Generation of a Vero-Based Packaging Cell Line to Produce SV40 Gene Delivery Vectors for Use in Clinical Gene Therapy Studies

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INTRODUCTION

After small molecules and therapeutic proteins, gene therapy will be the next wave of medicines potentially capable of curing today’s major diseases. Key to the success of gene therapy will be the safe and efficient delivery of the therapeutic genes into cells of the affected tissue or organ target cells of a patient’s body. Viruses evolve to efficiently transfer and express their genes into host cells. This ability renders them ideally suited for use as gene delivery vectors. Among the viral vector systems currently used for gene therapy, lentiviral (LV) and adeno-associated viral (AAV) vectors are the most popular because they transduce a wide variety of tissues and mediate long-term expression when administered to animals.1

they transduce a wide variety of tissues and mediate long-term expression most likely have never been infected with AAV, and thus were immunologically naive to the RD vector used in the study. For these reasons, the efficacy of AAV vector-based in vivo gene therapies is limited.12,13

LD simian virus 40 (SV40) vectors could be an attractive alternative to AAV vectors for clinical gene therapy.14 SV40 is a polyomavirus with icosahedral capsids of 45 nm in size containing a 5.25 kb-long circular double-stranded DNA. The virus strictly replicates in its natural host, macaques, where it causes chronic asymptomatic infections.15 SV40 vector particles enter infected cells via the caveolar-endosomal route, but in contrast with other viruses are able to avoid the lysosomal degradation, thereby evading exposure to the host immune system.15–21 RD SV40 vectors have been generated by deleting the coding region of the two early non-structural proteins named large T antigen (LTag) and small T antigen (STag), giving 2.7 kb of space for cloning the transgene encoding the therapeutic protein or RNA. SV40 vectors are highly instable, rapidly degraded when administered in vivo, and the development of stable packaging cell lines has remained challenging.11 For these reasons, LV vectors are mainly used for the ex vivo transduction of leukocytes and/or their progenitors to treat blood-related genetic disorders, lysosomal storage diseases, or cancer. AAV vectors are mainly used for in vivo gene therapies. However, the majority of the human population encountered wild-type AAV together with its helper virus (adenovirus, causing the common cold) and developed a strong humoral and cellular immune memory for the AAV capsid proteins. Clinical studies using recombinant AAV vectors revealed that administration of vector particles elicits innate and adaptive immune responses against the viral and transgene-encoded proteins in the vast majority of treated patients, leading to decreasing expression levels of the therapeutic transgenes over time and elimination of the transduced cells from the body, compromising re-administration of the vector. The few treated patients who showed long-term transgene expression most likely have never been infected with AAV, and thus were immunologically naive to the RD vector used in the study. For these reasons, the efficacy of AAV vector-based in vivo gene therapies is limited.12,13

LV vectors integrate randomly in the host genome, and thus increase the risk of insertional mutagenesis.15 Moreover, LV vector particles have shortcomings that limit their application.

Replication-defective (RD) recombinant simian virus 40 (SV40)-based gene delivery vectors hold a great potential for clinical applications because of their presumed non-immunogenicity and capacity to induce immune tolerance to the transgene products in humans. However, the clinical use of SV40 vectors has been hampered by the lack of a packaging cell line that produces replication-competent (RC) free SV40 particles in the vector production process. To solve this problem, we have adapted the current SV40 vector genome used for the production of vector particles and generated a novel Vero-based packaging cell line named SuperVero that exclusively expresses the SV40 large T antigen. SuperVero cells produce similar numbers of SV40 vector particles compared to the currently used packaging cell lines, albeit in the absence of contaminating RC SV40 particles. Our unique SV40 vector platform named SVac paves the way to clinically test a whole new generation of SV40-based therapeutics for a broad range of important diseases.

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transduce a wide range of cell types in vivo, and their therapeutic potential has been demonstrated in animal models of human disease.22–26 Because humans can be considered naive to SV40, it is expected that RD SV40 vectors are non-immunogenic or tolerogenic when applied in clinical settings. The non-immunogenicity in humans and capacity to induce immune tolerance to transgene proteins render SV40 vectors highly attractive for use in gene replacement and immunotherapies.

However, the translation of SV40 vectors to the clinic has been hampered by the lack of a packaging cell line that does not accumulate detectable amounts of replication-competent (RC) SV40 particles during vector production.29–32 To date, two cell lines have been mainly used for the production of SV40 vectors: COS-1 and COS-7. COS cell lines were generated by transformation of monkey CV1 cells harboring a transgene resulting in vector expression plasmids. This most likely occurs by sequence homology-dependent recombination between the chromosomally inserted SV40-specific DNA sequences and episomally replicating vector-specific DNA sequences.33

Other cell lines that express the SV40 T antigens in trans are COT18 and CMT4. Both CV1-derived cell lines were generated using SV40 DNA in which the T antigens were expressed under transcriptional control of the mouse metallothionein promoter. Because the complete early region of the SV40 genomic DNA is present in the chromosomal DNA, the ability that RC virus contaminants emerge during the vector production process has remained. Moreover, the vector yields of both packaging cell lines are not increased compared with those of COS cells.33–35

Polyomavirus-based virus-like particle (VLP) vector systems have been developed to prevent the occurrence of RC virus particles in the vector preparations. In vitro-generated VLPs consisting of circular double-stranded DNA encapsidated with polyomavirus VP1 lack VP2 and VP3 in the capsids and histones covering the encapsidated DNA molecules. Although these particles display a higher packaging capacity, the absence of VP2/VP3 in the VLPs has a negative impact on their entry in the nucleus.36

In order to overcome the generation of wild-type virus contaminants, we optimized the SV40 vector genome used to initiate the production of vector particles and generated a safe and efficient Vero-based SV40 vector packaging cell line named SuperVero. The SuperVero cells solely express the viral LTag and accumulate vector particles at high titers, comparable with those obtained in the conventional packaging cell lines COS-1 and COS-7. Because RD SV40 vectors are safe, highly efficient for gene delivery, and non-immunogenic/tolerogenic in humans, our improved SV40 vector platform named SVac holds promising qualities for developing effective treatments for the major diseases of our time.37

RESULTS

Construction of SV40 Vector Destination pSVac and Derivative Vector Expression Plasmids

In order to improve the efficacy and versatility of the SV40 vector system, we modified vector plasmid pSL-PL38 in a series of steps yielding SV40 vector destination plasmid pSVac (Figure 1) that is used to initiate the production of recombinant SV40 particles. The low copy number bacterial backbone of pSL-PL (pBR322) was replaced with a high copy number backbone (pBluescript SK –). The residual 3′-terminal LTag coding sequences present in pSL-PL were removed, and a Gateway gene cassette derived from pEFS/FRT/V5-DEST was introduced in the early region of the SV40 vector DNA, providing a versatile method to introduce transgenes encoding therapeutic proteins or RNAs into the vector destination plasmid by LR clonase-mediated recombination between pSVac DNA and that of an entry plasmid harboring a transgene resulting in vector expression plasmids. The bovine growth hormone (BGH) polyadenylation (pA) signal originating from pEF5/FRT/V5-DEST was cloned downstream of the Gateway cassette to facilitate transient expression studies by transfecting target cells with DNA from vector expression plasmids. Unique Ascl and SpeI restriction sites were introduced between the SV40 early promoter and the Gateway cassette to facilitate the introduction of tissue-specific promoters. We generated a series of SV40 expression plasmids containing different transgenes by Gateway recombination. The resulting expression plasmids encode the jellyfish green fluorescent protein (pSVGFP), the human activated blood clotting factor VII (pSVFVIIa), and a short hairpin RNA specific for the firefly luciferase (pSVshLuc).

For the generation of SV40 vector particles, the prokaryotic backbone of pSVac needs to be removed from the SV40 vector sequences. For this purpose, NotI restriction sites and loxP recombination sites were introduced between the Gateway cassette and BGH pA signal and between the SV40 pA signal and the pBluescript SK – bacterial backbone. Circular vector DNA used as starting material for the

Figure 1. Schematic Representation of the SV40 Vector Destination Plasmid pSVac
The Gateway gene cassette comprising the ccdB and the chloramphenicol resistance (CmR) genes present in pSVac is substituted by the transgene using Gateway recombination yielding an SV40 vector expression plasmid.
production of SV40 vector particles in packaging cells was generated by digesting a vector expression plasmid with NotI followed by re-circularization of the vector DNA using T4 DNA ligase. Alternatively, circular vector DNA can be generated by homologous recombination at the loxP sites using Cre recombinase.

**Generation of a New SV40 Vector Packaging Cell Line**

The SV40 T antigens are required for virus DNA replication and expression of the late gene encoding the viral capsid proteins. In order to verify whether the LTag by itself is sufficient for the generation of SV40 vector particles, we generated the expression plasmids pHY338 and pHY359 as described under Materials and Methods. Expression plasmid pHY338 encodes both STag and LTG; plasmid pHY359 exclusively encodes LTag (Figure 2A). Vero cells were selected as acceptor cells for both expression plasmids because these cells are permissive for SV40 and because Vero constitutes an accepted cell culture platform for vaccine production. Vero cells grown in serum-free cell culture medium were transfected with either pHY338 or pHY359 DNA, and cells were cultured for 1 month in serum-free culture medium supplemented with puromycin. Puroycin-resistant cell clones were subsequently transduced with SVLuc particles produced in COS-1 cells at an MOI of 100. As a positive control, COS-1 cells were transduced with SVLuc particles at the same MOI. Three days after transduction, the amount of SVLuc particles present in the culture medium of the transduced cells was determined by quantitative real-time PCR. The qPCR assay has been developed in such a way that only packaged vector genomes are measured (Figure 2B). Both the pHY338 and pHY359-transfected Vero cells produced vector particles at approximately 30% of the amount of vector particles produced in COS-1 cells. To verify that the vector particles produced in the transfected Vero cells are biologically active, we subsequently used the culture media from the SVLuc-transduced Vero and COS-1 cells for transducing fresh COS-1 cells. Three days after transduction, crude cell lysates were prepared and the luciferase activity was determined in these lysates by luminescence. Figure 2C shows that the transfected Vero cells produced vector particles that induced luciferase expression when transferred to COS-1 cells. These experiments demonstrated that the presence of SV40 LTG in Vero cells is sufficient for producing SV40 vector particles.

In order to generate a new Vero-based packaging cell line for the production of SV40 vector particles, we cultured cells transfected with pHY359 DNA in the presence of puromycin for 1 month. Individual puroycin-resistant cell colonies were picked and sub-cultured in culture platform for vaccine production. Vero cells grown in serum-free culture medium were transfected with either pHY338 or pHY359 DNA in the presence of puromycin for 1 month. Individual puroycin-resistant cell colonies were picked and sub-cultured in culture medium supplemented with puromycin. Puroycin-resistant cell clones were subsequently transduced with SVLuc particles produced in COS-1 cells at an MOI of 100. As a positive control, COS-1 cells were transduced with SVLuc particles at the same MOI. Three days after transduction, the amount of SVLuc particles present in the culture medium of the transduced cells was determined by quantitative real-time PCR. The qPCR assay has been developed in such a way that only packaged vector genomes are measured (Figure 2B). Both the pHY338 and pHY359-transfected Vero cells produced vector particles at approximately 30% of the amount of vector particles produced in COS-1 cells. To verify that the vector particles produced in the transfected Vero cells are biologically active, we subsequently used the culture media from the SVLuc-transduced Vero and COS-1 cells for transducing fresh COS-1 cells. Three days after transduction, crude cell lysates were prepared and the luciferase activity was determined in these lysates by luminescence. Figure 2C shows that the transfected Vero cells produced vector particles that induced luciferase expression when transferred to COS-1 cells. These experiments demonstrated that the presence of SV40 LTG in Vero cells is sufficient for producing SV40 vector particles.

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**Molecular Analysis of the SuperVero Packaging Cell Line**

In order to confirm the presence of a functional LTG-encoding gene in the chromosomal DNA of SuperVero cells and to investigate whether the cells harbor contaminating viruses, the transcriptome of SuperVero cells was analyzed by Massive Parallel Sequencing (MP-Seq).
than 1.6 million different cDNA sequences from SuperVero poly-A-positive RNAs were generated. In parallel, more than 800,000 different cDNA sequences were generated from poly-A-positive RNAs isolated from reference cell line Vero ATCC CCL-81.

Among the SuperVero cDNA sequences, 202 cDNA reads mapped to the LTag gene present in the genomic DNA of the cells (see Table 1). SV40 cDNA sequences specific for other parts of the SV40 genome were absent in SuperVero cells. As expected, Vero cells do not contain SV40-specific mRNAs (Table 1).

The 202 LTag-specific cDNA reads of SuperVero cells were aligned and are homologous to the DNA sequence of pHY359 used to generate the cell line. Sequence variants were not present among the 202 sequenced cDNAs (Figure 3A).

The remaining cDNA reads obtained from the SuperVero cells with the MP-Seq method were compared with a viral sequence database (ViralDB) that contains 964,619 sequences extracted from the complete GenBank nucleotide sequence database. The most significant homologies found in the comparison are shown in Table 1. In the SuperVero sample, cDNAs were found that show homology to human herpesvirus 5 DNA. This homology corresponds with a short transcribed region in the cytomegalovirus immediate early (CMVie) promoter that drives transcription of the pac puromycin-resistance gene.

All other sequence homologies found between cDNAs from the SuperVero cells and the ViralDB are also found when the cDNAs from the Vero ATCC CCL-81 cells were compared with the sequences in the database. The MP-Seq study has shown that the SuperVero cell line is not contaminated with known viruses during its generation from Vero ATCC CCL-81 cells.

The presence of the LTag protein in SuperVero cells was subsequently confirmed by western blot analysis. Both LTag and STag proteins can be detected using an antibody specific for the N-terminal part of the LTag protein. As expected, COS-1 cells accumulate both LTag and STag, whereas SuperVero cells express only LTag (Figure 3B). Immunocytochemistry of the SuperVero and COS-1 cells further confirmed the presence of the LTag in the nucleus of the cells (Figure 3C). A densitometric analysis of the bands on the western blots revealed that the relative amount of LTag in SuperVero cells was slightly lower than that in COS-1 cells (Figure 3D).

The SV40 late gene encoding the capsid proteins is exclusively expressed in cells permissive for SV40 that accumulate the viral LTag. This implies that the viral capsid proteins are produced only in the packaging cells during vector manufacturing, but absent in (human) target cells after transduction. The transcriptional silencing of the SV40 late gene in cells lacking the viral LTag was verified by transfecting SuperVero and HeLa cells with pSVGFP DNA. After 3 days, the presence of GFP, LTag, and the major viral capsid protein VP1 in both cell types was checked on western blots using monoclonal antibodies specific for GFP, LTag, and VP1, respectively. A monoclonal antibody specific for GAPDH was included as a protein loading control. Only SuperVero cells constitutively produce SV40 LTag. In both pSVGFP-transfected SuperVero and HeLa cells, GFP was detected. However, VP1 accumulated only in pSVGFP-transfected SuperVero cells, demonstrating that the SV40 late gene is not expressed in target cells lacking SV40 LTag (Figure 4).

**Production of SV40 Vector Particles in SuperVero Cells**

When COS-1 cells cultured in medium with fetal bovine serum and SuperVero cells cultured in serum-free medium are transduced with SV40 vector particles at an MOI of 400 vector particles per

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**Table 1. Comparison of the cDNA Reads of SuperVero and Vero Cells to a Viral Sequence Database**

| Original Accession No. Identified | SuperVero (1,673,145 Total Reads) | Vero ATCC CCL-81 (806,262 Total Reads) | Reference Genome Accession No. | Taxonomy |
|----------------------------------|------------------------------------|----------------------------------------|-------------------------------|----------|
|                                  | Total No. of ViralDB Wins | Total No. of Reads Mapped to Refseq| Total No. of ViralDB Wins | Total No. of Reads Mapped to Refseq | |
| NC_001669.1                      | 202a                          | 238b                                   | 0                            | 0 | NC_001669 | simian virus 40, complete genome |
| AC146999.1                       | 63                             | 97                                     | 0                            | 14 | NC_006273 | human herpesvirus 5 |
| EU410304.1                       | 23                             | 21                                     | 13                           | 3 | NC_006998 | vaccinia virus GLV-1b68 |
| X03922.1                         | 17                             | 97                                     | 0                            | 14 | NC_006273 | human herpesvirus 5 |
| HM143845.1                       | 12                             | 0                                      | 6                            | 0 | M11841 | simian retrovirus 1 |
| U552506.1                        | 9                              | 18                                     | 10                           | 14 | U85505 | simian endogenous retrovirus |
| Z54175.1                         | 7                              | 10                                     | 5                            | 5 | NC_001461 | bovine viral diarrhea virus 1 |
| AF104029.1                       | 6                              | 1                                      | 6                            | 0 | NC_002032 | bovine viral diarrhea virus 2 |
| FM212572.1                       | 4                              | 5,330                                  | 1                            | 2,926 | AB047240 | human endogenous retrovirus |
| JN134185.1                       | 3                              | 87                                     | 7                            | 18 | JN134185 | simian endogenous retrovirus SRVca |
| AF2990913.1                      | 1                              | 0                                      | 0                            | 1 | U13766 | murine leukemia virus |
| M15805.1                         | 1                              | 64                                     | 0                            | 21 | M15805 | feline sarcoma virus |

*Note:* A single read may map to multiple positions of the reference sequence and each mapping is counted independently.  
| Accession No. Taxonomy |
|-----------------------|
| Original Accession No. Identified | Reference Genome Accession No. | Taxonomy |
|-----------------------|
| AC146999.1 | | simian virus 40, complete genome |
| EU410304.1 | | vaccinia virus GLV-1b68 |
| X03922.1 | | human herpesvirus 5 |
| HM143845.1 | | simian retrovirus 1 |
| U552506.1 | | simian endogenous retrovirus |
| Z54175.1 | | bovine viral diarrhea virus 1 |
| AF104029.1 | | bovine viral diarrhea virus 2 |
| FM212572.1 | | human endogenous retrovirus |
| JN134185.1 | | simian endogenous retrovirus SRVca |
| AF2990913.1 | | murine leukemia virus |
| M15805.1 | | feline sarcoma virus |
cell, both cell lines secreted comparable numbers of vector particles in the culture medium (Figure 5A). The capacity of SuperVero cells to produce consistent amounts of SV40 particles was further tested by passaging vector particles in fresh SuperVero cells and measuring the number of vector particles after each passage. In SuperVero cells transduced with SVLuc particles obtained at subsequent passages, consistently $10^8$–$10^9$ vector particles per milliliter cell culture are found in the cell culture medium (Figure 5B). Moreover, approximately the same numbers of vector particles remain inside the cells and are not harvested after production (data not shown). The transduction efficacy (potency) of the produced vector particles after each passage was determined using a modified tissue culture infectious dose 50 (TCID50) assay. Because SV40 vector particles do not lyse transduced cells, we used SVLuc as a reporter vector and used luminescence as a readout in this limited dilution assay. These experiments revealed that the vector particles quantified by qPCR (Figure 5B) remain fully functional after passaging them in SuperVero cells as determined by TCID50 (Figure 5C). Furthermore, defective interfering vector particles do not accumulate during the vector production process as demonstrated by the stability of the TCID50 titer over subsequent passaging (Figure 5C).

We next produced SVLuc, SVGFP, SVFVIIa, and SVshLuc vector batches in SuperVero cells. The vector yields obtained after three passages consistently ranged between $10^8$ and $10^9$ particles per milliliter cell culture, demonstrating the robustness of the vector production process (Figure 5D).

SVLuc particles produced in SuperVero cells were stored for different periods of time at different temperatures. The numbers of vector particles were determined by qPCR (Figure 6A), whereas the potency of the particles was determined using the TCID50 assay (Figure 6B). Independent of the storage temperature, the amounts of vector particles did not significantly decrease after 6 months. These experiments...
demonstrate that SV40 vector particles stored at 4°C or at lower temperatures remain fully functional for at least 6 months.

**Detection of RC SV40 Particles**

It has been found that the production of SV40 vectors in COS cells results in the emergence of RC SV40 particles. In order to sort out whether the virus contamination problem is solved by using the SuperVero cell line (Figure 7), we designed a set of primers to detect RC SV40 particles by qPCR (Figure 8).

In all the samples tested so far using these primers, including samples from highly concentrated vector preparations and samples from subsequent vector passages (up to 10), the number of RC SV40 particles remained below the detection limit of the assay. This means that after passaging the vector particles in SuperVero cells, RC SV40 particles certainly do not overgrow the RD vector particles.

**DISCUSSION**

SV40 gene delivery vectors have shown therapeutic efficacy in numerous preclinical studies, and because of their predicted non-immunogenic/tolerogenic potential in humans these vectors are an attractive alternative to the currently used vector systems for developing safe and efficient gene therapies. To date, however, SV40 vectors have not been tested in clinical trials because an efficient packaging cell line that does not accumulate RC SV40 particles during the vector production process is lacking. Here we report on the development of an SV40-based gene delivery platform, which we named SVac, that is well suited for producing RD SV40 vector particles free of contaminating RC revertants, and thus eligible for use in clinical gene therapy studies.

We developed a versatile SV40 vector destination plasmid (pSVac) that allows the evaluation of promoters, transgenes, and other regulatory elements for safety and efficacy in animals and humans. Vero cells were selected for the generation of a new packaging cell line because this cell line is permissive to SV40 and is recommended by the World Health Organization for vaccine production for human use. RD SV40 vector genomes lack the T antigen region. In order to produce SV40 vector particles, T antigen must be provided by the packaging cell line. The SV40 early gene encodes LTag and STag. LTag is a replicase-associated protein required for viral DNA replication and for activation of the viral late promoter. STag inhibits cellular protein synthesis from capped messenger RNAs (a process denoted host shutoff), thereby promoting the synthesis of viral capsid proteins. Simultaneous expression of LTag and STag results in immortalization of primary mammalian cells, transformation of established mammalian cell lines, and induction of tumors in immuno-compromised young-born hamsters. However, it has been shown that expression of the individual T antigens in mammalian cells does not lead to immortalization, transformation, or tumor induction. We here show for the first time that expression of the LTag in the absence of STag in Vero cells is sufficient for the replication and packaging of SV40 vector particles. A Vero-based SV40 packaging cell line denoted SuperVero was generated that expresses the viral LTag, and thus lacks the viral oncogene and oncoproteins. Because sequence overlap between chromosomally inserted SV40 LTag coding sequences and episomally replicating SV40 vector sequences is lacking,
the emergence of RC particles during the vector production process is highly unlikely.

We demonstrate that the LTag in SuperVero cells activates transcription from the viral late promoter, resulting in the accumulation of capsid proteins during the vector production process. However, in target cells lacking SV40 LTag transduced with an RD SV40 vector, only the transgene is expressed from the viral early promoter.

From the previous attempts to generate an SV40 vector packaging cell line it was found that the amount of the LTag is directly correlated with the number of SV40 vector particles, but inversely correlated with cell viability. This could be the reason why we had to screen more than a hundred independent puromycin-resistant clones to identify one cell clone that is capable of producing vector particles to levels comparable with those in COS cells. Remarkably, this cell clone accumulates LTag to levels similar to COS-1 cells. Apparently there is a narrow margin of LTag expression tolerated by Vero cells that supports SV40 vector replication. We initiated experiments to further increase the amount of LTag in the packaging cells during the production process to even improve the vector yields.

The SV40 vector particles remain fully functional after passaging them in SuperVero cells, indicating that defective interfering particles do not accumulate during the production process. Furthermore, the produced vector particles are highly stable upon storage at 4°C or lower temperatures. The major advantage of SuperVero packaging cells above COS cells for producing RD SV40 vector particles is the absence of contaminating RC SV40 particles during the vector production process.

The circular vector DNA molecules used in our studies to start the production of SV40 vector particles in SuperVero cells are similar, with the same genetic elements, to those used in previous studies to produce vector particles in COS cells. Because both Vero and CV1 cell lines are derived from kidney tissue obtained from African green monkeys (Cercopithecus aethiops), it is assumed that the in vivo transduction efficacy of SV40 vector particles produced in a Vero-derived packaging cell line is similar to that of particles produced in CV1-derived packaging cell lines such as COS. Indeed, ongoing in vivo studies with SVGFP and SVLuc particles produced in SuperVero cells show high transduction rates and transgene expression levels of different organs and/or tissues depending on the mode of administration (unpublished data).

Overall, RD SV40 vectors are efficient, non-immunogenic/tolerogenic in humans, highly stable, safe to use, and can be produced in large quantities in SuperVero packaging cells. The generation of the SVac platform described here paves the way for developing the next wave of medicines. SV40 vectors expressing functional gene products can
be used in gene replacement therapies to treat genetic disorders such as, for example, low-density lipoprotein receptor deficiency, primary hyperoxaluria type 1, or hemophilia. In addition, SV40 vectors expressing T cell receptors (TCRs) or chimeric antigen receptors (CARs) can be used as vaccines to treat cancer. Last but not least, because of their capacity to induce immune tolerance to the transgene products in humans, SV40 vectors expressing primary self-antigens of degenerative/autoimmune diseases can be used as reverse vaccines to treat the major diseases of our time, such as neurodegenerative and psychiatric diseases, atherosclerotic cardiovascular disease, diabetes mellitus, arthritis, and chronic obstructive pulmonary disease (COPD).

MATERIALS AND METHODS

Plasmid Construction

Expression plasmid pHY338 was constructed using a pBluescript SK+ (Stratagene; Agilent Technologies) backbone by adding a gene cassette comprising the 5' end of the LTag coding sequence. Second, with forward primer 5'-AGGAATGTTGTACACCATGCATTTG-3' covering the splice site, a first PCR fragment was obtained. Expression plasmid pHY338 was constructed using a pBluescript SK+ backbone by adding a gene cassette comprising the 5' end of the LTag coding sequence. Second, with forward primer 5'-AGGAATGTTGTACACCATGCATTTG-3' covering the splice site, a first PCR fragment was obtained. Expression plasmid pHY339 exclusively expressing the SV40 LTag was generated by deletion of the large intron from the SV40 coding sequence by fusion PCR from plasmid pHY338 as follows.

**Molecular Therapy: Methods & Clinical Development Vol. 6 September 2017 131**

**Figure 8. Primers and Probes Used for the qPCR Analysis to Detect RC and RD SV40 Vector Particles**

(A) Schematic representation of the SV40 genome showing the position of the primers and probe for the detection of RC SV40 particles. (B) The sequences of primers and probes used in the qPCR analysis.

Cell Line Generation

Vero cells (ATTC, CCL-81) were cultured in serum-free OptiPRO (GIBCO, Thermo Fisher Scientific) medium supplemented with 4 mM glutamine (GIBCO, Thermo Fisher Scientific). The pBluescript SK+ backbone was removed from the pHY339 DNA, and Vero cells were transfected with the DNA fragment containing the pac and LTag gene cassettes using ExGen 500 in vitro Transfection Reagent (BIOMOL) following the manufacturer’s instructions. Two days after transfection, the cells were divided into culture medium containing 2 μg/mL puromycin (Sigma-Aldrich) and kept under the antibiotic pressure for 1 month.

**Construction of SV40 Destination Vector Plasmid pSVac**

DNA of pSL-PL was subjected to PCR using primers 5'-GACAGTACATAAAGATACATTG-3' and 5'-ATAGTTTATGGCAGCGCAATGTTAGCAATGTTGAATCC-3'. The resulting PCR fragment comprising the SV40-pA signal was cloned into pBluescript SK+. The resulting PCR fragment comprising the pac puromycin resistance gene flanked by the human CMVie promoter and the BGH pA (CMVie-pac-BGH pA) was cloned into pAM004, resulting in pAM005.

Expression plasmid pHY339 exclusively expressing the SV40 LTag was generated by deletion of the large intron from the SV40 coding sequence by fusion PCR from plasmid pHY338 as follows. First, with forward primer 5'-GACAGTACATAAAGATACATTG-3' homologous to the 5' end of the LTag coding sequence and reverse primer 5'-ATAGTTTATGGCAGCGCAATGTTAGCAATGTTGAATCC-3' covering the intron splice site, a first PCR fragment was generated comprising the 5' end of the LTag sequence. Second, with forward primer 5'-AGGAATGTTGTACACCATGCATTTG-3' covering the intron splice site and reverse primer 5'-AGGAATGTTGTACACCATGCATTTG-3' covering the NotI site in the center of the LTag coding domain, a second PCR fragment was generated. Third, the two PCR fragments were used to perform a fusion PCR using the two external primers. The generated PCR fragment comprising the 5' end of the LTag coding sequence without the large intron was cloned into the NotI-NsiI sites of expression plasmid pHY339 yielding expression plasmid pHY359.
culture medium containing the vector particles was collected and replaced by fresh medium. In order to further increase the number of vector particles, we used the pooled vector harvests for at least two subsequent transduction rounds. In each transduction round, Super- Vero cells were transduced with 400 SV40 vector genomes per cell. Three days posttransduction, the vector particle-containing culture medium was collected and replaced by fresh media. Vector harvests were clarified and concentrated by ultracentrifugation, and stored at 4°C or other temperatures as indicated.

SV40 Vector Quantification

The concentration of the vector particles in the produced vector batches was determined by quantitative real-time PCR. Samples were treated with DNase I (Sigma-Aldrich) to remove naked DNA. The vector DNA was isolated from particles by incubating the samples at 37°C for 1 hr in a protease K (Sigma-Aldrich) working solution (2.75 mg/mL in 8.6 mM Tris-HCl, 86 mM NaCl, 0.43% w/v SDS, pH 8). Magnetic beads (MagneSil Blue; Promega) were used to selectively bind vector DNA. Isolated DNA was used as a template for amplification of the VP2 gene region using AmpliTaq Gold (Thermo Fisher Scientific) polymerase and primers and probes shown in Figure 8B. The qPCR was set up according to the manufacturer’s recommendations (7300 Real-Time PCR System; Applied Biosystems) under the thermal cycling conditions of 2 min at 50°C and 10 min at 95°C, proceeding with 40 cycles of denaturation at 95°C for 1 s and annealing or extension at 60°C for 1 min. Data analyses were performed on the Applied Biosystems. Based on the signal obtained with the non-template control samples, we defined the limit of detection of the assay to be 60 RC SV40 particles per milliliter.

Luciferase Activity Measurements

Luciferase activity was measured from cells transduced with SVLuc by using the Luciferase Assay System (Promega). 200 μL of vector stock was used to transduce 1 × 10^4 cells in a 24-well plate format. All transductions were performed in triplicate. Three days after transduction, cells were harvested and luciferase activity was analyzed in a GloMax 96 Microplate Luminometer (Promega) according to the manufacturer’s recommendations.

Western Blot Analysis

Cells were lysed with SAM buffer (50 mM Tris-HCl, pH 6.8, 3% of SDS, 5% of β-mercapto-ethanol) containing protease inhibitor cocktail (SIGMAFAST Protease Inhibitor Tablets; Sigma-Aldrich), resolved by SDS-PAGE (10% polyacrylamide gels under reducing conditions) and electro-transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat milk and probed with different primary antibodies for 1 hr at room temperature: (1) mouse anti-SV40 LTag diluted in PBS, 0.2% of Triton X-100. The secondary antibody (Alexa Fluor 488 donkey anti-goat; Life Technologies, Invitrogen) was incubated for 1 hr at room temperature: (1) mouse anti-SV40 LTag 0.5 mg/mL (BD Biosciences), dilution 1:200; (2) rabbit anti-VP1 (Abcam), dilution 1:5,000; (2) mouse anti-GFP (Sigma-Aldrich), dilution 1:1,000; mouse anti-α-tubulin antibody (Sigma-Aldrich), dilution 1:5,000; mouse anti-GAPDH (Millipore), dilution 1:5,000. Finally, the membrane was hybridized with a goat anti-mouse or a goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich), dilution 1:5,000. The blot was developed by chemiluminescence (Chemidoc MP Imaging System; Bio-Rad Laboratories). To quantify the amounts of protein, we scanned the blots by densitometry using the Image Lab software version 5.2.1 (Bio-Rad Laboratories).

Immunocytochemistry

SuperVero, Vero ATCC CCL-81, and COS-1 cells were cultured on coverslips and were fixed with 4% paraformaldehyde and blocked with 2% bovine serum albumin for 1 hr at room temperature. We incubated the cells overnight at 4°C with a solution of 2.5 μg/mL mouse antibody to the SV40 LTag diluted in PBS, 0.2% of Triton X-100. The secondary antibody (Alexa Fluor 488 donkey anti-goat; Life Technologies, Invitrogen) was incubated for 1 hr at room temperature diluted at 1:800 in PBS, 0.2% Triton X-100. Nuclear staining was performed with a solution of 5 μg/mL DAPI (Life Technologies, Invitrogen), and the slides were finally mounted using fluorescent mounting medium (DAKO, Agilent Technologies). Fluorescence microscopy images were acquired using a Leica DM6000B microscope (Leica Microsystems).
Statistical Analysis

All experiments were performed with a minimum of n = 3. All the statistical analyses were performed with the GraphPad Prism 5 software. We applied the two-tailed unpaired Student t test.

AUTHOR CONTRIBUTIONS

Conceptualization, P.d.H., I.M., and M.G.T.; Methodology, P.d.H., I.M., and S.v.d.W.; Investigation, J.v.d.V., M.O., A.R., R.J.C.-G., and I.G.H.-G.; Writing – Original Draft, M.G.T., I.M., and P.d.H.; Writing – Review & Editing, M.G.T., I.M., and P.d.H.; Funding Acquisition, P.d.H.; Resources, P.d.H. and M.G.T.; Supervision, P.d.H., I.M., and M.G.T.

CONFLICTS OF INTEREST

S.L. and B.V. are employed or sponsored by Amnara Therapeutics. The company is the owner of patents and patent applications on the production and use of polyomaviral vector particles.

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