

## Characterization of liver specific promoters in a foamy viral vector pMD09

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**Summary.** – Foamy viruses (FVs) or spumaviruses are retroviruses that are explored as vectors for gene therapy. The good feature of foamy viruses is its broad tropism; however, their infections result in non-targeted gene expression. Here, we attempted to design the liver targeted viral gene delivery by employing liver specific gene promoters like albumin (ALB), transthyretin (TTR) and hepatitis B virus (HBV) promoters. We compared the relative gene expression of liver specific promoters versus the U3 promoter in liver cell line (HepG2) and non-liver cell lines: human fibrosarcoma cell line (HT1080), baby hamster kidney cell line (BHK), human embryonic kidney cell line (HEK 293T) and cervical cancer cell line (HeLa). We have found that the promoter exchange didn't affect viral assembly. The ability to drive gene expression was best with TTR promoter which was followed by HBV and ALB promoter. The use of TTR, HBV and ALB promoters are helpful in achieving liver specific gene expression.

**Keywords:** foamy virus; gene therapy; liver; albumin; transthyretin promoter; HBV promoter

### Introduction

Gene therapy is still mostly an experimental technique. Several gene-therapy trials have been performed in the past two decades for inherited diseases, cancer and chronic infections, but only a few reported clear clinical benefits. On the other side, in some cases, individuals experienced severe adverse events related to the vectors. Gene transfer must overcome complex cellular and tissue barriers to deliver new genetic information into the target cell to drive proficient expression of a therapeutic molecule without disrupting essential regulatory mechanisms (Naldini, 2015). Despite all the obstacles, some recent clinical trials of gene therapy have shown remarkable therapeutic benefits and safety. Improved vector designs that enable the safe delivery of therapeutic

genes to specific cells, technologies for editing genes and correcting inherited mutations and the engagement of stem cells to regenerate tissues has already been studied. However, there is a still need to revisit the investigation of gene therapy.

Adeno associated virus based vectors are in the forefront of successful gene therapy clinical trials (Nathwani *et al.*, 2011) However, major issues involved with adeno associated virus vector are immune mediated responses, limited cargo capacity and transient gene expression (Mingozzi and High, 2013).

In case of integrating viral vectors, such as lentiviruses, antibodies against the transgene and cell mediated immunity against the cells expressing the transgene compromise the success of gene therapy. Integrating virus such as retrovirus and lentivirus can be genotoxic because of its propensity to integrate to actively transcribed genes and their promoters (Rethwilm, 2007). Therefore, there is a need to develop new vectors and strategies for safer and effective gene therapy.

Foamy virus (FV) infects a wide range of mammals, including primates, felids, equines, ruminants and bats. En-

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**Abbreviations:** ALB = albumin; FV(s) = foamy virus(es); HBV = hepatitis B virus; TTR = transthyretin

ogenous FV sequences in sloths, the Cape golden mole, prosimians and the fossil fish coelacanth, indicate an FV–host relationship for over 400 million years (Trobbridge, 2009). It may be noted that anti-FV antibodies are less prevalent in humans, since FVs or spuma-retroviruses are not usual in humans, although they are prevalent in nonhuman primates and in other mammals. They show efficient horizontal transmission in their host species but without pathological changes. FV vectors have several unique properties that make them a better choice for therapeutic gene transfer including a desirable safety profile, a broad tropism, a large transgene capacity, the ability to persist in quiescent cells and efficient and stable gene transfer (Erlwein and McClure, 2010, 2011).

Analysis of FV vector integration sites *in vitro* and in hematopoietic repopulating cells shows that they have a unique integration profile, proposing their better safety than in gamma-retroviruses or lentiviral vectors (Naldini, 2015). In a recent study comparing lentivirus and foamy virus, foamy vector proviruses were observed less often near to a gene or proto-oncogene transcription start sites compared to lentiviral vectors. The foamy vectors were more polyclonal with fewer dominant clones than the lentiviral vector group, and only lentiviral vectors integrated near known proto-oncogenes in dominant clones. Foamy retroviral vectors have a promising integration profile and are less prone to read-through transcription than gamma retroviral or lentiviral vectors (Everson *et al.*, 2016; Rethwilm, 2007).

Foamy viruses can infect a wide range of tissues and therefore lack liver specificity. Liver gene therapy has implications in inherited liver diseases such as familial intra hepatic cholestasis,  $\alpha$ 1 anti-trypsin deficiency, Wilson's disease, glycogen storage diseases, lipid storage diseases, Crigler Najjar syndrome, urea cycle disorders, familial dyslipidaemias, kidney diseases such as primary hyperoxaluria and bleeding disorders such as haemophilia (Geng *et al.*, 2014). We have therefore chosen to test the promoters of two liver specific genes, albumin and transthyretin, as well as the promoter of hepatotropic HBV.

## Materials and Methods

**Vector constructs.** The pMD09 vector is derived from the prototype (formerly human) FV and contains eGFP reporter gene under the control of retroviral U3 promoter. Three different vectors namely pMD09TTR, pMD09ALB and pMD09HBVCORE were constructed using standard recombinant DNA techniques by replacing the U3 promoter with Eco47III and BamHI restriction enzymes (Fig1). Promoters ALB and TTR were amplified from the genomic DNA isolated from HEK 293T cell lines and HBV-CORE promoter was amplified from the plasmid pCF80 which contains tetramer of HBV genome using the specific sets of primers (Table1). In the first round of PCR for albumin promoter the

**Table 1. List of oligonucleotide primers**

Name	Primer
ALB promoter F	5'-TATGCCTGGTGAAGGTCAAG-3'
ALB promoter R	5'-GGTACCCTTCATTGTGC-3'
ALB F Eco47III	5'-GCTAGCGCTGTTTGCATCTGAGGCAACATGAG-3'
ALB R BamHI	5'-GCTAGCGCTGTTTGCATCTGAGGCAACATGAG-3'
TTR F out	5'-GTCCTCTCCTGCACATTCTC-3'
TTR R out	5'-GTGACCTCTCCTCTACCAAG-3'
TTRfw Eco47III	5'-GCTAGCGCTTGTGACCCATGGAACCATCAAG-3'
TTR rv out BamHI	5'-CGGATCCGAGTGGACTTCTGTGATGCTG-3'
HBV cpfw Eco47III	5'-GCTAGCGCTGAACGCCACCACTTCTTGC-3'
HBV cprvBamHI	5'-GCGGATCCTGGTGC GCAAGACCAATTATGC-3'

specific primer set used were 5'-TATGCCTGGTGAAGGTCAAG-3' forward primer and 5'-GGTACCCTTCATTGTGC-3' reverse primer. The product of first round of PCR was used as substrate for second round of PCR using the primer set 5'-GCTAGCGCTGTTTGCATCTGAGGCAACATGAG-3' forward primer and 5'-GCTAGCGCTGTTTGCATCTGAGGCAACATGAG-3' reverse primer, which finally amplified the albumin promoter of 327 bp. The primer set used for the first round of PCR amplification for TTR promoter was 5'-GTCCTCTCCTGCACATTCTC-3' forward primer and 5'-GTGACCTCTCCTCTACCAAG-3' reverse primer. The first round of PCR product was further amplified by using 5'-GCTAGCGCTTGTGACCCATGGAACCATCAAG-3' forward primer and 5'-CGGATCCGAGTGGACTTCTGTGATGGCTG-3' reverse primer which finally amplified the TTR promoter of 529 bp. HBV core promoter was amplified from plasmid pCF80 containing four copies of HBV genome in a single round of PCR using the primer set 5'-GCTAGCGCTGAACGCCACCACTTCTTGC-3' forward primer and 5'-GCGGATCCTGGTGC GCAAGACCAATTTATGC-3' reverse primer, which amplified the core promoter of 232 bp. After the amplification the liver specific promoter was ligated into pMD09 vector (without U3 promoter) using the T4 DNA ligase. Further, the clones were confirmed by restriction digestion of different vectors with specific restriction enzymes AflII, EcoNI, Psp14061.

**Cell lines.** We used five different cell lines HepG2 (human liver carcinoma cell line), Huh7 (human liver carcinoma cell line), HT1080 (human fibrosarcoma cell line), BHK (baby hamster kidney cell line), HEK 293T (human embryonic kidney cell line), and HeLa (cervical cancer cell line). HEK 293T cell lines were used to produce foamy viral vectors, BHK cell lines were used as transduction control (best for transduction by foamy viruses), HepG2 and Huh7 were used as liver specific cell lines and HT1080, HeLa were used as positive controls for human cancer cell lines. All the cell

lines were provided by Institut für Virologie und Immunbiologie, Universität Würzburg, Germany.

**Cell culture experiments.** HepG2 and Huh7 cell lines were maintained in DMEM (Life Technologies, Germany) containing 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). All other cell lines were maintained in MEM (Life Technologies) containing 10% fetal bovine serum and 1% penicillin/streptomycin. For transfection experiments,  $1 \times 10^5$  cells were plated in 24-well plates and co-transfected in triplicates with equimolar amounts of either pMD09TTR, pMD09ALB and pMD09CORE and pMD09 by using PIE (Polyethylenimine linear MW 25,000) (Polysciences Europe GmbH, Germany) at 80% of the cell confluency. After 48 h of transfection, fluorescence intensity was measured using fluorimeter FLUOstar Omega (BMG Labtech, Germany).

**Foamy viral vector production (pMD09 with U3 promoter and pMD09TTR, pMD09ALB and pMD09HBVCORE).** Into a 20 cm culture plate  $14.5 \times 10^6$  of HEK293T cells were seeded with 15 ml of media. The following day confluent cells were transfected with four packaging plasmids, with the ratio of 10:5:1:1 (pMD09: pCZi-gag2: pCZi-pol: pCZHF-env) (Supplementary Fig. S1). In another set of experiments, plasmids pMD09TTR, pMD09ALB and pMD09HBV-CORE were used for the transfection of cells containing the packaging constructs instead of pMD09. On the 5<sup>th</sup> day, the transfection efficiency was determined under the fluorescence microscope (Fluorescence Microscope DMIRE S<sup>2</sup>, Germany). Supernatants were collected and filtered through 45 µm filter and stored in the deep freezer. Different virus dilutions (1:10, 1:100, and 1:1000) were prepared and stored.  $3 \times 10^4$  cells (BHK, HT1080, HepG2, and HeLa) were seeded in to 12-well plates for the transduction with different foamy viral vectors produced by above mentioned experiment. On the second day transduction was performed with different dilutions of vectors and incubated at 37°C. After 72 h, the proportions (%) of fluorescent cells were calculated using Flow jo software inflow cytometry (Becton Dickinson FACScan, BD Biosciences, Germany).

**Flow cytometry analysis.** After 48 h of transfection media was removed from all the cells. The cells were washed with PBS and detached with trypsin for ten minutes. Media was added and cells were centrifuged at 1200 rpm and resuspended in 100 µl of 2% formaldehyde. The percentage of GFP positive cells were analyzed on a Becton Dickinson FACScan (BD Biosciences, Germany) using the Flow jo software against FL1-H.

## Results

### Construction of liver specific FV constructs

To generate the construct of foamy virus vectors with liver specific promoter, we have used three different liver specific promoters: transthyretin (TTR), albumin (ALB) and hepatitis B virus basal core promoter (HBV CORE) (Fig. 1). Liver specific promoter was exchanged with U3 promoter of foamy

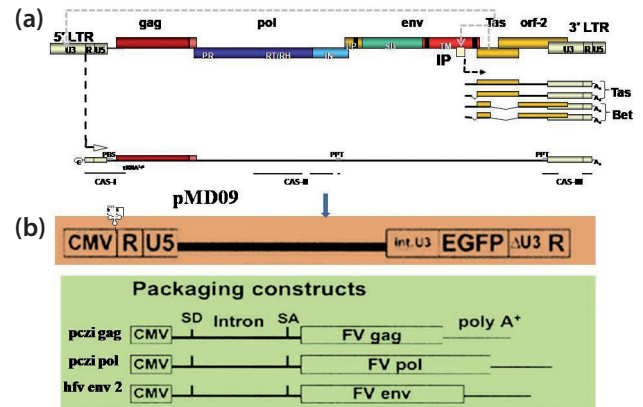


Fig. 1

### Foamy virus and constructs used for transduction experiments.

Representation of the arrangement of genes and transcripts of prototype foamy virus (PFV) (a) FV genome. Long terminal repeat (LTR), internal promoter (IP), cis-acting sequences (CAS), pol encapsidation signal (PES). (b) Plasmid and packaging constructs. pMD09 plasmid with CMV promoter and eGFP. Packaging constructs pCZi-gag2 containing FV gag gene for viral capsid, pCZi-pol containing FV pol gene for polymerase, pCZHFV-env containing FV env gene for envelope protein.

viral vector pMD09 using the standard cloning techniques (Fig. 2a,b). TTR and ALB promoters were amplified from genomic DNA isolated from HEK 293T cell using nested PCR approach. The albumin and transthyretin promoters were amplified in two rounds by polymerase chain reaction using specific primer sets. The product of first round of PCR was used as substrate for second round of PCR which finally amplified the albumin promoter of 327 bp (Fig. 2c) and TTR promoter of 529 bp (Fig. 2e). HBV core promoter was amplified from plasmid pCF80 containing HBV genome in a single round of PCR yielding the core promoter of 232 bp (Fig. 2d). After the amplification the liver specific promoter was ligated into pMD09 vector (without U3 promoter) using the T4 DNA ligase. The clones were confirmed by restriction digestion of different vectors with specific restriction enzymes (Fig. 2f).

### Transduction efficiency of foamy virus constructs in different cell lines

The transduction efficiency of foamy virus was similar in all cell lines (BHK, HT1080, HepG2, and HeLa) tested, however the differences were not statistically significant (Fig. 3).

### Efficiency of liver specific promoters in different cell lines

The promoter of the liver specific genes TTR, ALB and HBV versus U3 promoter were tested in cell lines (BHK, HEK, HT1080, HepG2 and HeLa) by measuring the expression of GFP which was expressed under the control of the

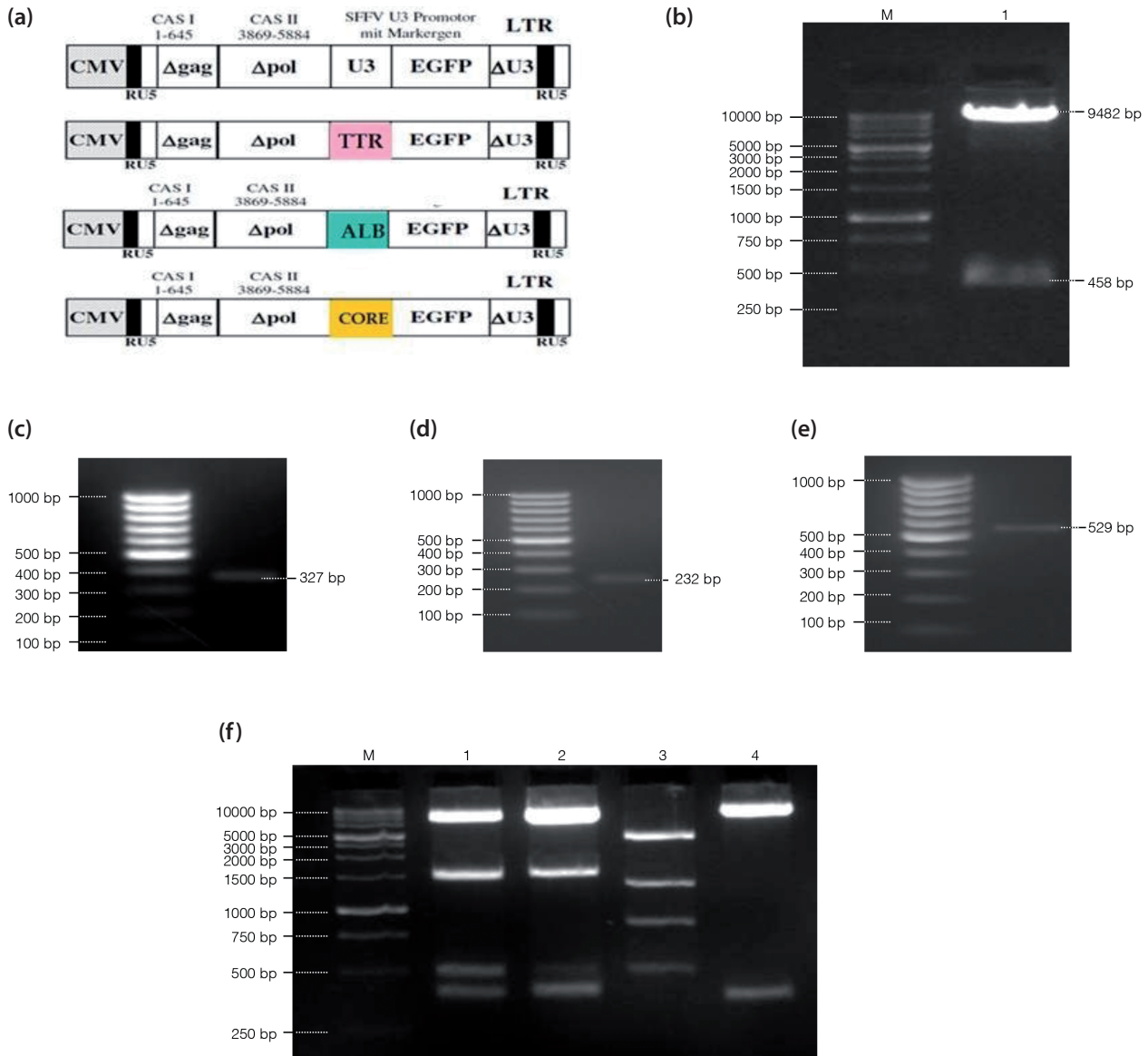


Fig. 2

#### Construction of liver specific foamy virus constructs

(a) Schematic diagram of foamy virus vectors and constructs. (b) Restriction digestion of pMD09 vector to replace U3 promoter. M = DNA marker from 250 bp to 10000 bp, lane – pMD09 vector digested with BamHI and Eco47III restriction enzyme to yield U3 promoter (458 bp). (c) Amplification of ALB promoter from genomic DNA. M = DNA marker from 100 bp to 1000 bp; lane – amplified ALB promoter (327 bp). (d) Amplification of HBV-CORE promoter from plasmid pCF80. M = DNA marker from 100 bp to 1000 bp; lane – amplified core promoter (232bp). (e) Amplification of TTR promoter from genomic DNA. M = DNA marker from 100 bp to 1000 bp; lane – amplified TTR promoter (529 bp). (f) Restriction digestion of different vectors to confirm the clones. M = DNA marker from 250 bp to 10000 bp; lane 1 – pMD09U3 vector digested with AflII restriction enzyme, lane 2 – pMD09CORE vector digested with AflII restriction enzyme, lane 3 – pMD09TTR vector digested with EcoNI restriction enzyme, lane 4 – pMD09ALB vector digested with Psp14061 restriction enzyme.

afore mentioned promoters. As expected, the liver specific promoters resulted in a stronger expression of GFP in liver cell lines (HepG2 and HuH7) compared to BHK, HT1080, and HeLa (Fig. 4a,b). Among the liver specific promoters, TTR was relatively stronger than HBV and ALB. The promoter

activity of TTR was about 8 times stronger when compared to U3 promoter in HepG2 cells. The HBV and albumin promoter activities were, about 7 and 6 times stronger respectively, compared to U3 promoter activity in HepG2 cells. The expression of GFP was about 30% and 60% respectively, in

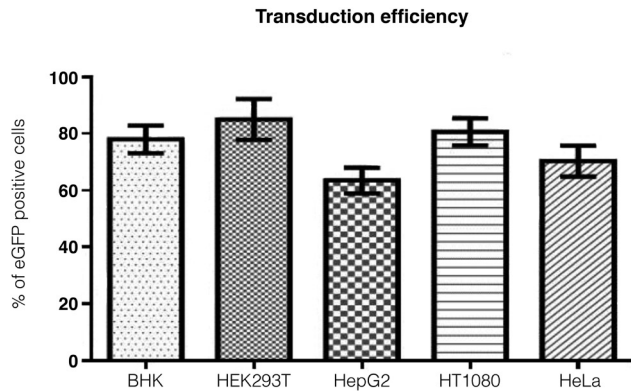


Fig. 3

#### Transduction efficiency of foamy viral vector in different cell lines

The transduction efficiency of foamy virus was similar in all cell lines (BHK, HT1080, HepG2, HEK293T and HeLa) tested. The differences were not statistically significant.

HeLa cells compared to the GFP expression in HepG2 cells under TTR and U3 promoters. The GFP expression was very weak in BHK, HEK and HT1080 cell lines under liver specific promoters compared to U3 promoter. However, the U3 promoter mediated expression was about 5 times stronger in BHK cell line than TTR promoter mediated GFP expression in liver cancer cells.

#### Promotor exchange did not affect viral packaging

The introduction of new promoters can affect the packaging efficiency of new vectors. To verify this, we produced the foamy viral vectors containing U3 (pMD09), TTR (pMD09TTR), HBV (pMD09HBVCORE), albumin (pMD09ALB) promoters in HEK 293T cells and transduced the BHK and HT1080 with these vectors and after 72 h of transduction, fluorescent cells were analyzed by flow cytometry. Results showed no significant difference between liver specific promoters with reference to the virus packaging efficiency (Fig. 5).

### Discussion

Foamy virus vectors were shown to have an efficient gene delivery in variety of target cells (Lindemann and Rethwilm, 2011). In this study, we have characterized different promoters for liver specific gene therapy in foamy virus vector pMD09 (Kramer *et al.*, 2003). In terms of the ability to drive gene expression it was found that TTR promoter was strongest followed by HBV promoter and albumin promoter. They were relatively liver (hepatocyte) specific because the relative gene expression driven by these promoters was 3 to 4-fold more in hepatocyte cell line HepG2 compared to

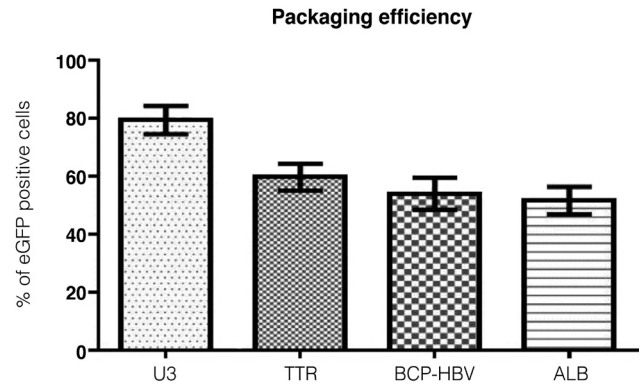


Fig. 5

#### Packaging efficiency of liver specific promoters

There was no significant difference between liver specific promoters with reference to the virus packaging efficiency. Proportions (%) of fluorescent cells were calculated by flow cytometry. pMD09U3 showed 79% GFP positive cells whereas pMD09TTR, pMD09ALB and pMD09CORE constructs showed from 53% to 58% GFP positive cells.

BHK, HeLa, HEK and HT1080. It may be interesting to note that strong promoter U3 expression in HeLa cells is only 2 folds higher than that of TTR, HBV and ALB mediated gene expression. This may be explained by certain similarities which the cervical cancer cell line HeLa might have acquired over many decades of evolution. In fact, Chang cell line, which is used in Bio-Artificial Liver (BAL) reactors is derived from HeLa cells. The cell line Chang was originally thought to be derived from normal liver. The cell line has since been found to be indistinguishable from HeLa cells by STR PCR DNA profiling (Masters *et al.*, 2001). Earlier reports also showed that liver targeted gene expression using liver-specific foamy virus promoters can infect a wide range of species and a wide range of tissues and found to be successful in gene therapy (Bauer *et al.*, 2008; Erlwein and McClure, 2010; Trobridge, 2009; Weber *et al.*, 2013). In future it may be possible to derive foamy virus which is organ specific through genetic engineering. However, currently, making use of liver specific promoters is the only way to make foamy viral gene therapy liver specific. Similarly, Geng *et al.* (2014) also showed liver specific gene therapy by using hepatocyte-specific  $\alpha$ -fetoprotein (AFP) enhancer/albumin promoter. We conclude that the use of TTR, HBV and ALB promoters can make foamy virus mediated gene expression relatively liver specific.

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**Supplementary information** is available in the online version of the paper.

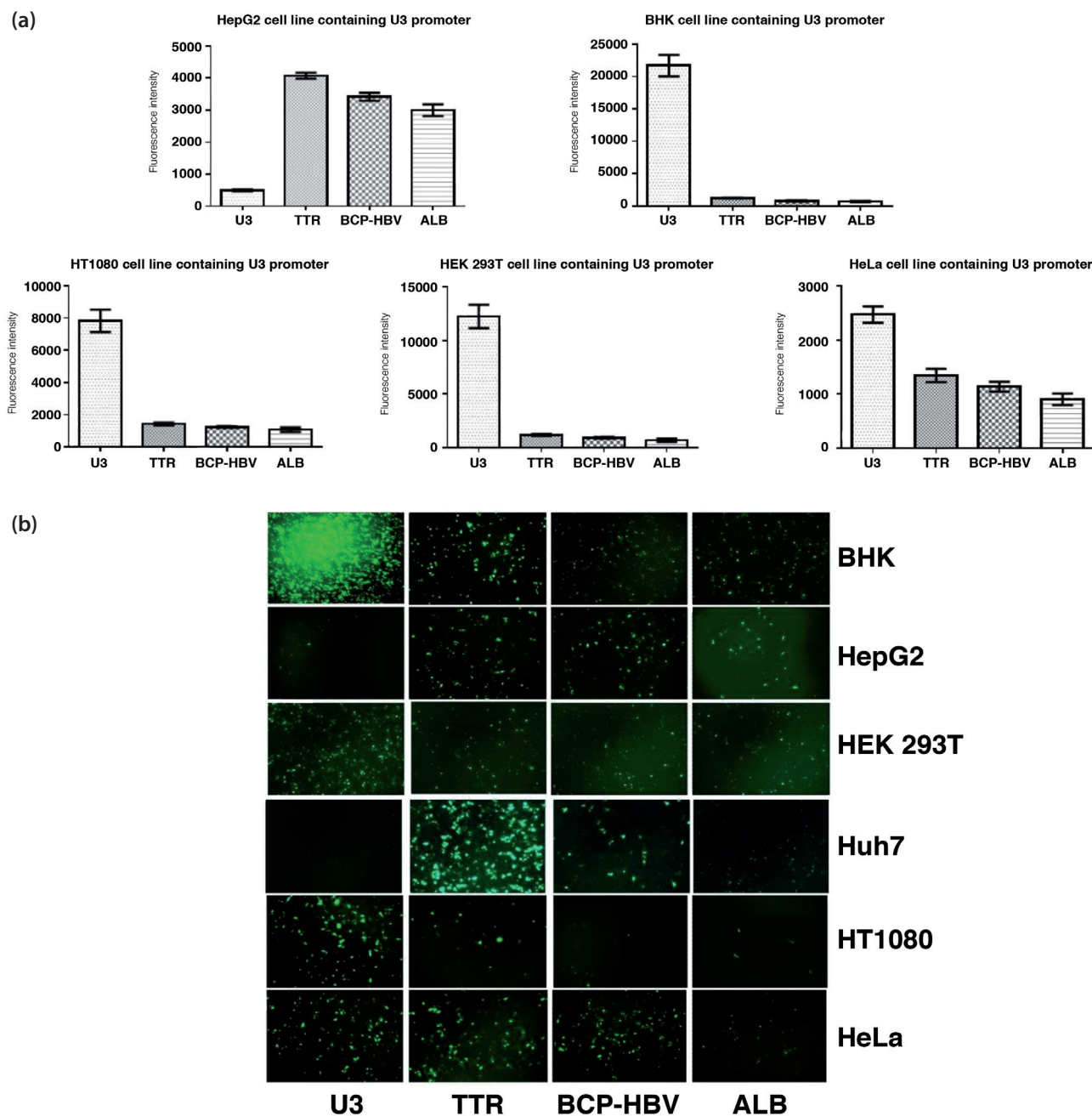


Fig. 4

#### Transfection efficiency of liver specific promoters

(a) The promoter of the liver specific genes transthyretin (TTR), albumin (ALB) and hepatitis B virus (HBV) versus U3 promoter expression were tested in cell lines (BHK, HEK 293T, HT1080, HepG2 and HeLa) using flow cytometry. (b) Liver specific promoters resulted in a higher transfection efficiency correlated with expression of GFP in HepG2 and Huh7 compared to BHK, HT1080, HEK 293T, and HeLa analysed using fluorescence microscope.

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## Supplementary information

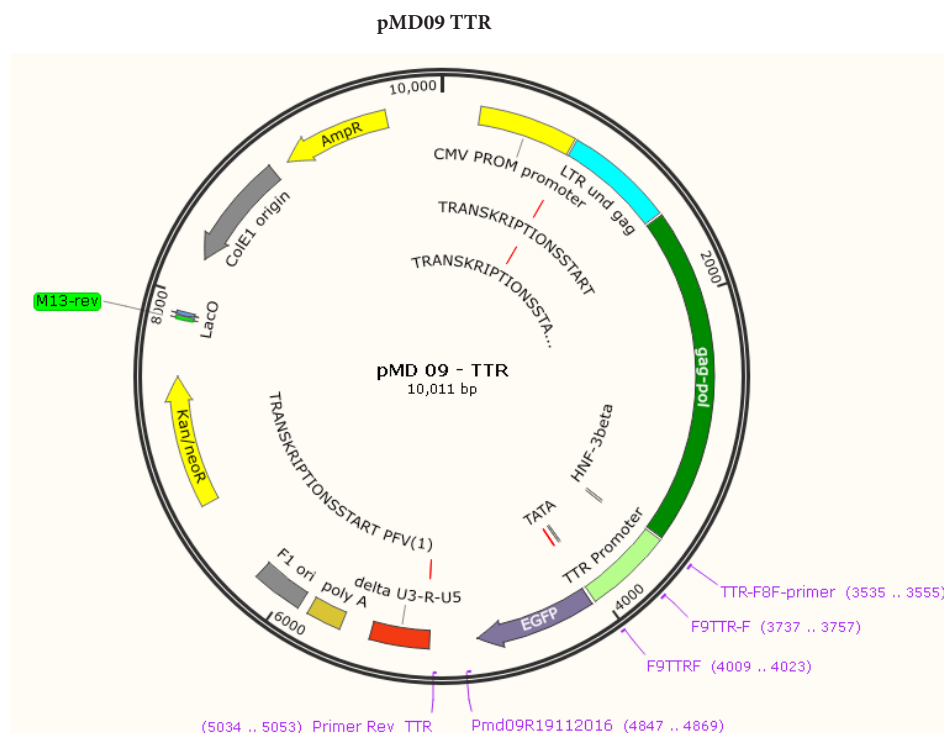
### Characterization of liver specific promoters in a foamy viral vector pMD09

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Fig. S1. Foamy virus vector constructs



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