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Baculovirus: An Insect-derived Vector for Diverse Gene Transfer Applications

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Insect-derived baculoviruses have emerged as versatile and safe workhorses of biotechnology. Baculovirus expression vectors (BEVs) have been applied widely for crop and forest protection, as well as safe tools for recombinant protein production in insect cells. However, BEVs ability to efficiently transduce noninsect cells is still relatively poorly recognized despite the fact that efficient baculovirus-mediated *in vitro* and *ex vivo* gene delivery into dormant and dividing vertebrate cells of diverse origin has been described convincingly by many authors. Preliminary proof of therapeutic potential has also been established in preclinical studies. This review summarizes the advantages and current status of baculovirus-mediated gene delivery. Stem cell transduction, preclinical animal studies, tissue engineering, vaccination, cancer gene therapy, viral vector production, and drug discovery are covered.

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INTRODUCTION

Gene therapy has matured to the point whereby the high potential of this therapeutic modality can now be realized for treating diseases with unmet needs, such as severe immune deficiencies,[1,2] ocular diseases,[3] and cancer.[4] Several virus-derived vectors and nonvirus gene transfer agents have been used to address these applications. However, the safety, generation, immune response, duration of expression, and gene delivery capacity, still limits the use of these gene drugs.[5] Thus, better vectors are still needed. Since first developed for overexpression of heterologous proteins,[6] baculoviruses have been applied widely in biotechnology due to their versatility and the close resemblance between insect cell and mammalian cell protein posttranslational modification systems.[7] Studies conducted over the last decade have also established the ability of baculoviruses to deliver genes into a wide range of species and types of vertebrate cells.[7–9]

The *Baculoviridae* are large, oblong-shaped (30–60 × 250–300 nm) enveloped particles with a circular, double-stranded DNA genome, approximately 80–180 kb, that naturally infect mainly Lepidoptera (butterflies and moth) larvae hosts.[10] The family consists of about 700 known members of which the genome is characterized for almost 60 species.[11] Baculoviruses are common in nature and in our food crops and are well known as pesticides and convenient tools for recombinant protein production in insect cells.[6] No diseases have been linked to these viruses in any organism outside the phylum *Arthropoda*.[12,13] Classically, baculoviruses were divided into two morphologically distinct genera: nuclear polyhedrosis viruses (NPVs) and granulosis viruses. However, baculovirus phylogeny follows the classification of the hosts more closely than the morphological traits, and a new classification divides baculoviruses into four genera: α baculovirus (lepidopteran-specific NPV), β baculovirus (lepidopteran-specific granulosis viruses), γ baculovirus (hymenopteran-specific NPV) and Δ baculovirus (dipteran-specific NPV).[14] α baculoviruses are further divided into group I and II by their essential envelope glycoproteins gp64 and F-protein, respectively. NPVs obtain their envelope from the nuclear membrane and are occluded within a polyhedrin protein matrix, forming large (1–15 μm) polyhedral inclusion bodies. NPVs can be further distinguished on the basis of whether they contain in the polyhedral inclusion bodies a single nucleocapsid or multiple nucleocapsids. Unlike NPVs, granulosis viruses are embedded only as a single virion into a small inclusion body.[11]

Baculoviruses have many attractive features. They are easy to manipulate,[11] able to carry large (at least 38 kbp)[15] and multiple DNA inserts,[16] and can be readily produced and purified at high titers.[11] The inherent inability of baculoviruses to replicate in mammalian cells and low cytotoxicity make them potentially safe candidates for therapeutic gene delivery.[12,13] Hepatocytes were the first noninsect origin cell type shown to be susceptible to baculovirus transduction in the mid 1990s.[17,18] However, today it is evident, with only a few known exceptions, virtually all types and species of cells, including primary and stem cells, can be transduced efficiently.[7–9,19] Baculovirus expression vectors (BEVs) can also be applied for the safe and efficient generation

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of induced pluripotent stem cells.[20] Gene delivery efficacy can be boosted by enhancing vector design and optimizing transduction conditions.[19] Translation of this technology to therapeutic use has begun and is supported not only by the availability of the latest generation vectors,[21,22] but also by membrane technology-based virus purification protocols amenable to high-scale and good manufacturing practice quality virus generation.[23,24] Baculovirus-mediated gene transfer has already demonstrated therapeutic efficacy in *ex vivo* and *in vivo* gene therapy studies.[25–28] The approval of baculovirus insect cell produced human vaccine components (Cervarix, GlaxoSmithKline, Rixensart, Belgium and Provenge, Dendreon, Seattle, WA) by the European Medicines Agency and US Food and Drug Administration sets an important precedent for future regulatory approval[29] of baculovirus-based gene therapy.

Autographa californica multiple nucleopolyhedrovirus (*Ac*MNPV), often considered the prototypic baculovirus, has a circular double-stranded DNA genome (~134 kb) inside a rod-shaped nucleocapsid (25 × 260 nm).[10] The genome of *Ac*MNPV has been sequenced, whereas the structure of virion is only known by protein composition (no 3D structure available).[30] The transgene capacity of *Ac*MNPV in theory is unlimited as the capsid can freely extend.[11] Multiple high-titer viruses can be generated and titered in parallel in a relatively short time frame in insect cells.[11,31] Insect cells are easy to cultivate as adherent cells or in suspension and adapt well to growth in serum-free media.[11] The recombinant viruses may be stored for long periods at 4 °C or within cryopreserved infected cells (titerless infected-cells preservation and scale-up).[32,33] *Ac*MNPV can be manipulated in biosafety level 1 facilities because it is strictly an insect pathogen and not known to cause disease in vertebrates.[12,13]

Progeny production of baculoviruses is biphasic (**Figure 1**).[34] The budded virus (BuV) spreads the infection within the host and the occlusion-derived virus between hosts. The two forms of virus differ only in their envelopes. The envelope of BuV derives from the cell membrane, whereas the occlusion-derived virus derives its envelope from the nuclear membrane.[35] The BuV form is the most widely used in biotechnology and enters insect and other host cells by endocytosis. The portal into mammalian cells has been shown recently to resemble phagocytosis more closely than pinocytosis.[36–39] However, the receptor(s) or cell-signaling events mediating attachment and entry are largely unknown, and consensus as to the exact endocytic pathway(s) involved is lacking. Common cell surface ligands consisting of heparan sulphate proteoglycan, phospholipid, integrin, or lectin, are likely receptor candidates.[9] Receptor binding and early steps of endocytosis are followed by an acid-induced fusion event, which releases the nucleocapsid into the cytoplasm.[40–42] The capsids travel through the cytosol towards the nucleus by actin polymerization behind the nucleocapsid.[43,44] *Ac*MNPV equipped with a gene under the control of a promoter active in the target cell is able to transduce both nondividing and dividing mammalian cells[40,45] which implies that nucleocapsid is able to transport the genome across the intact nuclear membrane through the nuclear pore complex.[46] Indeed, electron microscopy has revealed that nucleocapsids dock at the nuclear pore complex and apparently intact DNA filled nucleocapsids localize to the nucleoplasmic space[45,47] where the genome is released. However, the detailed molecular basis of transduction remains poorly understood in vertebrate cells and further studies are needed to resolve the pathway involved in efficient gene delivery.

GENE DELIVERY INTO VERTEBRATE CELLS

The first strong evidence of *Ac*MNPV's capability to enter a wide range of cells was gained while its safety for pest control was studied in the early 1980's.[48] The experiments showed that many vertebrate cells internalized *Ac*MNPV particles; however, no viral replication occurred. The lack of replication indicates that only the population of initial cells exposed to virus are transduced, with no cell-to-cell spread of the virus, and nontarget cells remaining unaffected. The early experiments were carried out with a wild-type virus lacking a vertebrate-compatible expression cassette. When Hofmann[17] and Boyce,[18] with their coworkers, in the mid 1990's outfitted *Ac*MNPV with an expression cassette driving a marker gene via a strong viral promoter (active in the target cells), they could detect transgene expression, especially in hepatocytes. These pioneering studies boosted interest in baculoviruses as potential vectors for gene therapy. Many subsequent studies have confirmed the safety and feasibility of *Ac*MNPV for efficient gene delivery in vertebrate cells, including cells other than those of hepatic origin.[8,9,25] The list of permissive cells is extensive, and still expanding.[7,9] It includes cell lines, primary cells, progenitor, and stem cells. Cells derived from human, monkey, porcine, rabbit, rat, feline, mouse, hamster, fish, avian, frog, and shrimp tissues have been transduced successfully.[7,9] Many cell types can be transduced by outfitting the *Ac*MNPV genome with a suitable expression cassette.[49] These modified vectors are often referred to as BacMam viruses. In those instances in which the transduction efficiency is low or higher rates of transgene expression are desirable, modified viruses along with optimized transduction conditions can be used.[19,50] Culture medium can have a role in transduction efficiency. Dulbecco's modified Eagle medium has been reported to support transduction poorly in some cell types and should be replaced with media such as RPMI1640, HyClone Classical Media, or Freestyle 293 Expression Medium.[51–53] Transduction in phosphate-buffered saline buffer has also been shown to be effective with cell types which can tolerate short-term removal from complete medium.[50] Transduction time is also important and virus treatment for at least 8 hours is recommended. Transient gene expression can also be extended by supertransduction[20] or applying episomal replication and transposon systems, such as Sleeping Beauty or PiggyBac, within baculovirus hybrid viruses.[54] The Woodchuck hepatitis virus posttranscriptional element provides significant benefit for baculovirus-mediated gene delivery by boosting substantially transgene expression.[55] This can reach efficacy comparable to enhancement achievable with histone deacetylate inhibitors,[56] such as a sodium butyrate, trichostatin A, or valproic acid, but without HDAI associated toxicity. Finally, the virus surface can be modified to augment *Ac*MNPV effectiveness in gene delivery.[57] Vesicular stomatitis virus G (VSV-G) transmembrane glycoprotein[58,59] or surface display of its truncated transmembrane fragment (VSV-GED)[60] are examples of successful pseudotyping. Surface display of avidin, biotin, and a lymphatic homing peptide or polymer coating with polyethyl glycol or polyethylenimine have also been used to improve the *in vitro* and *in vivo* transduction efficiency of *Ac*MNPV.[19,61]

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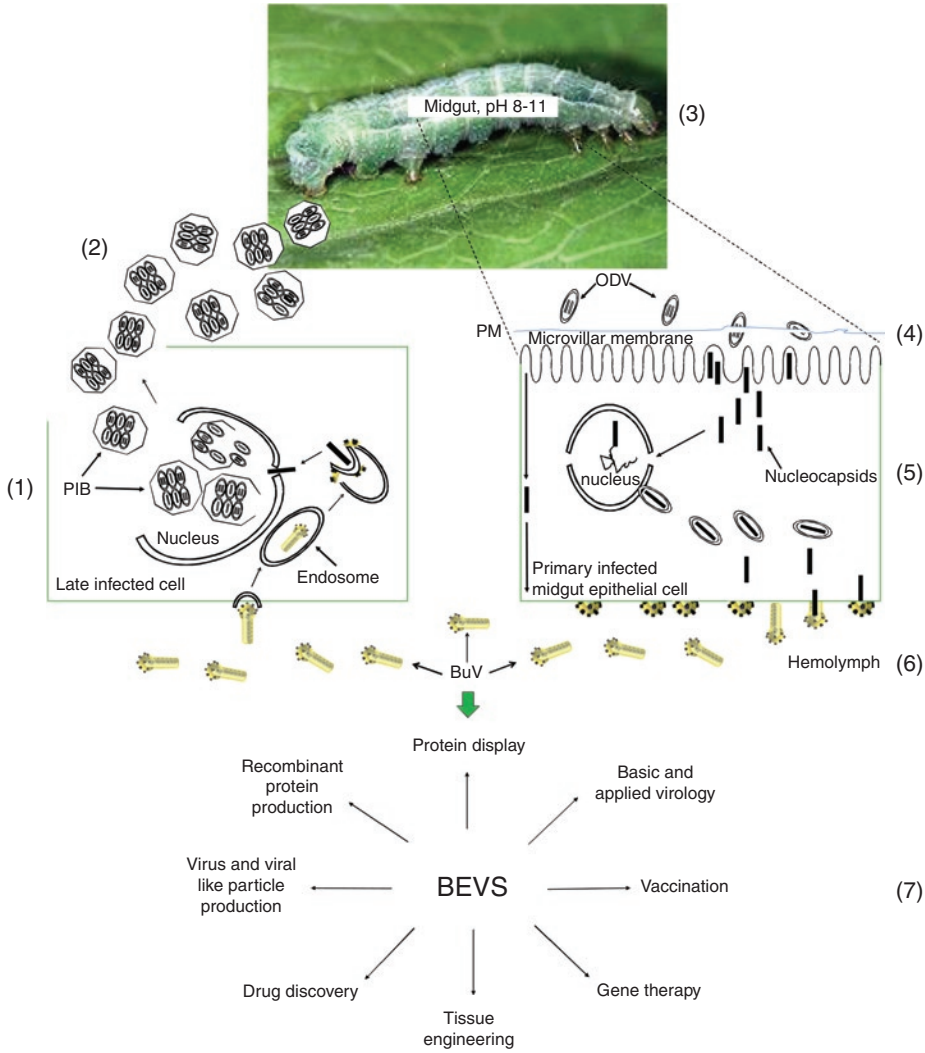

Figure 1 Life cycle and applications of baculoviruses. Baculoviruses have a biphasic life cycle in which two different forms of the virus spread through vertical and horizontal infection. Infection between hosts is mediated by virions occluded in PIBs late in infection (1). PIBs subsist in nature for years awaiting larva to ingest them (2). PIBs dissolve and release the ODVs only in the unique alkaline environment of the larval midgut (3). ODVs then penetrate the peritrophic membrane and infect epithelial cells by direct fusion of the virion and cell membrane (4). Nucleocapsids travel to the nucleus and initiate replication (5). To evade host defense mechanisms, some nucleocapsids traverse the cell directly to speed up the infection. The infected midgut cells produce the BuV form of the virus which spreads the infection within the larva through the tracheal system and hemolymph (6). The BuV can be used as a versatile tool for a plethora of biotechnological applications (7). BEVS, baculovirus expression vector system; BuV, budded virus; ODV, occlusion-derived virus; PIB, polyhedral inclusion body; PM, peritrophic membrane.

PRECLINICAL STUDIES

*Ac*MNPV is a promising vector not only for *in vitro* gene delivery, but also for *ex vivo* and *in vivo* gene delivery (**Figure 2**).[9,25–28] The studies carried out in mice, rat, and rabbit models have revealed the central nervous system, eye (see **Figure 2d–f**), and testis as good targets (for a recent comprehensive review see).[25] These targets represent immunoprivileged areas with an intrinsic ability to prevent the activation of adaptive and innate immune responses to antigens.[61] Indeed, although pre-existing immunity does not restrict baculovirus-mediated gene delivery,[62] both alternative and classical complement pathways are restrictive.[63,64] This fact delayed the first successful *in vivo* applications of baculovirus-mediated gene transfer to the beginning of the new millennium,[65,66] although the use of these viruses in an *ex vivo* context in mice was reported soon after the discovery that *Ac*MNPV can efficiently transduce hepatocytes.[67] Innate immunity-related problems can be avoided in *ex vivo* protocols, and promising results have been observed in cartilage and bone tissue engineering (see below). The low and transient immune response in the *ex vivo* context could also be useful in organ transplantation.[68]

Despite the complement-mediated inactivation of *Ac*MNPV, a meaningful angiogenic response has recently been reported in immunocompetent rabbit skeletal muscle[22] and rat heart[69] after intramuscular and intramyocardial delivery, respectively. Conversely, the immunogenicity of *Ac*MNPV can be taken advantage of in cancer therapy[27] and vaccination (see below).[26] Suppression of human epidermal-derived tumor growth in mice by programmed cell death gene delivery[70] and induction of lung cancer and melanoma antitumor immunity using baculovirus-infected bone marrow-derived dendritic cells,[71] represent the most recent examples. Other

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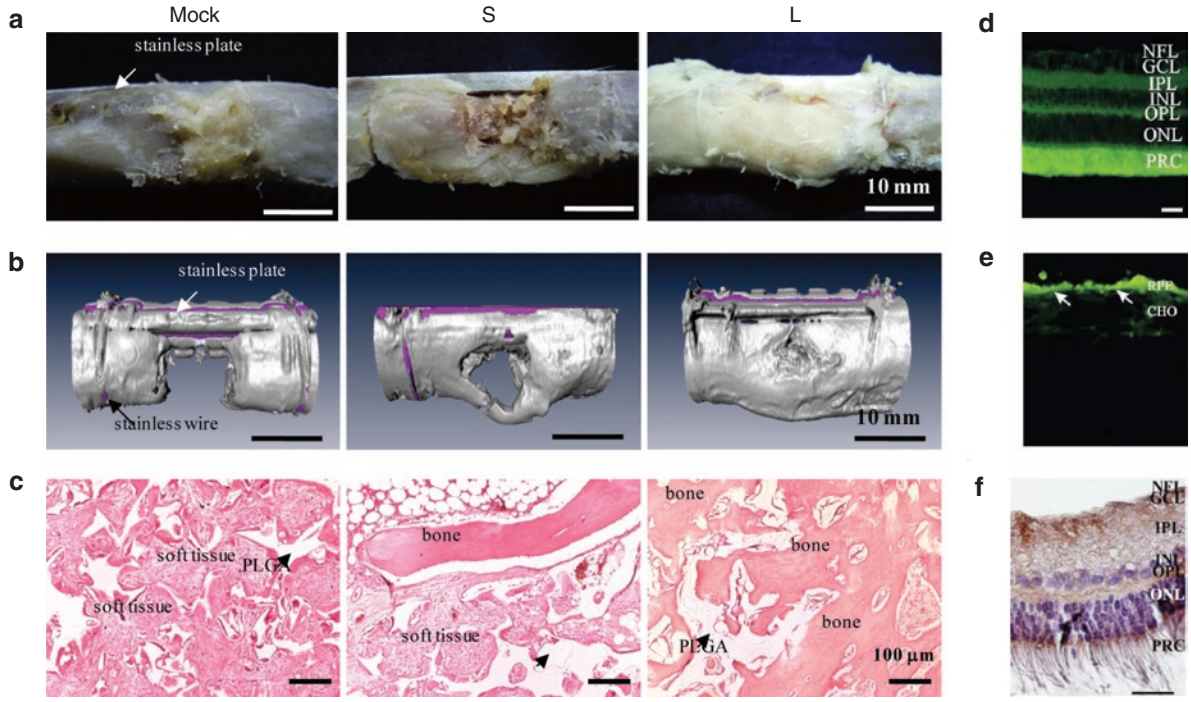

Figure 2 ***Ac*MNPV shows a high transduction efficacy *ex vivo* and *in vivo*.** (**a**–**c**) ASCs genetically modified by the hybrid baculovirus ameliorate the healing of segmental bone defects in rabbits. The ASCs derived from the subcutaneous fat pad of NZW rabbits were transduced with hybrid baculovirus vectors conferring sustained expression of *BMP-2* or *VEGF*, mixed with cells at a 4:1 ratio, loaded into cylindrical poly(l-lactide-co-glycolide) scaffolds (1.5×10^6 cells/scaffold) and transplanted into the critical-sized segmental defects at the femora of NZW rabbits (two scaffolds/defect, designated L group). The S group comprised PLGA scaffolds and ASCs that were transduced with the conventional baculoviruses transiently expressing *BMP-2* or *VEGF* and transplanted in an identical manner. The mock group consisted of PLGA scaffolds and mock-transduced ASCs as the negative control. After 12 weeks transplantation, (**a**) gross appearance examination, (**b**) μCT analyses and hematoxylin & (**c**) eosin (H&E) staining collectively demonstrated that the L group (persistently expressing *BMP-2* and *VEGF*) gave rise to significant new bone formation and bridging of bone defect in comparison with the S group (transiently expressing *BMP-2* and *VEGF*) and mock group. (**d**–**f**) Intravitreal transduction leads to expression of (**d**,**e**) green fluorescent protein and (**f**) vascular endothelial growth factor D also in the deeper layers of a rabbit eye. The arrows in **e** indicate RPE layer. ASCs, Adipose-derived stem cells; BMP-2, bone morphogenetic protein-2; CHO, choroidea; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; NFL, nerve fiber layer; NZW, New Zealand White; ONL, outer nuclear layer; OPL, outer plexiform layer; PLGA, poly(l-lactide-co-glycolide); PRC, photoreceptor cells; RPE, retinal pigment epithelium; VEGF, vascular endothelial growth factor. Scale bar = 20 μm. **a** and **b** were adapted from Lin *et al.*,[95] **c** shows C.Y. Lin and Y.-C. Hu; unpublished data, and **d**–**f** were adapted from Kinnunen *et al.* (2009).[80]

examples of preclinical efficacy include glioma treatment with p53 bearing *Ac*MNPV in combination with sodium butyrate[72] and the use of *Ac*MNPV as an antiangiogenic agent in model systems to treat prostate and human ovarian cancer.[54]

To overcome complement-related problems, complement inhibitors including antibody to the complement component 5, cobra venom factor,[63] a soluble complement receptor type 1,[73,74] inhibitors for protease activating the complement, FUT-175[58] and compstatin,[64] and positively charged polyethylenimine[75] have been used. Direct inoculation of baculovirus into various tissues such as rodent brain,[58,66,76,77] rodent skeletal muscle,[78] rodent testis,[58] rodent eye,[79] and rabbit eye[80] or mouse cancerous tissue including melanoma, lung cancer, brain cancer,[81] and hepatoma[82] have been reported. In some cases, baculoviruses possessing the VSV-G protein showed significantly higher gene expression than the unmodified virus in mice.[58,60,78] By using a silastic collar to avoid exposure to complement, rabbit carotid artery[65] was also amenable to baculovirus transduction. Furthermore, baculoviruses bearing chimeric GP64 fused with CD55/decay-accelerating factor (which block complement) showed some resistance to complement inactivation.[74] It has also been shown that GP64 can interact with decay-accelerating factor in a lipid raft and confer resistance to serum inactivation.[83] More recently, recombinant baculovirus displaying several complement regulatory proteins (decay-accelerating factor, factor H-like protein-1, C4b binding protein, and membrane cofactor protein) fused with the membrane anchor of VSV-G protein induced lower levels of inflammatory cytokines interleukin 1β (IL-1β), IL-6, and IL-12p40 in macrophages resulting in attenuation of liver inflammation in mice.[84] These results indicate that decay-accelerating factor display confers protection to baculovirus against complement inactivation and alleviates complement-mediated inflammation injury. Although baculoviruses bearing VSV-G protein have increased resistance to complement inactivation compared with the unmodified virus,[58,84] GP64 bearing lentiviral vectors, including feline immunodeficiency virus, exhibited more resistant to human and mouse complements than those bearing VSV-G protein.[85,86]

VACCINATION

Numerous efforts have been made to develop baculoviruses as viral vaccine vectors possessing adjuvant activity.[26,87,88] One of the advantages of using a recombinant baculovirus to carry a foreign antigen is that this method allows efficient gene delivery into immune competent cells and confers protective immunity *in vivo* without severe cytotoxicity. The absence of pre-existing antibodies to baculovirus is

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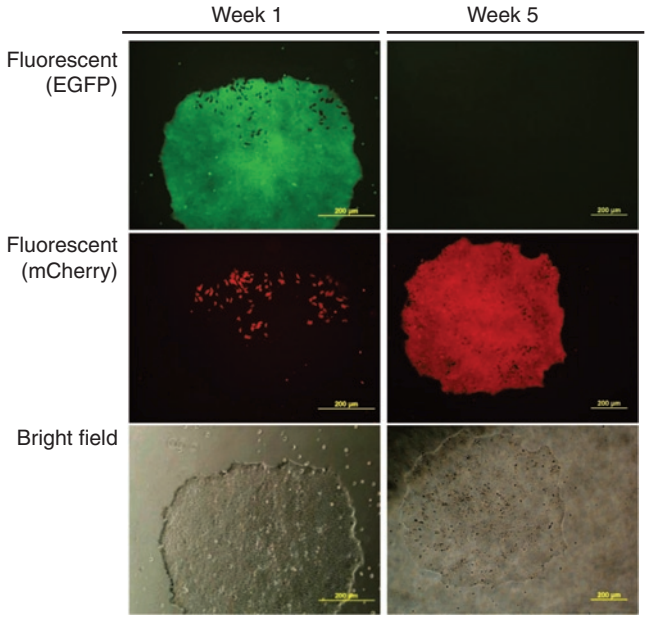

Figure 3 Cre recombinase-mediated cassette exchange in human embryonic stem cells (hESCs) by *Ac*MNPV. Homologous recombination was used to introduce the EGFP gene and heterospecific *loxP* sites into the *AAVS1* locus in hESCs. Two *Ac*MNPV vectors, one to express Cre recombinase and another containing the *mCherry* gene driven by the EF1α promoter, were used for Cre recombinase-mediated cassette exchange. mCherry-positive cells were visible at week 1 and increased by mechanical selection at the time for normal hESC subculture. After three rounds of selection (week 5), almost the entire hESC colony was mCherry-positive. *Ac*MNPV, *Autographa californica* multiple nucleopolyhedrovirus; EGFP, enhanced green fluorescent protein; hESCs, human embryonic stem cells; mCherry, red fluorescent protein. Scale bar = 200 µm. (unpublished data).

also an advantage of using baculoviruses for foreign gene transduction *in vivo*.[62] Although recombinant baculoviruses are capable of inducing antigen-specific humoral immune responses in mice, the induction of cellular immune responses are crucial for elimination of pathogens that cause chronic disease, such as hepatitis C virus and human immunodeficiency virus, because these viruses escape from humoral immune responses.[89] Further studies are needed to improve induction of the cellular immune response by baculoviral vectors to confer sufficient protective immunity *in vivo*.

Ligands for pattern recognition receptors, including toll-like receptor (TLR), retinoic-acid-inducible protein I (RIG-I)-like receptor (RLR), and the nucleotide binding oligomerization domain, are capable of stimulating innate and adaptive immune cells and have been proposed as promising adjuvant candidates. It has previously been shown that intranasal inoculation of mice with *Ac*MNPV induces protective immunity from a lethal challenge with influenza virus[90] and that *Ac*MNPV produces type I interferons in the immune cells of mice via a TLR-independent pathway.[91] Recent studies have further confirmed that *Ac*MNPV induces a host antiviral immune response through a TLR/RLR-independent pathway.[92] These observations suggest that a novel innate immune signaling pathway in addition to the TLR/RLR signaling pathway may contribute to the induction of the host antiviral immune response induced by baculoviruses. Further studies are needed to clarify the precise mechanisms involved, such as that of the DNA-sensing machinery, for the induction of type I interferons. The ability of baculoviruses to induce innate immunity makes them promising candidates for future adjuvant-containing vaccine vehicles. Benefits of *Ac*MNPV as an adjuvant are discussed below in the context of cancer vaccine therapy.

BACULOVIRAL TRANSDUCTION OF STEM CELLS

Advances in the identification, isolation, and derivation of human stem cells, especially the generation of human induced pluripotent stem cells, have raised great enthusiasm for regenerative medicine therapies. Among viral vectors capable of transducing stem cells, baculoviral vectors possess a unique feature in mediating high-level transient expression of transgenes, offering an attractive alternative to chemical or physical transfection methods and lentivirus-based transduction methods for stem cell genetic engineering.

As demonstrated with mesenchymal stem cells, baculoviral vectors can transduce multipotent stem cells obtained from different sources, including umbilical cord tissue,[93] bone marrow,[93,94] adipose tissue,[95] and human embryonic stem cells (hESCs).[96] Neural stem cells are another type of multipotent stem cells that can be effectively transduced by baculoviral vectors,[97,98] in which baculovirus-mediated transgene expression may last for up to 3 months.[97] Baculoviral vectors are also capable of transducing pluripotent stem cells such as hESCs[99–101] and induced pluripotent stem cells.[102,103] Currently, baculovirus transduction has been used to generate stem cells carrying therapeutic payloads of interest suitable for cartilage and bone tissue engineering and targeted glioma gene therapy. In these environments baculoviral transduction provides transgene expression from days to weeks fitting the needed therapeutic window. Baculovirus-mediated transient transduction may be useful as well in controlling the differentiation and stimulating the expansion of stem cells for medical applications.

In addition to providing transient transgene expression, baculoviral vectors can be tailored to permit stable transgene expression in stem cells. As baculovirus is a nonintegrating virus, a mechanism that promotes genomic integration needs to be incorporated into the vectors. In an initial study,[99] the *rep 78/68* genes and inverted terminal repeat sequences from adeno-associated virus (AAV) were incorporated into a baculoviral vector for hESC transduction to achieve Rep-mediated nonhomologous recombination at the AAVS1 locus of human chromosome 19q13.3-qter,[104] a site with open chromatin structure and native insulators that may prevent transgene silencing. Although hESC lines with stable transgene expression can be generated with the hybrid AAV/baculovirus vector and the hESC lines maintain transgene expression during their prolonged self-renewal and differentiation processes, the study was unable to confirm site-specific transgene integration due to the low frequency of such events.[99] A recent study has adopted a two-step process to target the AAVS1 locus in hESCs (**Figure 3**).[101] Homologous recombination was first used to introduce heterospecific loxP sites into the locus, followed by baculoviral transduction to deliver Cre recombinase and a floxed transgene. The transgene can be efficiently and specifically inserted into the AAVS1 locus via recombinase-mediated cassette exchange, providing targeting efficiency of up to 100% in the master hESC line containing the loxP-docking sites. In another recent study, baculoviral vectors have been used to deliver zinc-finger nucleases together with a DNA donor template for targeting the CCR5 locus in hESCs,[100] although with a relatively low targeting efficiency of

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5%. The development of these site-specific integration methods has taken advantage of the large cloning capacity of baculoviral vectors that allows the inclusion of multiple genomic elements. These methods provide safer alternative technologies to modify stem cells for downstream therapeutic applications, for example deriving unlimited numbers of functionally enhanced or genetically corrected adult cells for cell replacement therapy.

BACULOVIRUS FOR CARTILAGE AND BONE TISSUE ENGINEERING

Articular cartilage is a weight-bearing tissue that protects bones, but is limited in self-repair capacity. Hu and coworkers first demonstrated that baculovirus effectively transduced rat articular chondrocytes.[105] However, chondrocytes tend to de-differentiate during subculture, thus hindering their *in vitro* expansion and subsequent transplantation back to the host. Sung *et al.* demonstrated that transduction of de-differentiated chondrocytes with a baculovirus vector (Bac-CB) expressing bone morphogenetic protein-2 (BMP-2) not only restores the differentiation status of passaged chondrocytes *in vitro* but also increased the cartilage-specific extracellular matrix accumulation.[106] These activities were further augmented by baculovirus-mediated coexpression of *transforming growth factor β*.[107] Moreover, when the Bac-CB–transduced chrondrocytes were seeded onto a polymeric scaffold and cultured dynamically for 3 weeks in a rotating-shaft bioreactor, the transduced cell/scaffold constructs grew into cartilage-like tissues.[108] Eight weeks after such constructs were implanted into osteochondral defects of New Zealand White (NZW) rabbits, hyaline cartilages had regenerated and integrated well with adjacent host cartilage.[109] These studies collectively demonstrated the potential of baculovirus in cartilage tissue engineering.

Baculovirus also efficiently transduces human bone marrow-derived mesenchymal stem cells (BMSCs)[93] and progenitors originating from human BMSCs without obstructing their proliferation and differentiation potential. Transduction efficiencies can be elevated to ~95% under optimized conditions.[110] As BMSCs can differentiate into osteoblasts and BMP-2 is a potent osteogenic factor, Hu and coworkers genetically engineered human BMSCs with Bac-CB and demonstrated that Bac-CB transduction directed osteogenesis of naïve BMSCs. After implantation, the transduced BMSCs induced ectopic bone formation in nude mice and promoted calvarial bone repair in immunocompetent rats.[111]

In addition to osteogenesis, massive segmental bone healing necessitates coordinated vascularization to rebuild blood vessels. Lin *et al.* recently constructed a recombinant baculovirus (Bac-CV) expressing vascular endothelial growth factor (VEGF).[112] Implantation of Bac-CV– and Bac-CB–transduced rabbit BMSCs into critical-size segmental bone defects at the femora of NZW rabbits accelerated and ameliorated bone healing, thanks to the *in vivo* coexpression of *BMP-2/VEGF* and ensuing improved osteogenesis/angiogenesis.[112]

Adipose-derived stem cells (ASCs) are another promising cell source for bone regeneration but ASCs were suggested to be inferior to BMSCs in osteogenesis potential. To use ASCs for massive bone repair, Hu and coworkers hypothesized that sustained expression of genes promoting osteogenesis (*BMP-2*) and angiogenesis (*VEGF*) is necessary.[95] Therefore, they used a dual baculovirus vector system constituting two baculoviruses: one expressing the *flippase* recombinase, whereas the other accommodating the *BMP-2* or *VEGF* cassette flanked by the flippase recognition target (Frt) sequences. Cotransduction of mammalian cells with the hybrid vectors resulted in gene cassette excision of the baculovirus genome, formation of episomal circles, and prolonged transgene expression.[110] Such flippase/Frt-mediated recombination occurred efficiently in the NZW rabbit ASCs, enabling persistent transgene expression for >28 days.[95] Allotransplantation of the NZW rabbit ASCs transduced with the hybrid baculoviruses expressing *BMP-2/VEGF* into the critical-size femoral segmental defects accelerated the healing, improved the bone quality and angiogenesis when compared with transplanting ASCs engineered with the conventional baculoviruses (**Figure 2a–c**). The same technique was also exploited to promote the healing of critical-size calvarial defects in rabbits.[113] These data altogether support the feasibility of using baculovirus for stem cell engineering and bone regeneration.[95]

The safety issues pertinent to the application of baculovirus-engineered stem cells were also addressed. It was uncovered that baculovirus transduction of BMSCs upregulated certain inflammatory genes (e.g., *IL-1β, interferon α*, and *IL-6*) and stimulated transient, low level upregulation of human leukocyte antigen I (*HLA-I*). However, baculovirus neither altered *HLA-II* expression nor impaired the immunosuppressive property of BMSCs. Microarray analysis further revealed that baculovirus perturbed the expression of 816 genes in BMSCs and activated the TLR3 pathway, leading to robust secretion of IL-6 and IL-8. Nonetheless, the induction was transient and no other inflammatory cytokines or interferon β were provoked. Furthermore, baculovirus transduction of BMSCs neither integrated the transgene into the host chromosomes nor disrupted the karyotype.[114] These studies thus supported the safety of baculovirus-engineered BMSCs for cell therapy.[114]

CANCER GENE THERAPY

Effective transduction and high-level transgene expression mediated by baculoviral vectors are especially suited for cancer gene therapy. Baculoviral vectors armed with suicide genes, tumor suppressor genes, proapoptotic genes, immunopotentiating genes, and antiangiogenesis genes have been tested *in vivo* in animal tumor models in many different anticancer regimens.[27,54,115] Early studies investigated transcriptional targeting and transgene expression in directly transduced glioblastomas in the brain, an "immunoprivileged" site where complement-mediated inactivation is prevented by the blood–brain barrier. Transgenes under the control of a tissue-specific promoter, a tumor-selective promoter and a tissue-specific promoter and microRNA target sequences were evaluated (see for example, **Figure 2d–f**).[27] More recently, *ex-vivo* gene therapy studies have been conducted using baculoviruses equipped with suicide genes to transduce stem cells.[94,96,98] This approach benefits from the tropism exhibited by many adult stem cells for primary, solid, and metastatic tumors.

Cancer immunotherapy through vaccination with baculovirus-expressed tumor-associated antigens has been a promising area of research as well. For example, the majority of mice vaccinated with infected insect cells expressing colon carcinoma-specific antigen/major histocompatibility complex class I complexes were protected when challenged subsequently with colon tumor cells.[116] Perhaps

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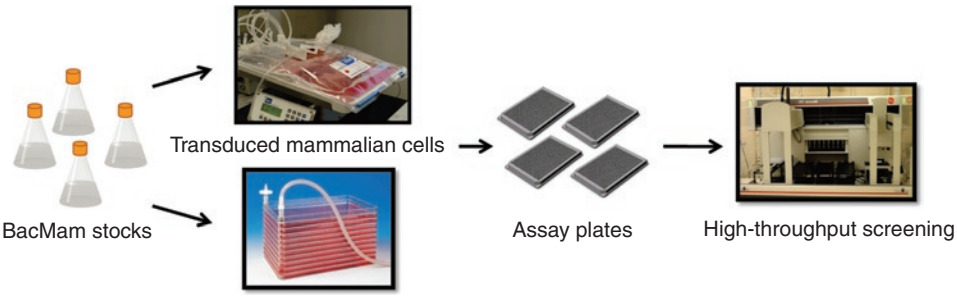

Figure 4 Application of BacMam transduction for assay development and automated high-throughput drug screening. Large quantities of either suspension or anchorage-dependent mammalian cells can be transduced by addition of single or multiple BacMam viruses at various titers. The transduced cells can be either dispensed directly using automated systems into 384 or 1536 well microtiter plates and subsequently incubated for 24–48 hours before assay or incubated for the desired period, harvested and stored frozen in liquid nitrogen for future use. In many instances this eliminates the need to develop stable cell lines for screening assays.

more surprising, vaccination with wild-type baculovirus devoid of any foreign genes elicited potent antimelanoma immunity equivalent to that achieved by five doses of immunostimulatory cytokine IL-12.[82] This intrinsic immunostimulatory property of baculovirus was attributed primarily to natural killer cell proliferation and associated antitumor activity, but could also involve enhanced tumor-specific cytotoxic T lymphocyte responses and tumor-specific antibody production.[71,117] Baculoviral transduction of antigen-presenting dendritic cells yielded promising results for melanoma therapy as well.[118] Specifically, baculovirus transduction of dendritic cells stimulated the upregulation of CD1d (a nonclassical class I-like major histocompatibility complex molecule) that, in turn, induced a proinflammatory cytokine profile and enhanced the priming of naïve $CD8^+$ T cells against a melanoma antigen.[118]

ASSAY DEVELOPMENT AND DRUG SCREENING APPLICATIONS

Viral-based gene delivery systems comprise an important part of the tool box for mammalian cell-based assay development and mechanism of action studies in drug discovery programs.[119,120] Baculovirus-infected insects cells are used routinely for producing recombinant proteins for biochemical assay development and structural chemistry efforts.[121,122] The development of versatile vector systems such as MultiBac have served to enhance the system for the production of multiprotein complexes.[21] More recently, baculoviruses modified to express proteins in mammalian cells have also begun to fulfill an important role in drug discovery.[123,124] In particular, drug discovery studies often require the development of robust, reproducible, readily transferable, and automatable cell-based assays for screening chemical libraries to identify starting points for chemical structure activity relationship studies. Historically, stable cell lines have been developed for this purpose. However, many attributes of BacMam-based gene delivery position this technology as an attractive alternative to stable cells and transient transfection methods. BacMam viruses can carry large DNA inserts, transduce a wide variety of mammalian cell types with little to no obvious cytotoxicity and are readily produced in insect cells. The viruses can be stored for long periods of time at 4 °C in the dark with little to no drop in virus titer. Transductions for cell-based assay applications are carried out simply by the addition of virus solution to cells grown either in suspension or attached culture. The transduced cells can be readily dispensed into microtiter culture plates for drug screening in automated drug screening facilities (**Figure 4**). BacMam viruses cannot replicate in mammalian cells, providing a unique low risk biosafety profile.[13] The viruses can be used to support a variety of assay formats for different targets proteins, such as G protein coupled receptors,[125,126] transporters,[127,128] ion channels,[129,130] histones,[131] enzyme fragment complementation,[132] and others. A particularly appealing aspect of using these viruses for developing assays requiring the expression of multiple proteins is that BacMam can be titrated reproducibly according to the amount of target protein desired simply by adding more or less virus.[133,134]

Two other important applications of BacMam gene delivery include the ability to launch viral infections and produce secreted proteins in mammalian cells. The delivery of hepatitis B virus genomes into hepatocytes has proven particularly useful for antiviral studies,[135] and recently, the system has been used for the delivery of a Semliki Forest virus replicon[136] and a human bocavirus.[137] BacMam transduction of suspension culture cells has been shown to yield efficient expression of secreted proteases[138] and membrane glycoproteins.[139] Further developments in BacMam technology will continue to expand the use of this versatile gene delivery vector within drug discovery programs.

LARGE-SCALE GENERATION OF OTHER GENE THERAPY VECTORS

Kotin and coworkers have extended the versatility of the baculovirus insect cell expression system to include the production of faithfully assembled and packaged recombinant AAV (rAAV) vectors suitable for *in vivo* gene therapy applications.[140] To achieve this goal, they developed a set of serotype-specific BEVs that produce stoichiometric amounts of the three AAV capsid proteins, VP1, VP2, and VP3 (**Figure 5**), which self-assemble within BEV-infected insect cells to form icosahedral virus-like particles. Additional BEVs were constructed that express the major AAV nonstructural genes, *Rep78* and *Rep52*, which are essential for AAV genomic replication and packaging. When cap- and rep-expressing BEVs are introduced into insect cells along with a transgene of interest flanked by AAV inverted terminal repeat elements, efficient replication and encapsidation of linear, single-stranded AAV vector genomes occurs.[140,141] The development of insect cell-based rAAV production was predicated on the unexpected finding that AAV vector genomes are efficiently replicated

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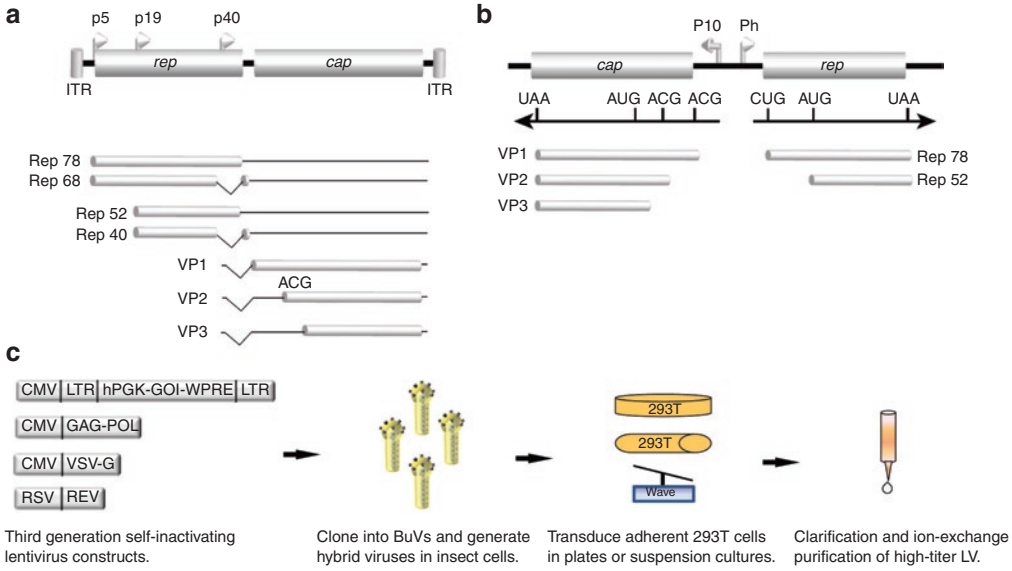

Figure 5 AAV and lentivirus generation by baculoviruses. (**a**) Schematic diagram of the AAV genome. The prototypical AAV-2 genome is 4.7 kb in length. Two major open reading frames encoded by the rep and cap genes are indicated. The protein coding sequences are flanked at each end of the genome by short (<0.15 kb) ITR elements, which provide cis-acting sequences necessary for replication and packaging of the viral genome. Three separate promoters (at map units 5, 19, and 40) give rise to a nested set of 3′ coterminal transcripts, which share a common polyadenylation signal. The translated portions of the Rep- and Cap-encoding mRNAs are indicated by cylinders. The "V-shape" indicates a differential splicing event. (**b**) Representation of AAV *Rep* and *Cap* coding sequences within a "second-generation" rAAV-producing BEV. (Right) The two major AAV nonstructural proteins, Rep78 and Rep52, are translated from a single species of mRNA via a leaky ribosomal scanning mechanism (see text). Rep78 proteins initiate at a CUG codon, whereas Rep52 proteins initiate at a downstream AUG codon. (Left) The AAV structural proteins, VP1, -2, and -3, are expressed from a single species of mRNA via leaky ribosomal scanning using a combination of AUG and ACG codons as indicated. RNAs are indicated by solid arrows. Expressed polypeptides are indicated by cylinders. VP, virion protein. (**c**) The constructs for the third generation self inactivating lentivirus production *i.e.,* the transfer construct in which gene of interest is driven by hPKG promoter, the packing construct expressing HIV *gag* and *pol* driven by CMV promoter, the envelope construct providing the envelope G glycoprotein of the VSV-G, and HIV *Rev* under RSV promoter are cloned into *Ac*MNPV genome and baculoviruses are generated in insect cells. Adherent 293T cells or suspension cultures are transduced by hybrid viruses to produce small or larger batches of LV, respectively. High-titer lentiviruses are collected from the medium and purified to homogeneity by ion-exchange chromatography. *Ac*MNPV, *Autographa californica* multiple nucleopolyhedrovirus; BEV, baculovirus expression vector; BuV, the budded form of baculovirus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; hPKG, human phosphoglycerate kinase; ITR, inverted terminal repeat; LTR, long terminal repeat; LV, Lentiviruses; P10, baculovirus p10 promoter; Ph, baculovirus polyhedrin promoter; RSV, Rous sarcoma virus; UAA, ocher stop codon; VSV-G, vesicular stomatitis virus G; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element.

in lepidopteran insect cell lines, such as Sf9 and Sf21, in a Rep-dependent fashion.

Engineering of AAV transcription units was required for efficient baculovirus-mediated rAAV production in insect cells. During wild-type AAV infection, four nonstructural proteins (Rep78, -68, -52, and -40) are produced from the single open reading frame of the AAV *rep* gene via differential promoter selection and alternative splicing (**Figure 5a**). Rep78 and -68 participate in AAV genomic replication, whereas Rep52 and -40 have been implicated in packaging of progeny genomes.[142,143] Recombinant AAV generation in insect cells requires production of at least one large (Rep78 or -68) and one small (Rep52 or -40) Rep protein, in addition to the three structural AAV capsid proteins. A strategy was developed to achieve expression of *Rep78* and *Rep52* from a single open reading frame without the need for an internal promoter element or splicing[144] (**Figure 5b**). The AUG initiation codon of the full-length *rep* open reading frame was altered, along with its immediate flanking sequences, to yield a non-AUG codon presented in the context of a strong translation initiation motif (known as a Kozak consensus sequence). Internal AUG triplets intervening between the *Rep78* and *Rep52* initiation codons were changed to yield either silent mutations or conservative amino acid substitutions, depending on the reading frame. Intermittent translational initiation at the context-enhanced non-AUG triplet coupled with ribosomal scanning and translational initiation at the AUG codon of *Rep52* resulted in efficient expression of both *Rep78* and *Rep52* from a single mRNA species. Combination of the translationally modified *rep* expression cassette with a similarly modified AAV coat protein expression cassette into a single recombinant baculovirus facilitated robust, scalable, high-titer production of rAAV in insect cells

Development of a baculovirus-mediated rAAV production method has facilitated large-scale preparation of rAAV vectors. Sf9 insect cells can be readily grown in serum-free suspension culture, thus providing a more cost-effective and easily scalable production platform than adherent mammalian cell-based strategies. In addition, delivery of the cis and trans elements needed for rAAV production via viral infection of producer cells is an efficient and robust process. However, the use of recombinant baculovirus seed stocks presents additional production issues, particularly upon scale-up. First, reliable titering of recombinant baculovirus stocks by plaque assay is a time consuming process. Second, recombinant baculovirus stocks loose infectivity upon prolonged storage in serum-free medium. These issues have been addressed by adapting a process for recombinant baculovirus-mediated protein production developed by Wasilko and colleagues,[33] known as titerless infected-cells preservation and scale-up, that uses cryopreserved

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recombinant baculovirus-infected insect cells in batch production processes. Upon thawing, the number of infectious baculovirus particles released from viable rBEV-infected cells remains relatively unchanged, thus obviating the need for periodic plaque titering of cell-free baculovirus stocks. Inoculating Sf9 cell culture at a defined cell density with a predetermined optimal fractional volume of baculovirus-infected insect cells stocks produced consistent rAAV yields.[145] An average yield of approximately 7×10^{13} purified, vector genome-containing rAAV particles per liter of batch culture has been reported for a wide range of process scales, resulting in total rAAV vector yields in excess of 10^{16} from 200 l scale bioreactor preparations.[145] The baculovirus – Sf9 cell system has provided a scalable and economic method for large-scale rAAV production.

Lentiviruses (LVs) have become widely used for gene delivery and gene therapy when sustained gene expression is required.[146] The popularity rests on the fact that lentiviruses show better safety and efficacy in gene delivery compared with conventional murine γ retroviruses.[146,147] Preclinical and clinical applications, however, are still hampered by the difficulty of producing lentiviruses in sufficient quantity and purity.[148] To answer this unmet need, BEVs was recently applied for lentivirus generation.[52,53] In contrast to AAV production, human embryonic kidney cells (293T) were used for virus propagation instead of insect cells. The production relies on four novel hybrid baculoviruses (**Figure 5c**). The BAC-transfer virus expresses a transgene and BAC-gag-pol, BAC-VSVg, and BAC-rev viruses contain the elements required for safe production of the third generation lentiviruses.[149] The results show that, in optimized production conditions, *Ac*MNPV can successfully transduce both adherent and suspension cell cultures.[52,53] High-titer LV stocks were readily produced and the generated virions performed similarly *in vitro and in vivo* as the viruses produced by conventional means. The same procedural problems related to seed stocks as with AAV concern baculovirus-based LV generation. However, the purification of LV from the contaminating baculoviruses is less cumbersome because baculoviruses do not replicate in vertebrate cells and they will be thus cleared after transduction by the producer cells. A scalable and cost-effective capture purification based on a diethylaminoethyl monolithic column enables 65% recovery of highly purified lentiviruses.[52] BEVs are thus a promising platform for large scale LV production and further development and scale-up is in progress. The baculovirus system has also been exploited for AAV vector production in HEK-293 cells.[150] Hu *et al*. have constructed a baculovirus system that comprises three vectors: Bac-LacZ carries the *lacZ* gene flanked by AAV inverted terminal repeats, Bac-RC harbors AAV *rep* and *cap* genes, and Bac-Helper carries helper genes derived from adenovirus. Cotransduction of HEK-293 cells with these three baculoviruses resulted in successful production of rAAV. Using a packed-bed reactor for cell culture and transduction processes significantly improved the AAV vector yield, thereby rendering this system an economically viable approach for AAV production.

CONCLUSION

Baculovirus technology has matured to the state in which it can be applied for numerous applications. Transduction of a plethora of mammalian cell types in addition to infection of insect cells establishes these viruses as a versatile gene delivery and protein expression platform. The first studies of therapeutic gene delivery in the context of immunoprivileged tissues, cancer, and *ex vivo* applications are encouraging and support further development of *Ac*MNPV from preclinical applications to human studies. The application of baculovirus for producing AAV and lentiviruses facilitates the development of the gene therapy field from the bioprocess perspective. A deeper understanding of the molecular basis of target cell transduction and the antigenic properties of *Ac*MNPV will help guide the development of improved vectors further enhancing the use of this unique and powerful gene delivery system.

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