

A Transient Three-plasmid Expression System for the Production of Hepatocytes Targeting Retroviral Vectors

Peng QI, Jinxiang HAN*, Yanqin LU, Chuanxi WANG, and Bo ZHU

Key Laboratory of Ministry of Health for Biotech-Drug, Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Science, Jinan 250062, China

Abstract Targeting of retroviral vectors to specific cells was attempted through modifying the surface protein of the murine leukemia viruses (MLVs), but in many cases the protein function was affected, and it is difficult to achieve the targeted delivery. In this study, we have tried to engineer ecotropic Moloney murine leukemia viruses (MoMLV)-based retroviral vectors to transduce hepatocytes. A chimeric envelope (Env) expression plasmid was constructed containing the hepatitis B virus PreS2 peptide fused to aa +1 at the N-terminus of Env. Following simultaneous transfection of pgag-pol, pLEGFP and chimeric env plasmids into 293T cells, helper-free retrovirus stocks with the titer of approximately 10^4 infectious units/ml were achieved at 48 h post-transfection. These pseudotype vectors showed the normal host range of retrovirus, infecting host NIH 3T3 cells, although the efficiency was reduced compared with that of virions carrying wild-type ecotropic MoMLV envelope. In addition, the resultant pseudotype viruses could transduce human hepatoma cells mediated by polymerized human serum albumin with relatively high titers in comparison with those transductions without polymerized human serum albumin. This approach can be used to target hepatocytes selectively.

Keywords gene therapy; Moloney murine leukemia virus; targeting vector; envelope; polymerized human serum albumin

The liver is an important target organ for gene therapy because it plays a central role in the metabolism and production of serum proteins. There are many lethal metabolic diseases resulting from the defect or deficiency of hepatocyte-derived gene products [1–5]. Acquired diseases such as hepatoma and viral hepatitis are also likely to be a target of hepatic gene therapy. Advancements in hepatic gene therapy depend to a large degree on the development of a delivery system capable of efficiently introducing genes into the hepatocytes. Because retroviruses integrate their genomes into the target cell chromosomes, retrovirus-mediated gene transfer theoretically provides for long-term expression of the therapeutic gene in the transduced cells [6]. However, a major limitation to the clinical use of such vectors is the

lack of specificity of delivery to defined target cells.

Ecotropic Moloney murine leukemia virus (MoMLV) envelope (Env) glycoproteins, expressed on the surface of virions, are responsible for the specific attachment of viral particles to rodent cell surface receptors and for the subsequent fusion between the viral and cell membranes [7,8]. During the past few years, a series of different targeting strategies has been developed. Among the initial strategies was the host range extension to specific human cells of murine retroviruses. For this purpose, the envelope protein was modified by the addition of single-chain antibody variable fragments [9–15] or peptide ligand sequences [16–20]. For most of these methods, the transduction efficiencies achieved have been low, even when high-affinity binding to the targeted receptor has been reported [12,17].

In previous reports, several receptors for either natural hepatitis B virus (HBV) particles or genetically engineered viruses have been described, whereby endocytosis

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*Corresponding author: Tel, 86-531-82919888; Fax, 86-531-82951586; E-mail, jinxianghan@sina.com

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represents a putative uptake mechanism for HBV particles [21–27]. The envelope of the HBV particle is composed of three related surface (S) proteins. The major or small S protein is 226 amino acids in length. The two other S proteins include the small S sequence at their carboxyl termini. In addition, the middle S protein contains an amino-terminal extension to the small S protein of 55 amino acids (PreS2). The large S protein contains a further amino-terminal extension to the middle S protein of 108 to 119 (depending on subtype *ay* or *ad*, respectively) amino acids (PreS1). It has been suggested that the middle protein, which binds to polymerized human serum albumin (pHSA) [28–31], might use pHSA to attach to hepatocytes, which express albumin receptors [32,33].

In this study, we investigated whether MoMLV-based retroviral vector that contains the HBV PreS2 peptide fused to aa +1 at the N-terminus of Env could be applied to target hepatocytes. Following simultaneous transfection of pgag-pol, pLEGFP and chimeric Env plasmids into 293T cells, helper-free retrovirus stocks with the titer of approximately 10^4 infectious units/ml were achieved 48 h post-transfection. These pseudotype vectors showed the normal host range of retrovirus, infecting host NIH 3T3 cells, although the efficiency was reduced compared with that of virions carrying wild-type ecotropic MoMLV envelope. In addition, the resultant pseudotype viruses could transduce human hepatoma cells mediated by pHSA with relatively high titers in comparison with those transductions without pHSA. We show that this approach can be used to selectively target hepatocytes.

Materials and Methods

Cell lines

HepG2 cells (ATCC HB8065) were cultured in H medium [75% minimal essential medium (Gibco, Grand Island, USA), 25% medium 199 (Gibco), insulin (5 mg/ml), bovine serum albumin (1 mg/ml), antibioticantimycotic (Gibco)] supplemented with 5×10^{-7} M hydrocortisone hemisuccinate, 2% dimethyl sulfoxide, and 10% fetal bovine serum. NIH 3T3 (ATCC CRL1658), 293T (ATCC CRL 11268) and human embryonic kidney (HEK) cells (ATCC CRL1573) were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum.

Construction of chimeric envelope expression plasmid

The coding sequence of PreS2 was synthesized from

pHBV315 [34] by polymerase chain reaction (PCR) using 5' and 3' primers, with *Hind*III and *Xho*I restriction sites (**Table 1**). In addition, two PCR-derived DNA fragments with *Kpn*I and *Hind*III or *Xho*I and *Xba*I restriction sites encoding respective parts of the envelope were generated with a wild-type envelope expression plasmid (pCEE) template [35] and corresponding primers (**Table 1**). The chimeric envelope expression plasmid was assembled by cloning both the PCR fragments and the *Hind*III-*Xho*I PreS2 fragment to multiple cloning sites of eukaryotic expression plasmid pcDNA3.1(-) digested with *Kpn*I and *Xba*I restriction enzymes (**Fig. 1**).

Table 1 Primers used in this study

Primer	Sequence (5'→3')
1a	CCAAGCTT ATGCATGCAGTGGAAATCC (<i>Hind</i> III)
1b	CC CTCGAG TCACGCGAGGGTCCCCAA (<i>Xho</i> I)
2a	TAG GTACCT TCGAGGGTCCAGCGTTCT (<i>Kpn</i> I)
2b	CCAAGCTT AGTACTGACCCCTCTGAG (<i>Hind</i> III)
3a	CC CTCGAG GCTTCGCCCGGCTCCAGT (<i>Xho</i> I)
3b	G CTCTAG ACTATGGCTCGTACTCTA (<i>Xba</i> I)

Matched primers 1a and 1b, 2a and 2b, and 3a and 3b were used to amplify hepatitis B virus preS2, envelope (Env) upper region (signal peptide, SP) and Env lower region, respectively. The restriction enzyme sites are indicated in bold.

Transient transfections, infections and determination of viral titer

Transient transfections were carried out on 293T cells by overnight calcium phosphate treatment [36] on 10 cm dishes seeded the previous day to give a maximum of 70% confluence/plate on the day of transfection. Ten micrograms of each plasmid (30 μ g in total) was used for each transfection. Chimeric envelope expression plasmid, including the control plasmid pCEE, was co-transfected into 293T cells with the MoMLV Gag-Pol expression plasmid pgag-pol [37], and the retroviral vector plasmid pLEGFP (preserved by our laboratory). Transfection efficiencies of the 293T cells were routinely 70%–90%, as determined by observing the expression of enhanced green fluorescent protein under fluorescence microscope. The transfected cells were incubated for 15–17 h, after which the medium was replaced with 5 ml fresh medium. The viral supernatants were harvested 48 h after transfection, filtered through 0.45 μ m filters and titered by infection on NIH 3T3 cells with or without pHSA.

NIH 3T3 cells were plated into wells of 6-well culture plates at a density of $1-2 \times 10^5$ cells/well the day before

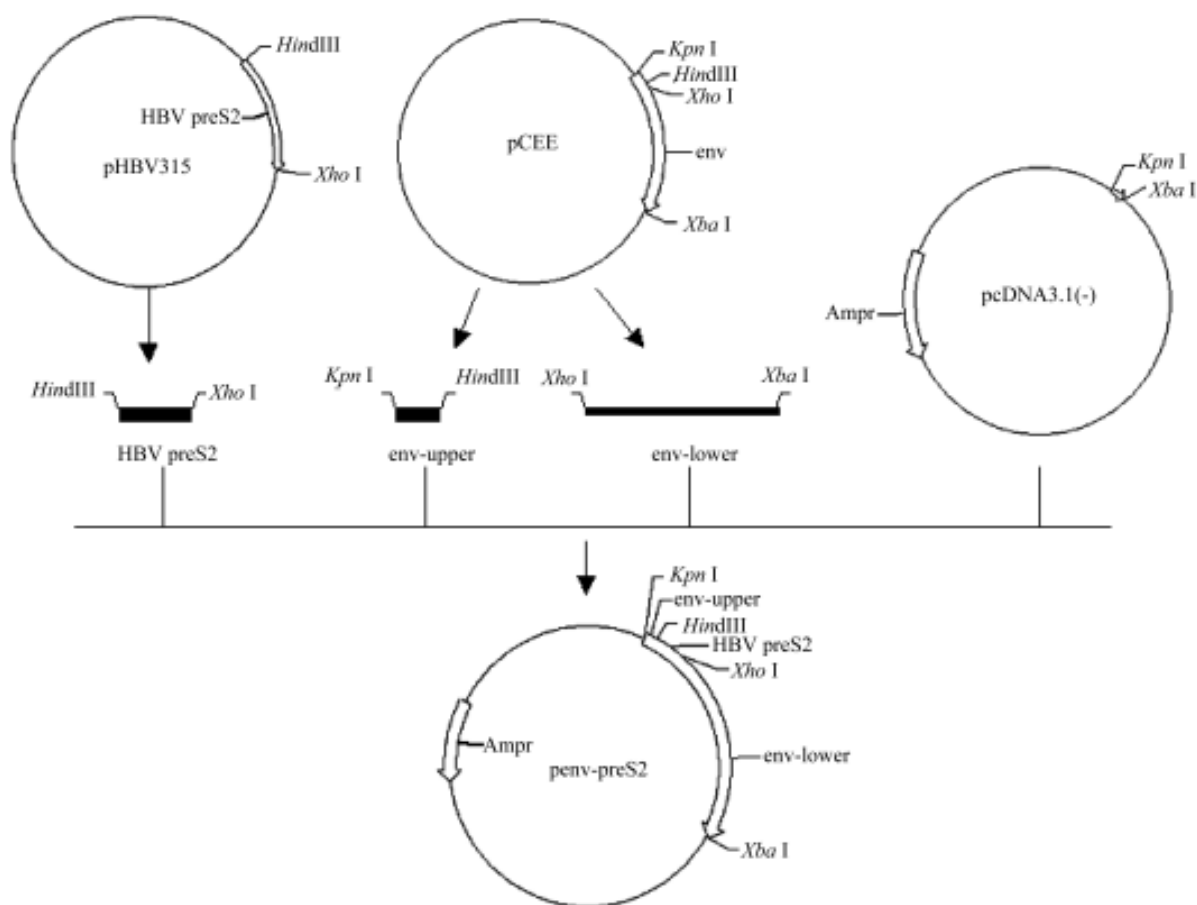


Fig. 1 Construction of chimeric envelope (env) expression plasmid

The coding sequence of PreS2 with *Hind*III and *Xho*I restriction sites was synthesized from pHBV315 [34]. In addition, two polymerase chain reaction (PCR)-derived DNA fragments with *Kpn*I and *Hind*III or *Xho*I and *Xba*I restriction sites encoding respective parts of the envelope were generated with a wild-type envelope expression plasmid (pCEE) template [35]. The chimeric envelope expression plasmid was assembled by cloning three PCR fragments to multiple cloning sites of eukaryotic expression plasmid pcDNA3.1(-) digested with *Kpn*I and *Xba*I restriction enzymes. HBV, hepatitis B virus.

infection. One milliliter of individual undiluted supernatants was added to each well with 8 µg/ml Polybrene (Sigma-Aldrich, St.Louis, USA) and incubated for 2 h, followed by the addition of 1 ml fresh medium. Twenty-four hours later the cells were split 1:4 and seeded onto 5 cm dishes. Viral titer was determined by selection in 400 µg/ml G418 (Gibco). For G418 selection, the medium was replaced every 3 or 4 d and the resistant colonies were counted 10–15 d post-infection by fixing and staining with 0.3% crystal violet in 70% methanol. Virus titer was determined by multiplying the total number of G418-resistant colonies by two, to account for the 2-fold increase in cells during the infection period and the 1:4 split.

For pHSA-mediated experiments, pHSA (5 µg/ml) was added to viruses, followed by incubation at 4 °C for 4 h and then viral infection.

Helper virus assays

Transfections were carried out on 293T cells as described above and individual undiluted viral supernatants were used to infect NIH 3T3 cells. All infections were carried out in the presence of 8 µg/ml Polybrene. The NIH 3T3 cells were passaged every 4–5 d, when the culture supernatants were filtered and used to infect fresh NIH 3T3 cells. These cells were selected in G418 for up to 10–15 d to assay for the presence of replication-competent virus.

Analysis of chimeric envelope in cell lysates and retroviral vector particles

Virus producer cells were lysed in 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% sodium dodecyl sulfate (SDS), 5 mg/ml sodium deoxycholate, 150

mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 10 min at 4 °C and were centrifuged for 10 min at 10,000 g to pellet the nuclei. Supernatants were then frozen at -70 °C until further analysis. Virus samples were obtained by ultracentrifugation of viral supernatants (10 ml) in an SW41 Beckman Rotor (Beckman Coulter, Fullerton, USA) at 40,000 g for 1 h at 4 °C. Pellets were suspended in 100 μ l of phosphate-buffered saline and frozen at -70 °C. Samples (50 μ g for cell lysates, or 10 μ l for purified viruses) were mixed 5:1 (V/V) in 375 mM Tris-HCl (pH 6.8) buffer containing 6% SDS, 30% β -mercaptoethanol, 10% glycerol and 0.06% bromophenol blue, boiled for 3 min then run on 10% polyacrylamide (SDS) gels. After protein transfer onto nitrocellulose filters, immunostaining was carried out in Tris-based saline (pH 7.4), with 5% milk powder and 0.1% Tween 20. Antibodies were goat antisera raised against either MoMLV gp70 surface (SU) protein or MoMLV p30 (CA) capsid protein and were diluted 1/1000 and 1/10,000, respectively. Blots were developed with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin antibodies (Sigma-Aldrich) and an enhanced chemiluminescence kit (Sigma-Aldrich).

Retroviral vector transduction

The resultant viruses were tested for transduction of HepG2 and HEK cells, in both cases with or without pHSA. Target cells were plated into wells of 6-well culture plates at a density of $1-2 \times 10^5$ cells/well the day before infection. One milliliter undiluted supernatant was added to each well with 8 μ g/ml Polybrene and incubated for 3-5 h at 37 °C, followed by the addition of 1 ml fresh medium. Viral titer was determined by selection in 400 μ g/ml G418, as described above.

Results

Construction of chimeric envelope

The MLV Env protein is cleaved from a precursor (pr85) into two subunits, a SU subunit (gp70) and a transmembrane subunit (p15e) (Fig. 2). It has been shown that the N-terminal portion of SU is sufficient for receptor recognition [38-40] and that the transmembrane subunit contains sequences necessary to catalyze fusion between the viral and host cell membranes [41,42]. In our study, HBV PreS2 peptide was fused to aa +1 at the N-terminus of Env. The choice of site was based on consideration of the data from previous attempts to engineer MoMLV Env [18-20]. These positions of insertion were previously shown to allow the functional display of chemical addition at the surface of virions.

Three-plasmid co-transfections to generate retrovirus stocks

We transfected pgag-pol, pLEGFP and Env plasmids into 293T cells simultaneously and titered the resulting viruses on NIH 3T3 cells. The original packaging components used were pgag-pol plasmid, MoMLV gag-pol expression vector and pCEE/chimeric envelope expression vector, and pCEE and chimeric envelope expression vector expressed wild-type ecotropic envelope and HBV PreS2-Env chimeric envelope, respectively. The retroviral genome vector used was pLEGFP, which could express enhanced green fluorescent protein. When these plasmids were co-transfected into the highly transfectable 293T cells, we produced wild-type retrovirus stocks with the titer of 0.9×10^6 cfu/ml with pHSA and 0.8×10^6 cfu/ml without

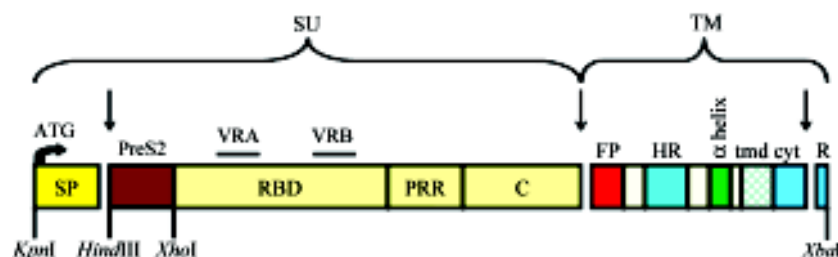


Fig. 2 Schematic representation of chimeric retroviral envelope glycoproteins

The surface subunit (SU, orange) consists of the receptor-binding domain (RBD), the proline-rich region (PRR) and the C-terminal domain (C). The transmembrane subunit (TM, multicolored) consists of an ectodomain comprising the fusion peptide (FP, red), a heptad repeat region (HR, blue) and a downstream α -helix (green) followed by a transmembrane domain (tmd), a cytoplasmic tail (cyt, blue) and the R peptide. The positions of some envelope glycoprotein subdomains are shown: variable regions A (VRA) and B (VRB). The black arrows mark the positions of cleavage of the envelope precursor. Hepatitis B virus PreS2 peptide was fused to aa +1 at the N-terminus of Env. SP, signal peptide.

pHSA, pseudotype retrovirus stocks with the titer of 3.6×10^4 cfu/ml with pHSA and 3.1×10^4 cfu/ml without pHSA.

Analysis of helper virus

We examined the supernatants from transfected 293T cells for the presence of contaminating replication-competent virus or helper virus. Undiluted supernatants of the retroviral stocks produced by co-transfection of 293T cells were used to infect NIH 3T3 cells and these cells were then passaged for 4 weeks to allow virus spread. At each passage, the culture supernatants were harvested, filtered and used to infect fresh NIH 3T3 cells. These cells were selected in G418 for up to 10 d to assay for the presence of replication-competent virus harboring the *neo* gene. Although replication-competent virus was found following three-plasmid co-transfection into 293T cells [43], G418-resistant colonies were undetected at 10 d post-infection using the above assays.

Expression and incorporation of chimeric envelope into virions

Chimeric envelope containing HBV PreS2 peptide fused to aa +1 at the N-terminus of Env were generated and the chimeric protein was assessed for its ability to be processed and incorporated into retroviral vectors. Supernatants of the 293T-transfected cell lines were ultracentrifuged to pellet viral particles. Pellets were then analyzed on immunoblots for their Gag (p30 CA) and envelope protein contents (Fig. 3). Both a precursor and a processed SU product were detected at ratios similar to those for wild-type envelope, suggesting that the mutant was correctly expressed and processed. Additionally, viral SU could be detected for both the wild-type and chimeric envelope. These data showed that insertion of large polypeptide at the N-terminus of the MoMLV SU did not impair expression, processing or viral incorporation of the mutant envelopes.

Transduction of human cells by pseudotype viruses mediated by pHSA

Hepatocytes are the only confirmed site of replication for all members of liver-tropic virus families such as HBV. As human hepatocytes are not easily available, human hepatoma cell line HepG2, which was shown to have the biosynthetic capabilities of normal liver parenchymal cells [44,45] and binds to HBV [46], was used for the subsequent transduction study. Fig. 4 shows that HBV PreS2 pseudotype viruses could transduce human hepatoma cells mediated by pHSA with a relatively high titer in compari-

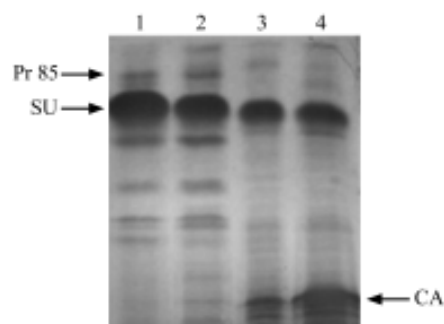


Fig. 3 Western blot analysis of Moloney murine leukemia virus envelope (Env) proteins

1 and 2, cell lysates. Wild-type Env protein (lane 1) and chimeric Env protein (lane 2) were expressed in 293T cells. Pr85 is the precursor form and the SU protein is the mature processed form of Env. 3 and 4, virions. Wild-type viral particles (lane 3) and pseudotype viral particles (lane 4) were harvested from the supernatant of 293T cells and partially purified by pelleting through 20% sucrose. The SU and capsid (CA) proteins were detected with appropriate antibodies.

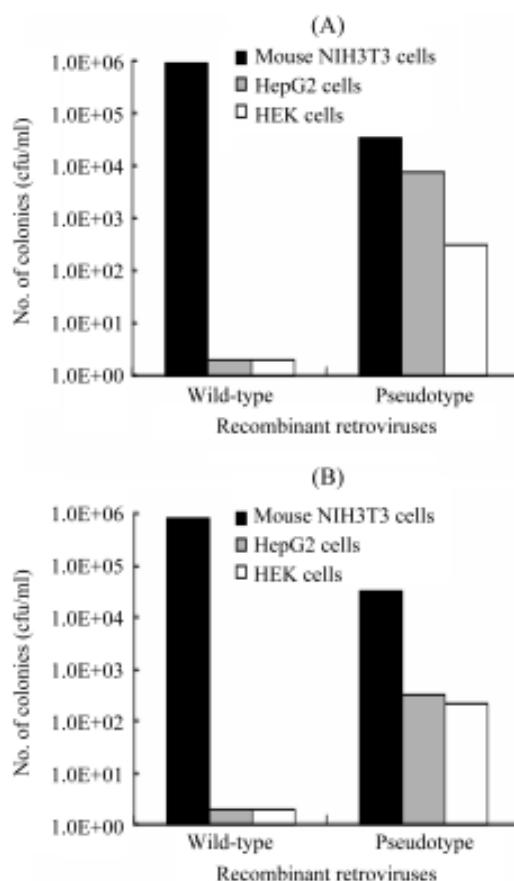


Fig. 4 Titers of recombinant retroviral vectors

NIH 3T3 cells, HepG2 human hepatoma cells, and human embryonic kidney (HEK) cells were infected with wild-type viruses and pseudotype viruses, with polymerized human serum albumin (pHSA) (A) or without pHSA (B). Cells were selected with G418 for approximately 2 weeks, fixed, and stained with crystal violet, and the colonies were counted.

son with those transductions without pHSA. The titers of retrovirus stocks were 0.8×10^4 cfu/ml [Fig. 4(A)] and 2.5×10^2 cfu/ml [Fig. 4(B)], respectively. In contrast to HepG2 cells, it was difficult for pseudotype viruses to transduce HEK cells with or without pHSA, the titers of retrovirus stocks were 3.1×10^2 cfu/ml [Fig. 4(A)] and 2.2×10^2 cfu/ml [Fig. 4(B)], respectively.

Discussion

A conceptual breakthrough in gene therapy would be the development of a gene transfer vector that could be systemically applied, allowing targeted gene transfer into a predetermined cell type. Hepatocytes are interesting as target cells for several reasons. The receptors expressed in hepatocytes are abundant and some of them are well-characterized, and its interaction with a ligand leads to endocytosis. Furthermore, the liver is the central organ of several genetic, acquired and viral diseases amenable to gene therapy [47,48]. Successful *in vitro* targeting of hepatocytes has been shown in many cases, with variable (10–10²-fold) selectivities and experimental conditions [49–55].

Several strategies can be used to engineer retroviral envelopes with altered host range. Previous study on the extension of the ecotropic MoMLV vectors to specific human cell types has been accomplished by several methods, including the chemical addition of sugar molecules to MoMLV Env to allow binding to the asialoglycoprotein receptor [56], cross-linking of MLV retroviral vectors to target cells using an antibody bridge [57–59], and the genetic modification of MoMLV Env by the addition of single-chain antibody variable fragments [9–15] or peptide ligand sequences [16–18]. However, such attempts to redirect MLV vectors to specific cell types by attaching additional cell-binding ligands to the ecotropic MLV Env have so far met with little success.

The search for an Env integration site that allows both binding and the induction of the fusion process is continuing. Recently, reports have described the insertion of short, nondisruptive peptides, such as RGD-containing peptides (ligand for the integrin receptor), short peptide ligands for gastrin-releasing protein and ligands for the human epidermal growth factor receptor family, into different regions of Env. The successful redirection of ecotropic vectors to selectively infect human cells overexpressing the respective receptors has been described [19,20]. Alternatively, an approach using a retroviral library based on feline leukemia virus subtype A with random

modifications in the receptor binding site in the Env molecule has been successfully used to change the viral host range [60].

Several receptors on liver cells have been previously suggested that could bind either to wild-type HBV particles or to genetically engineered virus [21–27]. HBV DNA transfer into hepatocytes using the ASGPR was reported by Wu and Wu in 1988 [61]. Other pseudotype retroviral vectors that show liver-specific transduction are those containing the HBV large and small surface antigens [62], although with low titers. It has been reported that HBV assembles at the post-endoplasmic reticulum-pre-Golgi membranes, and this prevented the incorporation of its envelope proteins into retroviral vectors budding at the plasma membrane. Sung and Lai [62] overcame this problem by overexpressing the large and small surface antigens at levels high enough to result in sufficient expression on the cell surface for incorporation into MLV vectors. These pseudotype vectors showed the normal host range of HBV, transducing only primary hepatocytes from certain primate species, including human. The viral envelope proteins PreS1 and PreS2 were found to bind to hepatocellular membranes [63]. Whether or not recombinant retrovirus carrying HBV envelope protein PreS2 domain could target hepatocytes mediated by pHSA, was not revealed.

In this study, a chimeric envelope expression plasmid that contains the HBV PreS2 peptide fused to aa +1 at the N-terminus of MoMLV Env was constructed. Following simultaneous transfection of pgag-pol, pLEGFP and env plasmids into 293T cells, helper-free pseudotype retrovirus stocks with titers of approximately 10⁴ infectious units/ml were achieved 48 h post-transfection. These pseudotype vectors showed the normal host range of retrovirus, infecting host NIH 3T3 cells, although the efficiency was reduced compared with that of virions carrying wild-type ecotropic MoMLV envelope. In addition, the resultant pseudotype viruses could transduce human hepatoma cells mediated by pHSA with relatively high titers compared to those transductions without pHSA. Our results indicate that pHSA could act as an “intermediate receptor”, attaching to liver plasma membranes.

Hepatocytes are the only confirmed site of replication for HBV. It has been reported that a subset of cells in the kidneys might also be a target of infection [64]. In our study, it was difficult for pseudotype viruses to transduce HEK cells with or without pHSA. Our results indicate that HBV might infect cells in the kidney through another pathway, perhaps because of the interactions between HBV PreS1 and receptors on the cell surface [65], rather than

the polymerized human serum albumin receptor pathway. Notably, pseudotype viruses still could transduce HEK cells with or without pHSA with a similar titer, although the titer was lower, approximately 10^2 cfu/ml. Franco *et al.* has proposed that transferrin receptor on the cell surface might interact with the HBV PreS2 peptide [26]. Whether the transduction of HEK cells was caused by interactions between HBV PreS2 peptide and transferrin receptor or other receptors on the cell surface, awaits further investigation.

If, indeed, the PreS2 peptide sequence is involved in virus attachment *in vivo*, our recombinant retroviral vector could be used as a gene therapy vector targeting hepatocytes. Appropriate animal models are needed that will show the feasibility of vector targeting and will permit the translation of the vectors into new treatment options in the clinic.

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Edited by
Minghua XU