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BACULOVIRUS VECTORS FOR GENE THERAPY

Yu-Chen Hu

Department of Chemical Engineering, National Tsing Hua University
Hsinchu, Taiwan 300

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ABSTRACT

Since the discovery that baculoviruses can efficiently transduce mammalian cells, baculoviruses have been extensively studied as potential vectors for both *in vitro* and *in vivo* gene therapy. This chapter reviews the history of this research area, cells permissive to baculovirus transduction, factors influencing transduction and transgene expression, efforts to improve transduction, mechanisms of virus entry and intracellular trafficking, applications for *in vivo* and *ex vivo* gene therapy, as well as advantages, limitations, and safety issues concerning use of baculoviruses as gene therapy vectors. Recent progress and efforts directed toward overcoming existing bottlenecks are emphasized.

I. BACULOVIRUS TRANSDUCTION OF MAMMALIAN CELLS

A. *Historical Overview*

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is one of the most well-studied baculoviruses. In 1983, baculoviruses

were first explored as vectors for the expression of human interferon β (IFN- β) in insect cells (Smith *et al.*, 1983). Since then, the potential of the baculovirus–insect cell expression system has been fully exploited for the production of numerous recombinant proteins (reviewed in Beljelarskaya, 2002; Luckow and Summers, 1988; Patterson *et al.*, 1995) and baculovirus research advanced insect cell culture as a force in the field of biotechnology. One of the reasons for the increasing popularity of the baculovirus–insect cell expression system is safety, because baculoviruses are regarded as nonpathogenic to humans, and the baculovirus host range is restricted to insects and invertebrates.

In 1983, however, Tjia *et al.* (1983) first found that baculoviruses can be internalized by mammalian cells and at least some of the viral DNA reached the nucleus. The nuclear DNA, however, did not persist and there was no evidence that baculovirus DNA was transcribed in mammalian cells (Tjia *et al.*, 1983). Later, Volkman and Goldsmith (1983) demonstrated that baculoviruses can be internalized by nontarget vertebrate cells such as human lung carcinoma cell line A427. Carbonell *et al.* (1985) further confirmed that baculoviruses entered mammalian cells and mediated very low-level expression of *Escherichia coli* chloramphenicol acetyltransferase (CAT) under the control of polyhedrin and *Rous sarcoma virus* (RSV) promoters. However, the significance of these findings was not widely noted until a decade later.

In the mid-1990s, two pioneer groups reported that recombinant baculoviruses harboring a cytomegalovirus (CMV) promoter-luciferase gene cassette (Hofmann *et al.*, 1995) or an RSV long terminal repeat (LTR) promoter- β -galactosidase (β -gal) gene cassette (Boyce and Bucher, 1996) efficiently expressed the reporter genes in mammalian cells. The data suggested a strong preference of baculovirus vectors to transduce hepatocytes of different origins (e.g., human and rabbit), because efficient transduction and high-level expression were only observed in primary hepatocytes and hepatoma cells. Significantly, lower reporter gene expression was observed in several other cell lines (e.g., COS-1 and 293) and little to no expression was observed in other cell types including A549, CHO, NIH-3T3, and CV-1 cells. The authors suggested that the block to expression in less susceptible cells might be subsequent to viral entry, rather than the ability to be internalized by the target cells, because high- and low-expressing cell lines internalized similar amounts of virus (Boyce and Bucher, 1996). One factor accounting for the low apparent transduction efficiency in certain cell types is promoter strength; Shoji *et al.* (1997) showed that cells that were not transduced by a baculovirus-expressing β -gal under

the control of the CMV promoter could be efficiently transduced by a baculovirus expressing the same reporter protein under the transcriptional control of the stronger CAG promoter. Furthermore, [Shoji *et al.* \(1997\)](#) compared the gene expression by baculovirus and adenovirus vectors using the same expression unit and observed the same level of expression in HepG2, HeLa, and COS-7 cells by both vectors. They even demonstrated efficient expression and proper processing of hepatitis C virus (HCV) protein mediated by a baculovirus vector. These pioneering studies paved the way for use of baculoviral vectors as tools for gene delivery into mammalian cells.

B. Cells Permissive to Baculovirus Transduction

The list of cells permissive to baculovirus transduction has rapidly expanded. These cells include cell lines originating from cells of human (e.g., HeLa, Huh-7, HepG2, keratinocytes, bone marrow fibroblasts), rodent (e.g., CHO, BHK), porcine (e.g., CPK, PK15), bovine (e.g., BT), and even fish (e.g., EPC, CHH-1) origin ([Table I](#)). In addition, baculoviruses are capable of transducing nondividing cells, such as PK1 cells arrested in S phase ([van Loo *et al.*, 2001](#)). Transduction of primary cells, such as human neural cells ([Sarkis *et al.*, 2000](#)), pancreatic islet cells ([Ma *et al.*, 2000](#)), and rat articular chondrocytes ([Ho *et al.*, 2004](#)), has also been observed. In addition, [Wagle and Jesuthasan \(2003\)](#) showed that baculoviruses successfully transduced the embryos of zebrafish. EphrinB2a is normally expressed in the posterior region of developing somites and baculovirus-mediated misexpression by injection of the baculovirus expressing ephrinB2a into specific tissues, caused abnormal somite boundary formation ([Wagle and Jesuthasan, 2003](#)). Moreover, we demonstrated that baculoviruses are capable of transducing mesenchymal stem cells (MSC) derived from human umbilical cord blood and bone marrow ([Ho *et al.*, 2005](#)), as well as MSC-derived adipogenic, osteogenic, and chondrogenic progenitor cells ([Ho *et al.*, 2006](#)). Despite the rapidly growing list of cells permissive to baculovirus transduction, however, baculovirus transduction of cell lines of hematopoietic origin, such as U937, K562, Raw264.7 ([Condreay *et al.*, 1999](#)), LCL-cm, and Raji ([Cheng *et al.*, 2004](#)), is inefficient. As the spectrum of cell types permissive to baculovirus transduction expands, the potential applications of baculovirus vectors are receiving increasing attention. [Table II](#) lists some of the applications of baculoviruses that have been explored (see also chapter by [Condreay *et al.*](#), pp. 255–286; [van Oers](#), pp. 193–253; and [Mäkelä and Oker-Blom](#), pp. 91–112, this volume).

TABLE I
SOME CELL TYPES THAT ARE PERMISSIVE TO BACULOVIRUS TRANSDUCTION

Cell type	References
Human cells	
HeLa	Boyce and Bucher, 1996; Condreay <i>et al.</i> , 1999; Hofmann <i>et al.</i> , 1995
Huh-7	Boyce and Bucher, 1996; Condreay <i>et al.</i> , 1999; Hofmann <i>et al.</i> , 1995
HepG2	Boyce and Bucher, 1996; Hofmann <i>et al.</i> , 1995
HEK293	Sollerbrant <i>et al.</i> , 2001
WI38	Condreay <i>et al.</i> , 1999
MRC5	Palombo <i>et al.</i> , 1998; Yap <i>et al.</i> , 1998
MG63	Condreay <i>et al.</i> , 1999
ECV-304	Airenne <i>et al.</i> , 2000
HUVEC	Kronshnabl <i>et al.</i> , 2002
PC3 (prostate cancer)	Stanbridge <i>et al.</i> , 2003
KATO-III (gastric cancer)	Shoji <i>et al.</i> , 1997
Osteosarcoma SAOS-2	Condreay <i>et al.</i> , 1999; Song <i>et al.</i> , 2003
Pancreatic β cells	Ma <i>et al.</i> , 2000
Keratinocytes	Condreay <i>et al.</i> , 1999
Bone marrow fibroblast	Condreay <i>et al.</i> , 1999
Primary foreskin fibroblast	Dwarakanath <i>et al.</i> , 2001
Primary neural cells	Sarkis <i>et al.</i> , 2000
Primary hepatocytes	Boyce and Bucher, 1996; Hofmann <i>et al.</i> , 1995
Mesenchymal stem cells	Ho <i>et al.</i> , 2005
Nonhuman primate cells	
COS-7	Condreay <i>et al.</i> , 1999
Vero	Poomputsa <i>et al.</i> , 2003
CV-1	Tani <i>et al.</i> , 2001
Porcine cells	
CPK	Aoki <i>et al.</i> , 1999
FS-L3	Shoji <i>et al.</i> , 1997
PK15	Aoki <i>et al.</i> , 1999
Bovine cells	
MDBK	Aoki <i>et al.</i> , 1999
BT	Aoki <i>et al.</i> , 1999

(continues)

TABLE I (continued)

Cell type	References
Rodent cells	
L929	Airenne <i>et al.</i> , 2000; Cheng <i>et al.</i> , 2004
PC12	Shoji <i>et al.</i> , 1997
CHO	Condreay <i>et al.</i> , 1999; Hu <i>et al.</i> , 2003a
BHK	Condreay <i>et al.</i> , 1999; Hu <i>et al.</i> , 2003a
Rat hepatic stellate cells	Gao <i>et al.</i> , 2002
Mouse pancreatic β cells	Ma <i>et al.</i> , 2000
Primary rat hepatocytes	Boyce and Bucher, 1996
Primary mouse osteoblasts and osteoclast	Tani <i>et al.</i> , 2003
Rat articular chondrocyte	Ho <i>et al.</i> , 2004
Fish cells	
EPC	Leisy <i>et al.</i> , 2003
CHH-1	Leisy <i>et al.</i> , 2003
Embryo	Wagle and Jesuthasan, 2003
Rabbit cells	
Rabbit aortic smooth muscle	Raty <i>et al.</i> , 2004
RK 13 (kidney)	Nakamichi <i>et al.</i> , 2002

TABLE II
APPLICATIONS OF THE BACULOVIRUS/MAMMALIAN CELL SYSTEM

Application	References
<i>In vitro</i> and <i>in vivo</i> gene therapy	Airenne <i>et al.</i> , 2000; Boyce and Bucher, 1996; Hofmann <i>et al.</i> , 1995; Pieroni <i>et al.</i> , 2001; Sarkis <i>et al.</i> , 2000; Tani <i>et al.</i> , 2003
Cell-based assays	Ames <i>et al.</i> , 2004; Jenkinson <i>et al.</i> , 2003; Katso <i>et al.</i> , 2005
Studies of gene function	Clay <i>et al.</i> , 2003; Pfohl <i>et al.</i> , 2002
Studies of virology	Delaney and Isom, 1998; Dwarakanath <i>et al.</i> , 2001; Lopez <i>et al.</i> , 2002; McCormick <i>et al.</i> , 2002; Zhou <i>et al.</i> , 2000
Protein production	Chen <i>et al.</i> , 2005; Ojala <i>et al.</i> , 2004; Ramos <i>et al.</i> , 2002
Virus vector production	Cheshenko <i>et al.</i> , 2001; McCormick <i>et al.</i> , 2002; Poornputsa <i>et al.</i> , 2003; Sollerbrant <i>et al.</i> , 2001
Surface display	Ernst <i>et al.</i> , 1998; Grabherr and Ernst, 2001
Vaccine candidates	Abe <i>et al.</i> , 2003; Aoki <i>et al.</i> , 1999; Facciabene <i>et al.</i> , 2004; Tami <i>et al.</i> , 2000; Yoshida <i>et al.</i> , 2003

C. Mechanisms of Baculovirus Entry into Mammalian Cells

1. Importance of the Envelope Glycoprotein gp64

The baculovirus gp64 glycoprotein is a major component of the budded virus envelope and is essential for virus entry into insect cells by receptor-mediated endocytosis (Wickham *et al.*, 1990). Following virus entry, gp64 further mediates the acid-induced endosomal escape, thus allowing for nucleocapsid transport into the cytoplasm and nucleus (Blissard and Wenz, 1992). Similarly, gp64 is essential for virus attachment and endosomal escape in mammalian cells (Hofmann *et al.*, 1998). In support of the importance of gp64 is the finding that a monoclonal antibody specific for gp64 abolishes the capability of baculovirus vectors to transduce mammalian cells (Gronowski *et al.*, 1999). A baculovirus overexpressing gp64 from an additional gp64 gene can incorporate ~1.5 times the normal amount of gp64 on the virion surface and exhibit 10- to 100-fold more reporter gene expression in a variety of mammalian cells when compared to the control baculovirus (Tani *et al.*, 2001). The importance of gp64 for virus transduction is further substantiated as a mutant virus lacking gp64 on the viral envelope failed to transduce mammalian cells (Abe *et al.*, 2005). Furthermore, gp64 of AcMNPV was shown to rescue transduction of mammalian cells by HaSNPV (*Helicoverpa armigera* single nucleopolyhedrovirus), a virus that does not transduce mammalian cells. The range of mammalian cell types transduced by HaSNPV expressing the gp64 of AcMNPV was consistent with those transduced by AcMNPV (Lang *et al.*, 2005).

2. Surface Molecules for Virus Docking

Although the importance of gp64 for virus entry has been documented, the nature of the cell surface molecule that interacts with the virus is unclear in both insect and mammalian cells (Kukkonen *et al.*, 2003). Initially, it was suggested that baculovirus transduction was liver specific and that asialoglycoprotein could be involved in virus binding (Boyce and Bucher, 1996; Hofmann *et al.*, 1995). However, van Loo *et al.* (2001) showed that Pk1 cells, which do not express asialoglycoprotein receptors, can be successfully transduced, and hence asialoglycoprotein is not a key determinant. It was also shown that electrostatic interactions may be necessary for baculovirus binding to the mammalian cell surface because preincubation of 293 cells with polybrene, a cationic compound that neutralizes negatively charged epitopes on the cell membrane, resulted in a rapid decrease in virus binding (Duisit *et al.*, 1999). The same group also suggested that heparan sulfate may act as an important docking motif for baculovirus binding because removal of heparan

sulfate from the cell surface by heparanase I or III prior to transduction reduced transgene (*LacZ*) expression by $\approx 50\%$ (Duisit *et al.*, 1999). Aside from heparan sulfate, phospholipids on the cell surface were suggested to serve as an important docking point for gp64, thus facilitating viral entry into mammalian cells (Tani *et al.*, 2001).

On the other hand, by transient depletion of calcium using EGTA pretreatment, Bilello *et al.* (2003) demonstrated that paracellular junction complexes are important barriers for baculoviral entry into primary hepatocytes. In contrast, we found that EGTA treatment of Huh-7 cells and chondrocytes does not significantly enhance transduction efficiencies although disruption of cell junctions was apparent (unpublished data). Despite the discrepancies in identification of the surface receptors, multiple lines of evidence suggest that baculoviruses are internalized by endocytosis (Condreay *et al.*, 1999; van Loo *et al.*, 2001). By electron and confocal microscopy, Matilainen *et al.* confirmed that baculoviruses enter HepG2 cells via clathrin-mediated endocytosis. However, baculovirus attachment to clathrin-coated pits seemed to be a relatively rare phenomenon, and therefore other internalization mechanisms (possibly via macropinocytosis) may also exist (Matilainen *et al.*, 2005). Virus attachment does not appear to be limiting, because baculoviruses can efficiently bind to NIH-3T3, a cell line less susceptible to baculovirus transduction, even at 4 °C (Stanbridge *et al.*, 2003).

3. Endosomal Escape and Intracellular Trafficking

Having entered the mammalian cell, budded virus is transported to the endosome, followed by acid-induced endosomal escape of the nucleocapsid mediated by gp64. Endosomal escape was first uncovered by treating baculovirus-transduced mammalian cells with a lysosomotropic agent (e.g., chloroquine), which inhibits endosomal maturation and subsequent baculovirus-mediated gene expression (Boyce and Bucher, 1996; Hofmann *et al.*, 1995). The importance of endosomal escape was further confirmed by treating HepG2 cells with monensin, which blocked early endosome acidification and trapped the nucleocapsids in the endosome (Kukkonen *et al.*, 2003). Therefore, it is generally assumed that escape from the endosomes blocks baculovirus transduction of some mammalian cells (Barsoum *et al.*, 1997; Boyce and Bucher, 1996). However, Kukkonen *et al.* (2003) suggested that the block may lie not in escape from the endosome, but rather in cytoplasmic trafficking or nuclear import of the nucleocapsids. In cells nonpermissive to baculovirus-mediated transduction (e.g., EAHY, MG63, and NHO cells), virus is internalized and routed to the endosome 30 min posttransduction, and escapes from the endosome by 4 h posttransduction, but the nucleocapsid

does not enter the nucleus efficiently. Accordingly, no detectable transgene expression is observed even with a very high virus load. In contrast, baculoviruses are capable of entering HepG2 cells (which are highly permissive to baculovirus transduction), escaping from the endosome and entering the nucleus 4 h after transduction. Consistent with this notion is that direct injection of nucleocapsids into the cytoplasm does not affect the translocation of nucleocapsids into the nucleus, demonstrating that endosomal escape is not necessarily a critical step (Salminen *et al.*, 2005). Nucleocapsids are transported into different subcellular compartments in different cells (Abe *et al.*, 2005). In 293T cells, the nucleocapsids reached the nucleus where the transgene was efficiently transcribed following uncoating. However, in the nonpermissive macrophage RAW264.7 cells, the nucleocapsids appeared to be trapped by the phagocytic pathway, and degraded viral DNA was then transported into toll-like receptor 9 (TLR9)-expressing intracellular compartments (Abe *et al.*, 2005).

In the cytoplasm, the nucleocapsids seem to induce the formation of actin filaments which probably facilitate the transport of nucleocapsids into the nucleus. Cytochalasin D, an agent causing reversible depolymerization of actin filaments, strongly inhibits reporter gene expression, but does not prevent the uptake of enveloped virions inside cytoplasmic vesicles, or prevent their escape into the cytoplasm (van Loo *et al.*, 2001). More recently, it was shown that disintegration of microtubules by microtubule-depolymerizing agents (e.g., nocodazole and vinblastine) significantly enhanced the nuclear transport of virus and subsequent transgene expression in HepG2 cells (Salminen *et al.*, 2005), suggesting that intact microtubules constituted a barrier to baculovirus transport toward the nucleus. The viral genome, major capsid protein, and electron-dense capsids were also found inside the nucleus, suggesting that the nucleocapsid was transported through the nuclear pore (van Loo *et al.*, 2001). All of these studies highlight the importance of intracellular trafficking for transgene expression. The proposed route of baculovirus entry and intracellular trafficking is illustrated in Fig. 1.

D. Transduction Efficiency and Level of Transgene Expression

1. Dependence on Cell Types

Baculovirus transduction efficiencies vary considerably according to the cell type and can range from 95% for BHK cells (Wang *et al.*, 2005) to lower than 10% for NIH-3T3 cells (Cheng *et al.*, 2004). Baculovirus

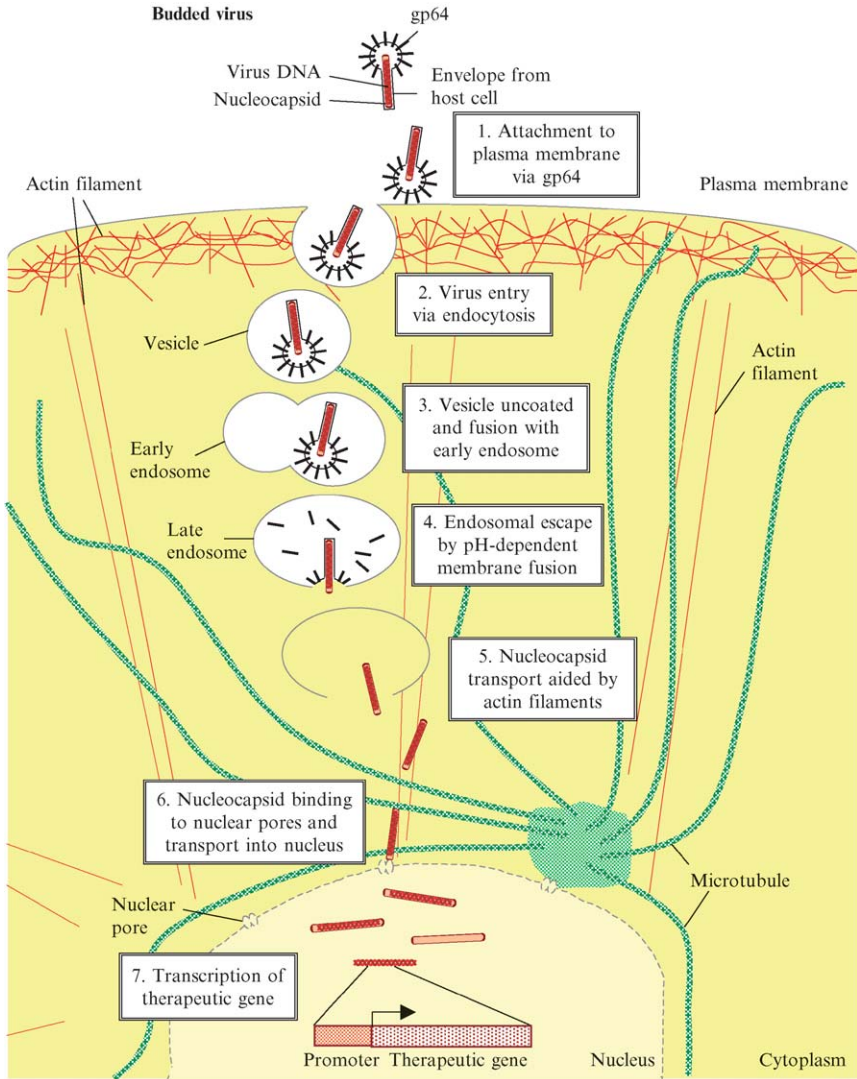


FIG 1. Proposed entry and intracellular trafficking of a baculovirus vector for expression of a therapeutic gene in a mammalian cell.

transduction of hepatocytes (e.g., HepG2, Huh-7) is particularly efficient, with efficiencies of up to 80% (Wang *et al.*, 2005). Because plasmid transfection of hepatocytes, a common approach for gene delivery into liver cells, is notoriously difficult (with a typical delivery

efficiency of 5–30%), the highly efficient baculovirus-mediated gene delivery has been exploited to study hepatitis B virus replication in HepG2 cells (Delaney and Isom, 1998) and to produce hepatitis delta virus-like particles (HDV VLP) in hepatocytes (Wang *et al.*, 2005).

The transduction efficiency may also be dependent on cellular differentiation state because transduction efficiency is only $\approx 30\%$ for undifferentiated human neural progenitor cells, but can be up to $\approx 55\%$ for differentiated neural cells at a multiplicity of infection (MOI) of 25 (Sarkis *et al.*, 2000). Likewise, transduction efficiency (21–90%), transgene expression level and duration (7–41 days) vary widely with the differentiation state and lineage of the adipogenic, osteogenic, and chondrogenic progenitors originating from human MSCs (Ho *et al.*, 2006). The transduction efficiency is very high for adipogenic and osteogenic progenitors, but is relatively low for chondrogenic progenitors (Fig. 2).

2. Effects of Promoter

The transduction efficiency is also promoter-dependent because Shoji *et al.* (1997) demonstrated that baculovirus-mediated luciferase expression driven by the CAG promoter was tenfold higher than that driven by the CMV promoter. Thus it is of interest to examine the efficiency of different promoters of viral and cellular origins in baculovirus vectors in mammalian cells. Although various promoters have been cloned into baculovirus vectors to drive gene transcription (Table III), only recently have Spenger *et al.* (2004) systematically compared the transgene expression driven by *Simian virus 40* (SV40), CMV, RSV, and a cellular promoter (human ubiquitin C) in CHO, COS-1, and HEK293 cells. The CMV and RSV promoters were the most active in all cell lines tested, followed by the ubiquitin C promoter. SV40 promoter was the weakest among these four promoters.

3. Effects of Drugs

The transduction efficiency can be markedly enhanced by the addition of sodium butyrate, trichostatin A (Condreay *et al.*, 1999), or valproic acid (Hu *et al.*, 2003a). These compounds are histone deacetylase inhibitors that induce histone hyperacetylation and lead to a relaxed chromatin structure (Kramer *et al.*, 2001). The use of these drugs enhances baculovirus-mediated gene transcription, thereby highlighting the importance of the chromatin state of the baculovirus genome in the transduced cells for transgene expression. Note, however, that cytotoxicity is often associated with the use of these drugs (Hu *et al.*, 2003a) and the extent to which gene expression is upregulated is dependent on the promoter and the particular cell line (Spenger *et al.*, 2004).

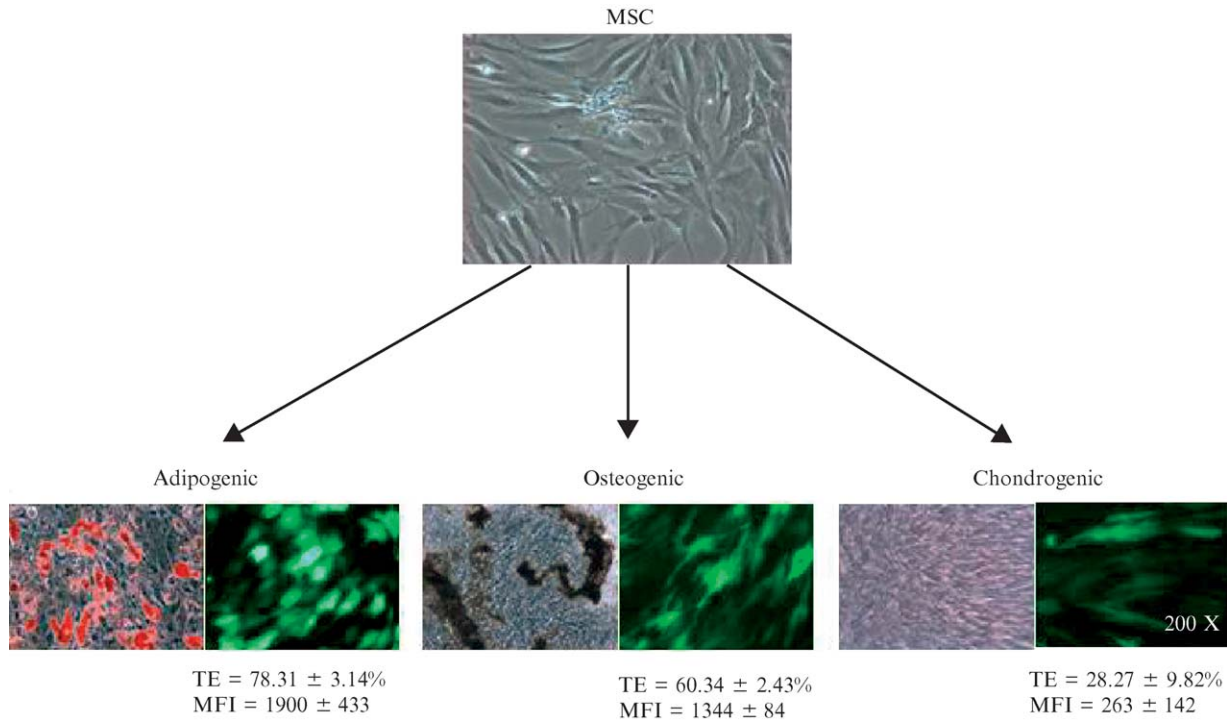


FIG 2. Baculovirus transduction of adipogenic, osteogenic, and chondrogenic progenitors. Human mesenchymal stem cells (MSC) were induced to differentiate into adipogenic, osteogenic, and chondrogenic pathways. The adipogenic, osteogenic, and chondrogenic progenitors were revealed by staining with oil red-O, von Kossa, and safranin-O staining, respectively, two weeks postinduction (left panels of each lineage pathway). The progenitors were transduced with baculoviruses expressing enhanced green fluorescent protein (EGFP) 2 weeks postinduction and exhibited different degrees of EGFP expression (right panel of each lineage pathway). The transduction efficiency (TE) and mean fluorescence intensity (MFI) were measured by flow cytometry.

TABLE III
MAMMALIAN PROMOTERS INSERTED INTO BACULOVIRUS VECTORS

Promoter	References
<i>Rous sarcoma virus</i> long terminal repeat (RSV-LTR) promoter	Boyce and Bucher, 1996
Cytomegalovirus immediate early promoter CMV-IE	Hofmann <i>et al.</i> , 1995; Sollerbrant <i>et al.</i> , 2001
<i>Simian virus 40</i> (SV40) promoter	Spenger <i>et al.</i> , 2004
Hybrid chicken β -actin promoter (CAG)	Shoji <i>et al.</i> , 1997; Stanbridge <i>et al.</i> , 2003
<i>Hepatitis B virus</i> (HBV) promoter/enhancer	Delaney and Isom, 1998
Human α -fetoprotein promoter/enhancer	Park <i>et al.</i> , 2001
Human ubiquitin C promoter	Spenger <i>et al.</i> , 2004
Hybrid neuronal promoter	Li <i>et al.</i> , 2004; Wang and Wang, 2005
<i>Drosophila</i> heat shock protein (<i>hsp70</i>)	Viswanathan <i>et al.</i> , 2003

4. Effects of Baculovirus Genomic Enhancer

Another factor influencing the transduction efficiency and expression level in certain cell lines is the activation of mammalian promoters (e.g., the CMV promoter and the heat shock promoter) by a homologous region (*hr*) in the baculovirus (AcMNPV) genome (Viswanathan *et al.*, 2003). One of the *hr* regions, *hr1*, enhances transcription from the polyhedrin and the *Drosophila* heat shock protein (*hsp70*) promoters in insect cells (e.g., Sf9) *in trans* (Venkaiah *et al.*, 2004). Yet *hr1* also functions in mammalian cells (e.g., Vero and HepG2) as an enhancer when present *in cis* and *in trans* (Viswanathan *et al.*, 2003). The upregulation of gene expression by *hr1* probably stems from binding of *hr1* with high affinity and specificity to nuclear factors in mammalian cells, thereby stimulating transcription (Viswanathan *et al.*, 2003). The insertion of an additional copy of the *hr1* region into the AcMNPV genome thus represents an attractive approach for overexpression of foreign proteins in mammalian cells (Venkaiah *et al.*, 2004). The additional *hr1* also improves the genetic stability of the bacmid-derived baculovirus and consequently prolongs expression of the heterologous protein, because spontaneous deletion of the heterologous gene(s) in the foreign bacterial artificial chromosome sequences readily occurs (Pijman *et al.*, 2001, 2004).

5. Transduction Protocols

Another approach to enhancing the efficiency of baculovirus transduction of mammalian cells is to alter the transduction protocol. For transduction, typically the baculovirus is concentrated by ultracentrifugation

and resuspended in phosphate-buffered saline (PBS). The cells are then incubated with the virus for 1 h at 37 °C in growth medium (e.g., DMEM) (Boyce and Bucher, 1996; Shoji *et al.*, 1997; Tani *et al.*, 2003). We developed a protocol by which incubation of unconcentrated virus (i.e., virus supernatant harvested from infected cell culture) with cells at lower temperature (e.g., 25 °C or 27 °C) for 4–8 h in PBS resulted in gene transfer into HeLa, chondrocytes (Ho *et al.*, 2004; Hsu *et al.*, 2004), and human MSC (Ho *et al.*, 2005) with efficiencies comparable or superior to those using traditional protocols. Specifically, the transduction efficiencies of human MSC derived from umbilical cord blood can be elevated from 42% (using the conventional protocol) to 73% (using the modified protocol). This protocol eliminates the need for virus ultracentrifugation, and hence not only represents a simpler approach but also reduces the chance for virus loss or inactivation during ultracentrifugation.

A key determinant for the improved transduction efficiency is the incubation medium. We found that PBS is superior to DMEM or TNM-FH (the medium for baculovirus production) in terms of transduction efficiency and transgene expression (Ho *et al.*, 2005; Hsu *et al.*, 2004). Comparison between the major components in PBS and medium revealed that NaHCO₃ present in DMEM or TNM-FH significantly reduced transduction efficiency (unpublished data), but the reason for this is unknown.

6. Modifications of the Baculovirus Vector for Improved Gene Delivery

The tropism and transduction efficiency of baculoviruses has been manipulated by modifying the envelope protein. Modification can be performed by fusing a heterologous gene in frame at the 5' end of the gp64 gene under the control of the polyhedrin or p10 promoter. The fusion protein, after expression as an additional copy, is translocated to the plasma membrane and incorporated into the viral envelope on virus budding. Use of this approach was first demonstrated by fusion of human immunodeficiency virus-1 (HIV-1) envelope proteins to gp64 and the modified budded virus bound to the CD4 receptor on T cells (Boublik *et al.*, 1995). A similar strategy was applied to construct avidin-displaying baculoviruses, which showed a 5-fold increase in transduction efficiency in rat malignant glioma cells and a 26-fold increase in transduction efficiency in rabbit aortic smooth muscle cells compared to the wild-type baculovirus (Raty *et al.*, 2004). Baculoviruses displaying heterologous envelope proteins, such as vesicular stomatitis virus G protein (VSVG), have also been constructed. These vectors transduce human hepatoma and rat neuronal cells at efficiencies roughly 10- to 100-fold greater than baculoviruses lacking VSVG

(Barsoum *et al.*, 1997). This pseudotyped virus also transduced cell lines that are transduced at very low levels by the unmodified baculovirus, thus broadening the tropism. The enhanced transduction efficiency and wider tropism are attributed to the increased transport of baculovirus DNA into nuclei rather than to the increased binding or virus uptake (Barsoum *et al.*, 1997).

In contrast, specific targeting of baculoviruses to mammalian cells by displaying a single-chain antibody fragment specific for the carcinoembryonic antigen (CEA) or synthetic IgG-binding domains was also demonstrated (Mottershead *et al.*, 2000; Ojala *et al.*, 2001). Such viral targeting could reduce the virus dose required for *in vivo* gene therapy regimes, if baculoviruses can be engineered to bind efficiently to specific cell types.

II. BACULOVIRUS VECTORS FOR GENE THERAPY

A. In Vivo Gene Therapy

Given the highly efficient gene delivery into many cell types, baculoviruses have captured increasing interest as vectors for *in vivo* gene delivery. Tissues that have been targeted include rabbit carotid artery (Airenne *et al.*, 2000), rat liver (Huser *et al.*, 2001), rat brain (Lehtolainen *et al.*, 2002; Sarkis *et al.*, 2000; Wang and Wang, 2005), mouse brain (Sarkis *et al.*, 2000), mouse skeletal muscle (Pieroni *et al.*, 2001), mouse cerebral cortex and testis (Tani *et al.*, 2003), and mouse liver (Hoare *et al.*, 2005). For baculovirus-mediated *in vivo* gene therapy, however, the complement system appears to be a significant barrier because systemic or intraportal application as well as direct injection into the liver parenchyma fail to result in detectable gene expression (Sandig *et al.*, 1996). This failure stems from inactivation of the baculovirus vector in the presence of native serum, because baculoviruses activate the classical pathway of the complement system (Hofmann *et al.*, 1998). Hoare *et al.* (2005) further showed that both classical and alternative pathways are involved in the inactivation and suggested that naturally occurring IgM antibodies with high affinity for baculoviruses may be partially responsible for the inactivation.

Various strategies have been employed to avoid complement inactivation. Hofmann and Strauss (1998) demonstrated that the survival of a baculovirus vector in human serum can be enhanced through treatment with a functional antibody-blocking complement component 5 (C5). Meanwhile, the complement inhibitor sCR1 (soluble complement

receptor type 1) protects baculoviruses from serum inactivation *in vitro* and coadministration of sCR1 along with intraportal administration of the baculovirus vector leads to hepatic expression in mice (Hoare *et al.*, 2005). Inactivation of baculoviruses in human plasma and whole blood can be prevented by treatment with cobra venom factor (CVF), an inhibitor of the complement system (Hofmann and Strauss, 1998). By injecting CVF into mice one day prior to baculovirus administration, Sarkis *et al.* (2000) demonstrated that the baculovirus was not inactivated by the complement system and could transduce neural cells (mainly astrocytes) *in vivo* when directly injected into the brain of rodents. Surprisingly, they observed the same level of expression in animals without treatment of CVF, suggesting that the baculovirus was not inactivated by the complement system. These contradictory observations were probably caused by the particular immunological characteristics of the brain (Sarkis *et al.*, 2000) or differences in stereotaxic coordinates and injection pressure, or speed (Lehtolainen *et al.*, 2002).

The complement inactivation problem can also be minimized by avoiding contact of the baculovirus vectors with blood components. By using a silastic collar, transduction of adventitial cells in rabbit carotid arteries was achieved and the efficiencies were comparable to those obtained with adenoviral vectors (Airenne *et al.*, 2000). Gene expression was transient remaining high level for 1 week but disappearing by day 14, and the arterial structure and endothelium remained intact after baculovirus transduction. Baculovirus vectors have also been injected into the rodent brain where complement proteins may be absent because of the blood–brain barrier, or the complement level in the brain may be insufficient to affect gene transfer (Lehtolainen *et al.*, 2002). After *in vivo* injection into the brain, baculoviruses specifically transduced the epithelium of the choroids plexus in ventricles and the transduction efficiency was as high as $76 \pm 14\%$. In contrast, adenovirus vectors showed preference to corpus callosum glial cells and ventricular ependymal lining. Hence, baculovirus vectors are especially useful for targeting of choroids plexus cells (Lehtolainen *et al.*, 2002).

A more cutting-edge approach to alleviate complement inactivation is the generation of complement-resistant baculoviruses by display on the viral envelope of decay-accelerating factor (DAF), a regulator that blocks complement at the central step of both the classical and alternative pathways (Huser *et al.*, 2001). Such complement-resistant baculovirus vectors allow for a substantial improvement of gene transfer into neonatal rats *in vivo* after local injection into the liver parenchyma.

Expression of the transgene (human coagulation factor IX, hFIX) was transient probably as a result of the generation of antibodies directed against the transgene product hFIX, which might lead to clearance of either expressed hFIX protein and/or positively transduced cells. Alternatively, baculoviruses can be pseudotyped by displaying VSVG on the envelope. The VSVG-modified virus enhanced gene transfer efficiencies into mouse skeletal muscle *in vivo* and the transgene expression lasted 178 days in DBA/2J mice and 35 days in BALB/c and C57BL/6 mice (Pieroni *et al.*, 2001). The VSVG-modified baculovirus also exhibited greater resistance to inactivation by animal sera and could transduce cerebral cortex and testis of mice by direct inoculation *in vivo* (Tani *et al.*, 2003).

In addition to expressing therapeutic proteins, it has been shown that transduction of Saos-2, HepG2, Huh-7, and primary human hepatic stellate cells with a baculovirus expressing shRNAs (short-hairpin RNAs) targeting lamin A/C effectively knocked down expression of the corresponding mRNA and protein (Nicholson *et al.*, 2005). More recently, baculoviruses have been used to mediate RNA interference (RNAi) using a novel hybrid promoter consisting of the CMV enhancer and polymerase III H1 promoter. The recombinant baculovirus was capable of suppressing expression of the target gene by 95% in cultured cells and by 82% *in vivo* in rat brain (Ong *et al.*, 2005). These data suggest that baculoviruses may be used as delivery vectors for triggering RNA interference for *in vivo* gene therapy.

B. Ex Vivo Gene Therapy

To date, most gene therapy studies using baculovirus vectors have focused on *in vivo* applications, yet relatively little is known about the potential of baculoviruses for *ex vivo* therapy. One relevant study was performed by establishing an *ex vivo* perfusion model for human liver segments (Sandig *et al.*, 1996). The recombinant baculovirus was perfused through the liver segments for 15 min and reasonable transduction rates were achieved in all perfused parts of the liver tissue. This study verified for the first time that baculovirus-mediated gene transfer is possible in liver tissue and is encouraging for future studies including *in situ* perfusion of intact livers with baculovirus vectors in animal models.

In addition, we have demonstrated highly efficient baculovirus-mediated gene transfer into articular chondrocytes (Ho *et al.*, 2004), human MSCs (Ho *et al.*, 2005), and MSC-derived progenitors (Ho *et al.*, 2006), all being candidate cell sources for the treatment of disorders in

connective tissues, particularly cartilage and bone. Importantly, differentiation states of chondrocytes, MSC, and MSC-derived progenitor cells were not affected after baculovirus transduction. Further, the transduction of primary rabbit articular chondrocytes with baculoviruses expressing BMP-2 significantly improved the secretion of extracellular matrix (ECM) and promoted the expression of chondrocyte-specific genes (unpublished data), thus implicating the potential use of baculoviruses for delivery of genes encoding growth factors for cartilage and bone tissue engineering.

The ECM represents a barrier to baculovirus entry into chondrocytes because treatment of rat articular chondrocytes that had been cultured for 10 days with enzymes (hyaluronadase and heparanase) effectively removed the ECM and enhanced virus uptake and gene expression (unpublished data). Unfortunately, articular chondrocytes and osteoblasts are embedded in the ECM *in vivo*. Therefore, the dense ECM surrounding the target cells constitutes a formidable barrier for *in vivo* baculovirus-mediated gene therapy for tissue engineering. As such, *ex vivo* gene therapy may be a more appropriate choice in the context of bone and cartilage tissue engineering. For instance, differentiation of MSCs or progenitors toward a specific lineage pathway (e.g., osteogenic) may be modulated by *ex vivo* transduction with recombinant baculoviruses expressing appropriate growth factors (e.g., BMP-2). Given the efficient transduction of the partially differentiated progenitors, these cells may be transduced again with baculoviruses expressing identical (or different) factors with high efficiency, followed by seeding into scaffolds and implantation into animal models. The transduced cells may continue to express the appropriate factors *in vivo*, thereby stimulating cell differentiation and tissue (e.g., bone) regeneration in an autocrine or paracrine fashion.

III. ADVANTAGES AND LIMITATIONS OF BACULOVIRUSES AS GENE THERAPY VECTORS

A. *Advantages*

To date, vectors used for gene therapy are divided into two categories: nonviral and viral. Nonviral vectors comprise polymers (or liposomes) conjugated with polycations or other targeting molecules. However, the application of nonviral vectors is often restricted by the poor efficiency of delivery and transgene expression (Verma and Somia, 1997). In contrast, viral vectors, such as retroviral, lentiviral,

adenoviral, and adeno-associated viral (AAV) vectors are in common use due to the more efficient cellular uptake and transgene expression. Despite this, each vector has intrinsic advantages and disadvantages (Table IV). For example, retroviruses can mediate integration of viral DNA into the host chromosome for permanent genetic modification; however, transcriptional silencing often occurs and results in transient expression. More critically, the random integration could lead to activation of oncogenes or inactivation of tumor suppressor genes and has resulted in unfortunate leukemia-like diseases in two X-linked SCID patients treated with retroviral vectors (Check, 2002). Lentiviral vectors, derived from *Human* or *Simian immunodeficiency virus* (HIV or SIV), are emerging vectors capable of long-term expression in dividing and nondividing cells. However, the pathogenic nature of HIV or SIV raises serious concerns about the safety of these vectors, and the production of high-titer virus stock is inefficient (Lundstrom, 2003). Adenoviruses can effectively infect dividing and nondividing cells and mediate high-level transgene expression, but the transgene expression is often transient due to the elicitation of strong humoral and cellular immunity, which has also resulted in the death of a patient (Marshall, 1999). AAV vectors can mediate sustained expression, but the packaging capacity is restricted and large-scale vector production is difficult. Furthermore, the preexisting immunity to human AAV vectors is comparable to that of adenoviral vectors (Thomas *et al.*, 2003).

In comparison with these common viral vectors, baculoviruses possess a number of advantages:

1. *Lack of Toxicity and Replication*

Baculovirus transduction is nontoxic to mammalian cells and does not hinder cell growth even at high MOI (Gao *et al.*, 2002; Hofmann *et al.*, 1995). Our studies again confirmed this notion because transduction with a wild-type baculovirus did not cause any observable adverse effects to chondrocytes or human MSC (Ho *et al.*, 2004, 2005). Cell proliferation, however, may be slightly retarded by transgene products, such as EGFP (Ho *et al.*, 2004), which could be toxic and might even induce apoptosis in some cells (Detrait *et al.*, 2002; Liu *et al.*, 1999). Fortunately, the cell growth rate was restored after several passages as EGFP expression attenuates (Ho *et al.*, 2005). Moreover, baculoviruses do not replicate in transduced mammalian cells (Hofmann *et al.*, 1995; Kost and Condreay, 2002; Sandig *et al.*, 1996; Shoji *et al.*, 1997).

The nonreplicative and nontoxic attributes of baculoviruses are particularly important because retroviruses, lentiviruses, and adenoviruses

TABLE IV
COMPARISON OF BACULOVIRUS AND OTHER VIRAL VECTORS

Features	Retroviral	Lentiviral	Adenoviral	AAV	Baculoviral
Ease of preparation	No	No	Yes	No	Yes
Packaging capacity	7–7.5 kb	7–7.5 kb	Up to 30 kb	3.5–4 kb	>38 kb
Route of administration	<i>Ex vivo</i>	<i>Ex/in vivo</i>	<i>Ex/in vivo</i>	<i>Ex/in vivo</i>	<i>Ex/in vivo</i>
Vector genome forms	Integrated	Integrated	Episomal	Episomal ^a	Episomal
Gene expression duration	Short	Long	Short	Long	Short
Tropism	Dividing cell	Broad	Broad	Broad	Broad
Immune response	Low	Low	High	Unknown	Unknown
Preexisting immunity	Unlikely	Unlikely	Yes	Yes	Unlikely
Safety	Integration may induce oncogenesis	Integration may induce oncogenesis	Inflammatory response, toxicity	Inflammatory response, toxicity	High ^b

^a AAV can mediate site-specific integration into human chromosome 19, but common AAV vectors do not contain the *rep* gene and thus cannot mediate integration. However, random integration may occur.

^b Baculoviruses are considered safe, but more studies are required to confirm the safety in *in vivo* and *ex vivo* applications.

are human pathogens, and hence emergence of replication-competent viruses (RCV) raises serious safety concerns. In contrast, baculoviruses are not pathogenic to humans, and hence the emergence of RCV is not an issue for baculovirus-mediated gene therapy.

2. *Large Cloning Capacity*

The baculovirus (AcMNPV) genome is large (≈ 130 kb) and the maximum cloning capacity is at least 38 kb because the adenovirus genome has been cloned into a baculovirus vector (Cheshenko *et al.*, 2001). Such a large cloning capacity provides flexibility for multiple genes or large inserts. This flexibility is particularly advantageous in comparison with retroviral and AAV vectors whose cloning capacities are limited to 7–7.5 kb and 3.5–4 kb, respectively, and prohibit the cloning of regulatory sequences or large gene fragments (e.g., dystrophin).

3. *Ease of Production*

The production of retroviral, lentiviral, and AAV vectors requires transfection of plasmids encoding essential genes into packaging cells (Thomas *et al.*, 2003). The transfection process, however, is cumbersome, costly, and difficult to scale up. In sharp contrast, baculovirus propagation can easily be achieved by infecting insect cells in suspension culture (e.g., in spinner flasks or bioreactors) and harvesting the supernatant 3–4 days postinfection. Scale-up of the production process is straightforward because large-scale insect cell culture processes are well-established. The production phase is initiated simply by adding virus solution to cultured cells. Furthermore, the construction, propagation, and handling of baculoviruses can be performed readily in Biosafety Level 1 laboratories without the need for specialized equipment.

4. *Lack of Preexisting Immunity*

One of the problems associated with adenoviral and AAV vectors is that most people are exposed to these viruses and develop corresponding neutralizing antibodies. Circulating virus-neutralizing antibodies can preclude efficient transduction with the viral vector. In contrast, it is unlikely that people develop such preexisting immunity against baculoviruses. The use of baculovirus vectors in gene therapy, therefore, may avoid the problem of preexisting immunity.

B. Limitations

Despite the promising aspects, baculoviruses have a number of disadvantages as gene therapy vectors.

1. Transient Expression

In vitro, baculovirus-mediated expression usually lasted from 7 to 14 days for common cell lines such as CHO, HeLa, and BHK cells (Hu *et al.*, 2003a), although expression continued for 41 days in adipogenic progenitor cells (Ho *et al.*, 2006). *In vivo*, transgene expression typically declines by day 7 and disappears by day 14 (Airenne *et al.*, 2000; Lehtolainen *et al.*, 2002). The duration of *in vitro* transgene expression can be enhanced by prolonging the transduction period (e.g., upto 8 h) (Hsu *et al.*, 2004) or by supertransduction (Hu *et al.*, 2003a). Nonetheless, the extent to which expression can be prolonged is limited because expression is generally restricted to less than 1 month, which is significantly shorter than expression (in the range of months) mediated by retroviral, lentiviral, and AAV vectors.

One key difference between baculoviral and other viral vectors is that the genes carried by other vectors can persist in the host nucleus, either in an integrated or episomal form, for a longer period. However, Tjia *et al.* (1983) demonstrated that baculoviral DNA persists in the nuclei of transduced mammalian cells for only 24–48 h, as determined by Southern blot (Tjia *et al.*, 1983). We also found that the total transgene (*egfp*) copy number within baculovirus-transduced chondrocytes declined 11-fold (as determined by quantitative real-time PCR) while cell number increases 3.5-fold in 11 days, indicating that baculoviral DNA degrades over time (Ho *et al.*, 2004). The declining *egfp* copy number was concomitant with the decrease in mRNA transcription level (unpublished data) as well as fluorescence intensity.

To prolong transgene expression, Palombo *et al.* (1998) designed hybrid baculovirus–AAV vectors which contained a transgene cassette composed of the β -gal reporter gene and hygromycin resistance gene (*Hyg^r*) flanked by the AAV-inverted terminal repeats (ITR), which are necessary for AAV replication and integration into the host genome (Palombo *et al.*, 1998). Hybrid baculovirus–AAV vectors were derived with or without the AAV *rep* gene (whose gene products are essential for viral DNA replication and integration) cloned in different positions with respect to the baculovirus polyhedrin promoter. Transduction of 293 cells with the hybrid vector expressing the *rep* gene resulted in specific integration of ITR-flanked DNA into the AAVS1 site of chromosome 19 (Palombo *et al.*, 1998). A similar baculovirus–AAV hybrid vector

incorporating an ITR-flanked luciferase gene under a neuron-specific promoter was also employed for *in vivo* studies (Wang and Wang, 2005). Even without the help of *rep* gene expression, the viral vector was able to provide transgene expression for at least 90 days when tested in rat brains. These studies demonstrate an effective methodology for engineering of baculoviral vectors for sustained transgene expression.

2. Inactivation by Serum Complement

As described earlier, contact between baculoviruses and serum complement results in rapid inactivation. Despite various attempts to minimize complement inactivation, to date the number of successful baculovirus-mediated *in vivo* gene therapy experiments in complement-competent animals is limited (Hoare *et al.*, 2005). However, the complement system is also a potent barrier to *in vivo* administration of other gene delivery systems such as liposomes (Marjan *et al.*, 1994), murine retrovirus (Takeuchi *et al.*, 1996), and various synthetic DNA complexes (Plank *et al.*, 1996).

3. Inhibition of Transduction by Intercellular Junctions

Intercellular junctions may be an additional hurdle to baculovirus-mediated gene therapy because transient disruption of these junctions by EGTA treatment prior to transduction improved gene delivery efficiency into long-term cultures of primary hepatocytes (Bilello *et al.*, 2001). Bilello *et al.* (2003) also suggested the importance of the basolateral surface for virus entry at least for some cell types. In our laboratory, however, transient disruption of cell junctions failed to effectively enhance baculovirus-mediated gene transfer into chondrocytes and HepG2 cells that were cultured to overconfluence (unpublished data), implying that other factors in addition to the paracellular junction complexes might be involved in transduction of these cells.

4. Fragility of Budded Virus

Another drawback associated with baculoviruses as gene delivery vectors is that the nucleocapsid is enveloped with lipids derived from the host cell membrane. The envelope structure is essential for virus infectivity due to the anchored gp64 (Blissard and Wenz, 1992), but it also renders virus vulnerable to mechanical force and results in relatively low virus stability, a common problem also observed for other enveloped viruses such as retrovirus (Wu *et al.*, 2000). Typically, baculoviral vectors are concentrated by ultracentrifugation after harvesting from cell culture and resuspended in PBS prior to use. However, ultracentrifugation often leads to significant loss of infectivity probably due

to damage to viral envelopes. Ultracentrifugation also tends to result in virus aggregation (Barsoum, 1999). To alleviate these problems, we constructed a recombinant baculovirus with a hexahistidine (His₆) tag displayed on the viral envelope, which enables virus purification by a simple immobilized metal affinity chromatography (IMAC) (Hu *et al.*, 2003b). The IMAC methodology results in high purity (87%) and obviates the need for successive ultracentrifugation steps. However, the recovery yield in terms of infectious titer is lower than expected (<10%), probably because of damage to the viral envelope during the binding, washing, and elution steps. One possibility to alleviate virus loss during chromatographic purification steps is to display VSVG protein on the baculoviral envelope. Display of VSVG on the retrovirus envelope has been shown to enhance virus stability and the same strategy may be applied to enhancing baculovirus stability.

Besides sensitivity to mechanical force, the half-life of baculoviruses is drastically decreased from 173 h at 27 °C to 7–8 h at 37 °C (Hsu *et al.*, 2004). Such labile thermal stability, in conjunction with the tendency to be inactivated by serum complement, may further restrict the *in vivo* application of baculovirus gene delivery vectors.

IV. SAFETY ISSUES CONCERNING THE USE OF BACULOVIRUSES FOR GENE THERAPY

A. DNA Integration and Viral Gene Expression

As mentioned earlier, baculoviruses are nonpathogenic to humans and are nonreplicative in mammalian cells. Also, baculovirus DNA tends to be degraded in mammalian cells. However, Condreay *et al.* (1999) demonstrated that a recombinant baculovirus containing two expression cassettes (one harboring GFP under the control of the CMV promoter and the other harboring neomycin phosphotransferase under the control of the SV40 promoter) is capable of mediating stable expression. When transduced cells were selected with the antibiotic G418, cell lines that stably maintain the foreign expression cassettes can be obtained at high frequency and exhibit stable, high-level expression of the reporter gene for at least 25 passages. The frequency ranged from one clone in 39 transduced cells to one clone in 109 transduced cells, indicating that stable transduction is an efficient event. Stably transduced derivatives have been selected from a substantial number of cell types (e.g., CHO, Huh-7, HeLa, K562), suggesting that stable cell lines can be derived from any cell type that exhibits transient expression (Condreay *et al.*, 1999).

Such a stably expressing derivative (CHO cell) was later confirmed to stem from the integration of baculovirus DNA into the host cell genome as small, discrete single-copy fragments (Merrihew *et al.*, 2001). These fragments, ranging in size from 5 to 18 kb, had randomly distributed breakpoints outside the selected region, suggesting an illegitimate mode of integration (little or no homology between recombining DNA molecules). Such integration resulted in at least two clones that expressed GFP for up to 5 months.

Since leukemia-like conditions developed in two of the 11 SCID patients treated by retrovirus-mediated gene therapy, safety issues regarding whether and/or how vector DNA integrates into the genomic DNA are under scrutiny. Although these stably expressing cell clones are obtained under antibiotic selection, and the integration occurs in a way different from that of retroviruses (which encode a viral integrase directing nearly full-length, single-copy integration events), the possibility that baculoviruses mediate spontaneous integration into genomic DNA cannot be excluded. To date, there is no direct evidence showing that spontaneous integration of baculoviral DNA occurs in the absence of an antibiotic resistance gene and selective pressure, but extensive studies examining the state and fate of introduced viral DNA are necessary to further prove the safety of baculovirus gene therapy vectors.

Another concern regarding the use of baculoviruses is whether baculovirus endogenous genes are expressed. As long ago as 1983, Tjia *et al.* (1983) showed that baculovirus endogenous gene transcription is absent in transduced HeLa cells. Using RT-PCR, Stanbridge *et al.* (2003) assessed the expression of a number of baculovirus genes after transduction of human cells, and found no baculovirus gene transcripts in human cells. However, a study demonstrated that the baculoviral genomic early-to-late (ETL) promoter is active and able to drive reporter gene expression in mammalian cells (Liu *et al.*, 2006). Although gene expression does not equate to virus replication, the possibility that other baculoviral promoters are also active in the transduced mammalian cells cannot be excluded. Whether and how expression of baculoviral proteins at basal levels in the mammalian cells induces immune responses and how this may influence cellular gene expression and physiological state requires further investigation.

B. Immune Response and Potential as a Vaccine Vector

The “Achilles heel” of gene therapy is that immune responses used to tackle wild-type infections are activated against the vectors and/or the new transgene products (Thomas *et al.*, 2003). Although baculoviruses

were found capable of entering mammalian cells as early as in 1983 (Tjia *et al.*, 1983; Volkman and Goldsmith, 1983), the host response to baculovirus uptake, either *in vitro* or *in vivo*, was not evaluated until 1999 when Gronowski *et al.* (1999) reported that administration of baculoviruses *in vitro* induced the production of IFN- α and IFN- β from human and murine cell lines. The IFN-stimulating activity of baculoviruses required live virus and was not due to the presence of viral RNA or DNA. Furthermore, administration of baculoviruses induced *in vivo* protection of mice from encephalomyocarditis virus infection (Gronowski *et al.*, 1999). A subsequent study discovered that baculovirus transduction of cultured rat hepatocytes disrupted phenobarbital (PB) gene induction, a potent transcriptional activation event characteristic of highly differentiated hepatocytes, and repressed expression of the albumin gene (Beck *et al.*, 2000). But neither cAMP nor PKA activities were affected by the virus. Baculovirus transduction also induced the expression of cytokines, such as TNF- α , IL-1 α , and IL-1 β , in primary rat hepatocytes, however, TNF- β , IL-2, IL-3, IL-4, IL-5, IL-6, and IFN- γ were not detected in any of the baculovirus-exposed hepatocytes (Beck *et al.*, 2000). Airene *et al.* (2000) found that *in vivo* administration of baculovirus to rabbit carotid artery resulted in signs of inflammation. More recently, Abe *et al.* (2003) demonstrated that inoculation of baculovirus induced the secretion of inflammatory cytokines, such as TNF- α and IL-6, in a murine macrophage cell line, RAW264.7. In the same study, they also demonstrated that intranasal inoculation with a wild-type baculovirus elicited a strong innate immune response that protected mice from a lethal challenge of influenza virus (Abe *et al.*, 2003). This protective immune response was induced via the TLR9/MyD88-dependent signaling pathway (Abe *et al.*, 2005). The production of inflammatory cytokines was severely reduced in peritoneal macrophages (PECs) and splenic CD11c⁺ dendritic cells (DCs) derived from mice deficient in MyD88 or TLR9 after stimulation with baculovirus. In contrast, a significant amount of IFN- α was still detectable in the PECs and DCs of these mice after stimulation with baculovirus, suggesting that a TLR9/MyD88-independent signaling pathway may also participate in the production of IFN- α (Abe *et al.*, 2005). The induction of cytokines required gp64, however, gp64 itself did not directly participate in the TLR-mediated immune response. Instead, the authors concluded that internalization of viral DNA via gp64-mediated membrane fusion and endosomal maturation which released the viral genome into TLR9-expressing cellular compartments were necessary for the induction of innate responses (Abe *et al.*, 2005). As mentioned earlier, membrane fusion and endosomal

escape via gp64 are essential for nucleocapsid transport into the nucleus. Hence it appears that transgene expression may be coincident with induction of the TLR9/MyD88-signaling pathway.

Taken together, these findings suggest that baculoviruses may induce various immune responses *in vitro* and *in vivo* as for other viruses (e.g., adenovirus), thus raising questions as to whether this will compromise the use of baculovirus vectors for *in vivo* human gene therapy. Which cytokines are induced by baculoviruses, and how cytokines modulate cellular and humoral immunities *in vivo* are not completely understood. The question of whether baculovirus-mediated *ex vivo* gene therapy elicits the immune response is also of interest. All of these questions need to be answered with more in-depth investigations to ensure the safe application of baculoviral gene therapy vectors.

The immune response induced by baculoviruses makes it a promising candidate as a novel vaccine vehicle against infectious diseases (see chapter by van Oers, this volume, pp. 193–253). The ability of baculoviruses to induce immune responses was first exploited by [Aoki *et al.* \(1999\)](#), who found that a recombinant baculovirus-expressing glycoprotein gB of pseudorabies virus induced antibodies against gB protein in mice, suggesting that this recombinant baculovirus could serve as a vaccine candidate for pseudorabies. The feasibility of using baculoviruses as vaccine carriers was also demonstrated by [Abe *et al.* \(2003\)](#), who found that intranasal inoculation with a recombinant baculovirus expressing hemagglutinin (HA) of the influenza virus under the control of the CAG promoter elicited the innate immune response and provided mice with a high level of protection from a lethal challenge of influenza virus. The level of protection is dependent on the route of administration, and intranasal administration is considerably superior to intramuscular administration although the latter induces significantly higher anti-HA IgG levels. More recently, [Facciabene *et al.* \(2004\)](#) demonstrated that intramuscular injection of a baculovirus expressing carcinoembryonic antigen (CEA) induced a measurable anti-CEA-specific CD4⁺ T cell response. The immunogenic properties of baculoviruses are not restricted to CEA because intramuscular injection of another baculovirus (Bac-E2) expressing the E2 glycoprotein of HCV induced an anti-E2 CD8⁺ T cell response as well as the innate immune response such as natural killer (NK) cell cytolytic activity ([Facciabene *et al.*, 2004](#)). Interestingly, when Bac-E2 is pseudotyped to display VSVG on the envelope, the minimal dose required to elicit a measurable T cell response was tenfold less, indicating that the VSVG-pseudotyped Bac-E2 was a more potent vaccine carrier than the unmodified virus. This finding agrees with the previous statement that baculoviruses

displaying VSVG provide for more efficient immunogen expression in transduced cells.

Baculoviruses can also provoke an immune response against an antigen when it is displayed on the viral surface. For instance, immunization with adjuvant-free baculovirus displaying rodent malaria *Plasmodium berghei* circumsporozoite protein (PbCSP) on the envelope induced high levels of antibodies and IFN- γ -secreting cells against PbCSP, and protected 60% of mice against sporozoite challenge (Yoshida *et al.*, 2003). A more recent study further showed that baculovirus displaying severe acute respiratory syndrome-coronavirus (SARS-CoV) spike protein on the envelope induced the release of IL-8 in lung cells (Chang *et al.*, 2004). These studies substantiate the potential of baculoviruses displaying immunogens as vaccine candidates.

V. CONCLUSIONS AND PROSPECTS

The broad range of mammalian cells permissive to baculovirus transduction, the nontoxic and nonreplicative nature, large packaging capacity, and ease of production make baculoviruses promising tools for gene therapy. Despite these advantages, baculoviruses are inactivated by serum complement, which restricts the application of baculovirus vectors for *in vivo* gene therapy. Additionally, the duration of transgene expression is generally short, thus baculoviruses may not be suited for long-term gene therapy unless a hybrid vector capable of integrating the expression cassette into the host genome (e.g., baculovirus-AAV) is employed. Nonetheless, baculoviruses, in conjunction with other viral vectors (e.g., adenoviral or lentiviral), may be administered sequentially to escape either preexisting or therapy-induced antiviral immunity. Additionally, baculoviruses may serve as delivery vectors for triggering RNA interference. Baculoviruses carrying tumor-suppressor or suicide genes may also be used in combination with other treatments for cancer therapy (Song and Boyce, 2001; Stanbridge *et al.*, 2003). Given the highly efficient gene transfer to chondrocytes and MSCs, baculoviruses expressing appropriate growth factors may be used for *ex vivo* genetic modification of cells prior to transplantation into animals. The growth factors, acting in either an autocrine or a paracrine fashion, potentially accelerate tissue regeneration *in vivo*. Unlike the treatment of chronic disease, it is neither necessary nor desirable for transgene expression to persist beyond the few weeks or months needed to achieve healing (Huard *et al.*, 2003; Lieberman *et al.*, 2002). As a result, long-term transgene expression

is not critical in tissue engineering. Hence, the combination of baculovirus-mediated gene therapy and tissue engineering may hold great promise. Of course, to address the safety issues of employing baculoviruses in gene therapy, the DNA integration, expression of baculovirus endogenous genes, and baculovirus-induced immune responses should be investigated.

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