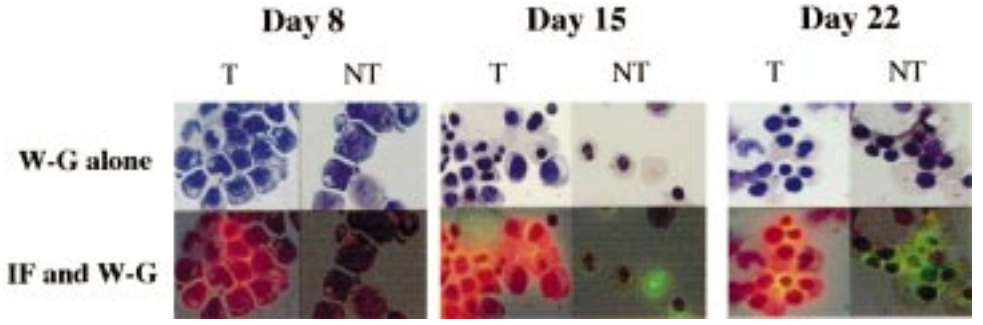

Figure 4 *Immunofluorescence microscopy of erythroid cells generated from UCB and transduced with LuvNM. T, transduced; NT, nontransduced controls. At each indicated time point, the upper panels are stained with Wright–Giemsa (W–G) and directly below them are the identical fields stained with both W–G and anti-NGFR-PE (red), anti-E6 FITC (green). Similar results were obtained with LuvGM (data not shown). Data are representative of five experiments.*

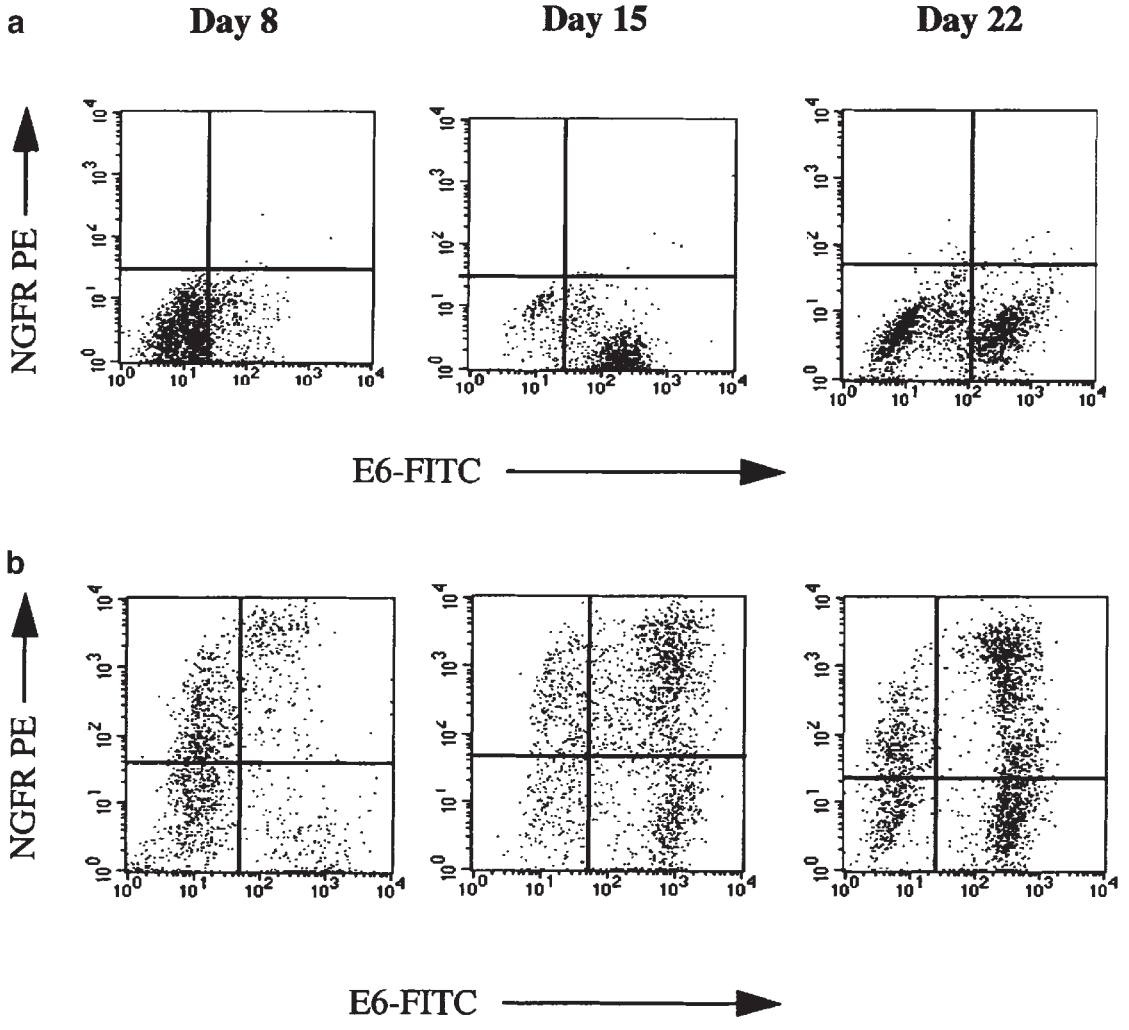

Figure 5 *FACS analysis of erythroid cells generated from UCB transduced with LuvNM. Erythroid cells transduced with LuvNM as described in Materials and methods and then analyzed at progressive time points for staining with the erythroid specific antibody E6 and anti-NGFR. Panel a is the mock infected controls; panel b is the LuvNM transduced cells. Data are representative of five experiments.*

The techniques and reagents described in this report could be used for a variety of applications. First, Luv derivatives expressing alternative β-globin expression cassettes could be easily engineered and tested in primary human erythroid cells. Second, gene therapy strategies aimed at repairing mutant Hb DNA or RNA could also be easily tested. For example, we have been developing a strategy for correcting sickle cell disease via trans-splicing ribozymes which convert the β^s-globin RNA transcript to γ-globin.[11] The ability to generate and

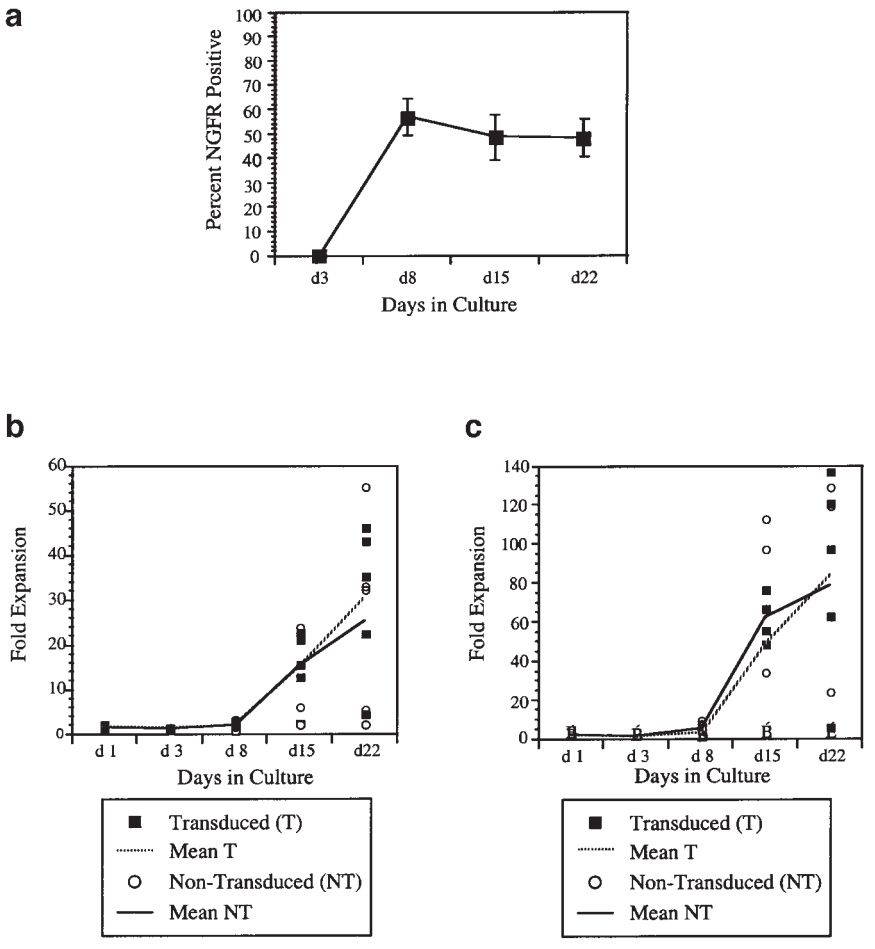

Figure 6 *Transduction of UCB-derived cells. (a) The mean percentage of UCB-derived erythroid lineage cells expressing NGFR. (b) Comparison of total cell expansion from transduced and non-transduced samples based on viable cell counts. (c) Comparison of erythroid cell expansion from transduced and non-transduced samples based on viable cell counts multiplied by the proportion of cells staining positive for E6. All data are the mean of five experiments.*

transduce primary human erythroid cells efficiently allows the comparison of a variety of constructs designed to enhance ribozyme expression, specificity and efficiency in cells which have a variety of critical biologic differences relative to immortalized cell lines. Third, in addition to gene therapy applications, the culture conditions described in this report may be useful for studying various aspects of erythroid cell biology including globin RNA trafficking and processing. In addition, these procedures may provide a facile method for studying Hb gene switching since the umbilical cord blood cells convert from expressing hemoglobin F to hemoglobin A during the 3 weeks in culture.

There are several caveats to the results described in this report. First, there was wide variability in the total cell and erythroid cell expansion in the culture conditions. While this variability was seen regardless of the source of mononuclear cells, it was more pronounced in the peripheral Hb SC cultures where two of the five cultures showed no expansion at all. The reasons behind this wide variability are not clear but may relate to donor age, other disease processes as well as inherent genetic differences which govern hematopoietic stem cell content and biology. In addition, we observed that peripheral mononuclear cells from sickle cell patients being treated with hydroxyurea tended to show minimal expansion (data not shown), presumably due to cytotoxic effects of the hydroxyurea on erythroid progenitors. A second caveat to these studies is that the culture conditions were optimized to generate primarily nucleated red blood precursors rather than mature anucleate red blood cells. This was done because our primary interest is in developing trans-splicing ribozymes for correcting β^s-globin RNA transcripts. Consequently, we focused on red cell precursors which are rich in mRNA, the target for the trans-splicing ribozyme, as opposed to mature anucleate cells. For studies focusing on globin protein biology, it may be feasible to generate higher proportions of mature RBCs by adding higher levels of erythropoietin and serum to the cultures. A third caveat to these studies is that the culture and transduction conditions were not systematically tested using mononuclear cells obtained from bone marrow or from patients with sickle cell disease or any of the thalassemias. Consequently, it is not clear whether these techniques may be applied in these other settings without additional modifications.

Despite these caveats, the techniques and reagents described in this report provide a simple and efficient methodology for evaluating alternative gene transfer strategies aimed at red cell disorders in more relevant cells than immortalized cell lines. The ability to assess multiple variations of the standard Luv vector or other

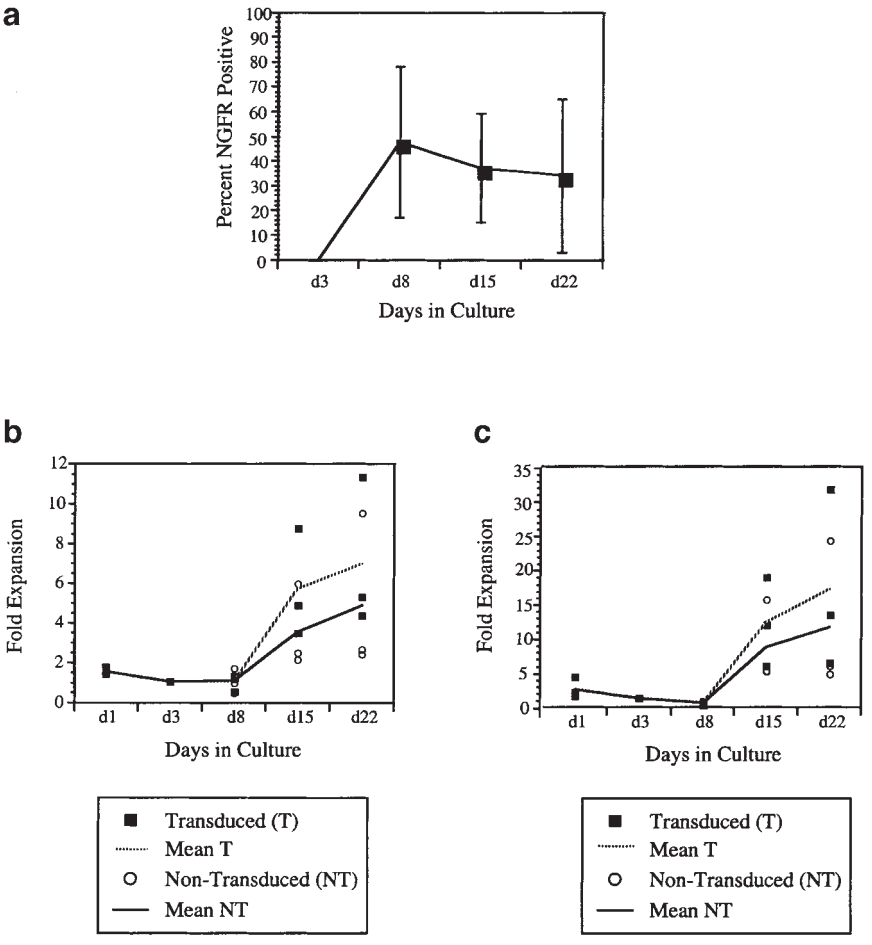

Figure 7 *Transduction of Hb SC PBMC derived cells. (a) The mean percentage of Hb SC PBMC derived erythroid lineage cells expressing NGFR. (b) Comparison of total cell expansion from transduced and non-transduced samples. (c) Comparison of erythroid cell expansion from transduced and non-transduced samples. All data are the mean of three experiments.*

gene delivery systems, combined with a quick and easy method of screening erythroid transduction efficiency will be a valuable tool in developing gene therapy for the treatment of sickle cell anemia and other hemoglobinopathies.

Materials and methods

Retroviral vector construction

The Luv series of retroviral vectors was constructed by using PCR to clone modular elements from a molecular clone of proviral Moloney murine leukemia virus (MoMLV). The overall design of the Luv series and its relationship to the parental MoMLV sequence is depicted in Figure 3. Directional assembly of proviral plasmids was accomplished using both mutagenesis of the PCR fragments via mismatched primers and by adding specific sequences to the ends of PCR fragments. The Luv vector is composed from three major fragments. Fragment 1 contains the entire 5′ LTR and extends to position 1041 of MoMLV with modification of the Pr65 gag initiating methionine to a TAG stop codon. Fragment 1 was generated in two steps from Fragments 1A and 1B using the PCR primer pairs described below. Fragment 2 extends from the wild-type splice acceptor at position 5403 to the envelope start codon at position 5779 with modification of the A nucleotide at position 5775 to a C nucleotide. Fragment 3 begins with the envelope stop codon at position 7772 and extends through the entire 3′ LTR. The MoMLV sequence is printed in bold type and the restriction sites used for directional cloning of the three PCR fragments are underlined in Figure 3. The PCR primers used to generate the three fragments are listed below:

Fragment 1A:
(upstream) 5′ ggcgcggcaagcttg**aatgaaagaccccacctg** 3′
(downstream): 5′ **ggtaacagtcttggcc**cta**attctcagacaaatacag** 3′
Fragment 1B:
(upstream) 5′ ct**gtatttgtctgagaat**tag**ggccagactgttaccact** 3′
(downstream) 5′ ggcgcggcg**agatct**c**atatggcgcctagagaagg** 3′

Fragment 2:
(upstream) 5′ ggcgcggc**agatctt**a**tatggggca** 3′
(downstream) 5′ ggcgcggccc**atggcagtctagaggatggtcc** 3′

Fragment 3:
(upstream) 5′ ggcgcggcccatggcgcggatccca**tagataaaaataaaag** 3′
(downstream) 5′ ggcgcggcgaatt**caatgaaagacccccgctgacg**

Hot start PCR reactions were performed in 100 ml of a mixture containing 100 mM dNTPs, 0.5 mM each primer, 10 ng template DNA, 10 ml 10 × Pfu buffer and 2.5 units Pfu DNA polymerase (Stratagene, La Jolla, CA, USA).

The reaction was initially denatured by incubating at 95°C for 4 min followed by two cycles of (95°C, 30 s; 50°C, 30 s; 72°C, 2 min); 25 cycles of (95°C, 30 s; 60°C, 30 s; 72°C, 2 min) then held at 72°C for 7 min. PCR products were purified using Centricon-100 columns (Amicon, Bedford, MA, USA) according to the manufacturer's directions. Each fragment was individually ligated into the plasmid puc19 and sequenced. A multiple cloning site was introduced into the unique *Nhe*I site within the U3 region for subsequent generation of double copy vectors. Individual PCR fragments were combined and ligated into the unique *Hin*dIII/*Eco*RI site of pBR322 in which the *Bam*HI site was destroyed to generate the LuvM proviral plasmid. To generate the LuvNM retroviral vector, the NGFR marker gene[27–29] was inserted into the *Not*I site in frame with the original Moloney envelope start codon (ATG). To generate the LuvGM vector, the GFP marker gene (Clontech, Palo Alto, CA, USA) was also inserted into the *Not*I site. Recombinant amphotropic LuvNM and LuvGM virus were produced in the AM12 cell line and titered on NIH3T3 cells as previously described.[27,28]

Isolation and culture of mononuclear cells from umbilical cord blood and peripheral blood of patients with Hb SC disease

Peripheral blood intended for disposal was obtained from patients with hemoglobin SC disease undergoing scheduled phlebotomy in accordance with IRB approved protocols. Umbilical cord blood was collected in accordance with IRB approved protocols from labor and delivery into citrate-phosphate-dextrose anticoagulant (Abbott Laboratories, North Chicago, IL, USA). Mononuclear cells were isolated by Ficoll–Hypaque gradient separation (American Red Cross, Washington DC, USA), washed three times with Dulbecco's phosphate buffered saline (PBS) (Gibco BRL, Rockville, MD, USA) and suspended at a concentration of 1×10^6 cells per milliliter in serum-free conditions consisting of Iscove's modified Dulbecco's medium with 1% bovine serum albumin, 10 μg/ml insulin, 200 μg/ml transferrin (BIT9500 media, Stem Cell Technologies, Vancouver, BC), 40 μg/ml low density lipoprotein (Sigma, St Louis, MO, USA), 2 μM glutamine (Life Technologies), and 5×10^{-5} M β-mercaptoethanol (Life Technologies), supplemented with Flt-3 ligand (25 ng/ml, Immunex, Seattle, WA, USA), IL-3 (2.5 ng/ml, R&D Systems, Minneapolis, MN, USA), Erythropoietin (1 U/ml, R&D Systems), and penicillin G (100 U/ml)/streptomycin(100 μg/ml) (Gibco BRL). One million cells per well were incubated in 24 well plates at 37°C in a 5% CO_2 incubator overnight, then transferred to fresh plates to eliminate adherent cells. Cells were then cultured in the same serum free media at 37°C for further analysis and manipulation as described below.

Retroviral transduction of erythrocyte precursors

On day 3 of culture, cells were counted and the volume was adjusted so that the cell concentration was 5×10^5 cells in 375 μl of serum-free media. Cells were transferred to a 24-well plate previously coated with 25 μg/ml RetroNectin (PanVera, Madison, WI, USA). LuvNM or LuvGM retroviral vector supernatant was added in a 3:1 cell to supernatant ratio by volume and incubated at 37°C in a 5% CO_2 incubator. An equal volume of retroviral supernatant was also added on days 4 and 5.

Flow cytometric and morphologic analysis of cultured erythrocyte precursors

Samples were counted weekly and medium, was added as needed to maintain the cell concentration at no greater than 5×10^5 cells per milliliter, with a maximum volume of 1 ml per well. An aliquot of cells was analyzed by FACS analysis and immunofluorescent microscopy on days 1, 3, 8, 15 and 22. Briefly, the cells were washed with PBS, and resuspended in 100 ml versene (Gibco BRL) with 4% fetal calf serum. Cells were incubated with E6, an antibody to red cell surface protein band 3 (a generous gift of Marilyn Telen, Duke University Medical Center, Durham, NC, USA), washed with PBS, and then stained with a secondary antibody conjugated to FITC (Jackson ImmunoResearch Lab, West Grove, PA, USA). For gene transfer experiments, cells were then washed, and stained with an anti-NGFR monoclonal antibody conjugated with phycoerythrin. 7-AAD was added to exclude dead cells and all samples were run on a FACSCalibur (Becton Dickenson, San Jose, CA, USA). For morphologic analysis, an aliquot of the stained cells was cytocentrifuged (Shandon Lipshaw, Pittsburgh, PA, USA) and fluorescent images were acquired using a digital video microscope (Olympus BX60 microscope, Optronics Engineering DEI-750 video camera, Scion Image 1.60 video capture software, NIH, Bethesda, MD, USA). The same slides were then stained with Wright–Giemsa using an automated stainer, the original cell field was located using bright field microscopy and the image was digitally acquired. The Wright–Giemsa and fluorescent images were then superimposed using Adobe Photoshop (Adobe Systems, Seattle, WA, USA). Cellulose acetate electrophoresis analysis of cultured cells was performed according to kit instructions modified for smaller sample sizes (Helena Laboratories, Beaumont, TX, USA).

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