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Antibody-directed lentiviral gene transduction in early immature hematopoietic progenitor cells

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Abstract

Background—The specific and efficient transduction of retroviral particles remains problematic for *in vivo* and *ex vivo* gene therapy studies, where the targeting cell population is a heterogeneous bulk population.

Methods—Pseudotyping lentiviral particles with Sindbis virus Env proteins modified with an immunoglobulin Fc-binding domain presents a method of selecting cells within a mixed population through antibody (Ab)-mediated targeting. Conditions were tested for targeted lentiviral gene delivery to hematopoietic progenitor cells via Ab-conjugated envelopes independent of CD34.

Results—Conditions to optimize the efficiency of gene delivery were established using the ABCG2 multidrug resistance protein, associated with stem cell phenotypes, as the cell surface target. By varying the proportion of ABCG2 expressing cells in a population, ABCG2-targeted gene delivery was detectable by flow cytometry when ABCG2⁺ cells comprise greater than 5% of the population. Conditions that increased the efficiency of gene transfer including cholesterol independent Env proteins and pH, increased non-specific gene delivery. The feasibility of this cell-Ab-virus sandwich system in targeting transduction in a mixed population was tested in cells derived from human cord blood (CB). Conjugation of viral particles with anti-CD133 and anti-ABCG2 hematopoietic stem cells-associated Ab resulted in targeted gene transfer into early immature hematopoietic progenitor cells. Enhancement was found when the hematopoietic progenitor cells were enriched from CB cells via the depletion of lineage⁺ committed cells.

Conclusions—Gene transfer to lineage[−] early immature hematopoietic progenitors from human umbilical CB was obtained using CD133, ABCG2 or HLA-1 antibodies conjugated to lentiviruses pseudotyped with modified Sindbis viral Env proteins.

Keywords

Human cord blood; hematopoietic progenitor cells; targeted viral entry; Sindbis Env pseudotyped lentiviral particles; CD133; ABCG2

Introduction

The safety and efficacy of gene delivery can be improved through targeted content release, minimizing the potential of imparting detrimental effects on irrelevant cells or tissues. Several approaches have been explored in order to alter and/or narrow the viral tissue

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tropism and host range, facilitating specific transduction. These approaches include mediating infection by inserting ligands [1], random libraries [2–4], or single-chain antibodies into the receptor binding site of retroviral Envelope (Env) proteins [5–9]. Pseudotyping alternative viral Envs onto viral vectors provides an additional means of changing the tissue and host specificity. Pseudotyping lentiviral particles with vesicular stomatitis virus G (VSV-G) Env provides an extremely stable particle with a broad range of infectivity [10,11]. However, the property of broad spectrum limits the use of VSV-G Env pseudotype in *in vivo* studies due to safety concerns. Recent advances in pseudotyping modified Sindbis virus Env onto lentiviral particles have proven effective for targeted gene transfer due to the high levels of expression, high-titer transduction efficiencies and the relative ease for molecular engineering these constructs [12–19].

Sindbis virus, a member of the *Alphavirus* genus, can infect a broad range of insect and vertebrate cells due to the wide distribution of the cellular receptors (laminin and heparin) [20,21]. Sindbis virus infection of dendritic (DC) and reticuloendothelial cells is associated with the presence of DC-SIGN and L-SIGN surface molecules [22]. In order to reduce non-specific binding and increase selective targeting, a wide range of modifications have been incorporated into the Sindbis Env. These modifications include deletion of the laminin receptor binding domain [17] and/or replacement of the laminin receptor binding site with biotin-adaptor peptides [23] or the protein A immunoglobulin G (IgG) recognition domain (ZZ domain) [24]. Introduction of the ZZ domain allows for targeted viral infection via conjugation with a specific antibody [24]. Further mutations of the Sindbis Env modified the undesirable non-specific heparin-binding sites [15] and mediated fusion in the absence of cholesterol [25]. Several systems pseudotyping modified Sindbis Env onto lentiviral vectors have significantly enhanced the specificity of viral infection. Using lentiviral particles pseudotyped with the modified Sindbis Env (m168)-antibody conjugate, lung metastatic melanoma cells were targeted by *in vivo* tail vein viral injection [15]. The use of a variety of antibody molecules has been shown to be effective in targeting specific cell types [19,26,27]. Alternatively, a system has been developed utilizing a Sindbis Env that is negative for receptor binding but positive for membrane fusion. Viral binding is mediated through particles expressing CD20, which binds to target cells expressing anti-CD20 surface immunoglobulins. Lentiviral pseudotypes bearing this dual binding/fusion system are effective both *in vitro* and in live animals [17,18,25].

For many gene transfer protocols, the target cells are within a heterogeneous population of cells ranging in their potential for differentiation and self-renewal. Of particular interest is the ability to target the human hematopoietic stem cells (HSCs), which represent a small subpopulation in the cord blood (CB) cells. The success of selective transduction of HSCs in CB cells would be a highly significant advance in clinical translational research. Studies using Sindbis Env (m168) conjugated with CD34 antibodies were capable of targeting CD34⁺ progenitor cells from human fetal liver and non-purified peripheral blood mononuclear cells [19], however CD34[−] cells have also been reported to function as long-term repopulating cells [28–31]. Alternative putative cell-surface markers on HSCs include ABCG2 and CD133. The mRNA of the multidrug-resistance protein ABCG2 was highly expressed in primitive murine HSCs and associated with cells with stem cell-like properties including side population (SP) cells [32,33]. Transduction of the ABCG2 gene in cord-blood-derived early human hematopoietic progenitor cells increased the number of clonogenic progenitors and enhanced the proportion of CD34⁺ progenitors *in vivo* [34]. Similarly, CD133 was identified as a marker on human HSCs and progenitor cells [35,36]. In the current study, m168 Sindbis Env-Ab conjugates, pseudotyped onto lentiviral particles [15], were optimized and tested for their ability to target early hematopoietic progenitor cells within CB cells utilizing the cell surface markers ABCG2 and CD133.

Materials and Methods

Cell lines

293T cells purchased from American Type Culture Collection (ATCC; Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) containing 10% fetal bovine serum (FBS; Atlanta Biologicals), antibiotics and antimycotics in a 5% CO₂ atmosphere. Saos2-ABCG2 and Saos2-pcDNA are human osteosarcoma cell lines transduced with the human breast cancer resistant protein (ABCG2/BCRP) cDNA expressed in a pcDNA3 vector and a control pcDNA3 vector, respectively. Both cell lines were kindly provided by Dr. B. Sorrentino (St. Jude Children's Research Hospital) [33] and cultured in McCoy's 5A Medium (GIBCO) supplemented with 10% FBS in the presence of 400 µg/ml G418. Mouse melanoma cell line B16F10-MDR5 overexpressing human multidrug resistance protein 1 (MDR1/ABCB1), was kindly provided by Dr. I. Chen (UCLA) [15] and grown in DMEM containing 10% FBS. The corresponding wild type cell line B16F10 was purchased from ATCC.

Plasmids

Chimeric Sindbis virus envelope m168 was kindly provided by Dr. I. Chen (UCLA) [15]. The ZZ domain was excised from m168 by digestion with BstEII and followed by self-ligation, producing the construct m168ΔZZ. The mutation AK226-227SG in E1 [25,37], generating cholesterol independent infection, was incorporated in m168ΔZZ. Briefly, two PCR fragments were amplified from m168 with the following two sets of primers: 1a): 5'-cctttggccgctttcatcg-3', 1b): 5'-catgcacgttccggaggagagcttg-3'; 2a): 5'-caagccttctccgggaacgtgcatg-3', 2b): 5'-gtcagcatcatgctgcaagc-3' (mutations underlined). After overlapping PCR, mutations were fragment-exchanged into m168ΔZZ through the NarI sites, and the ZZ domain was reintroduced through the BstEII sites, generating m168-SG. Human immunodeficiency virus (HIV)-1 based vector HR'CMVGFPW [38], HIV-2 based vectors HIV-2gtPGIPW [39] and HIV-2 packaging vector HIV-2CGP-AC were a gift from Dr. J. Dougherty (UMDNJ). The HIV-1 packaging vector pCMVd8.2dVPR, bearing a deletion of env and vpr was purchased from Addgene (www.addgene.org). HIV-2-CGP-AC is similar to HIV-2-CGP [39] (Ψ⁻), except that it maintains the accessory proteins in addition to rev and tat. The HIV-1 and HIV-2 [39] expression vectors express the GFP reporter gene from an internal promoter (CMV and pGK, respectively) and maintain an intact LTR. Feline immunodeficiency virus (FIV) based vector pGINWF [40] (also containing the GFP gene) and the corresponding packaging construct pFP93 [41], were provided by Dr. E. Poeschla (Mayo Foundation).

Virus preparation

Transfection on 293T cells was performed using Lipofetamine™ 2000 (Invitrogen) following the manufacturer's instructions. 293T cells were cultured in DMEM with 10% ultra-low IgG FBS (GIBCO) during the transfection. Briefly, 4 × 10⁶ 293T cells were transfected with a combination of DNA plasmids, including the lentiviral reporter vector, envelope and viral packaging plasmid. Five micrograms of each plasmid were used in the transfection. The day after transfection, 10 mM of sodium butyrate was added to the medium and the cells were incubated at 37°C for 6 hours. Supernatant was collected 48 hours post-transfection, filtered through a 0.45 micron filter, and stored at -70°C.

Monoclonal antibodies (mAbs)

In order to utilize the antibodies with the highest affinity to the ZZ domain for targeting transduction, subclass IgG_{2a} or IgG_{2b} were chosen, if available [42]. Mouse anti-human HLA ABC (HLA class I; clone W6/32) was purchased from Sigma. Mouse anti-human

purified ABCG2 (clone 5D3) was obtained from Millipore (Billerica, MA) and anti-ABCG2 (clone BXP53) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The former recognizes the epitope of ABCG2 protein on the cell surface while the latter recognizes a cytoplasmic epitope of ABCG2 protein. Anti-P-glycoprotein (P-gp) antibody (MRK-16) recognizing MDR1/ABCB1 protein was obtained from Kamiya Biomedical Company (Seattle, WA). For the detection of GFP⁺/ABCG2⁺ cells, phycoerythrin (PE) conjugated anti-ABCG2 antibody (clone 5D3; Millipore) was used. Mouse anti-human CD133/2 (293C3) was purchased from Miltenyi Biotec. (Auburn, CA).

Benzonase treatment on lentiviral vectors

Lentiviral vectors were mixed with 1× Benzonase buffer (50 mM Tris-HCl pH 8.0, 1 mM MgCl₂, and 0.1 mg/ml bovine serum albumin [BSA]), freshly diluted benzonase (50 U/ml; Sigma, St. Louis, MO), and incubated at 37°C for 15 min. The reaction was terminated by freezing the virus at -70°C.

Transduction of various cell lines by lentiviral vectors

The titer of m168 or m168-SG-pseudotyped lentiviral vectors was determined by infecting 293T cells with the virus conjugated with anti-HLA ABC antibody (1 µg/ml). The cells were infected by viral serial dilution. Three days post-infection, the percentage of GFP⁺ cells was measured by flow cytometry. The calculation of titer is based on the following formula: Viral titer = (number of infected cells × %GFP⁺ cells × viral dilution factor) / volume of viral supernatant (ml). An MOI of 1~2 (based on infection of 293T cells using the HLA ABC antibody) was used in all of the remaining transductions.

For studies examining the effect of acid buffer on the induction of viral entry, target cells were first infected by lentiviral vector via antibody. The cells were then rinsed with PBS and treated with citric acid buffer (0.131 M citric acid, 0.066 M Na₂HPO₄, pH 5) at 37°C for 10 min. The buffer was then replaced by culture medium. GFP expression and cell-surface expression were analyzed by flow cytometry, 3 days post-infection.

Flow cytometry

Flow cytometry was performed at the Rutgers/UMDNJ FACS Core Facility with Beckman Coulter Cytomics FC 500 and the data was analyzed with CXP Software. Cell sorting was performed on a Beckman Coulter Epics Altra Cell Sorter and the data were analyzed using Expo32 Software.

Transduction of human CB and lineage-depleted CB cells

CB cells were obtained from Coriell, pretreated in Ficoll to enrich for mononuclear cells (MNCs) after approval of the UMDNJ IRB. The cells were recovered overnight in defined serum-free medium (StemSpan SFEM medium; Stem Cell Technology, Vancouver, Canada) complemented with recombinant human (rh) Flt-3 ligand, rh SCF, rh IL-3 and rh IL-6 (StemSpan CC100; Stem Cell Technology, Vancouver, Canada). The next day, the living nucleated cells were counted using trypan blue dye exclusion. 5×10^5 cells were infected with virus conjugated with specific antibodies (1 µg/ml) at a MOI of 2, and maintained on retronectin-coated 35 mm dishes (Takara Bio Inc.). To obtain lineage-depleted cells, 1×10^7 CB cells were first conjugated with biotin-labeled lineage cocktails and then combined with anti-biotin microbeads (Miltenyi Biotec). The cells were applied to the MS column (Miltenyi Biotec) and the flow-through was collected as lineage⁻ cells. 5×10^4 lineage⁻ cells were infected with virus as described above for the total CB cells. The cells were incubated with virus overnight. The next day, the cells were resuspended in Iscove's MDM with 2% FBS (Stem Cell Technology) and viable cells were counted using trypan blue dye

exclusion. The cells were mixed with methylcellulose medium (MethoCult; Stem Cell Technology) following the manufacturer's instruction. A triplicate assay was performed in every group. The dishes were incubated at 37°C, 5% CO₂ for 14 days. Colonies of colony forming unit-erythroid (CFU-E), burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte (CFU-G), colony forming unit-macrophage (CFU-M), colony forming unit-granulocytes and macrophage (CFU-GM) and colony forming unit-granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM) as well as GFP⁺ colonies were scored under a fluorescence microscope.

Statistic analysis

Student's t-test was used for statistic analysis and *p* values less than 0.05 were considered to be statistically significant.

Results

m168 pseudotyped lentiviral vectors enter cells via a specific antibody

The multidrug-resistance protein ABCG2 is reported to be heterogeneously distributed within primary cells and immortalized cell lines. Specifically, ABCG2 is expressed on murine HSC cells and expression in early human HSC enhanced the proportion of CD34⁺ cells detected after transplantation into NOD/SCID mice [33,34]. The ability to deliver genes through the ABCG2 protein as the viral receptor and select for a population with stem cell characteristics was therefore tested.

Successful retargeting has been achieved using the Sindbis Env m168 construct, bearing a replacement of the viral receptor binding domain with the protein A derived ZZ domain and elimination of three heparin binding sites [15]. Conjugation with a targeting antibody allows for binding and entry of the Env pseudotyped lentiviral particles. In order to investigate whether the m168 Sindbis Env-Ab conjugate pseudotyped lentiviral vector could enter cells via an anti-ABCG2 antibody, human osteosarcoma cells expressing high levels of surface ABCG2 (Saos2-ABCG2) were chosen as a target cell. The Saos2 cell line in the absence of ABCG2 expression (Saos2-pcDNA) and the mouse melanoma cell line B16F10 expressing the MDR1 multidrug-resistance protein (B16F10-MDR5), previously characterized in the Sindbis Ab-conjugate system [15], were used as control cells. To establish this system, populations of cells that express high and low levels of ABCG2 were obtained through cell sorting of Saos2-ABCG2 and Saos2-pcDNA, respectively (Fig. 1). Viral transduction was measured through transfer of the GFP gene.

The m168 pseudotyped lentiviral vectors mediated delivery of the GFP gene into the Saos2-ABCG2 cells through the monoclonal antibody recognizing the cell surface epitope of the ABCG2 protein (5D3). Conjugation of viral particles with the 5D3 anti-ABCG2 antibody yielded 19% of the Saos2-ABCG2 target cells expressing GFP (GFP⁺) as compared to 1.5% of the Saos2-pcDNA cells (Fig. 2, panels D and H). Productive gene transfer required the presence of an appropriate antibody. An ABCG2 monoclonal antibody that recognized an internal epitope within the cytoplasm of the cell (BXP53) resulted in a level of gene transfer (2.6%, Fig. 2, panel G) equivalent to the background of pseudotyped particles in the absence of antibody (3.5%, Fig. 2, panel E). On the control Saos2-pcDNA cells, the level of gene transfer was consistently between 1.4–1.6%, either in the presence of the ABCG2 antibody, or in the absence of antibody (Fig. 2, panels A, C, and D). Deletion of the immunoglobulin binding ZZ domain from the m168 construct also resulted in background levels of gene transfer, even in the presence of the appropriate antibody (data not shown). HLA class I is highly expressed on nucleated cells and therefore was used for Ab-mediated transduction as

a positive control [15]. GFP gene delivery through HLA class I was 20% and 29% on the Saos2-pcDNA and Saos2-ABCG2 cells, respectively (Fig. 2, panels B and F).

Additional studies examined the cross-reactivity between multi-drug resistance proteins. MDR1 expression in B16F10MDR5 cells was enriched to obtain a population of cells that were 70% positive via cell sorting. Although both MDR1 and ABCG2 belong to the ATP Binding Cassette (ABC) family and demonstrate similar transmembrane protein structure, m168-pseudotyped viral entry was not observed in MDR1 expressing cells (B16F10MDR5) in the presence of the 5D3 anti-ABCG2 antibody, showing only 0.06% of GFP⁺ cells (data not shown). The low level of infection indicates that no cross-reactivity of the 5D3 anti-ABCG2 antibody was observed with the MDR1 protein.

Evaluation of gene transduction using m168 pseudotyped lentiviral vectors

The source of the viral particle has implications relating to the safety of the viral vector, the viral titer and the host innate immune response to infection. To address this, the titers using three lentiviral vector backbones, HIV-1, HIV-2 and FIV were analyzed under conditions optimized for each system (Table 1). The analysis was performed on 293T cells with the m168 Sindbis Env in the presence of anti-HLA-I antibody. Flow cytometry showed that over 95% of 293T cells expressed HLA-I. Titration of the HLA-I antibody indicating maximal infection by the m168 pseudotyped virus was obtained at 5 µg/ml (data not shown). Using VSV-G pseudotyped particles, the viral titers of the HIV-1, HIV-2 and FIV vectors were 8.8×10^6 , 4.7×10^6 , and 3.6×10^4 /ml, for the transfer of GFP, respectively. The m168 Env was efficiently pseudotyped onto both the HIV-1 and HIV-2 particles, resulting in HLA-I antibody-mediated gene transfer of GFP at 3.75×10^4 and 3.5×10^4 GFP⁺/ml virus, at an equivalent ratio to that found for the VSV pseudotyped virus. A proportional level of HLA-I Ab-mediated gene transfer was detected from the m168 pseudotyped FIV particles (4.2×10^2 GFP⁺/ml), however, the overall production of viral particles was low in this system. Viral transduction in this study utilized the m168 Sindbis Env pseudotyped on the HIV-1 backbone, due to the highest titer, however direct quantitative comparisons of titers between the different viral systems would require additional studies. HIV-2 would be a suitable backbone if potential pathogenesis concerns were paramount.

In order to maximize viral particle assembly in 293T cells, the ratios of the three plasmids encoding the Sindbis m168 Env plasmid, the HIV-1-based packaging plasmid pCMVd8.2dVPR and the GFP-containing expression vector pHR'CMVGFPW (HIV-1 backbone), were varied during transfection into 293T cells. In the presence of lipofectamine, maximal viral particle production was obtained at a plasmid ratio of 1:1:1 using a total of 15 µg of plasmid in 4×10^6 cells, and incubation of the viral particles with target cells for 6 hours. Under these modified conditions, the 5D3 antibody mediated infection of Saos2-ABCG2 cells was increased to 60% of the cells on average (titer of 6×10^4 GFP⁺/ml), over 20-fold above the background of particles in the absence of antibody (Table 2).

Variations in the Sindbis pseudotyped lentiviral system have been reported to increase gene transduction, including the use of low pH pulses [37], mutant Sindbis Env displaying cholesterol independence (m168-SG; m168-2.2) [25,37], and treatment with Benzonase [43]. Viral transduction was not altered with Benzonase treatment (data not shown), confirming that the gene transfer was a result of replication of the viral RNA, rather than direct plasmid DNA transfer to the target cell. A transient low pH pulse is reported to enhance antibody mediated gene transduction for primary hematopoietic cells [37]. As shown in Table 2, in the presence of appropriate antibody, a low pH pulse following the m168-pseudotyped viral transduction yielded 48% of GFP⁺ cells at the average, whereas the average resultant GFP⁺ cells by using conventional transduction protocol (i.e. neutral pH)

was 23%. However, in the Saos2-pcDNA cells, non-specific gene transduction was also found to increase dramatically with a low pH pulse as compared to neutral pH. As a result, in low pH, there was only a 4-fold increase of gene transduction of Saos2-ABCG2 compared to Saos2-pcDNA cells, versus a 13-fold increase of %GFP⁺ cells at neutral pH. Similarly, mutations within E1 AK226/227SG double mutation (m168-SG; 2.2 [37]) which has been reported to increase infectivity [37], also resulted in a lower fold increase than m168 when comparing infection of Saos2-pcDNA versus Saos2-ABCG2 (Table 2). Therefore, infections were maintained at neutral pH using the m168 backbone for further experiments.

Sensitivity of mAb-mediated selective transduction in a mixed cell population monitored by flow cytometry

In order to analyze the sensitivity of the m168-pseudotyped lentiviral vector and detect infection of target cells within a heterogeneous population, Saos2-ABCG2 and Saos2-pcDNA cells were mixed at different ratios and lentiviral GFP gene transduction in the presence of 5D3 anti-ABCG2 antibody was analyzed by flow cytometry. Analysis of the ABCG2 positive cells within the population was confirmed by flow cytometry using ABCG2 Ab conjugated with PE three days post-infection (Fig. 3). In these experiments, the percent infection through the ABCG2 antibody was proportional to the percent of ABCG2⁺ cells (compare upper right quadrants, Fig. 3). Within the mixed populations, ABCG2 Ab-directed gene transfer could be detected at levels above background when the targeting cells were greater than 5% of the population. The presence of the antibody directing infection did not interfere with the expression level of ABCG2 using the PE-conjugated ABCG2 antibody (see Supporting Information, Table S1).

Viral targeting of early hematopoietic progenitor cells from cord blood (CB) utilizing HSC specific antibodies

Of key interest is the ability of the Sindbis antibody system to selectively infect stem cells within a heterogeneous mixed population. Studies were initiated to direct viral entry into hematopoietic progenitor cells derived from human CB MNCs utilizing two of the stem cell related markers: ABCG2 and CD133. Both proteins are early markers of HSCs/progenitor cells and may be involved in early human hematopoietic development [34–36]. ABCG2 is reported to be associated with HSC side population (SP) cells, and conditions were established for the ABCG2 antibody mediation of selective transduction by m168-pseudotyped virus (Figs. 2–4); CD133 expression was enriched in Huh7 SP cells (data not shown).

Cell surface expression of ABCG2 and CD133 markers on total human cord blood was initially performed to assess if m168 Env-Ab mediated GFP delivery could be detected by FACS analysis of transduced cells. ABCG2 and CD133 was represented in ≤0.1% of the cells. Analysis of mixed heterogeneous populations (Fig. 3) indicated that GFP⁺ transduced cells represented by <5% of the total cells could not be detected by FACS analysis. Due to the minor distribution of hematopoietic progenitor cells in CB MNCs, the sensitivity of flow cytometry was below the limit of detection. To circumvent this problem, the colony-forming cell (CFC) assay (also referred to as methylcellulose assay), a common *in vitro* assay employed for the quantification of committed hematopoietic progenitors was utilized. Since only the potential HSC/hematopoietic progenitor cells within a heterogeneous population could differentiate on methylcellulose medium as colonies, the GFP gene transfer directed by stem cell markers in the hematopoietic progenitor cells could be reflected by the resultant GFP⁺ colonies observed under fluorescence microscope (Fig. 4). Therefore, the CFC assay could overcome the sensitivity limit of the flow cytometer. Lineage depletion can enrich the hematopoietic progenitor cells in CB MNCs, yielding 2.8% of the CB MNCs purified as lineage[−] cells as found in our study. CD133⁺ cells represented 2.25% of the lineage[−]

population. In order to determine whether enrichment of hematopoietic progenitor cells from CB MNCs is necessary in the goal of improving the transduction efficiency, a comparison was performed between direct infection of nonpurified CB MNCs and infection of the cells post lineage depletion. Both lineage⁺ and lineage⁻ cells were infected with m168 pseudotyped virus conjugated with either anti-CD133 (clone 293C3), ABCG2 (5D3) or HLA class I antibodies. VSV-G pseudotyped and no antibody conjugated virus were used as controls. The assays were performed in triplicate for each group. Between 29–77 clones were scored in each assay. Non-specific entry of m168-pseudotyped virus was measured by infection in the absence of an antibody. The level of non-specific viral entry in lineage⁻ cells was similar to that in whole CB MNCs (3% vs. 2%, $p > 0.05$; Table 3). Similarly, viral particles pseudotyped with VSV-G, with broad-spectrum tropism, displayed no differences in transduction efficiency between the lineage⁻ cells and non-purified CB cells ($p > 0.05$; Table 3). In contrast, viral delivery directed by an anti-CD133 antibody yielded a dramatic improvement of transduction in the lineage⁻ cells (12%) when compared with that in CB MNCs (5%) ($p < 0.05$; Table 3). In addition, a statistically significant 2 fold increase of gene delivery mediated by ABCG2 antibody was found in the lineage⁻ cells over nonpurified CB MNCs (Table 3). A preferential bias of targeting transduction directed by anti-HLA-I also was observed on the lineage⁻ cells, as a significantly higher percentage of resultant GFP⁺ colonies were detected in the lineage⁻ cells (11%) than in the non-purified CB cells (5%) ($p < 0.05$; Table 3). This correlated with the higher mean fluorescence expression (4.8) of HLA-I on the lineage⁻ cells, as compared to the total population (2.36). Depletion of mature and differentiated cells from CB MNCs yielded a higher percentage of hematopoietic progenitor cells in the population and an increased ability for the potential stem cells to be targeted by the m168-pseudotyped virus through stem cell associated markers.

Figure 4 shows individual brightfield and fluorescence microscope images of individual colonies grown on methylcellulose and the transduction of GFP via lentiviral particles pseudotyped with m168 and conjugated with anti-CD133, ABCG2 antibodies or VSV-G envelopes. The figures display GFP⁺ signal in clonal expansions of BFU-E as well as variable-sized non-hemoglobinized cells that were transduced with either CD133 or ABCG2 antibody conjugated m168 lentiviral particles. VSV-G pseudotyped virus infected a variety of cells within the single CFU-GM colony.

Discussion

Antibody-directed gene delivery is based on the assumption that specific receptors are exclusively or preferentially expressed on target cells or tissues. For stem cells, achieving targeted delivery is met with an additional level of difficulty, in that they are of low abundance, and reside within heterogeneous populations. In these studies, conditions were sought to optimize targeted gene delivery to hematopoietic progenitor cells using alternative cell surface markers independent of CD34 [19]. Using the m168 Sindbis Env antibody bridging system, we examined the ability of anti-CD133 and anti-ABCG2 (BCRP) Ab to optimize the selective delivery of genes to specific cell types cells, including human early immature hematopoietic progenitors from CB.

HSCs are of high interest due to their properties of self-renewal and potential range of differentiation. Beyond the conventional stem cell marker CD34, alternative proteins, including CD133, ABCG2, Flk-2 and c-kit, have been shown to play an important role in both the short- and long-term engraftment in transplant, in *ex vivo* expansion, and in gene therapy strategies [33,35,44,45]. Indeed, various distinct markers are expressed during hematopoietic developmental pathways [46]. Within human fetal liver and non-purified peripheral blood mononuclear cells, gene delivery via Sindbis Env pseudotyped particles bound with anti-CD34 antibodies were capable of targeting the CD34⁺ progenitor cells [19].

Our studies extend this system to progenitor cells from human umbilical cord blood using alternative antibody complexes.

The concept that the CD34 antigen is necessary and exclusively expressed on HSCs has been challenged with reports that CD34⁺ cells can function as long-term repopulating cells and yield significantly higher levels of engraftment than CD34⁺ cells [28–31]. Overall, HSCs represent a relatively small subpopulation in the CB, peripheral blood and bone marrow. Based on the mixing experiments, cells consisting of less than 5% of the population would be below the detection limit of flow cytometry. Upon undergoing a differentiation assay such as CFC, only the hematopoietic progenitor cells with the ability to differentiate will form colonies. The GFP gene transfer in the resultant colonies can be easily detected by fluorescence microscopy. As expected, in the total cord blood population, VSV-G pseudotyped virus achieved the highest transduction efficiency (Table 3). However, antibody-mediated transduction in the presence of CD133, ABCG2 or HLA-I antibody was readily detectable and more importantly, was 2.5 fold above transduction in the absence of Ab. Thus, gene transfer in the absence of additional purification steps, including lineage depletion, can effectively target the early progenitor cell population. Lineage depletion does enrich for the progenitor cells and the efficiency of gene transfer using CD133, ABCG2 or HLA-I antibody-mediated gene transfer improved as a result to 3–4 fold above the no antibody controls. Significantly, using the CD133 antibody, 12% of the differentiated colonies were GFP positive, approaching the level of transduction achieved with the non-specific VSV-G Env (14%). Transduction via HLA-I is consistent with published reports of high expression of HLA ABC in lineage⁺ CB cells [47]. Experimentally, the expression level of HLA-class I in lineage⁺ CB cells was uniformly higher than in the total CB population (mean fluorescence value of 4.8 in Lin⁺ vs. 2.36 in the total CB).

Modifications in the Sindbis-pseudotyped lentivirus systems have been published which increase the transduction efficiency either through low pH pulse [37], mimicking the low pH found in the endosome [48,49], or mutations (m168 2.2 [19]) that improve the ability of the virus to infect cells in the absence of cellular cholesterol [25]. On the Saos2 cell line, increased titer was observed with the m168-SG mutation (equivalent to the m168 2.2 virus) as well as with a low pH pulse, however a concomitant increase in the non-antibody mediated transduction was observed. It is possible that the increased background may be related to the differences in cell types used in the study (primary hematopoietic cells [37] versus osteosarcoma cells). Similarly, decreasing the time (10 sec [19] versus 10 min in this study) of the pH pulse may limit the non-antibody mediated gene transfer. However, transient treatment of host cells in a low pH environment may not be technically applicable in *in vivo* targeting trials.

Every delivery system has its benefits and shortcomings. Many of the weaknesses of the m168-Ab conjugate system have been previously discussed [15,17,23], including the non-covalent nature of the Ab-Env interaction, allowing for release and possibly subsequent rebinding of alternative targeting agents. This non-covalent interaction can also contribute to the lower titer of the Ab-Env pseudotyped particles compared to the titers obtained with VSV-G. VSV-G can infect a broad range of cells within CB including activated T and B cells, macrophages and dendritic cells and thus show no selectivity in targeting progenitor cells. Compared to VSV-G mediated gene delivery, the efficiency of gene transfer via the CD133 Ab-complex is decreased in the total CB population, however the cost of this minor reduction in efficiency is compensated by the overall specificity derived from the Ab targeting. Similarly, HLA-1 positive cells represented 87% and 91% of the cells within the total CB and lineage⁺ cells, respectively. In contrast, CD133 represented 0.1% and 2.25% of these populations, respectively. The delivery of GFP through the CD133 antibody was at similar or greater levels to that detected using HLA-1 Ab mediated transfer, indicating this

minor population of CD133⁺ cells were efficiently marked and subsequently differentiated within the CFC assay.

Initial focus on ABCG2 stemmed from reports of side populations (SP) being associated with multidrug-resistance proteins, in particular with murine HSCs [33]. SP cells are enriched by Hoechst dye staining and demonstrate an active self-renewal and ability to differentiate [50] and are reported to have enhanced stem cell-like properties [32,33] independent of CD34⁺ expression. Although multidrug-resistance proteins have been associated with SP populations, the cell markers responsible for the SP phenotype are still controversial. The ABCG2 marker has been linked with the SP population, however, alternative multidrug resistance proteins may contribute equally to this phenotype in different cell types [32,33,51,52]. Analysis of SP populations of human hepatocellular carcinoma cell line Huh7 indicated enrichment of the ABCG2 over the total population (data not shown) consistent with previous reports [53]. Although many cells display the SP phenotype, no single multidrug resistance protein can be generalized to target this population and the targeted protein must be empirically determined for each cell line. Targeting via the ABCG2 gene remains of interest, as a transition through a Hoechst negative state correlating with expression of ABCG2 [54], occurs during formation of induced pluripotent stem cells.

The studies presented here further characterize the use of an antibody targeting system via pseudotyped lentiviral particles for *ex vivo* gene delivery to potential hematopoietic progenitor cells within a mixed population. In addition to CD34 as a targeting protein for HSCs, surface markers including CD133 and ABCG2 were identified that could function as targets for antibody-targeted gene delivery to CB populations. Lineage depletion to enrich the hematopoietic progenitor cells in the human CB cells improved the transduction efficiency via specific cell surface markers, but is not required for targeted gene delivery. The ability to identify and categorize a subpopulation of cells by determining the expression levels of cell surface markers such as CD133, has broad applications in the study of stem cells as well as cancer stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression of ABCG2. Cell surface expression of ABCG2 as determined by flow cytometry in the presence of antibody 5D3. Panel A. Saos2 cells were transduced with empty pcDNA vector designated Saos2-pcDNA. Panel B. Human osteosarcoma cell line Saos2 transduced with pcDNA vector containing ABCG2 gene (named Saos2-ABCG2). Cells with high expression of ABCG2 were sorted by flow cytometry, yielding 97% ABCG2⁺ of the cells. Panel C. Overlay of expression of Saos2-pcDNA and Saos2-ABCG2 cells.

Figure 2.

Specificity of targeted transduction of m168-pseudotyped lentiviral vector on the host cells. Saos2-ABCG2 cells and Saos2-pcDNA cells were infected by virus bearing m168 in the following conditions: no conjugation with antibody, conjugated with anti HLA class I antibody, clone BXP3 of anti-ABCG2 antibody and clone 5D3 of anti-ABCG2 antibody. In every group, 1×10^5 cells were infected with virus at a MOI of 1 via antibody (1 μ g/ml) if used. The MOI of 1 was based on infection of 293T cells using the HLA ABC antibody. Lentiviral particles were generated following the protocol published [37]. m168, HIV-1 expression vector and packaging vector were co-transfected in a total amount of 15 μ g at a ratio of 1:2.5:2.5 (m168: HR'CMVGFPW: pCMVd8.2dVPR) in 4×10^6 293T cells. The results are shown as %GFP⁺ detected by flow cytometry 3 days post-infection.

Ratio Saos2-ABCG2 : Saos2-pCDNA (% Saos2-ABCG2 cells)

Figure 3.

Targeting transduction of m168-pseudotyped virus in a mixed cell population. Saos2-ABCG2 and Saos2-pCDNA cells were mixed in different ratios with the total number as 2×10^5 cells. The mixed populations were infected with viruses bearing m168 conjugated with the 5D3 anti-ABCG2 antibody. Three days post-infection, the cells were stained with PE-conjugated anti-ABCG2 antibody and the %ABCG2⁺GFP⁺ cells are shown in the top right of each plot.

Figure 4.

Transduction of m168-pseudotyped virus packaging the GFP gene was performed on CB MNCs with or without lineage depletion. The infection was directed by anti-HLA class I, anti-ABCG2, anti-CD133 and in the absence of antibody conjugate. The cells were also infected by VSV-G pseudotyped virus. Following overnight infection, the cells were plated in methylcellulose medium for 14 days. The numbers of resultant colonies and GFP⁺ colonies were scored under bright-field (panels A, C, and E) and fluorescence (panels B, D and F) microscope, respectively. Representative pictures are shown. Panel A and B: BFU-E with surrounding variable sized non-hemoglobinized cells transduced with CD133 Ab-conjugate. Panel C and D: BFU-E with surrounding variable sized non-hemoglobinized cells transduced with ABCG2 Ab-conjugate. Panels E and F, CFU-GM transduced with VSV-G pseudotyped particles.

Table 1

Titer of m168 vs. lentiviral backbones

Env	Viral particle		
	HIV-1 ^{a,b}	HIV-2 ^{a,c}	FIV ^{a,d}
VSV-G	8.8×10^6	4.7×10^6	3.6×10^4
m168/HLA-I ^e	3.8×10^4	3.5×10^4	4.2×10^2

^aViral titer is determined by GFP⁺ cells/ml of virus. Viral titer was calculated from flow cytometry data: Viral titer = (infected cell number × %GFP⁺ × viral dilution factor) / volume of viral supernatant.

Background infection (no Ab) was subtracted from each sample. Results are an average of two infections.

^bViral particles were assembled with pCMVd8.2dVPR, pHR'CMVGFPW, and the corresponding Env plasmid.

^cViral particles were assembled with HIV-2-CGP-AC, HIV-2gtPGIPW and the corresponding Env plasmid.

^dViral particles were assembled with pFP93, pGINWF and the corresponding Env plasmid.

^e5 μg/ml of anti-HLA class I was used per infection.

Table 2

%GFP⁺ cells analyzed by flow cytometry in the cells transduced by lentiviral particles mediated with anti-ABCG2 antibody.

Treatments	Saos2-pcDNA	Saos2-ABCG2	Fold increase
Neutral pH ^a	1.68 ± 0.55	23.33 ± 3.79	13.69
Low pH ^a	11.67 ± 5.13	47.77 ± 16.25	4.09
m168 ^b	2.33 ± 0.25	52.44 ± 2.22	22.49
m168-SG ^b	5.57 ± 0.08	60.93 ± 2.01	10.93

^aThe total amount of plasmid DNA was 15 µg at a ratio of 1:2.5:2.5 of m168:HIV vector:packaging plasmid. The cells were incubated with the virus for 2 hours.

In low pH group, the cells were incubated in citrate acid buffer at 37°C for 10 minutes following the transduction.

^bThe total amount of plasmid DNA was 15 µg at a ratio of 1:1:1 of m168:HIV vector:packaging plasmid. The cells were incubated with the virus for 6 hours in neutral environment.

Table 3
Colony-forming cell assay of cord blood mononuclear cells with and without lineage depletion.

	No lineage depletion			Lineage depletion			p value
	Total colonies #	GFP ⁺ colonies #	%GFP ⁺	Total colonies #	GFP ⁺ colonies #	%GFP ⁺	
VSV-G	129	13	10	101	14	14	0.09
No Ab	127	3	2	171	5	3	0.34
CD133	145	7	5	132	16	12	0.005
ABCG2	109	5	5	169	15	9	0.004
HLA-I	159	8	5	205	23	11	0.01

1×10^4 nonpurified CB MNCs and 1×10^3 Lineage⁻ CB MNCs were used in CFC assay. The resultant colonies included CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. The number of total colonies and GFP⁺ colonies were the sum of three independent experiments.