A High-Capacity Adenoviral Hybrid Vector System Utilizing the Hyperactive Sleeping Beauty Transposase SB100X for Enhanced Integration

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For efficient delivery of required genetic elements we utilized high-capacity adenoviral vectors in the past allowing high transgene capacities of up to 36 kb. Previously we explored the hyperactive Sleeping Beauty (SB) transposase (HSB5) for somatic integration from the high-capacity adenoviral vectors genome. To further improve this hybrid vector system we hypothesized that the previously described hyperactive SB transposase SB100X will result in significantly improved efficiencies after transduction of target cells. Plasmid based delivery of the SB100X system revealed significantly increased integration efficiencies compared with the previously published hyperactive SB transposase HSB5. After optimizing experimental setups for high-capacity adenoviral vectors-based delivery of the SB100X system we observed up to eightfold and 100-fold increased integration efficiencies compared with the previously published hyperactive SB transposase HSB5 and the inactive transposase mSB, respectively. Furthermore, transposon copy numbers per cell were doubled with SB100X compared with HSB5 when using the identical multiplicity of infection. We believe that this improved hybrid vector system represents a valuable tool for achieving stabilized transgene expression in cycling cells and for treatment of numerous genetic disorders. Especially for in vivo approaches this improved adenoviral hybrid vector system will be advantageous because it may potentially allow reduction of the applied viral dose.

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Introduction

Gene therapy is an effort to cure diseases on a genomic level by utilizing nucleic acid as a drug. The development of this emerging medical field is ongoing for more than 20 years and has successfully reached the bedside which was demonstrated by recent pioneering studies for diseases either curable by gene transfer into hematopoietic stem cells or human liver. However, challenging problems remain which have to be addressed in the future and further improvements of the used vectors are desirable to continue this process.

Currently used vectors in the clinic are still lacking efficiency and safety for systemic in vivo delivery of large transgenes, and therefore high-capacity adenoviral vectors (HCAdV) represent attractive alternatives. They lack all sequences coding for viral proteins, leading to a transgene capacity of up to 36 kb which is remarkably higher compared with retroviruses and lentiviruses (~10 kb) or Adeno-associated virus (AAV) (~5 kb). By using HCAdV instead of early generation adenoviral vectors lacking only one or more of the early adenoviral genes, the toxicity problems associated with these vectors due to leaky expression of adenoviral genes in the transduced target cell have been significantly improved. The newest generation of adenoviral vectors offers the lowest immunogenicity and toxicity currently available. The HCAdV can be used for ex vivo treatment (cells are explanted from the patient, treated, and reimplanted) or for systemic in vivo administration. Since the production of the HCAdV using a helper virus to provide all viral proteins in trans is highly modular, it is possible to exchange the viral proteins in an easy way leading to a different tropism and escape from neutralizing antibodies of the vector. A novel cloning system was recently introduced by Mueck-Haeusel and colleagues using a platform based on bacterial artificial chromosomes and recombinering.

An attribute which sets back the use of adenoviral vectors for a broad range of diseases has been shown that HCAdVs are replication deficient due to the lack of a retention and replication mechanisms leading to loss of the therapeutic DNA during mitosis. This is especially of interest for the treatment of cells having a short generation time such as blood cells because copy numbers of the vector rapidly decline and the therapeutic effect is lost. Attempts have been made in the past to engineer mitotically stable adenoviral vectors either by somatic integration into the genome or by episomal persistence. Examples of genetic elements for integration of the therapeutic transgene into the host genome included PhiC31, retrovirus, adeno-associated virus, and the Sleeping Beauty (SB) transposase system. Two different attempts have been made to construct episomally persisting adenovirus hybrid vectors. One system was based on Epstein-Barr virus replication and retention and the other a nonviral plasmid replicon containing a scaffold matrix attachment region.

Here we evaluated a significantly improved version of an HCAdV hybrid system combining high transduction efficiencies of adenoviral vectors with the hyperactive SB
transposase system based on SB100X for enhanced somatic integration. The SB transposase system, derived from the TC1/mariner family of transposable elements, integrates the therapeutic transgene, cloned between two inverted repeat (IR) sequences, close to randomly at TA dinucleotides within the human genome in a copy and paste manner.24,28 Currently the SB transposase system is reaching the clinic in a trail using ex vivo engineered T-cells.29 Since the efficacy of the wild type transposase was relatively low, several attempts have been made to engineer hyperactive forms of the SB transposase molecule. This improvement of the SB transposase system after the awakening from its domestic sleep in the salmonid genome in 1997 was based on a rational approach leading to the hyperactive SB transposase versions SB11, HSB5, and SB100X.30–32 The latest version of SB transposase shows a 100-fold increased enzymatic activity compared with its original version. Since the systemic plasmid based delivery of SB transposase in large mammals remains a major challenge, several HCAdV-SB transposase hybrid vectors were constructed in the past.10,23 In this study, we are introducing the latest and most active version of a HCAdV-SB transposase hybrid vector based on hyperactive SB100X and show that this system is significantly enhanced.

Results
Principle and vectors used to establish the improved adenoviral hybrid vector system utilizing hyperactive transposase SB100X
In this study, we aimed at developing a significantly improved generation of a mitotically stable high-capacity adenoviral vector (HCAdV) hybrid system by taking advantage of enhanced SB-mediated integration. The hyperactive SB system is based on a two vector system which can either be delivered by plasmids or viral vectors. After viral or non-viral delivery into target cells and transport to the nucleus, the whole transposon cassette is excised and circularized by Flp recombinase and subsequently mobilized and integrated by SB transposase into the host cell genome between TA dinucleotides (Figure 1a). The detailed mechanism was shown and discussed elsewhere.10,23,33 Since efficient SB transposase mediated integration was demonstrated to work most efficiently from circularized constructs as shown in Figure 1a, the transposon needs to be excised from the HCAdV donor vector by Flp recombinase mediated recombination.10,23,33

To establish and optimize the system, we constructed various plasmids and HCAdVs which are displayed in Figure 1b and Figure 1c. We aimed to explore the hyperactive SB transposase SB100X in the context of an adenoviral hybrid vector and to compare this setup with the previously published adenoviral hybrid vector system utilizing the hyperactive SB transposase HSB5.10,24 As negative control an inactive version of the transposase (mSB) carrying a point mutation in the catalytic domain was used. To quantify integration events and to visualize transgene expression, we generated a transposon containing an internal ribosomal entry site based bicistronic construct expressing enhanced green fluorescent protein (eGFP) and the selection marker blasticidin (BSD) under the control of the CMV promoter. This expression cassette was either delivered as a plasmid (pTeGFP/BSD) or as a viral vector (HCAdV-TeGFP/BSD-luc).

Vectors HCAdV-SB100X and HCAdV-TeGFP/BSD-luc were produced using an established protocol.5,14 In brief, after release of the viral genome from the plasmid (see Supplementary Figure S1) vectors were amplified by serial passaging. After large-scale amplification vectors were purified by cesium chloride gradient ultracentrifugation and titrated (see Supplementary Figure S1). Note that the viral amplification production protocol was slightly modified and optimized for the HCAdV-SB100X vector (see also Materials and Methods section). Before performing further experiments the vector HCAdV-TeGFP/BSD-luc was evaluated for eGFP expression (see Supplementary Figure S1).

Functionality of DNA sequences contained in adenoviral vectors for achieving mitotic stability
To characterize and to show functionality of DNA sequences contained in the adenoviral hybrid vector system described in Figure 1, we performed plasmid-based colony-forming assays in human embryonic kidney cells (HEK293). We cotransfected the hyperactive SB100X encoding plasmid (pSB100X) in which transposase expression is driven by the cytomegalovirus promoter with either the transposon containing plasmid pTeGFP/BSD or the control plasmid p.ATeGFP/BSD lacking IRs at a molar ratio of 1:3 of transposase to transposon. In case of the control, plasmid stuffer DNA was added to assure that equal molar ratios were transduced into both groups, respectively. Before splitting the cells at different ratios (1:3, 1:10, 1:50, and 1:100), equal transfection efficiencies were evaluated by eGFP expression 48 hours after transfection (data not shown). Experiments were performed in triplicates and after blasticidin selection resistant colonies were stained with methylene blue (Figure 2a and Figure 2b). We observed more than 60-fold increased transposition efficiencies for SB100X compared with the control groups (Figure 2a and Figure 2b). Note that for groups that received SB100X, which were split 1:1 and 1:3, colonies were not countable anymore because they grew to complete confluency. As shown in Figure 2c vector genome copy numbers (VCN) per cell were increased twofold compared with surviving colonies in the control group.

To show that our results are cell line independent and to use a cell line which was also used for HCAdV based colony forming assays in our previous study,27 we aimed at confirming our findings in human glioblastoma cells line U87. Thus, U87 cells were cotransfected with the SB transposase encoding plasmids (pSB100X and pHSB5) and the transposon containing plasmid pTeGFP/BSD. The negative control group received the plasmid p.ATeGFP/BSD and stuffer DNA. As shown in Figure 2d SB100X resulted in the highest number of colonies also compared with the precursor SB transposase version HSB5.

Characterization and efficiencies of the high-capacity adenoviral vectors system utilizing SB100X for somatic integration
Consequently we performed experiments using the HCAdV hybrid-vector system as delivery platform for the transposase integration machinery in U87 cells. Initially we used plasmid
delivery of the transposon (plasmid pTeGFP/BSD) in concert with HCA dv delivery of SB100X. We transfected the transposon DNA followed by infection with the respective transposase encoding adenoviral vector (HCA dv-SB100X, HCA dv-HSB5, and HCA dv-mSB) using a multiplicity of infection (MOI) of 30. After the selection process was completed, cell colonies were stained. HCA dv-SB100X resulted in a higher number of colonies as compared to HCA dv-HSB5 (2.7-fold) or the negative control group which received mSB (fivefold) (Figure 3a and Supplementary Figure S2a). Later we tested different experimental settings for the adenovirus hybrid vector system for which the transposon donor and the transposase were delivered by the HCA dv. For the first experiment U87 cells were infected at MOIs 15 for the vectors HCA dv-SB100X and HCA dv-mSB, and the transposon encoding vector was applied at MOI 60 (Figure 3b). After the selection process was completed the analyses were performed based on the software CellProfiler enabling pixel based analysis. The software was used for colony forming assays performed with U87 cells, since efficiency was high and resulting colonies too close to be counted manually (see Supplementary Figure S2b). Between HCA dv-SB100X and HCA dv-mSB there was a nearly 100-fold difference based on pixel surface coverage (Figure 3b and Supplementary Figure S2b). Figure 3c shows an experiment in which we compared HCA dv-SB100X and HCA dv-HSB5 in U87 cells at MOI 15 for the transposase and MOI 30 for the transposon-donor vector HCA dv-TeGFP/BSD-FRT/IR. The efficiency of HCA dv-SB100 was > sevenfold higher compared with HCA dv-HSB5.

To determine VCN in virus based experiments we isolated genomic DNA from pools of selected cells and performed
quantitative polymerase chain reaction (PCR). The left panel of Figure 3d shows copy numbers of the transgene within the colonies of the experiment using the HAdV for transposase delivery in combination with plasmid delivery for the transposon donor. We measured up to 10 VCN per cell for this group and up to four VCN for a group which received the SB100X system using plasmid-based delivery. As shown in the right panel of Figure 3d, VCNs were also increased (> twofold) in U87 cells which were infected with the SB100X based hybrid vector system if directly compared with the previous version of the adenoviral hybrid vector system utilizing HSBS for somatic integration.

To rule out that this is not a cell line dependent phenomenon, we performed experiments in adenocarcinoma human alveolar basal epithelial (A549) cells (Figure 4). We infected cells at MOI 100, for the transposon donor vector and MOI 30, for the SB100X encoding HAdV vector. Two days later, infection cells were split at different ratios (1/5, 1/10, and 1/100) and blasticidin
selection was started. In concordance to our results obtained in U87 cells, HCAdV-SB100X showed significantly increased numbers of colonies compared with HCAdV-HSB5 (up to 10-fold for splitting ratio 1:10) (Figure 4a). We confirmed this trend in the second experiment performed in A549 cells when using a lower total MOI (MOI 30, for the transposon donor vector and MOI 10, for the SB100X encoding vector) as shown in Figure 4b. Representative single colonies stably expressing eGFP in A549 cells after selection are displayed in Supplementary Figure S2c. Finally, we doubled the MOI of the transposon vector (MOI 60) while keeping the MOI for transposase stable (MOI 10). This resulted in a significant increase in colony forming numbers (> eightfold) (Figure 4c). To show true transposition in surviving colonies we performed PCR analyses to prove that the selection process was sufficient to lose all episomal adenoviral genomes (right panel) and an experiment using solely HCAdV for delivery of transposon and transposase (right panel). Error bars indicate mean ± SD. The P-values are provided and a significant difference between the two groups was confirmed if the P-value was < 0.05. **P < 0.01, ***P < 0.001, ****P < 10−4, n.s.: not significant. MOI, multiplicity of infection; SB100X, hyperactive SB transposase; HSB5, hyperactive SB transposase; mSB, inactive version of SB transposase; TeGFP, transposon enhanced green fluorescent protein; BSD, blasticidin; Luc, luciferase; HCAdV, high-capacity adenoviral vectors.

**Figure 3 Quantification of colony forming numbers based on viral transduction of U87 glioblastoma-derived cells.** (a) This figure shows the results of a colony forming assay performed in U87 cells after cotransduction of the transposon containing plasmid pTeGFP/BSD and the transposase encoding viral vectors HCAdV-SB100X, HCAdV-HSB5, and HCAdV-mSB. (b) Results of a colony forming assay comparing HCAdV-SB100X and HCAdV-mSB at MOI 30 after coinfection with the transposon vector HCAdV-pTeGFP/BSD at MOI 60. After the selection process was completed remaining colonies were stained. Colored pixels were counted in a pixel based assay. There was ~100-fold difference between the HCAdV-SB100X and HCAdV-mSB. The number of absolute pixels is shown below the diagram as mean ± SD. (c) U87 cells were infected with the transposase vectors HCAdV-SB100X and HCAdV-HSB5 at MOI 15 and the transposon donor vector HCAdV-pTeGFP/BSD-luc at MOI 30. There was a sevenfold difference in transposition efficiencies for HCAdV-SB100X compared with HCAdV-HSB5. The number of absolute pixel numbers is shown as mean ± SD below the diagram. (d) Transgene copy numbers from an experiment using plasmid delivery for the transposon and viral delivery for the transposase (left panel) and an experiment using solely HCAdV for delivery of transposon and transposase (right panel). Error bars indicate mean ± SD. The P-values are provided and a significant difference between the two groups was confirmed if the P-value was < 0.05. **P < 0.01, ***P < 0.001, ****P < 10−4, n.s.: not significant. MOI, multiplicity of infection; SB100X, hyperactive SB transposase; HSB5, hyperactive SB transposase; mSB, inactive version of SB transposase; TeGFP, transposon enhanced green fluorescent protein; BSD, blasticidin; Luc, luciferase; HCAdV, high-capacity adenoviral vectors.

**Discussion**

The aim of this study was to construct and evaluate a new third generation high capacity adenoviral hybrid vector for somatic persistence in rapidly dividing cells. The integration machinery was based on SB transposase mediated adenoviral genome including inverted terminal repeat (ITR) and packaging signal (see Supplementary Figure S3a). The latter PCR should only be positive if the adenoviral DNA molecule itself was maintained during selection pressure. We analyzed six individual clones derived from single colonies which were either transduced with HCAdV-HSB5 or HCAdV-SB100X. As expected we detected the transgene blasticidin in all analyzed colonies. However, we could show absence of the adenoviral DNA in five of six analyzed samples indicating true transposition in most of our colonies. One of our colonies showed a positive signal for the adenoviral ITR and the packing signal (see Supplementary Figure S3b).
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included lentiviral vectors, 34 baculovirus for long-term gene vector systems for efficient delivery of the genetic cargo. This also used in other gene transfer studies utilizing other viral another study by Kolacsek and colleagues.37

tors have been explored in previous studies. 10,23 However, integration. SB transposase based adenoviral hybrid vectors have been explored in previous studies.10,23 However, increased efficacy may be desirable to further improve the system and to potentially decrease the required viral dose to achieve stabilized transgene expression at therapeutic levels. In this study, we tested the latest optimized form of SB transposase SB100X35 offering significantly increased efficiencies compared with precursor versions. Note that SB100X was also used in other gene transfer studies utilizing other viral vector systems for efficient delivery of the genetic cargo. This included lentiviral vectors,34 baculovirus for long-term gene expression in the eye35 and Adeno-associated virus (AAV).36 This study complements these previous studies showing that the hybrid HCAdV system is efficient in delivering SB100X leading to remarkable integration efficiencies. Nevertheless it remains to be evaluated which viral vector system may be the most efficient technology for a specific application in gene therapy. It is likely that the molecular design and the viral vector type used for delivery of the SB transposase system needs to be adapted to the specific application.

The total number of colonies in plasmid based experiments was lower for all experimental settings in U87 cells when compared with experiments performed in HEK293 cells. We speculate that this may have two reasons. The protocol for the colony forming assay performed in U87 cells was different since selection was performed without splitting cells after transfection. Furthermore, initial transfection efficiencies vary between different cell lines. The other reason could be that the cell line and the origin of the tissue may have an influence on transposition efficiencies which was also shown by another study by Kolacsek and colleagues.37

For our experiments using the HCAdV for delivery of the transposase we observed that SB100X was more efficient compared with the precursor HSB5 and the inactive SB transposase mSB. When compared with HCAdV-HSB5 the gain of efficiency was > sevenfold for the SB100X based system, demonstrating that SB100X is superior compared with HSB5. This might be of special interest for in vivo experiments for which transposon and transposase are codelivered via two HCAdVs. Higher efficiency may allow injection of decreased vectors doses which may improve the toxicity profile related to incoming viral particles and expression of recombinases such as SB100X and Fip recombinase. Overall, improved efficiency of the SB transposase hybrid vector system may also allow decreasing the required viral load for achieving therapeutic levels to treat a genetic disease, which may correspond to reduced vector-related toxicity.

Moreover, the VCN was increased for the SB100X based hybrid vector system in A549- and U87 cells. On first sight, this observation may be advantageous because it may lead to increased expression levels, but genotoxicity risks may be increased if more integration sites at different loci in the host genome are detected. Notably, in U87 cells adenoviral vector delivery of the transposon system was more efficient when performing colony forming assays. Plasmid delivery of the identical transposon led to decreased numbers of resistant colonies. This could be explained by limitations of the transfection protocols for the respective cell line. Overall experiments in both cell lines clearly indicated that the MOI seems to play a crucial role for transposition.

It was shown that recombinant adenovirus genomes can persist extrachromosomally for a prolonged time especially in

Figure 4 Performance of the viral hybrid vector system in human A549 adenocarcinoma cells. A549 cells were infected a two different total MOIs for transposon (HCAdV-TeGFP/BD-luc) and transposase vectors (HCAdV-SB100X and HCAdV-HSB5). Two days postinfection, cells were split at different ratios (1/5, 1/10, and 1/100), maintained under blasticidin selection pressure and stained with methylene blue. (a) A549 cells were infected at an MOI of 30 for the transposase vector and MOI 100 for the transposon vector. (b) Colony forming assay performed at lower MOI using MOI of 10 of the transposase vector and MOI 30 for the transposon vector. (c) Colony forming assay performed at an MOI of 10 for the transposase vector and an elevated MOI for the transposon vector (MOI 60). Error bars indicate mean ± SD. The *P-values are provided and a significant difference between the two groups was confirmed if the P-value was < 0.05. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 10−4, n.s.: not significant. MOI, multiplicity of infection; SB100X, hyperactive SB transposase; HSB5, hyperactive SB transposase; mSB, inactive version of SB transposase; TeGFP, transposon enhanced green fluorescent protein; BSD, blasticidin; Luc, luciferase; HCAdV, high-capacity adenoviral vectors.
quences of adenovirus DNA molecules are more stable compared with nonviral DNA and that HCAdV genomes can somatically integrate virus DNA molecules are more stable compared with nonviral DNA. Therefore, it can be hypothesized that in our experiments these features of HCAdV genomes may contribute to background transgene expression. PCR analyses revealed that one isolated cell clone maintained HCAdV DNA and it can be speculated that in this particular cell clone the HCAdV genome was integrated into the host genome.

Next steps should involve experiments which utilize the high-capacity adenoviral hybrid vector system utilizing SB100X in primary cells. In our previous studies we successfully applied the precursor version of the hybrid vector system utilizing hyperactive SB transposase HSBS5 instead of SB100X in primary cells in vivo in small and large animals. The only differences of both systems are the point mutations in the HCAdV genome was integrated into the host genome. In this study, we renounced from performing integration site analysis for SB100X and HSBS5-mediated transposition since this was already performed in depth in other studies. It can be concluded from these studies that no significant differences can be detected regarding the integration profile between the hyperactive SB transposase SB100X and its precursor versions. Moreover the transposition machinery itself is independent from the viral or nonviral delivery method and therefore, we expect a comparable integration profile as shown for the previous studies. Although SB100X transposase clearly improves efficacy of the adenovirus SB transposase hybrid-vector system, some challenges still remain to overcome. This includes the necessity of using Flp recombinase for circularization from the adenoviral vector genome and the requirement of using two vectors coinfecting a single cell simultaneously. Latter is necessary since coexistence of transposon and transposase in the production cell line would inhibit vector production. One strategy to overcome this limitation could be micro-RNA mediated suppression of SB-transposase in the HCAdV producer cell lines. Such an approach was previously shown for expression of cytotoxic zinc fingers from adenoviral vectors. Another strategy could be the usage of an inducible system driving expression of the SB transposase. In summary, in this study we produced, established and evaluated a novel, significantly improved high capacity adenoviral vector system for somatic integration and stabilized transgene expression. In a further step, this hybrid vector system can be evaluated in preclinical studies. Especially for direct in vivo gene therapy, we believe that this system will play an important role in developing novel concepts for therapeutic approaches.

**Materials and methods**

**Plasmids used in this study.** The plasmids pZac-SB100, pHM5-PMel, pHM5-Pepito-FRT, pHM5-Pepito-ΔS/MAR-FRT/pΔTeGFP, p cytomegalovirus -HSBS5, pEPito, and p phosphoglycerate kinase(PGK)-ΔTP were published previously and the plasmid pZAC2.1 was obtained from Jim Wilson (University of Pennsylvania, Philadelphia, PA).

To generate the HCAdV production plasmid pHmAdV-TeGFP/BSD-IR-luc, the following cloning steps were performed. The pHmAdV-TeGFP/BSD-IR-FRT-luc was generated in this study and is based on the pHmAdV-FRT/IR. A PCR using pEPito as template was used to amplify the transgene including cytomegalovirus promoter, eGFP cDNA, an IRES sequence and the blasticidin encoding DNA. To amplify the specific PCR product, primers GFP Forw (5'- GAT ACT CGA GTC GCC ACC ATG GTG AGC AAG-3') and Blast rev (5'- CTA GCG GCC GCT ATT TAG ATC CTT AGC CCT C-3') were used. The PCR product was digested using Ncol and Xhol and the resulting DNA fragment was inserted into the vector pZac2.1, which was opened using the same enzymes. The resulting plasmid was digested with Sma1 and the transgene cloned into the plasmid pHM5-TeGFP/BSD-IR/FRT. A luciferase expression cassette was added by digesting pHM5-FRT/IR using I-CeuI and cloning of a PCR product derived from pGL3-control vector (Promega, Madison, WI) using primers I-CeuI-luc-forward (5'-CTA AGG TAG CGA AGC TCG AGA TCT GGG ATC TGC-3') and PmeI-luc-reverse (5'- CGC CGT TTA AAC CGA TTT TAC CAC ATT TGT -3') as template for this PCR reaction the plasmid pcDNA-BioF-Luc was used. The PCR product was cloned into the plasmid pHM5-Pmel using the restriction enzyme Pmel resulting in the plasmid pHM5-Flp. Subsequently the cDNA of SB100X was PCR amplified from pZac-SB100 using Primers SB100X Forward (5'- GCC CTT CTA GAT TTA GCT GAT TTA CTT ATC CCT GCG TCT-3') and SB100X Reverse (5'- CTA GAG GTG CAA CCG CGT ATC AAT-3') and the fragment was then cloned into the Pmel site of pPGK-ΔTP. The SB100X expression cassette expressed under control of the PGK promoter was then PCR amplified using primers PGM-F and PGM-R resulting in the plasmid pHM5-FpL which was digested with Kpn1 and blunted with T4 DNA polymerase resulting in the plasmid pHM5-pfpL123. After Clal digest of this plasmid, homologous arms flanking the Flp-SB100X cassette for subsequent recombining into pBHA-galK-kan harboring the adenoviral 5' ITR, the adenoviral packing signal 'I', and the 3' ITR were generated by PCR using primers BHCA-Pepi Fra5 (5'- CCG TTA ATG CGC TAC AGG CGG CCG GTG GTG AGC GCG TCA ATT AAC CC-3') and BHCA-Pepi Fra3 (5'- CCG TTA ATG CGC TAC AGG CGG CCG GTG GTG AGC GCG TCA ATT AAC CC-3'). This PCR fragment was then inserted via galK based counter-selection recombining into pBHA-galK-kan43 replacing galK-Kan with the Flp-SB100X cassette. This resulted in the HCAdV production plasmid pHmAdV-SB100X.
Vector production. Viruses HCAdV-HSB5\textsuperscript{10} and HCAdV-mSB\textsuperscript{31} were described previously and recombinant vectors HCAdV-SB100X and HCAdV-TeGFP/BSD-IR were produced in this study. For virus production of HCAdV-SB100X and HCAdV-TeGFP/BSD-IR, a previously established protocol was applied.\textsuperscript{5,14} In brief, the HCAdV vector genomes were released by Pmel (HCAdV-SB100X) and NotI (HCAdV-TeGFP/BSD-IR) (see Supplementary Figure S1) and the linearized HCAdV genome was transfected into 116 cells producer cells.\textsuperscript{14} Pre-amplification steps were performed in tissue culture dishes using the previously published helper virus introduced by Palmer and Ng.\textsuperscript{14} For large scale amplification a spinner flask system was used and the vectors were purified using cesium chloride gradient ultracentrifugation as described previously.\textsuperscript{5,14} As first attempts to amplify HCAdV-SB100X failed until we infected with the helper virus 6 hours prior to the HCAdV infection during the serial amplification steps.

For titration and measurement of HCAdV transducing units, A549 cells were infected using different volumes of the final vector preparation. After 3 hours, cells were washed to eliminate noninfectious particles. Then DNA isolation was conducted and a quantitative PCR was performed using the primers TQ-eGFP-RH-fw (5’-GGA GGC CGA TCA CAT GGT-3’) and TQ-eGFP-RH-rv (5’-CCA TGC CGA GAG TGA TCC-3’) for EGFP containing vector and primers SBnf1 (5’-GGT GGC AGC ATC ATG TTG TG-3’) and SBnr2 (5’-CCT TCC TCA TGA TGC CAT CTA TT-3’) for SB transposase containing vectors. A quantitative PCR was performed using the following protocol: a 5 minute denaturation step at 95°C was followed by 39 rounds of 15 seconds denaturation at 95°C, annealing and extension at 60° for 60 seconds (SYBR mix Biorad, Thermocycler C1000 Touch (Biorad, Hummelgasse, Wien Austria)).

Cell culture. All experiments were performed in HEK293, human adenocarcinoma alveolar basal epithelial-derived A549 cells, and U87 glioblastoma cells. For production of the HCAdV vectors 116 cells were used. HEK293, U87, and A549 cells were cultured in Dulbecco’s modified eagle medium (DMEM, PAN-Biotech, Aidenbach, Germany). For culture of U87 cells dishes were collagenized with Collagen (Biochrom, Berlin, Germany) according to the manufacturer’s protocol. For 116 cells we used minimal essential medium (MEM Eagle, Pan-Biotech) additionally supplemented with 100 μg/ml hygromycin B for sustained selection for CRE expression during virus production. CRE expression is crucial to remove the packing signal from the helper virus used to supply essential viral proteins. In contrast to the helper virus the HCAdV genome contains a normal packaging not flanked by loxP sites. This assures that predominantly HCAdV genomes are packed into assembled virus particles. Fetal bovine serum (FBS, PAA, Pasching, Austria) was added to 10% together with 0,1 mg/ml penicillin-streptomycin (PAA).

Colony forming assay. For the plasmid based colony forming assays in HEK293, cells were grown to a density of ~70% in 6-well plates. Plasmid DNA was purified using a commercially available plasmid DNA isolation kit (Peqlab, Erlangen, Germany and Qiagen, Hilden, Germany). Transfection was performed using Fugene 6 (Promega). Before transfection was performed copy numbers were adjusted based on DNA concentration measured using a spectrophotometer (Eppendorf, Hamburg, Germany). After 2 days, transfection cells were split at different ratios and selection with blasticidin (Carl Roth, Karlsruhe, Germany) was started with a concentration of 4 μg/ml. Also medium was changed at average 3 times per week. For the viral and nonviral experiments using U87 cells, selection was started after 2 days by performing media exchange. For viral colony forming assays using A549 cells, cells cultured in 6-well plates were infected and 2 days later split at different ratios. Subsequently selection was started. After several weeks of successful selection, shown by 100% eGFP expressing colonies, cells were stained using methylene blue (Sigma-Aldrich, Munich, Germany) or DNA was isolated using a commercial DNA extraction kit (Peqlab).

Surface analysis for the percentage of covered dishes with cells was performed (in case of U87 cells) using the open source software CellProfiler available at www.cellprofiler.org from the Broad Institute\textsuperscript{44} and the analysis module “Measure Image Area Occupied”.

Molecular analyses of surviving cell clones after selection. After completion of the selection procedure genomic DNA was isolated from resistant cell pools derived from transduced HEK293- and U87 cells. To determine VCN, a transgene EGFP specific quantitative PCR was performed using the primers TQ-eGFP-RH-fw (5’-GGA GGC CGA TCA CAT GGT-3’) and TQ-eGFP-RH-rv (5’-CCA TGC CGA GAG TGA TCC-3’). A quantitative PCR was performed using the following protocol: a 5 minute denaturation step at 95°C was followed by 39 rounds of 15 seconds denaturation at 95°C, annealing and extension at 60° for 60 seconds (SYBR mix Biorad, Thermocyclyer C1000 Touch (Biorad, Hummelgasse, Wien Austria)).

To analyze surviving colonies derived from A549 cells on a molecular level, we isolated and amplified single cell colonies after the initial selection procedure was completed. When the colonies had grown to confluence in a 6cm tissue culture dish, we prepared genomic DNA using the Qiagen Blood and Tissue kit which were then analyzed by two PCR reactions. The first PCR amplifies the transgene blasticidin which should be detectable in all resistant colonies and the second PCR amplifies the left arm of the adenoviral genome including ITR and packaging signal which results in a PCR product specific for the adenoviral DNA molecule which was maintained during selection pressure. Subsequently we performed a standard PCR using Phusion High-fidelity Polymerase (NEB) according to the NEB protocol using ITR and packaging signal-specific primers ADITR5’ (5’-GGT GGC GGG TGA CGT AGT AGT GT-3’) and ADITR3’ (5’-GGT GGA AAC ACC TGA GAA AA-3’), and transgene-specific primers BSD5’ (5’-CCG CAT CTT CAC TGG TGT-3’) and BSD3’ (5’-GCT CAA GAT GCC CCT GTT CT-3’).

Statistical analysis. All experiments were performed using at least triplicates. All data are reported as mean ± SD unless otherwise noted. Statistical comparison was made using the two-tailed student’s t-test, and a value of $P < 0.05$ was
considered to be relevant compared to the respective control group.

Ethics statement. In the context of this study no research involving human subjects (including human material or human data) was performed.

Supplementary material

Figure S1. Production of the high-capacity vectors HCAdV-SB100X and HCAdV-TeGFP/BSD-luc.

Figure S2. Sample colonies of the performed experiments.

Figure S3. Molecular analyses of single colonies derived from A549 cells.

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Author contributions

P.B. performed the majority of shown experiments, participated in the design of the study and contributed to drafting the manuscript. W.Z. made substantial contributions to conception and design. M.S. helped in drafting the manuscript and E.S. was involved in producing viral vectors analyzed in this study. A.E. conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript. The authors declare that there are no competing interests.

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