Efficient gene delivery into cell lines and stem cells using baculovirus

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Baculovirus is a promising vector for transducing numerous types of mammalian cells. We have developed hybrid baculovirus vectors and protocols for the efficient transduction of a variety of cell lines, primary cells and stem cells, including bone marrow–derived mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ASCs). The hybrid vector enables intracellular minicircle formation and prolongs transgene expression. The advantages of this transduction protocol are that baculovirus supernatant alone needs to be added to cells growing in medium, and transduction occurs after only 4–6 h of incubation at room temperature (25 °C) with gentle shaking. The entire procedure, from virus generation to transduction, can be completed within 4 weeks. Compared with other transduction procedures, this protocol is simple and can confer efficiencies >95% for many cell types. This protocol has potential applications in tissue regeneration, as transduced cells continue to express transgenes after implantation. For example, transduction of rabbit ASCs (rASCs) with growth factor–encoding hybrid baculovirus vectors, as described as an example application in this protocol, enables robust and sustained growth factor expression, stimulates stem cell differentiation and augments tissue regeneration after implantation.

INTRODUCTION

Baculovirus (*Autographa californica* multiple nucleopolyhedrovirus, AcMNPV) is an insect enveloped virus and is highly infectious to cultured insect cells, and thus baculovirus is often genetically engineered to infect host insect cells for recombinant protein production. Since it was found that baculovirus is able to transduce mammalian cells[1,2], numerous permissive cells of human, rodent, porcine, canine, avian, feline and rabbit origins have been identified (for a review, see refs. 3–6). Baculovirus can transduce human embryonic stem cells[7], mouse induced pluripotent stem (iPS) cells[8] and mammalian cells cultured in 3D matrix[9] and in suspension[10]. Within the baculovirus-transduced cells, transgenes can be expressed as long as they are driven by an appropriate promoter (e.g., cytomegalovirus (CMV) immediate-early or hybrid CAG promoter). Therefore, baculovirus has captured growing attention as a gene delivery vector for a plethora of applications, ranging from cell-based assay development[11], protein production[12], virus production[10], virus-like particle production[9], eukaryotic protein display[13], RNA interference[14], vaccine development[15,16] and cancer therapy[17] to tissue engineering of bone[18,19], cartilage[20,21] and heart[22].

Comparison of baculovirus with other viral gene delivery systems

In comparison with other viral gene delivery vectors such as retrovirus, lentivirus, adenovirus and adeno-associated virus (AAV), baculovirus has a number of desirable features:

- The natural host of baculovirus is insect cells, and thus baculovirus is nonpathogenic to humans and baculovirus neither replicates nor is toxic inside transduced mammalian cells[23].
- Baculoviral DNA degrades in the cells over time[24,25], and baculoviral DNA integration into host chromosomes is extremely rare and cannot be found[26,27] unless selective pressure is applied[28].
- Humans do not possess pre-existing antibodies[29] and T cells[30] specifically against baculovirus, which enables baculovirus to circumvent the pre-existing immunity problem.
- Recombinant baculovirus can be readily constructed and propagated to high titers by infecting its natural host insect cells in biosafety level 1 (BSL-1) facilities[4].
- The baculovirus genome is large (~134 kb) and hence it confers a huge cloning capacity of at least 38 kb (ref. 31).

The first three features ease safety concerns regarding the use of baculovirus for gene delivery, whereas the last two features simplify baculovirus vector production and expand the potential applications of baculovirus vectors.

However, there are three possible drawbacks associated with baculovirus vectors:

- Owing to the nonreplication and nonintegration nature, baculovirus typically mediates transient (<7 d (refs. 3–6)) expression, which may hamper the applications of baculovirus vectors in some scenarios requiring long-term, stable expression (e.g., hemophilia and X-linked severe combined immunodeficiency).
- Baculovirus is an enveloped virus and the baculoviral enveloped protein gp64 is essential for infection of insect cells and transduction of mammalian cells[32]. Consequently, baculovirus is vulnerable to mechanical forces, and bioprocessing steps during the vector purification (e.g., ultracentrifugation and filtration) tend to attenuate the virus titer.
- Baculovirus is inactivated by serum complement system[33], which may hamper the baculovirus administration by direct injection.

The advantages and disadvantages of baculovirus compared with other viral gene delivery vectors are further summarized in **Table 1**.

Key differences between our baculovirus transduction protocol and other protocols

Incubation with unconcentrated virus. For baculovirus transduction of permissive cells, frequently baculovirus supernatant is concentrated by ultracentrifugation and resuspended in PBS. Cells are then incubated with the virus for 1 h at 37 °C, using

TABLE 1 | Pros and cons of baculovirus and other viral vectors.

Vector	Genetic material and packaging capacity	Advantages	Limitations
Baculovirus (BVs)[3,11,23]	dsDNA, ~134 kb	• Nonpathogenic to humans; nonreplicating and nontoxic in transduced cells • DNA degradation and lack of integration that improves safety • No pre-existing immunity • Ease of production by infecting insect cells in BSL-1 facilities • Large cloning capacity (at least 38 kb)	• Transient transgene expression • Vulnerable to mechanical force and loss of virus titer during virus concentration and purification • Inactivated by serum complement
Lentivirus (LVs)[64–66]	RNA, 8 kb	• Transduce nondividing and dividing cells • Stable transgene expression • Absence of pre-existing immunity	• Possible insertional mutagenesis • Limited insert size of the transgene • Requiring transfection of multiple plasmids into packaging cells for vector production • LVs should be handled at BSL-2 facilities
Retrovirus (RVs)[64,66]	RNA, 8 kb	• Theoretically stable transgene expression • Low immunogenicity • No (or very low) pre-existing immunity	• Transduce only dividing cells • Possible insertional mutagenesis and induction of leukemia in clinical trials • Limited insert size (8 kb) of the transgene • Requires transfection of multiple plasmids into packaging cells for vector production • RVs should be handled at BSL-2 or BSL-3 facilities • Transgene prone to silencing
Adenovirus (AdVs)[64,66]	dsDNA, 36 kb	• Transduce non-dividing and dividing cells • High-level transgene expression • Easy production with ready-to-use packaging cells • Vector particles produced at high titers (10^{10} pfu/ml) • High cloning capacity (for helper dependent AdV)	• Broad pre-existing immunity • High immunogenicity (yet immunogenicity can be reduced by using helper-dependent AdV) • Transient transgene expression
Adeno-associated virus (AAVs)[64,67]	ssDNA, <5 kb	• Transduce nondividing and dividing cells • Non-pathogenic • Wide cellular tropism • Capable of long-term transgene expression • Low immunogenicity	• Pre-existing immunity • Requiring transfection of multiple plasmids into packaging cells for vector production • Limited insert size (5 kb) of the transgene • Possible transgene integration

growth medium (e.g., DMEM) as the surrounding solution to adjust the final liquid volume[2,34–37]. However, ultracentrifugation may result in considerable loss in virus titer. Moreover, larger transduction processes require larger amounts of baculovirus, yet virus preparation by ultracentrifugation constitutes a formidable task upon process scale-up.

To simplify the transduction process, in our protocol cells are incubated with unconcentrated virus (i.e., virus supernatant harvested from the infected insect cell culture) at a lower temperature (e.g., 25 °C or 27 °C) for 4–8 h with mild shaking, using PBS as the surrounding solution[24,38]. This protocol enables and improves baculovirus-mediated gene transfer into HeLa[38,39], human BMSCs[40], BMSC-derived adipogenic progenitor cells[41] and primary chondrocytes derived from rats[24] and rabbits[21,26], with efficiencies comparable or superior to those using concentrated virus. For instance, the transduction efficiencies of human

BMSCs derived from umbilical cord blood can be elevated from ~42% (using concentrated virus) to ~73% (using our protocol)[40]. Our protocol also ameliorates AcMNPV-mediated transduction of BHK[9], Vero[39], canine MDCK[42] and chicken Df-1 cells[42], as well as augments the transduction of HEK293 and Schwann cells mediated by another baculovirus, *Bombyx mori* NPV[43]. Because our protocol obviates the need for virus ultracentrifugation, it represents a simpler approach and also considerably reduces possible virus inactivation during ultracentrifugation.

Surrounding solution during transduction. Baculovirus titer often falls in the range of 3×10^8 to 1×10^9 plaque-forming units per milliliter (pfu/ml). Transduction of cells cultured in six-well plates (e.g., 3×10^5 cells per ml) at a multiplicity of infection (MOI) of 100 (pfu per cell) only requires 30–100 µl of virus supernatant, and thus the virus supernatant needs to be diluted. For transduction in six-well plates, at least 500 µl of solution is required during the incubation period. Thus, virus supernatant can be diluted with insect cell medium to 100 µl and then mixed with the surrounding solution (400 µl) to a final volume of 500 µl. The volumetric ratio of diluted virus and surrounding solution can vary from 1:3 to 1:10, but in general a ratio of 1:4 is preferred (L.-Y.S., unpublished data).

The choice of surrounding solution has a considerable effect on transduction efficiency. We found that PBS as the surrounding solution confers better transduction efficiency and transgene expression than TNM-FH (the medium for baculovirus production) or DMEM[38,40]. Comparison between the major components in PBS and these media reveals that $NaHCO_3$ present in DMEM and TNM-FH markedly inhibits baculovirus transduction by reducing the intracellular virus number[44].

$NaHCO_3$ is a buffering agent that is commonly added to the medium during medium preparation. Owing to the key role of $NaHCO_3$ and concerns that incubation of certain cells (e.g., stem cells) in PBS for hours may damage the cells, we modified the protocol by using $NaHCO_3$-deficient medium in lieu of PBS as the surrounding solution. By using our protocol, baculovirus is able to transduce various cell lines (e.g., Huh-7, HeLa and BHK), BMSCs[27], ASCs from humans (hASCs)[45] and rabbits (rASCs)[46] and even cell sheets derived from rASCs[22], at efficiencies exceeding 95%.

Temperature and length of transduction. Temperature and the length of time that cells are incubated with baculovirus also dictate the transduction efficiency. Recombinant baculovirus is produced by infecting insect cells that are cultured using insect cell medium (e.g., TNM-FH) at 27 °C. We found that incubation of baculovirus and mammalian cells at 25 °C or 27 °C results in better transduction than at 4 °C or 37 °C, because the physical integrity of enveloped baculovirus is compromised at 37 °C, whereas virus particle adsorption to cell surface is less efficient at 4 °C (ref. 38). Incubation of cells with a virus supernatant for a prolonged period of time can enhance the transduction efficiency and transgene expression, as longer incubation time enables better virus entry[38]. However, excessive incubation time at 27 °C may undermine cell health, and the optimal incubation time varies for different cells. In general, incubation for 4–6 h is recommended for most cell types[24].

Other parameters influencing baculovirus transduction efficiency

Reagents enhancing transgene expression. Baculovirus-mediated transgene expression can be elevated by the addition of histone deacetylase (HDAC) inhibitors such as sodium butyrate[37]. HDAC inhibitors relax the chromatin structure by inducing histone hyperacetylation, which underscores the importance of epigenetic status of baculovirus genome for transgene expression. Notably, these drugs exert cytotoxicity at high concentrations[47], and the extent to which gene expression is upregulated hinges on cell lines[48]. Therefore, optimal sodium butyrate concentration needs to be pre-determined for different cells.

Choice of promoter. Transgene expression is also promoter-dependent. Viral promoters that are active in mammalian cells, such as SV40, CMV, RSV and the hybrid CAG promoter, have been used to drive baculovirus-mediated gene expression with different strengths (for a review, see ref. 4). In addition, baculovirus-mediated transgene expression can be augmented by appending woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to the 3′ end of transgene[49]. WPRE is a *cis*-acting RNA element that can stabilize mRNA and facilitate mRNA nuclear processing and export of mRNA into the cytoplasm, and thus it enhances transgene expression. A three- to tenfold increase in baculovirus-mediated enhanced green fluorescent protein (EGFP) expression is noted in several cell lines[49].

Hybrid baculovirus vectors for prolonged transgene expression.

As mentioned above, baculovirus mediates transient transgene expression (typically <7 d), which may preclude the applications of baculovirus in certain scenarios requiring long-term expression. To prolong the expression, attempts to incorporate AAV inverted terminal repeats[7,34] or the *Sleeping Beauty* transposon[14,50] into baculovirus vectors have been made. These elements result in transgene integration into the host chromosome, and they can be used for sustained expression in neuronal cells and cancer cells.

In addition to developing integrating vectors, we have developed baculovirus systems that enable episomal maintenance of transgenes. Development of these systems is based on the hypothesis that shedding transgene cargo from the baculovirus genome to form an extrachromosomal DNA minicircle may prolong the transgene expression. With this assumption in mind, we established an FLP/*Frt*-based hybrid baculovirus system that comprises one baculovirus-expressing FLP recombinase and one substrate baculovirus harboring the transgene cassette flanked by two *Frt* sequences[27]. Co-transduction with the two baculovirus vectors successfully extended the transgene expression in a number of mammalian cells, including rBMSCs[27] and rASCs[51]. The expression level and duration positively correlate with the recombination efficiency, presumably because minicircles confer stronger and longer transgene expression than their plasmid counterparts[52]. However, the FLP/*Frt*-mediated DNA minicircle formation occurs in only ~40–50% of rBMSCs and rASCs[51,53]. To further enhance the recombination efficiency, we have developed a new FLPo/*Frt* system[45] that explores the codon-optimized FLP (FLPo). The FLPo/*Frt* system enables

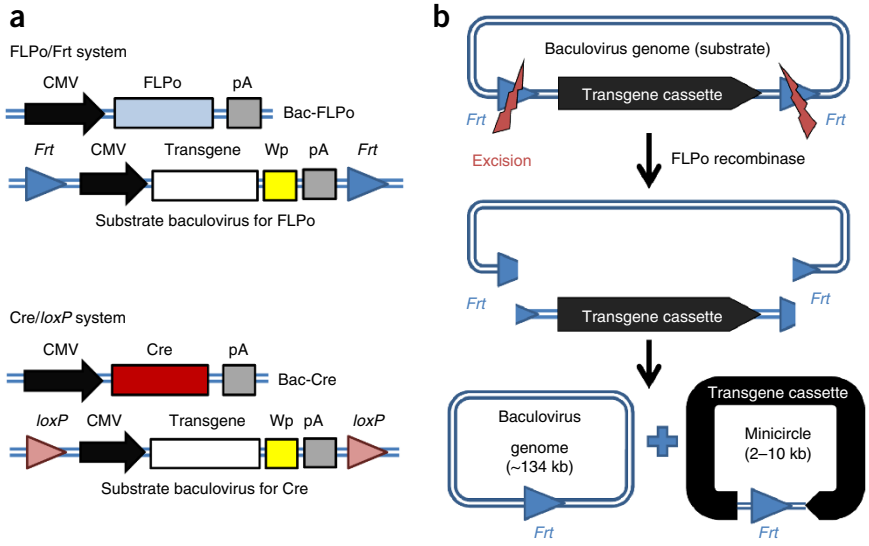


Figure 1 | Recombinase-based hybrid baculovirus system. (**a**) Illustration of the FLPo/*Frt*- and Cre/*loxP* sytems. (**b**) Illustration of the FLPo-mediated recombination and DNA minicircle formation. After co-transduction with Bac-FLPo and the substrate baculovirus, the expressed FLPo recognizes the *Frt* sites flanking the transgene cassette, leading to the excision of the transgene cassette, recombination and the formation of the DNA minicircle. CMV, cytomegalovirus immediate early promoter; Wp, WPRE element; pA, polyadenylation signal.

recombination and minicircle formation in different mammalian cells, rASCs and rBMSCs at efficiencies >90%, and it has been successfully used in rASCs to extend and promote the expression of growth factors such as bone morphogenetic protein 2 (BMP-2)[54], BMP-6 (ref. 20), transforming growth factor β3 (ref. 20) and vascular endothelial growth factor (VEGF)[22].

The new FLPo/*Frt*-based baculovirus system similarly consists of two baculoviruses: Bac-FLPo-expressing FLPo and the substrate baculovirus harboring the *Frt*-flanking transgene cassette (**Fig. 1a**). After co-transduction of cells, the expressed FLPo recognizes the *Frt* sites and excises the *Frt*-flanking cassette off the baculovirus genome, and hence it catalyzes the recombination and formation of episomal DNA minicircles encompassing the transgene cassette (**Fig. 1b**). The minicircle can persist in the cells for a longer term, whereas the baculovirus genome is rapidly degraded[45]. Aside from FLPo, two other site-specific recombinases, Cre and codon-optimized ΦC31 (ΦC31o), have been explored[45]. Cre catalyzes excision/recombination events between two identical *loxP* sites[55], whereas ΦC31o mediates excision/recombination between heterotypic sites *attP* and *attB*. Similarly to our work with the FLPo/*Frt*-based baculovirus system, we constructed a binary baculovirus vector system based on Cre/*loxP* (**Fig. 1a**) and ΦC31o/*attP*/*attB* (not shown). Upon co-transduction, the transgene cassette in the substrate baculovirus is excised by the recombinase (ΦC31o or Cre) expressed by a second baculovirus vector, and it recombines into minicircles[45].

Experimental design

Gene delivery into mammalian cells using baculovirus requires multiple stages: (i) construction of a transgene-containing donor plasmid, (ii) generation of recombinant bacmid, (iii) generation of recombinant baculovirus, (iv) virus amplification and (v) transduction. The construction of the donor plasmid is discussed here, but it is omitted from the procedure.

Construction of the donor plasmid. Baculovirus naturally infects insect cells, and recombinant baculoviruses are traditionally constructed by homologous recombination in two steps. A transgene is first cloned into a transfer plasmid under the control of a native baculovirus promoter (e.g., *polh* or *p10*). The second step involves co-transfection of the plasmid and linearized parental baculovirus DNA into insect cells, in which the overlapping sequences on the transfer plasmid and parental DNA lead to homologous recombination and hence transgene insertion into the baculoviral genome. Subsequent viral replication generates the recombinant baculovirus containing an integrated copy of the transgene. This baculovirus construction system is commercially available (e.g., BacPAK, Clontech).

To generate baculovirus vectors for transduction, we alternatively use the Bac-To-Bac system (Invitrogen), which exploits site-specific transposition of transgene cassette on the donor plasmid into the baculovirus shuttle vector (bacmid) in *E. coli*. The system offers a series of donor plasmids such as pFastBac 1 and pFastBac DUAL. The plasmids also contain the baculovirus promoter (e.g., *polh* or *p10*) upstream of the multiple cloning site and flanking attachment sites Tn7R and Tn7L. For expression in mammalian cells, the baculovirus promoter is simply replaced by a promoter (e.g., CMV) that is active in mammalian cells using standard cloning techniques[56] (designated as pBac-CMV5; **Fig. 2**), followed by cloning of the transgene into the donor plasmid.

To generate the FLPo/*Frt*-based hybrid baculovirus system, construction of two baculoviruses is required: Bac-FLPo and the substrate baculovirus harboring the *Frt*-flanking transgene cassette (**Fig. 1a**). Construction of the FLPo-expressing vector (Bac-FLPo) is straightforward, simply by cloning the optimized *flpo* gene from pPGKFLPobpA into the donor plasmid (pBac-CMV5) under the control of the CMV promoter. To construct the substrate vector harboring a transgene, a DNA fragment composed of a multiple cloning site flanked by two *Frt* sites is PCR-amplified from pLOI2226 (ref. 57). The amplicon (0.25 kb) is subcloned into pFastBac DUAL to yield pBac-Frt. Second, a CMV promoter (0.6 kb) is PCR-amplified from pcDNA3.1(+) and subcloned into pBac-Frt to yield pBac-Frt-CMV. Third, a WPRE sequence and an SV40pA are cloned into pBac-Frt-CMV to generate pBac-FCW. Fourth, the transgene is subcloned into pBac-FCW downstream of the CMV promoter.

To assay the recombination efficiency mediated by either ΦC31o, Cre or FLPo, we also generated a substrate baculovirus Bac-ALF, which harbors *d2egfp* (encoding destabilized EGFP, d2EGFP) flanked by tandem recombination sites (*attP-loxP-Frt* and *attB-loxP-Frt*)[45]. To construct the donor plasmid pBacALF, the DNA fragment encoding the tandem recombination sites (XhoI-*attP-loxP-Frt*-BamHI-StuI-*attB-loxP-Frt*-HindIII) is first

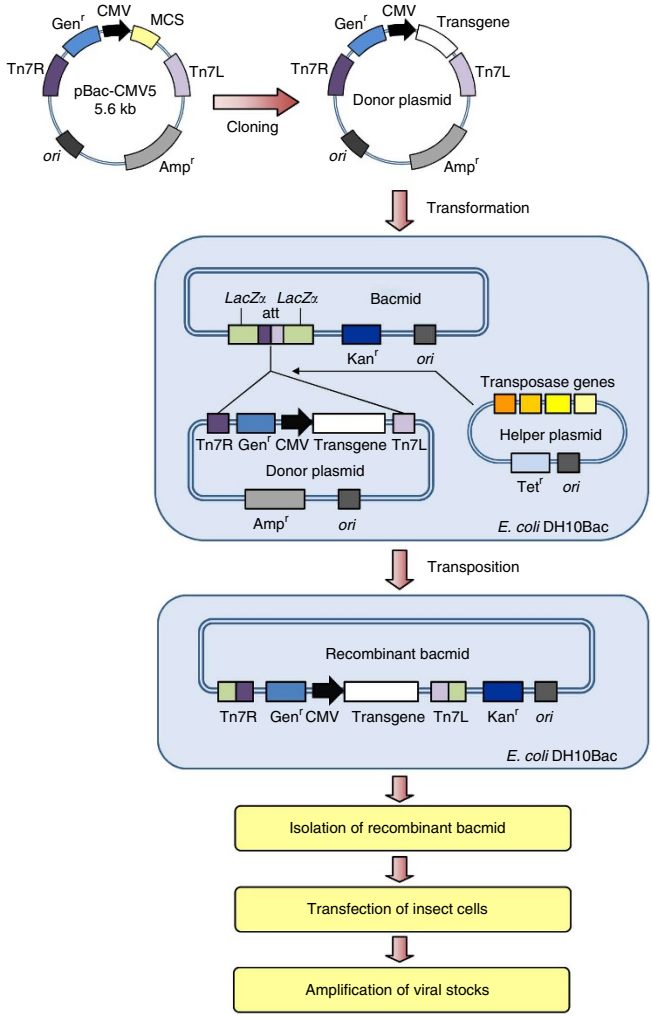


Figure 2 | Construction of recombinant baculovirus by site-specific transposition. The baculovirus promoter in the pFastBac vector is replaced by the CMV promoter to yield pBac-CMV5, and the transgene is cloned into the plasmid. The resultant donor plasmid is transformed into *E. coli* DH10Bac, in which the transposase expressed by the helper plasmid transposes the Tn7-flanking cassette from the donor plasmid to the Tn7 attachment site on the bacmid, resulting in transgene integration into the bacmid and disruption of the *lacZα* gene. In the agar plate containing antibiotics, IPTG and X-gal, the recombinant bacmid can be isolated from the white colonies. The recombinant bacmid is transfected into insect cells, in which the recombinant bacmid results in recombinant virus generation.

chemically synthesized and subcloned into pFastBac Dual to yield pALF. Second, the *d2egfp*-SV40pA fragment is PCR-amplified from pd2EGFP-N1 and inserted into the BamHI site of pALF to form pALF-dE. Finally, the CMV promoter (flanked by StuI/SmaI) is PCR-amplified from pcDNA3.1(+) and inserted into the StuI site of pALF-dE to yield pBacALF.

Generation of recombinant bacmid (Steps 1–33). To generate a recombinant bacmid, a donor plasmid is transformed into *E. coli* DH10Bac, which contains the bacmid and helper plasmid. The bacmid possesses a low-copy-number mini-F replicon (*ori*), a kanamycin resistance gene and a Tn7 attachment (*att*) site in frame within the *lacZα* gene. The helper plasmid within DH10Bac confers resistance to tetracycline and expresses the transposase (**Fig. 2**). After transformation of the recombinant donor plasmid into DH10Bac, the transposase transposes the Tn7-flanking cassette from the donor plasmid to the Tn7 attachment site, resulting in transgene integration into the bacmid and disruption of *lacZα* gene. The antibiotic selection and disruption of *lacZα* gene after transposition enable rapid selection/isolation of the recombinant bacmid from DH10Bac cultures. The recombinant bacmid is PCR-confirmed for the presence of transgene.

Production of recombinant baculovirus (Steps 33–44). The recombinant bacmid is used to transfect insect cells (e.g., Sf-9 derived from *Spodoptera frugiperda*), which leads to virus replication. Viral stocks collected from the transfected cells are defined as passage 0 (P0).

Box 1 | Titration of recombinant baculovirus ● TIMING 12–15 d

1. *Day 1.* Grow Sf-9 cells using Sf-9 medium in the spinner flask to log phase (1–2 × 10^6 cells per ml) with >95% viability.
2. Dilute the cells to 1 × 10^5 cells per ml with Sf-9 medium in a sterile 50-ml tube. For each virus to be titrated, 10 ml of diluted cells is needed. Keep the diluted cells at 27 °C and resuspend them before use.
3. Prepare tenfold serial dilutions of the virus stock. Add 135 µl of Sf-9 medium to each of nine sterile 1.5-ml microtubes (labeling from 10^{-1} to 10^{-9}). Mix 15 µl of the original virus stock to the first microtube (10^{-1}). Briefly vortex the mixture and transfer 15 µl to the next microtube (10^{-2}). Repeat the step until the dilution of 10^{-9}.
4. For virus dilution from 10^{-5} to 10^{-8}, add 1,215 µl of diluted cells to the microtube so that the total volume is 1,350 µl. Also add 1,215 µl of diluted cells to the microtube containing 10^{-1} diluted virus as the positive control. Mix the diluted cells and virus thoroughly.

▲ **CRITICAL STEP** In most cases, the titer of unconcentrated P2 virus can be determined using dilutions from 10^{-5} to 10^{-8}. If the virus titer is too low or too high to be determined in this dilution range, mix the cells with a lower or higher dilution range (e.g., 10^{-4} to 10^{-7} or 10^{-6} to 10^{-9}).

5. For each dilution, mix the cells and virus thoroughly and seed the mixture to 12 wells (100 µl per well) of a 96-well plate. Seed six wells (100 µl per well) with positive (10^{-1}) and negative (cells only) controls.
6. Incubate the 96-well plate at 27 °C for 11–14 d. To avoid dehydration, seal the plate in a plastic bag lined with damp paper towels.
7. *Days 12–15.* Check the cytopathetic effect in each well under the microscope. Score all wells with signs of infection as positive.

▲ **CRITICAL STEP** For recombinant baculovirus expressing a reporter protein, the signs of infection can be examined earlier by the respective reporter protein detection method.

(continued)

Box 1 | (continued)

8. Calculate the virus titer on the basis of the virus dilution that induces 50% infection of the cultured cells (i.e., 50% tissue culture infectious dose ($TCID_{50}$))[68]. The principle of this method is to assume that all cultures infected at a dilution would have been infected at lower dilutions, and that all cultures uninfected at that dilution would have been uninfected at all higher dilutions[58].

The following shows an example of typical results obtained from virus titration[58]:

Dilution	Infected (no. of wells)	Uninfected (no. of wells)	Total infected (no. of wells)	Total uninfected (no. of wells)	% Infected
10^{-5}	12	0	21	0	100.0
10^{-6}	8	4	9	4	69.2
10^{-7}	1	11	1	15	6.3
10^{-8}	0	12	0	27	0

In this example, 1, 12 and 8 wells are infected at the 10^{-7}, 10^{-5} and 10^{-6} dilutions, respectively. Assuming that all these wells would have also been infected by the 10^{-5} dilution, the total number of infected wells at 10^{-5} is 21 (12+8+1). Conversely, all 12 wells are uninfected at 10^{-8}, and thus it can be assumed that those uninfected at 10^{-6} and 10^{-7} would have also been uninfected at 10^{-8}. Therefore, the total number of uninfected wells at 10^{-8} is 27 (4+11+12). The percentage of infected wells at 10^{-5} is 100 (21/21). The percentage of infected wells at 10^{-6} is 69.2 (9/13), and the percentage at 10^{-7} is 6.3 (1/16).

In this case, the dilution that leads to 50% response lies between 10^{-6} and 10^{-7}. This dilution is calculated by linear interpolation between the infection rates observed at this assay. The proportionate distance (PD) of a 50% response from the response above 50% is calculated using the following formula:

$$\mathbf{PD} = (\mathbf{A} - 50) / \mathbf{A} - \mathbf{B})$$

where *A* is the % response above 50%, and *B* is the % response below 50%. In this example:

PD = (69.2–50)/(69.2–6.3) = 0.305

The $TCID_{50}$ is calculated as follows:

Log $TCID_{50}$ = log of the dilution giving a response above 50%—the PD of that response.

In this case,

Log $TCID_{50}$ = –6–0.305 = –6.305

$TCID_{50} = 10^{-6.305}$

The virus titer is the reciprocal of $10^{-6.305}$ (= 2.02×10^6 $TCID_{50}$ per 10 µl, because the volume of diluted virus added to each well is 10 µl)

For each milliliter, the virus titer is 2.02×10^6 $TCID_{50}$/10 µl × 1,000 µl/ml = 2.02×10^8 $TCID_{50}$/ml.

9. Convert the unit of virus titer ($TCID_{50}$/ml) to pfu/ml using the relationship[58]: pfu = $TCID_{50}$ × 0.69. In this case, the virus titer is 1.4×10^8 pfu/ml.

▲ **CRITICAL STEP** The titer of P0 virus stock produced from bacmid-transfected Sf-9 cells generally lies between 1×10^6 and 1×10^7 pfu/ml. The titers of descent P1 and P2 virus stocks usually range from 1×10^8 to as high as 1×10^9 pfu/ml.

Amplification of recombinant baculovirus (Steps 45–56). The P0 baculovirus stocks can be used to further infect fresh Sf-9 cells for P1 virus production (**Fig. 2**). P1 virus can be used as an inoculum to infect Sf-9 cells for the generation of P2 virus stock. The resultant baculovirus is titrated by end-point dilution method[58] (**Box 1**). To evaluate transduction efficiency, baculovirus vectors expressing EGFP[47] and dsRed[8] have been generated.

Transduction of mammalian cells (Step 57). In the PROCEDURE, we describe how to transduce cell lines (Step 57A, describing transduction of HEK293 as an example), iPS cells (Step 57B), rASCs (Step 57C) and a rASC sheet (Step 57D). The procedures for HEK293 and iPS cell culture are routine, and they have been described previously (see refs. 59 and 60 for iPS cells); thus, this protocol starts from the cell seeding. rASCs can be harvested s.c. from the inguinal fat pads surrounding the epididymis of 3–4-month-old New Zealand white (NZW) rabbits (see **Box 2** for a detailed protocol)[51].

For transduction, cells are seeded on to culture plates or flasks (e.g., six-well plates, 10-cm dishes, T-75 or T-150 flasks) and incubated overnight. At day 2, a certain volume (depending on the multiplicity of infection (MOI) and baculovirus titer) of unconcentrated baculovirus supernatant is diluted with fresh insect cell (Sf-9) culture medium, and it is then mixed with $NaHCO_3$-free culture medium (e.g., DMEM or α-MEM) at a volumetric ratio of 1:4. The final volume of virus solution should be sufficient to wet the surface. Larger volumes of virus solution may reduce the chance of virus contact with cells and may decrease the transduction efficiency. Transduction is initiated by directly adding the appropriate volume (e.g., 500 µl per well in six-well plates, 4 ml per 10-cm dish and 5 ml per T-75 flask) of virus solution to cells. The transduction continues by gentle shaking on a rocking plate at room temperature for 4–6 h. After the transduction period, virus solution is replaced by the culture medium containing sodium butyrate. After ~15–24 h of incubation at 37 °C, the medium is replaced by fresh medium. At day 3 (1 d post transduction, 1 dpt), cells are collected for analysis or applications (**Fig. 3**). For instance, cells transduced with Bac-CE (expressing EGFP) can be harvested at 1 dpt and analyzed by flow cytometry for the percentage of GFP^+ cells (i.e., transduction efficiency). Alternatively, the cells continue to be cultured with medium exchange every 2–3 d (**Fig. 3**). For mock-transduction

Box 2 | Isolation and culture of rASCs ● TIMING 21–23 d

Additional materials required:
- NZW rabbits (3- to 4-month-old male rabbits, 2–3 kg, Animal Health Research Institute, Taiwan).

! CAUTION All animal experiments must conform to appropriate governmental and institutional regulations. This protocol is in compliance with the Guide for the Care and Use of Laboratory Animals (National Science Council, Taiwan).
- Zoletil 50 (Virbac)
- Rompun (2% (wt/vol); Bayer)

Procedure

1. *Day 1*. To isolate rASCs, anesthetize a 3- to 4-month-old male NZW rabbit (~2–3 kg) by i.m. injection of Zoletil 50 (25 mg/kg body weight) and 2% (wt/vol) Rompun (0.15 ml/kg body weight). After anesthesia, place the rabbit into the induction chamber and asphyxiate by carbon dioxide for 10 min or until breathing movements cease for 2 min.
2. Sterilize the operating table with 70% (vol/vol) ethanol. Shave the rabbit from the inguinal region to the abdominal region. After shaving, swab the body using 70% (vol/vol) ethanol to remove hair. Make an incision around inguinal fat pads and collect s.c. the adipose tissues surrounding the epididymis. Wash the adipose tissues three times with PBS.
3. Transfer the adipose tissues to a 10-cm dish containing 10 ml of PBS. Mince the adipose tissues into pieces aseptically with a sterile scalpel. Transfer the 10-ml tissue slurry with a 10-ml pipette to a sterile 50-ml centrifuge tube. Add 0.375 ml of 2% (wt/vol) type I collagenase solution into the tube (final collagenase concentration = 0.075% (wt/vol)).
4. Place the tube on a rocking plate within a 37 °C, 5% CO_2 incubator. Shake it gently for 30 min for tissue digestion.
5. Centrifuge the cell suspension at 300*g* for 10 min at room temperature. Aspirate the floating tissue fragments and the supernatant.

▲ CRITICAL STEP Be careful not to disturb the cell pellet at the bottom of the tube. The cell pellet is gelatinous and it does not adhere firmly to the tube.

6. Resuspend the cell pellet with 10 ml of PBS and transfer it to a new sterile 50-ml centrifuge tube.
7. Centrifuge the mixture at 300*g* for 10 min at room temperature and then aspirate the supernatant. Resuspend the cell pellet in rASCs medium. Determine the cell viability and density by trypan blue exclusion and by using a hemocytometer.
8. Seed the cells into T-75 flasks (1×10^5 cells per cm^2) containing 5 ml of prewarmed rASC medium. Fill the flask with fresh medium to obtain a final volume of 10 ml. Culture the cells at 37 °C in a 5% CO_2 incubator for 2 d.

▲ CRITICAL STEP For each NZW rabbit, the cell slurry is usually sufficient to seed two to four T-75 flasks.

9. *Day 3*. Remove nonadherent cells and wash adherent cells gently with PBS twice. Remove PBS, add 10 ml of fresh rASCs medium and incubate at 37 °C, 5% CO_2. Exchange the medium every 3 d (at days 6, 9 and 12). Be gentle when changing the medium to avoid dislodging the cells.
10. *Day 14*. Aspirate the medium and wash the cells gently twice with 10 ml of PBS prewarmed to 37 °C.

▲ CRITICAL STEP The cells are usually ~80–90% confluent at this time, and they are mostly rASCs already (designated as P0 rASCs).

11. Remove PBS and add 1 ml of 0.05% (wt/vol) trypsin-EDTA. Incubate the mixture at 37 °C for 5 min. Check for complete cell detachment by examining cell morphology under a microscope.
12. Inactivate trypsin by adding 9 ml of fresh rASC medium. Detach the cells from the T-75 flask by pipetting or tapping the flask. Measure the cell viability and density.
13. Transfer the trypsinized cells into a sterile 15-ml centrifuge tube and centrifuge at 300*g* for 5 min at room temperature.
14. Aspirate the supernatant and resuspend the cell pellet with rASC medium to 1.5×10^5 cells per ml.
15. Seed 10 ml of cells into the T-75 flask (1.5×10^6 cells per flask) and culture them at 37 °C in a 5% CO_2 incubator. After 3–4 d of incubation, the cells are usually ~80–90% confluent and are designated as P1 rASCs.

▲ CRITICAL STEP The characteristics of rASCs can be verified by immunofluorescence labeling and flow cytometry analysis of surface markers ($CD44^+$, $CD29^+$, $CD90^+$, $CD31^-$, $CD45^-$ and $CD105^{Low}$).

16. *Day 17 (–18)*. Subculture the P1 cells by repeating Steps 10–15 to get P2 rASCs.
17. *Day 20 (–22)*. For cell banking in liquid nitrogen, resuspend P2 rASCs in fresh rASCs medium containing 7.5% (vol/vol) DMSO (1×10^6 cells per ml). Transfer the cell solution into cryogenic vials (1 ml per vial). Transfer the cryogenic vials into the cryo box (filled with absolute 2-propanol) and place the box in the –80 °C freezer overnight.
18. *Day 21 (–23)*. Transfer cryogenic vials into the liquid nitrogen container for long-term storage.

control, the procedure is the same, except that insect cell culture medium is used in lieu of virus supernatant.

Limitations of the protocol

The Bac-to-Bac system we commonly use eliminates the need for multiple rounds of plaque purification, which reduces the time required to obtain the P0 recombinant virus. However, the transgene in the genome of bacmid-derived baculovirus is more prone to spontaneous excision, leading to a rapid decrease in transgene expression in passages beyond P8 (ref. 61). Therefore, it is advised to keep the virus passage low.

Baculovirus transduction is highly efficient for a wide variety of cell types, yet it is reported that baculovirus transduction of hematopoietic cell lines such as U937, K562, Raw264.7, LCL-cm and Raji is inefficient (for a review, see ref. 6). In addition, baculovirus transduction efficiency for primitive stem cells such as

embryonic stem cells[62] and iPS cells[8] is relatively low (<40–50%). Furthermore, baculovirus-mediated growth factor expression is relatively inefficient in rat or mouse ASCs or BMSCs (K.-C.L. and C.-L.Y., unpublished data), although the expression is robust in rASCs or rBMSCs[53,54].

Last, although the transgene expression can be prolonged from <7 d to ~3 weeks using the FLPo/*Frt*-based hybrid baculovirus vectors, for longer transgene expression (months to years) integration of transgene cassette is required. In this regard, hybrid baculovirus vectors by incorporating AAV inverted-terminal repeats[7,34] or *Sleeping Beauty* transposons[14,50] have been developed to enable transgene integration into the chromosome and long-term expression.

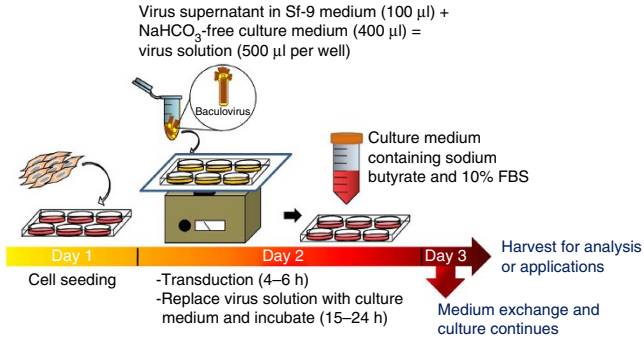


Figure 3 | Transduction of cells in a six-well plate.

MATERIALS

REAGENTS

- MAX Efficiency DH10Bac chemically competent cells (Invitrogen, cat. no. 10361-012)
- Sf-9 cells (ATCC, cat. no. CRL-1711)
- Mammalian cells to be transduced, for example, HEK293 cells (ATCC, cat. no. CRL-1573), iPS cells (we have used the mouse iPS cell line 20D17, supplied by S. Yamanaka, Center for iPS Cell Research and Application, Kyoto University) or rASCs (derived as described in **Box 2**). See INTRODUCTION for a full discussion of the cell types that we have successfully transduced
- S.O.C. medium (Invitrogen, cat. no. 15544-034)
- LB broth (BD Biosciences, cat. no. 244620)
- Agar (bacteriological; OXOID, cat. no. LP0011)
- Sf-900 II serum-free medium (SFM; Invitrogen, cat. no. 10902-088)
- TNM-FH insect cell medium (Sigma, cat. no. T3285)
- DMEM (high glucose; Sigma, cat. no. D5648)
- DMEM (low glucose; Gibco, cat. no. 31600-034)
- α-MEM (Gibco, cat. no. 12000-022)
- PBS without calcium or magnesium (pH 7.4; Invitrogen, cat. no. 14190-094)
- Hyclone FBS (Thermo Scientific, cat. no. SH30070.03)
- Sodium bicarbonate ($NaHCO_3$; Sigma, cat. no. S5761)
- Gentamycin (AMRESCO, cat. no. 0304)
- Kanamycin (AMRESCO, cat. no. 0408)
- Tetracycline (AMRESCO, cat. no. 0422)
- X-gal (AMRESCO, cat. no. 0428)
- IPTG (AMRESCO, cat. no. 0487)
- Absolute 2-propanol (Sigma, cat. no. I9516)
- Cellfectin II reagent (Invitrogen, cat. no. 10362-100)
- Trypsin-EDTA (0.05% (wt/vol); Gibco, cat. no. 25300)
- Tris-HCl (Sigma, cat. no. T5941)
- EDTA (Sigma, cat. no. E6758)
- Sodium hydroxide (NaOH; Sigma, cat. no. S8045)
- SDS (Sigma, cat. no. L3771)
- Potassium acetate (Sigma, cat. no. P1190)
- DMSO (Sigma, cat. no. D2650)
- Trypan blue stain (0.4%; APOLO, cat. no. APL-0167)
- Ethanol (70% (vol/vol); Sigma, cat. no. 02877)
- Gelatin (0.1% (wt/vol) solution) from porcine skin type A (Sigma, cat. no. G1890)
- MEM non-essential amino acids solution, 100× (Gibco, cat. no. 11140-050)
- 2-mercaptoethanol (55 mM; Gibco, cat. no. 21985-023)
- Leukemia inhibitory factor (10^7 U/ml; LIF, Millipore, cat. no. ESG1107)
- Type I collagenase (Gibco, cat. no. 17100-017)
- RNase A (Sigma, cat. no. R6513)
- Penicillin-streptomycin, 100× (10,000 U/ml; Gibco, cat. no. 15140-122)
- Penicillin-streptomycin-amphotericin B solution (Biological Industries, cat. no. 03-033-1B)
- Sodium butyrate (Sigma, cat. no. B5887)
- pFastBac vectors such as pFastBac 1 (Invitrogen, cat. no. 10360-014) and pFastBac Dual (Invitrogen, cat. no. 10712-024)
- Recombinant baculovirus expressing EGFP (Bac-CE), DsRed (Bac-ER), FLPo (Bac-FLPo) and BMP-2 (Bac-FCBW) under the control of the CMV promoter

EQUIPMENT

- Incubator, set at 37 °C, 5% CO_2 (Thermo Scientific)
- Incubator, 27 °C, no CO_2 (Firstek Scientific, RI-102)
- Shaking incubator, 27 °C, no CO_2 (Cocono)
- Upright ultra-low-temperature freezers, set at −80 °C (Forma 900 series, Thermo Scientific)
- Refrigerator, 4 °C (Daytime)
- Freezer, −20 °C (Elcold)
- Water bath (Firstek Scientific)
- Rotator (TAITEC, RT-50)
- Laminar flow cabinet (UBI)
- Sterilizer (Tomin, High-Pressure Steam Sterilizer)
- Inverted microscope equipped with a phase-contrast ring and fluorescence filters (Nikon, TE200)
- Upright microscope (Nikon)
- Microtube (1.5 ml; Axygen, MCT-150-C)
- Tubes (15 and 50 ml; Corning, cat. nos. 43079 and 35276, respectively)
- Disposable culture tube (Kimble Chase, cat. no. 73500-16100)
- Petri dish (Protech, cat. no. PT-PD-90)
- Tissue culture plates (6-, 12- and 96-well; Thermo Scientific, cat. nos. 140675, 167008 and 150628, respectively)
- Tissue culture flasks (T-75 and T-150; Thermo Scientific, cat. nos. 156499 and 159910, respectively)
- Tissue culture centrifuge (Hettich Universal 32)
- Microcentrifuge (Hettich Zentrifugen MIKRO-120)
- Flow cytometer (BD Biosciences, FACSCalibur)
- Rocking plate (TKS, RS01)
- Vortex mixer (Scientific Industries, cat. no. SI-0236)
- Multi-place magnetic stirrer (Thermolyne, Cellgro 45700)
- Spinner flask (250 ml; Bellco, cat. no. 1965-80255)
- Hemocytometer (Marienfeld-Superior, cat. no. 6300-30)
- Pipettes (5 and 10 ml; Costar, cat. nos. 4487 and 4488, respectively)
- Tips (10, 200 and 1,000 µl; MultiMax, cat. nos. 7230, 2947 and 2950, respectively)
- Pipettors (P2N, P10N, P20N, P200N and P1000N; Gilson, cat. nos. F144561, F144562, F144563, F144565 and F144566, respectively)
- Pipette aid
- Surgical scissors
- Surgical forceps
- Shaver
- Scalpel
- Induction chamber
- Glass cell spreaders
- Laboratory glass bottle (250 ml; Schott Duran, cat. no. 2180136)

• Cryogenic vials (Nalgene, cat. no. 5000-0020)
• Cryo box
• Syringes (BD Biosciences, 1-ml syringe with a 26-G needle for anesthesia)
• Bottle-top filters (0.22-μm; Sartorius, cat. no. 9.049 205)
• Syringe filter (0.22-μm; Nalgene, cat. no. 190-2520)

REAGENT SETUP

LB broth To prepare 1,000 ml of LB broth, dissolve 25 g of the LB broth powder in 1,000 ml of distilled water, and mix it thoroughly. Autoclave the medium at 121 °C for 15 min. Cool the broth to room temperature, and then store it at 4 °C for up to 3 months.

LB agar plate Dissolve 25 g of LB broth powder and 15 g of agar powder in 1,000 ml of distilled water and mix it thoroughly. Autoclave the solution at 121 °C for 15 min. When the temperature of the heated solution drops to 55 °C, add gentamycin, kanamycin, tetracycline, X-gal and IPTG to final concentrations of 7, 50, 10, 200 and 40 μg/ml, respectively. Divide the agar mixture into separate Petri dishes (20 ml per dish) and cool the plates to room temperature. Store the plates at 4 °C for up to 1 month.

Resuspension buffer To prepare 100 ml of resuspension buffer, dissolve 0.24 g of Tris-HCl and 0.29 g of EDTA in 80 ml of distilled water, and then adjust the pH to 8.0. Add 10 mg of RNase A and fill the container with distilled water to a final volume of 100 ml. Store the buffer at 4 °C and use it within 1 month.

Lysis buffer To prepare 100 ml of lysis buffer, dissolve 0.8 g of NaOH and 1 g of SDS in 100 ml of distilled water. Store the buffer at room temperature and use it within 3 months. Mix the buffer thoroughly before use. ▲ **CRITICAL** If precipitates form in the lysis buffer, warm the buffer in a 37 °C water bath until the precipitates totally dissolve.

Precipitation buffer To prepare 100 ml of precipitation buffer, dissolve 29.4 g of potassium acetate in 50 ml of distilled water and adjust the pH to 5.5. Fill with distilled water to a final volume of 100 ml. Store the buffer at room temperature and use it within 3 months.

Elution buffer To prepare 100 ml of elution buffer, dissolve 0.16 g of Tris-HCl and 0.03 g of EDTA in 80 ml of distilled water, adjust the pH to 8.0, and use distilled water to bring the final volume to 100 ml. Store the buffer at room temperature and use it within 1 month.

Sf-9 medium To prepare 1,000 ml of Sf-9 medium, mix 900 ml of TNM-FH medium (filtered through 0.22-μm) with 100 ml of FBS. Store the medium at 4 °C for up to 1 month.

HEK293 medium To prepare 500 ml of HEK293 medium, mix 450 ml of DMEM (high glucose) medium (filtered through a 0.22-μm filter) and 50 ml of FBS. Store the medium at 4 °C for up to 1 month.

Gelatin-coated culture flask and plate Add 0.1% (wt/vol) gelatin solution to a T-75 flask (5 ml) or a six-well plate (1 ml). Ensure that the entire bottom surface is covered with liquid. Incubate the flask or plate at 37 °C for 30 min. Aspirate the gelatin solution before use. Prepare the flask or plate immediately before use.

iPS medium To prepare 500 ml of iPS medium, mix 419 ml of DMEM (high glucose) medium (filtered through a 0.22-μm filter), 75 ml of FBS (15% (vol/vol), 5 ml of 100× non-essential amino acids, 0.9 ml of 2-mercaptoethanol and 50 μl of LIF. Store the medium at 4 °C and use it within 1 month.

rASC medium rASC medium can be DMEM- or α-MEM–based. To prepare 500 ml of rASC medium, mix 445 ml of DMEM (low glucose) medium or α-MEM medium (filtered through a 0.22-μm filter), 50 ml of FBS (10% (vol/vol) and 5 ml of 100× penicillin-streptomycin. Store the medium at 4 °C for up to 1 month.

rASC sheet fabrication medium To prepare 500 ml of rASC sheet fabrication medium, mix 395 ml of α-MEM medium (filtered through a 0.22-μm filter), 100 ml of FBS (20% (vol/vol)) and 5 ml of 100× penicillin-streptomycin. Store the medium at 4 °C for up to 1 month.

Surrounding solution Surrounding solution is culture medium containing 10% (vol/vol) FBS, without $NaHCO_3$. The surrounding medium should be stored at 4 °C and used within 1 month. ▲ **CRITICAL** The surrounding medium is prepared in a similar manner to the culture medium, except that 3.7 grams per liter $NaHCO_3$ is not added.

Type I collagenase solution To prepare 5 ml of 2% (wt/vol) type I collagenase, dissolve 0.1 g of type I collagenase powder in a final volume of 5 ml of PBS solution. Type I collagenase solution should be prepared freshly before starting the isolation process, and it should be filter-sterilized using a 0.22-μm syringe filter.

Sodium butyrate stock solution To prepare 100× stock solution of sodium butyrate (300 mM), dissolve 1 g of sodium butyrate in 30.3 ml of distilled water and sterilize the solution by passing it through a 0.22-μm filter. Freeze the stock solution in aliquots at −20 °C and use it within 6 months.

PROCEDURE

Generation of recombinant bacmid ● TIMING 5 d

▲ **CRITICAL** If a recombinant baculovirus stock is already available, Steps 1–44 can be skipped.

1| *Transposition of pFastBac donor plasmid, day 1.* Dilute the donor plasmid to 10 ng/μl using sterile water in a sterile 1.5-ml microtube.

2| Thaw one vial of MAX Efficiency DH10Bac competent cells on ice.

3| Prechill a sterile 1.5-ml microtube on ice.

4| Add 30 μl of DH10Bac competent cells into the prechilled microtube and keep it on ice.

5| Add 1 μl of the diluted donor plasmid into 30 μl of DH10Bac competent cells.

6| Incubate the competent cells on ice for 20 min.

▲ **CRITICAL STEP** Do not hold the bottom of the microtube with your finger. Hold the upper part of the microtube to avoid warming the competent cells.

7| Incubate the mixture in the 42 °C water bath for 60 s to perform heat shock.

8| Immediately transfer the mixture to ice and leave it on ice for 5 min.

9| Add 500 µl of S.O.C. medium that has been prewarmed to room temperature into the microtube containing competent cells. Shake the tube in a rotator (250 r.p.m.) at 37 °C for 4 h. Thirty minutes before the end of this 4-h incubation, prewarm an LB agar plate at 37 °C for 30 min.

10| Centrifuge the cells at 9,000*g* for 2 min at room temperature. Remove 470 µl of supernatant (leaving 60 µl). Resuspend the cell pellet by pipetting gently.

11| Spread 60 µl of the resuspended competent cells on the prewarmed LB agar plate using a glass cell spreader.

12| Incubate the LB agar plate at 37 °C for 48 h for colony formation. Store the plate at 4 °C before the next step if necessary.
▲ **CRITICAL STEP** Normally, blue and white colonies are formed after incubation. Successful transgene cassette transposition into the attachment site disrupts the *lacZα* gene, leading to interrupted expression of LacZα peptide and the formation of white colonies. Without transposition, *lacZα* gene remains intact, giving rise to LacZα peptide expression and the formation of blue colonies in the presence of X-gal in the LB agar plate. Therefore, colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid.
▲ **CRITICAL STEP** The process may be accelerated by incubating the agar plate for only 16–24 h. However, at this time, colonies are relatively small. For the following bacmid isolation, pick the largest, most isolated white colonies to avoid selecting false positives. Avoid picking colonies that appear gray or are darker in the center, as they can contain a mixture of cells with empty bacmid and recombinant bacmid.
■ **PAUSE POINT** The LB agar plate can be stored at 4 °C for 1 month.

13| *Day 3*. Prewarm the spread LB agar plate to room temperature.

14| Prewarm another fresh LB agar plate at 37 °C for 30 min and divide the fresh LB agar plate into three areas with a marker.

15| Pick a blue colony (as control) and two white colonies onto the spread LB agar plate and re-streak them on the three areas of the fresh LB agar plate.

16| Incubate the re-streaked LB agar plate at 37 °C for 16–24 h for colony formation. Store the plate at 4 °C before the next step if necessary.
▲ **CRITICAL STEP** The white colonies should contain the recombinant bacmid. Verify the integration of the transgene cassette into bacmid by restriction analysis or PCR if necessary.
■ **PAUSE POINT** The re-streaked LB agar plate can be stored at 4 °C for 1 month.

17| *Isolation of recombinant bacmid DNA, day 4*. Add 3 ml of LB broth containing gentamycin (7 µg/ml), kanamycin (50 µg/ml) and tetracycline (10 µg/ml) into a disposable culture tube.

18| Pick a white colony from the re-streaked LB agar plate, and then inoculate the cells into the disposable culture tube.
▲ **CRITICAL STEP** We recommend that you culture two white colonies individually in LB broth to ensure successful isolation of recombinant bacmids.

19| Place the culture tube on the rotator (250 r.p.m.) at 37 °C for 20 h.

20| *Day 5*. Transfer 1 ml of cultured bacterial cells to a 1.5-ml microtube and centrifuge the tube at 9,000g for 2 min at room temperature. Remove all of the supernatant.

21| Add 200 µl of resuspension buffer to the pellet. Resuspend the cells by pipetting or vortexing.

22| Add 200 µl of lysis buffer to the cell suspension. Mix it gently by inverting the microtube ten times.

23| Incubate the mixture at room temperature for 5 min.

24| Add 300 µl of precipitation buffer to the mixture. Mix it immediately by inverting the microtube ten times. Do not vortex.

25| Chill the microtube on ice for 10 min. Centrifuge the mixture at 15,000*g* at 4 °C for 20 min.

26| Transfer 600 µl of the supernatant to another 1.5-ml microtube.
▲ **CRITICAL STEP** Avoid pipetting any white precipitate in the supernatant. Pipette the clear supernatant into the microtube and centrifuge it at 15,000*g* for another 5 min at 4 °C to remove any contaminating precipitates.

27| Add 800 µl of absolute 2-propanol to the clear supernatant. Mix it gently by inverting the microtube ten times.

28| Chill the microtube on ice for 10 min. Centrifuge the tube at 15,000*g* at 4 °C for 15 min. Remove the supernatant.

29| Add 500 µl of 70% (vol/vol) ethanol to the pellet. Mix gently by inverting the microtube ten times.

30| Centrifuge the tube at 15,000*g* at 4 °C for 5 min. Remove the supernatant completely.

31| Open the cap of the microtube and air-dry the DNA pellet for 5–10 min until the pellet becomes half-transparent.

32| Resuspend the DNA pellet in 40 µl of elution buffer by gentle pipetting. Allow the pellet to dissolve completely on ice or in a 4 °C refrigerator.
▲ **CRITICAL STEP** To avoid shearing the bacmid DNA, do not pipette more than two times.

33| Divide the bacmid DNA into aliquots in separate sterile 1.5-ml microtubes, and store them at –20 °C to avoid more than one freeze-thaw cycle.
■ **PAUSE POINT** The bacmid DNA can be stored at –20 °C for up to 2 weeks.

Production of recombinant baculovirus ● **TIMING 5–7 d**

34| *Transfection of recombinant bacmid DNA, day 1*. Grow Sf-9 cells with Sf-9 medium (containing 10% (vol/vol) FBS) in suspension to log-phase (e.g., 1–2 × 10^6 cells per ml) with >95% viability.
▲ **CRITICAL STEP** Sf-9 cells can be readily cultured in monolayer or in suspension at 27 °C without CO_2.

35| Dilute Sf-9 cells to 3 × 10^5 cells per ml and seed 1 ml of cells to each well of a 12-well plate. Incubate the cells in a 27 °C incubator for at least 30 min to allow cell attachment.

36| For each bacmid that is ready for transfection, prepare solutions A and B (containing bacmid) in a sterile 1.5-ml microtube as follows:

Solution A		Solution B	
Reagent	**Volume (µl)**	**Reagent**	**Volume (µl)**
Cellfectin II reagent	2	Bacmid DNA	5
Sf-900 II SFM	30	Sf-900 II SFM	30

▲ **CRITICAL STEP** SFM is used in transfection because proteins in the FBS interfere with the Cellfectin reagent and inhibit the transfection.

37| Mix gently solutions A and B in a sterile 1.5-ml microtube, and incubate it at room temperature for 30 min.

38| Add 240 µl of Sf-900 II SFM to the DNA-lipid mixture and mix it thoroughly.

39| Wash the attached Sf-9 cells with Sf-900 II SFM twice to remove FBS. Discard the medium completely.

40| Add the DNA-lipid mixture to the 12-well plate (300 µl per well). Incubate the 12-well plate at 27 °C for 5 h for transfection.
▲ **CRITICAL STEP** Do not add antibiotics during transfection.

41| After transfection, discard the DNA-lipid mixture in the well. Add 1 ml of Sf-9 medium containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin-amphotericin B to each well. Incubate the 12-well plate at 27 °C for 4–6 d.

42| *Day 5–7*. Visually observe the cytopathic effect (CPE) of transfected Sf-9 cells under a microscope. When CPE is observed in ~30% of cells, collect the culture medium in a sterile 1.5-ml microtube.
▲ **CRITICAL STEP** At 3 d after transfection, visually check the CPE of transfected cells (e.g., cell lysis, for which cells appear lysed resulting in clearing plaques on the cell monolayer). If transfection efficiency is low, CPE may not be obvious until 4 or 5 d after transfection.
? TROUBLESHOOTING

43| Centrifuge the culture medium at 500*g* for 10 min at room temperature. Pipette the supernatant into a sterile 1.5-ml cryogenic vial as the P0 virus.

44| Divide the P0 virus into separate sterile 1.5-ml cryogenic vials. Store the working stock at 4 °C and the master stock at –80 °C for long-term storage.
▲ **CRITICAL STEP** Do not store the virus stock at –20 °C, which leads to more rapid titer loss[63]. Protect the stock from light.
■ **PAUSE POINT** The P0 virus can be stored for at least 3 months at 4°C and for years at –80 °C.

Amplification of recombinant baculovirus ● TIMING 10–12 d
45| *Day 1*. Grow Sf-9 cells using Sf-9 medium to log phase (~1 × 10^6 cells per ml). Seed 10 ml of Sf-9 cells to a T-75 flask. Incubate the cells at 27 °C for at least 30 min to allow cell attachment.
▲ **CRITICAL STEP** For baculovirus amplification, Sf-9 medium containing 10% (vol/vol) FBS is recommended because baculovirus is more stable in the presence of FBS.

46| Aspirate 5 ml of medium from the T-75 flask to a 15-ml centrifuge tube. Add 25 µl of P0 virus stock to the medium and mix it thoroughly.

47| Transfer the 5-ml medium containing P0 virus back to the T-75 flask. Continue the infection by incubating the flask in a 27 °C humidified incubator for 4–5 d.
▲ **CRITICAL STEP** Infecting cells at low MOI (e.g., 0.05–0.1) is recommended for baculovirus amplification. Infecting cells with higher MOI may generate noninfectious defective virus particles and it may reduce the quality of the baculovirus stock. At this stage, we do not routinely determine the virus titer, because it takes 12–15 d. Instead, we assume a virus titer of 2 × 10^7 pfu/ml according to our experience, and add 25 µl of virus stock to the T-75 flask. Do not directly add the virus stock into the T-75 flask. We recommend premixing the virus stock with 5 ml of Sf-9 medium before infection.

48| *Day 5 (–6)*. Check the CPE of infected Sf-9 cells. When CPE (e.g., cell lysis) is observed in ~70% of cells, collect the culture supernatant in a sterile 15-ml centrifuge tube.

49| Centrifuge the cells at 500*g* for 10 min at room temperature. Pipette the supernatant into another sterile 15-ml centrifuge tube as the P1 virus stock.

50| Store the P1 virus stock at 4 °C and protect it from light. For long-term storage, transfer the virus stock into separate 1.5-ml cryogenic vials and store them at –80 °C.
■ **PAUSE POINT** The P1 virus can be stored at –80 °C for years. When storing at 4 °C, the virus is stable for at least 3 months.

51| *Day 6 (–7)*. To generate P2 virus stock, grow 200 ml of Sf-9 cells in the 250-ml spinner flask to log phase (~1.0–1.2 × 10^6 cells per ml) using Sf-9 medium.

52| Transfer 10 ml of culture from the spinner flask to a 15-ml centrifuge tube. Add 50 µl of the P1 virus stock to the 10-ml culture.
▲ **CRITICAL STEP** The titer (pfu/ml) of baculovirus can be determined using either plaque assay[58] or end-point dilution assay, which is described in detail in **Box 1**. If the virus titer is not determined at this stage, a virus inoculum volume of 50 µl (assuming a P1 virus titer of 2 × 10^8 pfu/ml) is recommended. Premix the virus stock with 10 ml of culture.

53| Transfer the 10-ml culture containing P1 virus back to the spinner flask. Place the spinner flask on the magnetic stirrer (100 r.p.m.) in a 27 °C humidified incubator and incubate it for 4–5 d.

54| *Days 10–12*. Check the viability of infected Sf-9 cells by trypan blue dye exclusion with a hemocytometer. When the cell viability drops to ~70%, collect the culture in four sterile 50-ml centrifuge tubes.

55| Centrifuge at 500*g* for 10 min at room temperature. Transfer the supernatant to a sterile 250-ml laboratory glass bottle as the P2 virus stock.

56| Store the P2 virus stock at 4 °C and protect it from light. Titrate the virus as described in **Box 1**.
▲ **CRITICAL STEP** For mammalian cell transduction, in general, a batch of 100–200 ml of P2 virus stock is sufficient for experiments.
? TROUBLESHOOTING
■ **PAUSE POINT** The P2 virus can be stored at 4 °C for at least 3 months.

Transduction of mammalian cells with baculovirus

57| To transduce general cell lines (e.g., Huh-7, SNU-449, A549, HeLa and HEK293), follow option A. Option A describes the transduction of HEK293 cells in six-well plates using a baculovirus (Bac-CE) expressing EGFP as an example. To transduce iPS cells, follow option B, which describes the transduction of iPS cells using Bac-ER (containing a EF1α-DsRed transgene cassette) as an example. To transduce rASCs using the FLPo/*Frt*-based hybrid system, follow option C. To transduce rASC sheets, follow option D. Option D demonstrates the fabrication and transduction of rASC sheet using Bac-CE.

(A) Transduction of a general cell line ● TIMING 3 d

(i) *Day 1*. Trypsinize HEK293 cells from the T-75 flask and resuspend the cells with prewarmed HEK293 medium to a density of 1.5×10^5 cells per ml.
▲ **CRITICAL STEP** We prefer not to add antibiotics, as they mask bacterial contamination. Nonetheless, antibiotics do not affect baculovirus transduction, and they can be used in primary cells such as rASCs.

(ii) Seed 2 ml of cell suspension into each well of a six-well plate (3×10^5 cells per well). Incubate the cells overnight at 37 °C. After overnight incubation, HEK293 cells should reach 60–70% confluence.
▲ **CRITICAL STEP** The initial seeding cell may slightly vary from cell line to cell line, depending on the cell size.

(iii) *Day 2*. Calculate the required virus volume based on the virus titer (typically 3×10^8–1×10^9 pfu/ml for P2 virus) and cell number (3×10^5 cells per well). For each well, the virus volume required is calculated using the following formula: volume required (ml) = desired MOI (pfu per cell) × cell number (cells)/virus titer (pfu/ml).

(iv) For each well, add the required volume of Bac-CE into Sf-9 medium to a total volume of 100 µl. Next, mix 100 µl of virus solution with 400 µl of surrounding solution ($NaHCO_3$-free HEK293 medium).
▲ **CRITICAL STEP** For example, when 3×10^5 cells are transduced by Bac-CE with a titer of 5×10^8 pfu/ml at MOI 100, the volume required for each virus supernatant is 100 (pfu per cell) × 3×10^5 (cells)/5×10^8 (pfu/ml) = 0.06 ml (60 µl). Add 60 µl of Bac-CE to 40 µl of Sf-9 medium. Mix the 100 µl of virus mixture with 400 µl of surrounding solution. For mock transduction as control, simply mix 100 µl of Sf-9 medium with 400 µl of surrounding solution.
▲ **CRITICAL STEP** If the calculated total virus volume exceeds 100 µl, increase the surrounding solution volume with the fixed volumetric ratio (virus solution:surrounding solution = 1:4).
▲ **CRITICAL STEP** If a larger vessel (e.g., a T-75 or T-150 flask) is used, the volume of the final virus solution is proportionally increased according to the culture area.

(v) Discard the HEK293 medium and wash the cells twice with PBS (1 ml per well).

(vi) Remove PBS and initiate the transduction by adding 500 µl of the mixed virus solution into each well.

(vii) Put the six-well plate on a rocking plate. Continue the transduction process by gently shaking the plate (10 times per min) for 6 h at room temperature.
▲ **CRITICAL STEP** Transduction efficiency depends on cell type, MOI and incubation time, which may vary from 4 to 6 h. For HEK293, transduction at MOI 100 for 6 h at room temperature yields transduction efficiency >95% without compromising cell viability. Incubation at 37 °C results in reduced transduction efficiency because baculovirus integrity is compromised at 37 °C.

(viii) Aspirate the virus solution and wash the cells again. Add fresh HEK293 medium containing 3 mM sodium butyrate. Incubate the mixture at 37 °C for 24 h.
▲ **CRITICAL STEP** Sodium butyrate enhances transgene expression, but high sodium butyrate concentration is toxic to cells. We determine that 3 mM enhances the transduction of HEK293 without compromising cell viability.

(ix) *Day 3*. Observe the fluorescence-emitting cells under the fluorescence microscope. Trypsinize and resuspend cells in 0.5 ml of PBS. Analyze the percentage of fluorescence-emitting cells using a flow cytometer.
? TROUBLESHOOTING

(B) Transduction of iPS cells ● TIMING 3 d

(i) *Day 1*. Seed iPS cells to a gelatin-coated six-well plate (2×10^5 cells per well, 2 ml). Keep the cells at 37 °C overnight.

(ii) *Day 2*. Calculate the required virus volume as described in Step 57A(iii).

(iii) Remove the medium and gently wash the cells twice with PBS (1 ml per well).

(iv) For each well, add the required volume of baculovirus (Bac-ER) into Sf-9 medium to a total volume of 100 µl. Next, mix the 100 µl of virus solution with 400 µl of surrounding solution ($NaHCO_3$-free iPS medium).
▲ **CRITICAL STEP** The iPS cells express GFP under the control of the Nanog promoter. Therefore, here we transduce the cells with a baculovirus expressing DsRed (emitting red fluorescence).

(v) Remove PBS from the six-well plate. Add the mixed virus solution to the wells (500 µl per well).
(vi) Gently shake the six-well plate on a rocking plate (10 times per min). Continue the transduction process for 4 h at room temperature.
▲ CRITICAL STEP iPS cells do not adhere firmly to the plate. Treat the cells gently.
(vii) After transduction, aspirate the baculovirus-containing solution and gently wash the cells twice with 1 ml of PBS. Replenish with 2 ml of iPS medium containing 3 mM sodium butyrate. Incubate the cells at 37 °C for 24 h.
(viii) *Day 3*. Observe the DsRed expression under a fluorescence microscope. Trypsinize and resuspend the cells in 0.5 ml of PBS. Analyze the percentage of fluorescence-emitting cells using a flow cytometer (543 nm).
? TROUBLESHOOTING

(C) Transduction of rASCs ● TIMING 5–6 d

▲ CRITICAL STEP Here we describe the co-transduction of rASCs cultured in a six-well plate with the FLPo/*Frt*-based hybrid baculovirus system (Bac-FLPo and its substrate baculovirus) as an example. This procedure starts from the thawing of the primary rASCs, isolated as described in **Box 1**.

(i) *Day 1*. Move the cryogenic vial containing rASCs (1×10^6 cells per vial) from liquid nitrogen. Thaw the cells in a 37 °C water bath. Be sure not to immerse the cap.
(ii) Once the crystals melt (1–2 min), transfer the cells to a T-75 flask containing 9 ml of prewarmed rASC medium. Keep the cells at 37 °C in a 5% CO_2 incubator.
(iii) After 6 h of attachment, replace the DMSO-containing medium with 10 ml of rASC medium. Culture the cells at 37 °C for 2–3 d until ~80–90% confluency.
▲ CRITICAL STEP As rASCs tend to differentiate upon 100% confluency, do not allow the cells to grow to full confluency. We recommend subculturing rASCs at ~80–90% confluency.
(iv) *Day 3 (–4)*. Wash the cells with 10 ml of PBS twice, and discard the solution. Add 1 ml of prewarmed 0.05% (wt/vol) trypsin-EDTA to the T-75 flask and incubate it at 37 °C for 5 min to detach rASCs. Check for complete cell detachment by examining cell morphology under a microscope.
(v) Inactivate trypsin by adding 5 ml of rASC medium and gently resuspending the cells. Aspirate 50 µl of cell solution for counting (the concentration is generally $4–6 \times 10^5$ cells per ml).
(vi) Dilute the cell suspension to 1×10^5 cells per ml with rASC medium and seed 2 ml of cell solution to the six-well plate (2×10^5 cells per well or ~2×10^4 cells per cm^2). Incubate the cells at 37 °C overnight. After overnight incubation, the rASC density is ~2×10^5 cells per well (~60–70% confluency).
▲ CRITICAL STEP For rASC transduction in a larger scale, seed rASCs to T-75 or T-150 flasks with the same cell density (~2×10^4 cells per cm^2) and incubate them overnight in the same manner.
(vii) *Day 4 (–5)*. Calculate the required virus volume as described in Step 57A(iii). For example, when 2×10^5 cells are co-transduced by Bac-FLPo and the substrate baculovirus with the same titer (e.g., 5×10^8 pfu/ml) at the same MOI (e.g., 50), the volume required for each virus supernatant is 50 (pfu per cell) × 2×10^5 (cells)/5×10^8 (pfu/ml) = 0.02 ml (20 µl). Add 20 µl of Bac-FLPo and 20 µl of substrate baculovirus to 60 µl of Sf-9 medium. Mix the 100 µl of virus mixture with 400 µl of surrounding solution. For mock transduction as control, simply mix 100 µl of Sf-9 medium with 400 µl of surrounding solution.
(viii) For each well, add the required volume of Bac-FLPo and substrate baculovirus into Sf-9 medium to a total volume of 100 µl. Next, mix the 100 µl of virus solution with 400 µl of surrounding solution ($NaHCO_3$-free rASC medium).
(ix) Discard rASC medium and wash the cells twice with PBS (1 ml per well). Remove the PBS.
(x) Initiate the transduction by adding the mixed virus solution (500 µl) into each well.
(xi) Put the six-well plate on a rocking plate. Continue the transduction process by gently shaking the plate (10 times/min) for 6 h at room temperature.
(xii) After 6 h of transduction, remove the virus solution and add rASC medium (containing 3 mM sodium butyrate). Incubate the mixture at 37 °C for 24 h.
(xiii) *Day 5 (–6)*. At 1 dpt, replace the sodium butyrate-containing medium with fresh rASC medium.
? TROUBLESHOOTING

(D) Transduction of a rASC sheet ● TIMING 4 d

(i) *Day 1*. Seed rASCs (prepared as described in Step 57C(i–iii)) to a six-well plate (5×10^5 cells per well) with 2 ml of rASC sheet fabrication medium. Keep the cells at 37 °C in a 5% CO_2 incubator for 2 d.
▲ CRITICAL STEP To facilitate the cell sheet formation, higher seeding cell density (5×10^5 cells per well) is helpful. The 20% (vol/vol) of FBS in the fabrication medium also augments the secretion of extracellular matrix by rASCs to form the cell sheet.
(ii) *Day 3*. The rASC sheet should be visible. Remove the medium and wash the cell sheet gently with PBS (2 ml per well) twice.
(iii) Calculate the required virus volume.

(iv) For each well, add the required volume of baculovirus (e.g., Bac-CE) into Sf-9 medium to a total volume of 100 µl. Next, mix the 100 µl of virus solution with 400 µl of surrounding solution ($NaHCO_3$-free rASC medium).
(v) Initiate the transduction process by adding the virus solution to wells (500 µl per well). Put the six-well plate on a rocking plate. Continue the transduction process by gently shaking the plate (10 times per min) for 6 h at room temperature.
(vi) Terminate the transduction process by removing the virus solution.
(vii) Add the rASC sheet fabrication medium containing 3 mM sodium butyrate (2 ml per well) and incubate it at 37 °C for 15 h.
(viii) *Day 4*. Replace the medium with fresh rASC sheet fabrication medium and continue to incubate the cell sheet for 9 h.
(ix) Wash the cell sheet with PBS twice and statically incubate with 0.05% (wt/vol) trypsin-EDTA (0.5 ml per well) for 10 s.
▲ **CRITICAL STEP** The time for trypsin-EDTA treatment is crucial. Longer treatment time (e.g., 2 min) may dissociate the cell sheet. For rASC sheet, 10 s is sufficient to detach the cell sheet without compromising the sheet integrity.
(x) Remove trypsin-EDTA and wash the rASC sheet with PBS twice.
(xi) Remove residual PBS and replenish with 2 ml of PBS. Gently shake the six-well plate by hand until the transparent rASC sheet spontaneously detaches from the well. In general, gentle shaking for <1 min is sufficient to detach the cell sheet.
▲ **CRITICAL STEP** To quantify the transduction efficiency, the cell sheet can be dissociated by prolonging the trypsin-EDTA treatment time to 5 min. The dissociated cells are analyzed by flow cytometry.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
42	No CPE	Bacmid is in low quantity or is of low quality	Repeat the bacmid extraction steps
	Contamination	Bacmid is contaminated during the extraction procedure	Repeat the bacmid extraction steps aseptically within the laminar flow hood (especially during air-dry and resuspension, steps 33–34)
56	Low virus titer	Sf-9 cells are unhealthy or have been passaged too many times	Re-thaw the Sf-9 cell stock and grow Sf-9 cells to log phase for virus infection
57A(ix), 57B(viii), 57C(xiii)	Low transduction efficiency or transgene expression	Promoter is weak in the cell	Use a different promoter
		Virus dosage is insufficient	Increase the virus dosage
	Cell death	The overexpressed transgene is toxic to cells	Decrease the virus dosage

● TIMING

Steps 1–33, generation of recombinant bacmid: 5 d
Steps 34–44, production of recombinant baculovirus (P0): 5–7 d
Steps 45–56, amplification of recombinant baculovirus (P2): 10–12 d
Step 57, transduction of mammalian cells with baculovirus: 3–6 d
Box 1, titration of recombinant baculovirus: 12–15 d
Box 2, isolation and culture of rASCs: 21–23 d

ANTICIPATED RESULTS

The transduction procedures in Step 57 option A yield high transduction efficiencies and transgene expression levels in a wide variety of mammalian cell lines (**Fig. 4**). However, iPS cells are less permissive to baculovirus transduction. At MOI 100, the transduction efficiency was ~45% using option B (data not shown). For recombination efficiency analysis, the cells were co-transduced with the recombinase-expressing baculovirus (Bac-FLPo or Bac-Cre) and the substrate baculovirus Bac-ALF and

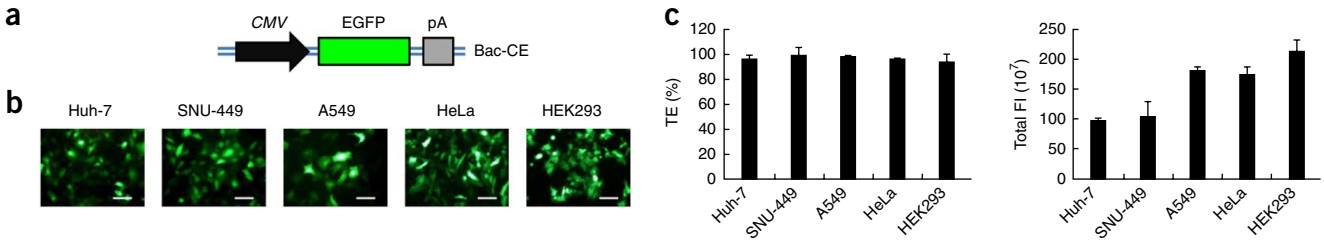


Figure 4 | Analysis of transduction efficiency in mammalian cell lines transduced with an EGFP-expressing baculovirus (Bac-CE). (**a**) Illustration of Bac-CE. (**b**) Fluorescence micrographs captured at 1 dpt. Magnification, 200×. Scale bars, 100 µm. (**c**) Transduction efficiency (TE) and total fluorescence intensity (FI). Huh-7, SNU-449, A549, HeLa and HEK293 cells were transduced with Bac-CE at MOI 100 and analyzed by flow cytometry. The data represent the means ± s.d. of three independent experiments.

analyzed by flow cytometry at 1 dpt. Co-transduction resulted in recombination efficiencies approaching 90%–95% in various cell lines (e.g., Huh-7, HeLa, BHK) and stem cells such as rASCs (**Fig. 5**)[45].

rASCs were co-transduced with Bac-FLPo (MOI 100) and a substrate baculovirus (Bac-FCBW, MOI 100) encoding BMP-2 (a growth factor that stimulates osteogenic differentiation of ASCs), as described in Step 57C. The co-transduction enhanced and prolonged the BMP-2 expression to ~21 d, which significantly exceeded the BMP-2 expression duration (~7 d) conferred by a conventional baculovirus vector Bac-CB (**Fig. 6**)[54]. In parallel, the co-transduced rASCs were collected at 1 dpt, seeded to scaffolds and implanted into critical-size (8 mm in diameter) calvarial defects in NZW rabbits[54]. To compare the calvarial bone healing effects, two types of scaffolds were used: (i) gelatin sponge that stimulates calvarial bone healing and (ii) PLGA (poly(lactic-co-glycolic acid)) scaffold that does not promote calvarial bone healing. The co-transduced rASCs/gelatin constructs successfully filled ~86% of the defect area and ~61% of the defect volume at 12 weeks post transplantation, whereas the control cell/scaffold constructs (mock-transduced rASCs/gelatin and co-transduced rASCs/PLGA) failed to heal the defects (**Fig. 7**)[54].

To treat myocardial infarction (MI), rASC sheets were co-transduced with Bac-FLPo and a substrate vector expressing VEGF (a growth factor stimulating angiogenesis), as described in Step 57D. Epicardial implantation of the VEGF-expressing rASC sheet to rabbit MI models reduced the infarct size, improved cardiac functions, prevented myocardial wall thinning, suppressed myocardium fibrosis and enhanced blood vessel

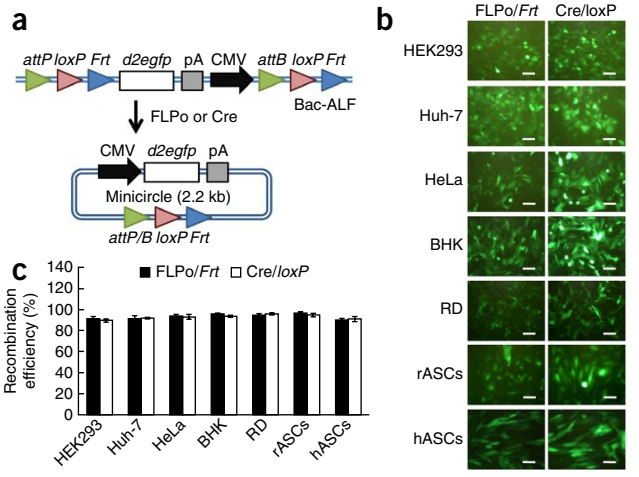
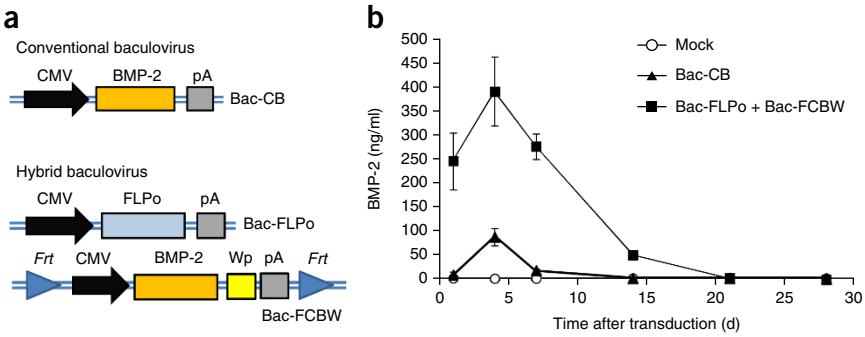


Figure 5 | Recombination efficiency in different cells. (**a**) Illustration of Bac-ALF vector and recombined minicircle. Co-transduction of cells with the recombinase-expressing baculovirus and Bac-ALF results in the transgene excision and recombination to form an ~2.2-kb minicircle and hence the placement of *d2egfp* downstream of the CMV promoter. Therefore, analysis of GFP+ cells yields recombination efficiency. (**b**) Comparison of Cre- and FLPo-mediated recombination in different mammalian cells. At 1 dpt, the d2EGFP expression was monitored with a fluorescence microscope. Scale bars, 100 µm. (**c**) Recombination efficiency analysis in different cells. HEK293, Huh-7, HeLa, BHK, RD, rASCs and hASCs were co-transduced with Bac-Cre or Bac-FLPo (MOI 50) and Bac-ALF (MOI 200). At 1 dpt, the cells were analyzed by flow cytometry. The data represent the means ± s.d. of three independent experiments. Panels **b** and **c** were adapted from ref. 45 under Creative Commons license 3.0.

Figure 6 | Prolonged BMP-2 expression via hybrid baculovirus. (**a**) Illustration of the BMP-2-expressing conventional baculovirus (Bac-CB) and hybrid baculovirus vectors (Bac-FCBW). (**b**) Time course of BMP-2 expression in rASCs. rASCs were mock-transduced, singly transduced with Bac-CB (MOI 100) or co-transduced with Bac-FLPo (MOI 50) and Bac-FCBW (MOI 100). The culture medium was collected at 1, 4, 7, 14, 21 and 28 dpt and analyzed by ELISA. The data represent the means ± s.d. of three independent experiments.



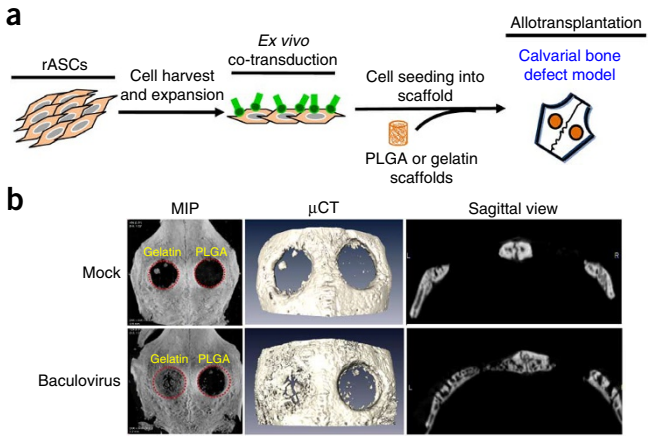


Figure 7 | rASCs engineered with the hybrid baculovirus augment the healing of bone defects. (**a**) Schematic illustration of experimental procedures including transduction, seeding and transplantation. rASCs were co-transduced with Bac-FLPo (MOI 100) and Bac-FCBW (MOI 100) and seeded into PLGA or gelatin sponges at 1 dpt, followed by transplantation into rabbit calvarial bone defects. (**b**) The maximum-intensity projection (MIP) images, microcomputed tomography (μCT) and sagittal-view images obtained at 12 weeks post implantation. This experiment was performed on five animals, and representative data are presented showing that baculovirus-transduced rASCs/gelatin scaffolds (persistently expressing BMP-2) significantly improved calvarial bone healing in comparison with the controls (mock-transduced rASCs/gelatin and co-transduced rASCs/PLGA). Panel **b** is adapted from: The use of ASCs engineered to express BMP2 or TGF-β3 within scaffold constructs to promote calvarial bone repair, C.-Y. Lin *et al.*, *Biomaterials* **34**, 9401–9412, Copyright 2013, with permission from Elsevier (ref. 54).

formation, implicating the potential of baculovirus-engineered, VEGF-expressing rASC sheet for future MI treatment[22]. Thus, these examples of the types of results optioned using baculovirus transduction demonstrate the potential of the hybrid baculovirus system and transduction protocol for stem cell engineering and tissue regeneration.

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1. Boyce, F.M. & Bucher, N.L.R. Baculovirus-mediated gene transfer into mammalian cells. *Proc. Natl. Acad. Sci. USA* **93**, 2348–2352 (1996).
2. Hofmann, C. *et al.* Efficient gene-transfer into human hepatocytes by baculovirus vectors. *Proc. Natl. Acad. Sci. USA* **92**, 10099–10103 (1995).
3. Chen, C.-Y., Lin, C.-Y., Chen, G.-Y. & Hu, Y.-C. Baculovirus as a gene delivery vector: recent understandings of molecular alterations in transduced cells and latest applications. *Biotechnol. Adv.* **29**, 618–631 (2011).
4. Hu, Y.-C. Baculovirus vectors for gene therapy. *Adv. Virus. Res.* **68**, 287–320 (2006).
5. Airenne, K.J. *et al.* Baculovirus: an insect-derived vector for diverse gene transfer applications. *Mol. Ther.* **21**, 739–749 (2013).
6. Hu, Y.-C. Baculoviral vectors for gene delivery: a review. *Curr. Gene Ther.* **8**, 54–65 (2008).
7. Zeng, J. *et al.* Baculoviral vector-mediated transient and stable transgene expression in human embryonic stem cells. *Stem Cells* **25**, 1055–1061 (2007).
8. Chen, G.-Y. *et al.* A graphene-based platform for induced pluripotent stem cells culture and differentiation. *Biomaterials* **33**, 418–427 (2012).
9. Chen, Y.-H. *et al.* Baculovirus-mediated production of HDV-like particles in BHK cells using a novel oscillating bioreactor. *J. Biotechnol.* **118**, 135–147 (2005).
10. Lesch, H.P. *et al.* Production and purification of lentiviral vectors generated in 293T suspension cells with baculoviral vectors. *Gene Ther.* **18**, 531–538 (2011).
11. Kost, T.A., Condreay, J.P. & Ames, R.S. Baculovirus gene delivery: a flexible assay development tool. *Curr. Gene Ther.* **10**, 168–173 (2010).
12. Liu, C.Y.-Y., Chen, H.-Z. & Chao, Y.-C. Maximizing baculovirus-mediated foreign proteins expression in mammalian cells. *Curr. Gene Ther.* **10**, 232–241 (2010).
13. Grabherr, R. & Ernst, W. Baculovirus for eukaryotic protein display. *Curr. Gene Ther.* **10**, 195–200 (2010).
14. Chen, C.-L. *et al.* Development of hybrid baculovirus vectors for artificial MicroRNA delivery and prolonged gene suppression. *Biotechnol. Bioeng.* **108**, 2958–2967 (2011).
15. Hu, Y.-C., Yao, K. & Wu, T.-Y. Baculovirus as an expression and/or delivery vehicle for vaccine antigens. *Expert Rev. Vaccines* **7**, 363–371 (2008).
16. Madhan, S., Prabakaran, M. & Kwang, J. Baculovirus as vaccine vectors. *Curr. Gene Ther.* **10**, 201–213 (2010).
17. Wang, S. & Balasundaram, G. Potential cancer gene therapy by baculoviral transduction. *Curr. Gene Ther.* **10**, 214–225 (2010).
18. Lu, C.-H. *et al.* Recent progresses in gene delivery-based bone tissue engineering. *Biotechnol. Adv.* **31**, 1695–1706 (2013).
19. Liao, Y.-H. *et al.* Osteogenic differentiation of adipose-derived stem cells and calvarial defect repair using baculovirus-mediated co-expression of BMP-2 and miR-148b. *Biomaterials* **35**, 4901–4910 (2014).
20. Lu, C.-H. *et al.* Regenerating cartilages by engineered ASCs: prolonged TGF-β3/BMP-6 expression improved articular cartilage formation and restored zonal structure. *Mol. Ther.* **22**, 186–195 (2014).
21. Chen, H.-C. *et al.* Combination of baculovirus-mediated BMP-2 expression and rotating-shaft bioreactor culture synergistically enhances cartilage formation. *Gene Ther.* **15**, 309–317 (2008).
22. Yeh, T.-S. *et al.* Baculovirus-transduced, VEGF-expressing adipose-derived stem cell sheet for the treatment of myocardium infarction. *Biomaterials* **35**, 174–184 (2014).
23. Kost, T.A., Condreay, J.P. & Jarvis, D.L. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat. Biotechnol.* **23**, 567–575 (2005).
24. Ho, Y.-C., Chen, H.-C., Wang, K.-C. & Hu, Y.-C. Highly efficient baculovirus-mediated gene transfer into rat chondrocytes. *Biotechnol. Bioeng.* **88**, 643–651 (2004).
25. Wang, K.-C. *et al.* Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. *Biotechnol. Bioeng.* **89**, 464–473 (2005).
26. Sung, L.-Y. *et al.* Modulation of chondrocyte phenotype via baculovirus-mediated growth factor expression. *Biomaterials* **28**, 3437–3447 (2007).
27. Lo, W.-H. *et al.* Development of a hybrid baculoviral vector for sustained transgene expression. *Mol. Ther.* **17**, 658–666 (2009).
28. Merrihew, R.V. *et al.* Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells. *J. Virol.* **75**, 903–909 (2001).
29. Luo, W.-Y. *et al.* Adaptive immune responses elicited by baculovirus and impacts on subsequent transgene expression *in vivo*. *J. Virol.* **87**, 4965–4973 (2013).
30. Strauss, R. *et al.* Baculovirus-based vaccination vectors allow for efficient induction of immune responses against *Plasmodium falciparum* circumsporozoite protein. *Mol. Ther.* **15**, 193–202 (2007).

31. Cheshenko, N., Krougliak, N., Eisensmith, R.C. & Krougliak, V.A. A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. *Gene Ther.* **8**, 846–854 (2001).
32. Zhou, J. & Blissard, G.W. Display of heterologous proteins on *gp64* null baculovirus virions and enhanced budding mediated by a vesicular stomatitis virus G-stem construct. *J Virol.* **82**, 1368–1377 (2008).
33. Hofmann, C. & Strauss, M. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. *Gene Ther.* **5**, 531–536 (1998).
34. Wang, C.-Y. & Wang, S. Adeno-associated virus inverted terminal repeats improve neuronal transgene expression mediated by baculoviral vectors in rat brain. *Hum. Gene Ther.* **16**, 1219–1226 (2005).
35. Abe, T. *et al.* Involvement of the Toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. *J. Virol.* **79**, 2847–2858 (2005).
36. Kukkonen, S.P. *et al.* Baculovirus capsid display: a novel tool for transduction imaging. *Mol. Ther.* **8**, 853–862 (2003).
37. Condreay, J.P., Witherspoon, S.M., Clay, W.C. & Kost, T.A. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl. Acad. Sci. USA* **96**, 127–132 (1999).
38. Hsu, C.-S., Ho, Y.-C., Wang, K.-C. & Hu, Y.-C. Investigation of optimal transduction conditions for baculovirus-mediated gene delivery into mammalian cells. *Biotechnol. Bioeng.* **88**, 42–51 (2004).
39. Peng, Y., Song, J., Lu, J. & Chen, X. The histone deacetylase inhibitor sodium butyrate inhibits baculovirus-mediated transgene expression in Sf9 cells. *J. Biotechnol.* **131**, 180–187 (2007).
40. Ho, Y.-C. *et al.* Transgene expression and differentiation of baculovirus-transduced human mesenchymal stem cells. *J. Gene. Med.* **7**, 860–868 (2005).
41. Ho, Y.-C. *et al.* Baculovirus transduction of human mesenchymal stem cell-derived progenitor cells: variation of transgene expression with cellular differentiation states. *Gene Ther.* **13**, 1471–1479 (2006).
42. Lu, L., Yu, L. & Kwang, J. Baculovirus surface-displayed hemagglutinin of H5N1 influenza virus sustains its authentic cleavage, hemagglutination activity, and antigenicity. *Biochem. Biophys. Res. Commun.* **358**, 404–409 (2007).
43. Kenoutis, C. *et al.* Baculovirus-mediated gene delivery into mammalian cells does not alter their transcriptional and differentiating potential but is accompanied by early viral gene expression. *J. Virol.* **80**, 4135–4146 (2006).
44. Shen, H.-C. *et al.* Baculovirus-mediated gene transfer is attenuated by sodium bicarbonate. *J. Gene Med.* **9**, 470–478 (2007).
45. Sung, L.Y. *et al.* Enhanced and prolonged baculovirus-mediated expression by incorporating recombinase system and in *cis* elements: a comparative study. *Nucleic Acids Res.* **41**, e139 (2013).
46. Lu, C.-H. *et al.* Improved chondrogenesis and engineered cartilage formation from TGF-β3-expressing adipose-derived stem cells cultured in the rotating-shaft bioreactor. *Tissue Eng. Part A* **18**, 2114–2124 (2012).
47. Hu, Y.-C., Tsai, C.-T., Chang, Y.-J. & Huang, J.-H. Enhancement and prolongation of baculovirus-mediated expression in mammalian cells: focuses on strategic infection and feeding. *Biotechnol. Prog.* **19**, 373–379 (2003).
48. Spenger, A. *et al.* Influence of promoter choice and trichostatin A treatment on expression of baculovirus delivered genes in mammalian cells. *Protein Expr. Purif.* **38**, 17–23 (2004).
49. Mahonen, A.J. *et al.* Post-transcriptional regulatory element boosts baculovirus-mediated gene expression in vertebrate cells. *J. Biotechnol.* **131**, 1–8 (2007).
50. Luo, W.Y. *et al.* Development of the hybrid *Sleeping Beauty*-baculovirus vector for sustained gene expression and cancer therapy. *Gene Ther.* **19**, 844–851 (2012).
51. Lin, C.-Y. *et al.* The role of adipose-derived stem cells engineered with the persistently expressing hybrid baculovirus in the healing of massive bone defects. *Biomaterials* **32**, 6505–6514 (2011).
52. Narsinh, K.H. *et al.* Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. *Nat. Protoc.* **6**, 78–88 (2010).
53. Lin, C.-Y. *et al.* Augmented healing of critical-size calvarial defects by baculovirus-engineered MSCs that persistently express growth factors. *Biomaterials* **33**, 3682–3692 (2012).
54. Lin, C.-Y. *et al.* The use of ASCs engineered to express BMP2 or TGF-β3 within scaffold constructs to promote calvarial bone repair. *Biomaterials* **34**, 9401–9412 (2013).
55. Turan, S. *et al.* Recombinase-mediated cassette exchange (RMCE): traditional concepts and current challenges. *J. Mol. Biol.* **407**, 193–221 (2011).
56. Jager, L. *et al.* A rapid protocol for construction and production of high-capacity adenoviral vectors. *Nat. Protoc.* **4**, 547–564 (2009).
57. Martinez-Morales, F. *et al.* Chromosomal integration of heterologous DNA in *Escherichia coli* with precise removal of markers and replicons used during construction. *J. Bacteriol.* **181**, 7143–7148 (1999).
58. O'Reilly, D., Miller, L. & Luckow, V. *Baculovirus Expression Vectors: a Laboratory Manual*. (W.H. Freeman, 1992).
59. Papapetrou, E.P. & Sadelain, M. Generation of transgene-free human induced pluripotent stem cells with an excisable single polycistronic vector. *Nat. Protoc.* **6**, 1251–1273 (2011).
60. Sugii, S., Kida, Y., Berggren, W.T. & Evans, R.M. Feeder-dependent and feeder-independent iPS cell derivation from human and mouse adipose stem cells. *Nat. Protoc.* **6**, 346–358 (2011).
61. Pijlman, G.P., Van Schijndel, J.E. & Vlak, J.M. Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells. *J. Gen. Virol.* **84**, 2669–2678 (2003).
62. Zeng, J.-M. *et al.* High-efficiency transient transduction of human embryonic stem cell-derived neurons with baculoviral vectors. *Mol. Ther.* **17**, 1585–1593 (2009).
63. Tsai, C.-T. *et al.* Factors influencing the production and storage of baculovirus for gene delivery: an alternative perspective from the transducing titer assay. *Enzyme Microb. Technol.* **40**, 1345–1351 (2007).
64. Vannucci, L. *et al.* Viral vectors: a look back and ahead on gene transfer technology. *New Microbiol.* **36**, 1–22 (2013).
65. Verma, I.M. & Weitzman, M.D. Gene therapy: twenty-first century medicine. *Ann. Rev. Biochem.* **74**, 711–738 (2005).
66. Kay, M.A. State-of-the-art gene-based therapies: the road ahead. *Nat. Rev. Genet.* **12**, 316–328 (2011).
67. Balakrishnan, B. & Jayandharan, G.R. Basic biology of adeno-associated virus (AAV) vectors used in gene therapy. *Curr. Gene Ther.* **14**, 1–15 (2014).
68. Reed, L.J. & Muench, H. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).