PRODUCTION AND PURIFICATION OF HSV-1 VECTORS AND ITS USE FOR GENE TRANSFER TO HUMAN CD34^{+} CELLS

by

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ABSTRACT

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PRODUCTION AND PURIFICATION OF HSV-1 VECTORS

AND ITS USE FOR GENE TRANSFER TO HUMAN $CD34^+$ CELLS

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The delivery of therapeutic DNA to patients with genetic or acquired disorders has evolved into a realistic alternate treatment to surgery, drug delivery, or as an adjuvant to therapies. A specific area of this research is to transduce $CD34^+$ cells with therapeutic DNA containing vectors developed from viruses. Explanted $CD34^+$ progenitor cells are exposed to these vectors and then are transplanted to a host. Despite the positive

advances, major limitations exist in the ability to efficiently transduce cells with current vector systems. In addition, if vectors are proven effective at ameliorating disease, the expense of manufacturing sufficient quantities required to treat large populations of patients may prohibit their widespread applications.

Our lab utilizes replication-defective herpes simplex virus type-1 (HSV-1) as a gene delivery vector. HSV-1 possesses the capability to deliver large or multiple genes and infects a wide variety of cell types.

In order to optimize transduction of human umbilical cord blood $CD34^+$ cells, I examined this population for presence and functionality of the three different HSV receptors required for virus binding and entry. Optimal transduction efficiencies of 75% were obtained by systematically varying vector concentration and adsorption time. Additionally, several vector constructs were developed in order to examine the effects of various promoter systems and additional gene deletions on gene expression and vector-associated toxicity.

Throughout these studies we were confronted with the difficulty of obtaining sufficient amounts of high quality vector needed for testing the efficacy of HSV-1 vectors in various applications. To further improve the vector yield, I examined the effect of temperature and media conditions on the stability of an HSV-1 vector. Applying a temperature shift to production conditions resulted in increased vector yields that remained stable at peak levels in cultures incubated at 33°C rather than 37°C.

To enhance vector recovery, alternative purification strategies were examined. A two-stage tangential flow filtration system coupled to a chromatography step was

developed to isolate vector from large debris such as cells and smaller molecules such as protein and DNA. This new system for vector concentration and purification proved superior to the standard protocols currently in use.

DESCRIPTORS

$CD34^+$ Cells

Cell Culture

Gene Therapy

Heparin Chromatography

Herpes Simplex Virus

Tangential-Flow Filtration

Vector Production

Vector Purification

Viral Vector

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NOMENCLATURE

AAV	adeno-associated virus
ACV	acyclovir
Ad	adenovirus
AD	Alzheimer's disease
ADA	adenosine deaminase
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
APC	allophycocyanin
bFGF	basic fibroblast growth factor
BM	bone marrow
BMT	bone marrow transplant
BSA	bovine serum albumin
cap	capsid
CB	cord blood
CD	cluster of differentiation
CF	cystic fibrosis
CFC	colony forming cell
CHO	Chinese hampster ovary
CNS	central nervous system
DISC	disabled infectious single cycle
DNA	deoxyribonucleic acid
DS	dextran sulfate

E	early
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EPC	endothelial progenitor cell
EPO	erythropoietin
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FDG	fluorescein di-B-D-galactoside
FITC	fluorescein isothiocyanate
FL	FLT3 ligand
FL	fetal liver
FLK-1	vegf-2 receptor (same as KDR)
g	glycoprotein
GC	glucoceribrosidase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/macrophage colony stimulating factor
GCV	ganciclovir
GFP	green fluorescent protein
GLV	Gibbon ape leukemia virus
HCF	host cell factor
HCMV	human cytomegalovirus

HHV	human herpesvirus
HIV	human immunodeficiency virus
hpi	hours post infection
HS	heparan sulfate
HSC	hematopoietic stem cell
HSV	herpes simplex virus
HUVEC	human umbilical vein endothelial cell
Hve	herpesvirus entry mediator receptor
ICAM-1	intercellular adhesion molecule-1
ICP	infected cell protein
IE	immediate early
Ig	immunoglobulin
IGF	insulin growth factor
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IN	integrase
ITR	inverted terminal repeat
Kb	kilobases
KDR	vegf-2 receptor (same as FLK-1)
L	late
LAP2	latency associated promoter 2
LAT	latency associated transcript
LDL	low density lipoproteins

Lin	lineage
LTC-IC	long term culture initiating cell
LTR	long terminal repeat
MD	muscular dystrophy
MEM	minimal essential medium
MHC	major hisocompatability complex
mL	milliliter
μm	micrometers
MMLV	Moloney murine leukemia virus
MOI	multiplicity of infection
MPB	mobilized peripheral blood
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NIH	National Institute of Health
nm	nanometers
NOD	non-obese diabetic
Oct1	octamer binding factor-1
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson’s disease
PE	phycoeretherin
PECAM-1	platelet-endothelial cellular adhesion molecule-1

pfu	plaque forming units
PI	propidium iodide
PSC	pluripotent stem cell
RAC	Recombinant DNA Advisory Committee
RCV	replication competent viruses
rep	replication
rhEPO	recombinant human erythropoietin
RNA	ribonucleic acid
RSV	Rous-Sarcoma virus
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SCA-1	stem cell antigen
SCF	stem cell factor
SCID	severe combined immunodeficiency
SP	side population
SRC	SCID repopulating cell
SV40	Simian Virus 40
TIU	total infectious units
TK	thymidine kinase
TNF	tumor necrosis factor
TPO	thrombopoietin
tRNA	transfer ribonucleic acid
UCB	umbilical cord blood

U_L	unique long
U_S	unique short
VE	vascular endothelial
VEGF	vascular endothelial growth factor
vhs	virion host shutoff protein
VP	virion protein
VSV-G	vessicular stomatitis virus glycoprotein
vWF	von Willebrand factor
VZV	Varicella-Zoster virus

1.0 BACKGROUND

1.1 Gene Therapy

1.1.1 Introduction

Delivery of therapeutic DNA, RNA, or protein to various tissues for the treatment of a wide range of disorders is emerging as an important route to therapeutics. Gene transfer to human cells has shown great promise for the prevention and/or treatment of both genetic and acquired diseases. Genetic diseases such as Adenosine Deaminase Deficiency (ADA)[1,2]*, Muscular Dystrophy (MD)[3-6], Cystic Fibrosis (CF)[7,8], Alzheimer's Disease (AD)[9-11], Parkinson's Disease (PD)[12-15], etc. that arise due to gene defects significantly affect the human condition and mortality, and have been examined for treatment by gene therapy. Researchers have isolated mutated genes that result in these disorders and delivering the correct gene sequences to the appropriate cells is one potential treatment. Another possibility is to deliver antisense RNA or a gene of which its product will block or degrade the mutated gene's product[16-18]. For acquired disorders such as cancer, other types of gene therapy strategies have been studied. Since elimination of these cells is preferred, the transfer of a gene coding for a cytokine to stimulate an immune response, or a gene that is toxic itself (suicide therapy) or in combination with conventional radio/chemical therapies have great potential[19-21].

According to data last updated 11/19/01 by the Recombinant DNA Advisory Committee (RAC) at the National Institutes of Health (NIH), Bethesda, MD, there are 500 clinical protocols that had been approved in the USA for human gene transfer. Out

* Parenthetical references placed superior to the line of text refer to the bibliography

of those approved, 458 were for therapy, 40 were for marking, and 2 were non-therapeutic. Sixty nine percent of therapy protocols were for the treatment of cancer, 12% were for genetic diseases such as cystic fibrosis, 8% were for infectious diseases (mainly AIDS), and 11% for other disorders. This information is available online at http://www4.od.nih.gov/oba/rac/documents1.htm.

There are two main types of vehicles that are used to transfer genes into cells: non-viral and viral. Non-viral methods involve transferring DNA to cells by a variety of methods such as naked DNA or as complexes with calcium chloride or lipid-based reagents. These types of vehicles account for 20% of the approved clinical protocols. The remaining 80% of clinical protocols use vectors developed from viruses[(22)]. Viruses have been extensively studied for decades in order to further the understanding of gene expression, DNA replication, virus pathology, and human disease. Basic understanding of how viruses function has greatly enhanced the advancement of genetically manipulating viruses in order to utilize natural parasitic systems as vehicles, or vectors, for efficient delivery of the therapeutic DNA. The main examples of genetically engineered viral vectors include; Retrovirus (MMLV and Lentivirus), Adenovirus, Adeno-Associated Virus (AAV), and Herpesvirus.

In developing or determining the appropriate vector for eventual *in vivo* use, several points must be considered.

1. Tropism- If using viral vectors, the natural tropism of the virus must be considered. Some viruses only infect specific cell types efficiently while others infect many cell types (ubiquitously).

2. Infectivity- (related to tropism) In some applications, an extremely high number of particles are needed to achieve the desired transduction numbers. Each delivery vehicle differs in the number and concentration of particles that can be produced.

3. Immunity- Immune responses can result from transduction with some viruses or DNA that may lead to the transduced cell being eliminated. Immune responses may also inhibit redosing due to cellular and/or humoral responses. Causes of these include viral proteins expressed, transgene, or contaminants such as proteins or DNA in the stock.

4. Expression- Therapeutic expression of a transgene may be needed transiently, required long-term, or regulated. This is dictated by the viral or plasmid background as well as the promoter system used. Episomal versus integrated genes can also affect the transgene-expressing cell numbers as well as the level of expression.

5. Capacity- The size of the transgene to be delivered must be within the capacity of the vehicle used. Some vectors can support large or multiple genes while others are not able to do so.

1.1.2 Non-Viral Vectors

Several methods have been developed to transfer genes to mammalian cells by non-viral vectors. These techniques include calcium phosphate co-precipitation, liposome complexation, microinjection, gene-particle bombardment, electroporation, and receptor-mediated gene transfer[23-26]. Interest in this area has developed due to potential problems faced by viral vectors that include viral tropism, packaging capacity, toxicity and/or immune responses, and production limitations. Non-viral vectors have been limited by their low efficiency and ability for direct *in vivo* use[27.28]. In addition, immune

responses have been a problem in some instances and long-term gene expression has been variable.

1.1.3 Retroviral Vectors

The retroviridae family are small, enveloped RNA viruses whose virions vary in size from 80-130 nanometers (nm). Their genome size ranges from 3.5-9 Kb and carry two copies of a (+) single-strand RNA genome in each virion (for a review see Fields[29]). An important feature of these viruses is their ability to reverse-transcribe and integrate into host cell DNA, thus resulting in its own genome permanently residing in subsequently produced daughter cells. Most retroviral infections do not kill the host cell, but have been shown to possess tumorigenic properties. This can result from integrations that disrupt cellular growth control genes, the activation of cellular oncogenes, or viral gene products that are oncongenic themselves.

Once integrated, the provirus is transcribed by cellular transcription factors directed by the viral promoter. Transcription results in one primary messenger RNA (mRNA) that is the genome for new virion production and codes for proteins upon splicing, translation, and proteolysis. The viral genome consists of 3 major genes: viral core proteins (gag), the polymerase (pol) genes which include reverse transcriptase and integrase enzymes, and envelope proteins (env). As virions are produced, they are assembled with specific proteins into capsids and eventually bud from the host cell. The genomic RNA has a packaging signal (ψ) at the 5' end of gag and the 3' end of the splice donor that is recognized by viral proteins to ensure that only viral RNA is packaged into virions.

Retroviruses were among the first viruses to be developed into gene therapy vectors. Retroviruses are divided into three subfamilies according to their pathogenicities: oncovirinae, lentivirinae, and spumavirinae. Although several virus groups have been examined as vectors, only two [Moloney murine leukemia oncovirus (MMLV) and HIV-1 Lentivirus] are extensively used for gene therapy research.

1.1.3.1 Moloney Murine Leukemia Viral Vectors

The initial retroviral vectors were derived from MMLV and have been most prevalently used to date[30,31]. There are two types of MMLVs, ecotropic and amphotropic. The major difference between these two is their infectable host cell range, or tropism, due to a variation in their surface binding protein (SU). Since ecotropic MMLVs can only infect rodent cells whereas the amphotropic form can infect human, rodent, and other cell types, the latter are primarily studied for human gene transfer[32-34].

In order to engineer safe MMLV vectors for gene therapy, the virus was rendered replication defective by deleting the gag, pol, and env genes and replacing them with a transgene[35]. In order to produce vector stocks, the deleted viral genes but not the packaging (ψ) signal, are provided *in trans* in packaging cell lines. This ensures that only vector RNA can be packaged and released. In order to minimize the potential for replication competent viruses (RCV) during production, the gag and pol genes are separated from the env gene in the packaging cell line. For vector stock production, packaging cells are grown in culture and then transfected with the desired vector plasmid construct. The desired virus is produced and assembled into particles that bud from the cells. The supernatant is harvested, purified, and concentrated to make vector stocks. This stock can be tested for wild-type virus and then used to infect target cells.

After an MMLV vector binds to the appropriate cellular receptor, viral uptake occurs by membrane fusion. Upon this entry, the capsid is released from the viral envelope. The capsid carries viral RNA, RT, IN, and a cellular transfer RNA (tRNA). The genome is reverse-transcribed into double-stranded DNA using the tRNA as a primer and the RT enzyme. During this conversion, unique long terminal repeated (LTR) sequences are present at each end of the DNA. This complex is then transported to the nuclear membrane where it requires cell division to obtain entry. Upon cellular division, the nuclear membrane is broken down and thus the complex is able to enter the nucleus. Integrase then mediates integration of the viral DNA to a random site of the host genome and is maintained by all daughter cells.

There are currently several limitations of MMLV vectors. First, vector stock titers have been limited to 10^7 pfu/mL[36,37]. Second, the virus is limited to only infecting cells that possess a receptor for its lone glycoprotein[32]. Third, the inability to integrate into non-dividing cells such as neurons or stem cells has limited their use[38]. Fourth, the virion is limited in the size of insert transgene that can be inserted into the genome. Lastly, the length of transgene expression in infected cells has been variable[39].

1.1.3.2 Lentiviral Vectors

The main primate infecting lentiviruses studied for vector use are the human immunodeficiency virus types 1 and 2[40]. These viruses are tropic for lymphocytes in particular and cause AIDS in humans. Lentiviruses have similar genomes to other retroviruses but encode numerous additional spliced RNA products (for a review, see Fields[41]). HIV vectors have received much attention recently due to their ability to reverse transcribe and integrate into non-dividing cells. Matrix protein (MA), a structural

protein present in the virus particle anchors the envelope proteins into the virion and has a nuclear localization signal that targets the viral DNA-protein complex to the nucleus[42,43], thus not requiring division as MMLV does. Another protein, Vpr, in addition to integrase has also been suggested to contribute to integration into non-dividing cells.

A variety of HIV based vector production systems have been engineered[44]. As with other retroviruses, the packaging signal, the primer binding site, a polypurine tract, and LTR sequences are present in the transfer construct. Several sustained or transient production schemes exist based on producer cell lines or co-transfection with one or two packaging constructs and a transfer plasmid. The main goal is to produce high levels of vector, while eliminating recombination that could produce replication competent viruses (RCVs).

The limited natural tropism of HIV had restricted the use of these vectors as a vaccine to patients already infected with HIV to provide protection to uninfected CD4 cells. To expand vector tropism, envelope genes from other viruses have been used to make a pseudotyped vector with a wider host range. Successful vectors have been engineered with the Vessicular Stomatitis Virus glycoprotein G (VSV-G) or the amphotropic MMLV env gene[40,45]. An added advantage that these vectors have over MMLV is the ability for high-level gene expression using the powerful Tat/LTR promoter combination found in HIV. Recent experiments have shown the ability of lentiviral vectors to maintain long-term transgene expression *in vitro* and *in vivo*[46,47]. HIV vectors are currently limited to titered stocks of 1×10^{8}/mL[48]. Additionally, its host range is limited to the envelope that is provided in the packaging cell line. The limit of

transgene insert is approximately 9 Kb. A major concern of working with these vectors is its reverting to a replication-competent vector.

1.1.4 Adenoviral Vectors

Adenoviridae are class I, non-enveloped, DNA viruses. The linear, double-stranded genome is approximately 36-38 Kb with a virion diameter is 70-90 nm (for a review, see Fields[49]). Adenoviruses (Ad) are subgrouped A through G, originally classified by hemagglutination patterns. A natural advantage of Ad is that it has been shown to infect a wide variety of cell types in culture. The subgroup that has been extensively studied for vector use is the non-oncogenic C types 2 and 5, which cause mild respiratory diseases in humans. Ad binds to the coxsackie and adenovirus receptor (CAR) on cell surfaces, interacts with clathrin-coated pits and is endocytosed. In the endosome, the pH drops and the virion is released into the cytoplasm where the capsid is then transported by microtubules to the nuclear membrane and the genome enters the nucleus.

The viral genes are classified as early-E (before DNA replication) and late-L (after onset of DNA replication). The E1 region of the genome is initially transcribed by cellular transcription factors and its gene products, E1a and E1b, transactivate E2–E4. The E2 gene products include a DNA binding protein (E2a) and viral DNA polymerase and terminal protein (E2b). The E3 region has been found to be non-essential for growth in culture but is involved in evading host defense mechanisms by interacting with major histocompatibility complexes (MHC). E4 gene products are involved in a number of functions including the regulation of late promoter activation and viral DNA replication. The late genes L1-L5 code for the structural proteins of the virion.

Adenoviral vectors are constructed by the deletion of a combination of early genes[50]. The vector must retain the 5' ITR and packaging signal. The deletions can then accommodate transgenes of similar size that have a promoter and a polyadenylation signal. A variety of ubiquitous promoters have been tested such as the Ad major late promoter[51] and the human cytomegalovirus immediate-early promoter (HCMV-IEp)[52]. Drug regulatable[53] and tissue-specific promoters[54] have also been used in Ad vectors. In order to propagate these vectors, complementing cell lines are needed that contain the essential deleted genes, such as 293 cells[55] that express E1a and E1b. After vector infection of the complementing cells, the virus is harvested. The cells are broken open and the virus is separated based on centrifugation and/or chromatography[56,57]. The Ad particles are stable over a wide range of pH and temperature compared to enveloped viruses and can result in 10^{11} pfu/mL or higher stocks. The major disadvantage presently associated with Ad vectors is the immune response to either the expression of low levels of viral genes or contaminating viral proteins associated with the viral preparations[58,59]. This latter is evident even in gutless vectors that have no viral genes remaining. The presence of helper viruses is a concern with this vector. These problems thus result in only short-term expression of the viral transgene. Another concern is that of generating replication competent viruses during propagation.

1.1.5 Adeno-Associated Viral Vectors

Adeno-Associated Virus (AAV) is a non-enveloped, class II parvovirus. Its genome is a single-stranded linear DNA of ~5 Kb, with a virion diameter of 18-26 nm (for a review, see Fields[60]). There are 4 human serotypes (1, 2, 3, and 5). AAV2 has been the most prevalently studied. AAV is not known to cause any disease in humans,

although it has been isolated in people with Ad infections. AAV also integrates its genome site specifically into the host DNA. These features have made AAV an attractive option for vector gene transfer.

AAV infects permissive cells by binding to heparan sulfate and subsequent endocytosis. As with Ad, the virion escapes during endocytolysis and the genome is transported into the nucleus. There are two outcomes of an AAV infection depending on the presence of a helper virus. If there is no helper virus (such as Ad or Herpesvirus), AAV will integrate as head to tail dimers of its genome in the host DNA or can be found as episomes. If the host cell is co-infected with a helper virus and AAV, AAV can replicate.

The AAV genome has inverted terminal repeats (ITRs) at both ends. The viral DNA transcribes two genes: rep (replication) and cap (capsid). Rep has two promoters, one internal. The first promoter encodes an unspliced Rep78 protein and a spliced Rep68 protein. Rep78 and Rep68 have a number of properties including aiding in viral DNA replication, processing, transcription, and integration. The internal promoter transcribes an unspliced Rep52 and a spliced Rep40 protein. Rep52 and Rep40 are required for accumulation of single-stranded DNA copies during productive infection. The cap gene is spliced to translate the three structural proteins of the virion VP1, VP2, and VP3.

AAV vectors are created by the deletion of some or all of the internal genome of the virus while minimally leaving the ITRs with a promoter and a transgene inserted between the ITRs[61,62]. In order to propagate this vector, the rep and cap genes must be provided to vector infected cells[63,64]. Packaged AAV vectors are extremely stable and can undergo several purification procedures without substantial losses resulting in vector

stocks of 1×10^{11} total infectious units/mL (TIU/mL). One noticeable disadvantage of AAV vectors is that they integrate much less frequently and not site specific as wild-type virus does due to the lack of Rep products[65]. This can cause the need to use high numbers of particles for adequate transduction. *In vivo* AAV vectors may evoke immune responses that results in the inability to redose[66]. Another problem is that of limited (5 Kb) transgene capacity.

1.1.6 Herpesvirus Vectors

There are two main classes of HSV-1 vectors being researched, amplicons and genomic. Amplicons were observed as replication defective particles naturally propagated during infections, in particular ones generated from high MOI cultures[67]. These particles have a viral origin of replication and a packaging sequence in addition to various repeated sequences, but lack several viral genes required for replication[68]. Amplicon plasmid vectors have been developed based on this observation[69,70]. Therapeutic genes cloned into plasmids can be packaged into virions due to the presence of the viral original packaging signals and propagated in bacteria. Packaging requires the coinfection of the amplicon with either a helper herpesvirus or complementing plasmids. A major problem is that stocks may be contaminated with helper virus and are limited to low-titer production[70,71]. The genomic vectors are either replication competent or replication defective. Those that are developed by the deletion of the expression of one or more non-essential viral genes such as ICP34.5[72] or Ribonucleotide Reductase (ICP6)[73], which affect neurovirulence, are replication competent and primarily used to deliver genes to neurons for CNS disorders. Another application for this vector is the destruction of tumor cells since they are extremely toxic to dividing cells. The second

type of replication-defective vector is based on HSV genomic manipulation to delete one or both of the essential immediate early (IE) genes ICP4 and ICP27[74-76]. A complementing cell line that expresses these deleted essential genes must be made to propagate the vector. Vector production and infected cell toxicity are often inversly related to the number of IE genes deleted[77,78]. In addition, approximately half of the 84 known genes are dispensable for growth in culture[79] and thus vectors are able to carry large or multiple transgene inserts due to the further deletion of these non-essential sequences. HSV-1 has been shown to efficiently infect most cell types, including nondividing cells, in culture[80,81] and has a natural property to become latent in neurons. The viral genome remains episomal in infected cells and shown to be maintained in neurons[82,83]. Various viral promoters are able to transiently express transgenes in the vector[84,85]. In neurons, latency-active promoter systems have demonstrated long-term transgene expression[86,87]. Disadvantages of HSV-1 vectors include either cytotoxicity from undeleted IE gene expression in non-neuronal cells or current low titers achieved from vectors crippled in multiple IE and other genes that show diminished toxicity. Purification of defective interfering particles in vector stocks is also currently lacking.

1.2 Herpesviridae

1.2.1 Classification of Herpesviruses

Herpesviridae are enveloped, class I, double-stranded linear DNA viruses[88]. The genomes range from 120-200 Kb and its virion diameter is 150-200 nm. Herpesviruses are grouped as α, β, or γ subfamilies according to infection characteristics and classed A-F according to sequence arrangements of their genomes. Eight different human herpesviruses have been isolated. The α subfamily includes Herpes simplex virus (HSV)

types 1 and 2, and Varicella-Zoster virus (VZV). The β subfamily includes Cytomegalovirus (CMV), Human Herpesviruses (HHV) 6 and 7. The γ subfamily includes Epstein-Barr virus (EBV) and HHV 8. Several herpesviruses have been studied for vaccines, such as EBV, VZV, and HSV-2, but HSV-1 has shown the most promise for gene transfer[89,90]. Some important characteristics of HSV-1, which is an α virus, include a large genome of 152 Kb, ease of gene manipulation, a wide host cell range, short reproductive cycle, rapid spread in culture, a natural tropism for neurons and the ability to become latent in these cells[79]. β viruses have a long reproductive cycle and progress slowly in culture. γ viruses have tropism for lymphocytes and become latent in this tissue.

1.2.2 HSV-1 Structure

A lipid bilayer membrane, obtained from the infected cell host during budding, surrounds the virus particle[79]. This envelope contains at least 10 different viral glycoproteins integrated in the lipid bilayer that function in binding to a variety of cell surfaces and aid in membrane fusion. The tegument is a matrix of at least 6 different proteins that connects the capsid to the envelope. These proteins include VP16, an Immediate Early (IE) viral gene transactivator, and U_L41, a virion host shutoff protein (vhs) that induces degradation of cellular mRNA, which aid the virus in efficiently infecting and replicating in cells. The nucleocapsid is an icosadeltahedron consisting of at least 5 proteins, with 960 copies of the major one, VP5, forming the hexamers and pentamers making up the shell. Inside the capsid is 152 Kb double-stranded linear DNA, seen as a torroidal shape in electron micrographs. The genome is designated as class E being that it is divided into a Unique Long (U_L) and Unique Short (U_S) region, with the

termini of each possessing inverted repeat sequences which make the regions reversible in orientation.

1.2.3 Biology of HSV-1

HSV-1 infection of susceptible cells involves attachment to ubiquitous cell surface glycosaminoglycans followed by secondary attachment to specific receptors. During initial adsorption, HSV-1 glycoproteins gB and gC bind to surface expressed heparan sulfate (HS) as a quasi-coreceptor[91-93]. Next, HSV-1 gD binds to one of two cellular receptors identified as Herpesvirus entry mediators (Hve) A and C. The gD receptor HveA[94-97] has been characterized as a member of the TNF/NGF receptor superfamily. The known natural ligands of HveA are LIGHT and lymphotoxin α, both of which are structurally similar to TNF[98,99]. The second major HSV-1 receptor, HveC (also termed nectin-1 or prr1), is a transmembrane cellular adhesion molecule[100,101] and a member of the immunoglobulin (Ig) superfamily[95,102,103]. In addition, gD can interact with 3-O-sulfotransferase (3-OST)-modified heparan sulfate on the cell surface[104]. It is one of these interactions which is believed to trigger fusion of the virus envelope with cell membranes resulting in virus entry.

The envelope fuses (independent of pH) at the cell membrane and the virion enters the cell. The tegument proteins and capsid are released into the cytoplasm. The capsid is transported via microtubules to the nuclear pores where the DNA is injected into the nucleus through the pores and circularizes. At this point, viral gene expression proceeds through a highly regulated temporal cascade. Viral IE protein transcription is initiated by VP16, after which a cascade of Early (E) and Late (L) gene expression follows, ending with virion production, packaging, and release, and host cell death. In

this cascade, the IE genes are generally transcription modulators, the E genes are regulated by the IE expression and are responsible for DNA replication. The L gene expression occurs following viral DNA replication and requires IE and E gene expression. The L genes are comprised mainly of structural proteins for the virions. It has been shown that approximately half of the viral genes are non-essential for viral production in culture.

There are 5 IE genes (ICP0, ICP4, ICP22, ICP27, ICP47), also designated α genes, that are transactivated and expressed immediately upon infection[105-108]. These IE genes are distinguished by the presence of TAATGARAT sequences in their promoters, which are recognized by VP16 and cellular factors octamer binding factor-1 (Oct1) and host cell factor (HCF). ICP4 is an essential gene for replication that is present in two copies, one at each U_S terminal repeat region. It is a major regulatory protein that acts as a repressor and activator of viral genes. In studies of viruses deleted for ICP4, the expression of E and L genes are greatly reduced while the remaining IE genes are overexpressed[109,110]. ICP27 is essential and present in 1 copy. ICP27 has been shown to aid in E and L gene expression[111-113]. ICP0 is non-essential and present in two copies in the inverted repeats, one each in the internal and external regions flanking the U_L. ICP0 is a powerful transactivator of viral promoters[114,115] and ICP0 mutants grow at reduced rates in culture[116,117]. It is also required for reactivation from latency in neurons. ICP22 is non-essential and ICP22 mutants have shown to be slightly reduced in viral yield in culture. ICP22 has been shown to affect late gene expression[118] and regulates stability and splicing of ICP0 mRNA[119]. ICP47 is non-essential and inhibits immune response *in vivo* by blocking the presentation of MHC class I antigen peptides[120].

The next class of genes expressed is the Early (E), or β genes, which are dependent upon efficient IE expression as mentioned above[109,110,116,117,121,122]. The products of this class are mainly enzymes that are involved in DNA replication. Replication involves a rolling circle mechanism that produces head-to-tail concatomers of the viral genome. There are three origins of replication in the genome, one in the U_L and two in the U_S region[79]. E genes include proteins such as Thymidine Kinase (TK or U_L24), dUTPase (U_L50), large and small Ribonucleotide Reductases (U_L39 and U_L40), and DNA polymerase (U_L30). TK is non-essential for growth in culture and its activity differs from cellular TKs. Upon addition of drugs such as Acyclovir (ACV) or Ganciclovir (GCV) to infected cells, TK converts them to 3' phosphorylated nucleotide analogs, which block subsequent nucleotide addition during DNA synthesis and thus prevent cellular DNA replication and cause cell death. The activated nucleotide analogs can pass through gap junctions to neighboring cells and likewise cause cell death, commonly referred to as the "bystander effect". Thus, HSV-TK has proven of great use in cancer therapy applications and numerous viral and non-viral approaches have been used to deliver HSV-TK to kill tumor cells. ACV or GCV is used to control reactivation of wild-type viral infections in patients.

The final set of genes expressed is designated as Late (L) or γ, which are primarily virion structural proteins. Late expression requires DNA replication[123,124] in addition to the IE gene products. Examples of L proteins include the major capsid protein ICP5, tegument proteins VP16 and U_L41, the glycoproteins of the viral envelope, and a protease, U_L26, required for packaging of DNA into capsids. DNA containing capsids with tegument proteins bud from the nucleus to the ER and obtain an envelope.

Two methods of viral release are postulated[79]. In one, the virion de-envelopes and enters the golgi where it reacquires a new envelope with mature glycoproteins. The virus is then transported to the cell membrane and released. The second directly transports the virion with envelope from the ER through the golgi where the immature glycoproteins present in the original envelope are processed. Then the virion is transported to the cell membrane to be released by exocytosis and/or cell lysis.

1.2.4 Latency

A wild-type HSV-1 infection *in vivo* begins with attachment to an epithelial skin cell[125,126]. Upon infection, the lytic cycle occurs as previously described[127-129]. After replication and release the virus spreads and invades local nerve endings. The virus is then transported to the cell body of the sensory ganglion. The cascade of viral gene expression in neurons begins as the lytic cycle does but IE gene expression is soon repressed and the virus enters a latent state. In this state, the genome is circular and non-replicating. During latency the normal cascade of gene expression is silenced, however, the latency-associated transcript (LAT) is expressed during this time and is the hallmark of HSV latency[130-132]. LAT is present in two copies, one each in the terminal repeat flanking the U_L. Following splicing of the primary LAT, two overlapping, nonlinear introns are produced of 2.0 and 1.5 Kb in length and accumulate in the neuron[133,134]. The functions of the LATs are not known. Mutant viruses deleted of LAT replicate and establish latency although they appear to be reduced in the ability to reactivate[135-137].

1.2.5 Current Replication-Defective HSV-1 Vectors

Replication-defective genomic HSV-1 vectors were initially developed by the deletion of both copies of the ICP4 essential gene[109]. The propagation of this vector *in*

vitro requires the protein be provided *in trans*. A complementing cell line was developed to provide this gene. However, this vector was toxic to both primary neurons and non-neuronal cells in culture due to the over-expression of other IE genes in ICP4 mutants. A second-generation vector was deleted for the essential ICP27 gene, which is required *in trans* as well[74-76]. This vector showed a decrease in toxicity. From this background, vectors have been developed with additional mutations in a combination of the remaining three IE genes (ICP0, ICP22, ICP47), with toxicity, production, and transgene expression decreasing in general as the number of IE deletions increases[77,78,138]. The deletion of all IE genes results in the inability of viral gene expression in noncomplementing cells (except for LAT in certain instances). The host protein shutoff gene, U_L41, has also been deleted in some backgrounds, which may aid in infected cell survival[139-141].

1.3 Stem Cells

1.3.1 Introduction

Cell-based therapy is the attempt to treat human disease by replacing or repairing the biological function of damaged tissues or organs with donor cells[142]. Traditionally this has been performed by organ or blood transplantation. Research in this area has begun to turn its attention toward the use of stem cells[143-145]. Stem cells of the blood[146] and skin[147,148], which have been studied for decades, have shown the ability to repopulate and/or repair their respective tissue under certain conditions. Recently stem cells have been identified in other tissue including muscle[149,150] and brain[151] that have the ability to become other cell types than that from which they were isolated.

The definition of a stem cell varies from each particular research area or application. One set of researchers has outlined a theoretical hierarchy based on the

limiting degrees of differentiation, proliferation, and the self-renewal potential of stem cell types[142,144,146]. The most primitive cells are named totipotent. Totipotent cells such as embryonic tissue, or zygotes, are able to give rise to cells of every lineage of the body. In adult tissue, the most primitive stem cells are defined as pluripotent. These cells are able to give rise to cells of one or more particular tissue types, but not all, as with totipotent stem cells. Examples of this type are muscle derived stem cells that are able to repopulate the hematopoietic system[149,150], mesenchymal stem cells that can become osteoblasts, fibroblasts, myoblasts, and other cell types[152], or hematopoietic cells that can incorporate into muscle[153] or endothelium[154]. The third type of stem cell is described as multipotent. These cells are less primitive and are committed to becoming various cell types of a predetermined tissue. Multipotent cells can be exemplified by certain hematopoietic cells that, as opposed to the pluripotent cells, can only give rise to the different types of blood cells. As all stem cells proliferate, mature, and differentiate, their daughter cells approach a terminal state that can no longer develop into new cell types. Some researchers have suggested that certain cells may be able to dedifferentiate or trans-differentiate[144,146]. This has not been formally proven and may be merely a matter of definitions or contaminating cell populations. An important feature of all three classes of stem cells is the ability for self-renewal, as well as the differentiating and proliferating potential each possesses. These features make stem cells very promising for many therapeutic applications.

Physical definitions also exist for describing and determining stem cell potential and have been extensively studied in the context of the hematopoietic system. One important method for determining primitive stem cells capable of reconstituting a

hematopoietic system is by transplantation of a population into a lethally irradiated SCID (severe combined immuno-deficient) mouse[155-161]. Survival of the mouse is dependent upon a reconstituted hematopoietic system derived from the donor cells. A comparative population of cells are then removed from this surviving mouse and transplanted to another lethally irradiated SCID mouse. If at least this secondary mouse and possibly any other serially treated mice from each previous mouse survive, the originally transplanted cells are considered to possess among them SCID repopulating cells (SRC) or primitive hematopoietic stem cells. Experiments of this nature have been performed with cells derived from umbilical cord blood, bone marrow, peripheral blood[160], and recently from cells of tissue such as muscle[150]. Initially all cells of the marrow or blood were used to obtain repopulating ability, but within the past several years researchers have begun to separate out and characterize various populations within these tissue. This can be achieved through the use of cell surface markers, which are identified by antibody labeling[157,162]. One of the earliest and most useful markers for hematopoietic cells is CD34. This marker is present on a wide population of primitive and lineage committed cells. Other surface markers have been discovered that can be of some use in further classifying sub-populations within the $CD34^+$ cells[163].

The absence of some or all surface markers associated with further differentiated states (lineage markers) on cells of the 34^+ population has been shown, in general, to indicate a more primitive cell with higher SRC or proliferative potential[164-166]. Some examples of common subsets of cells that are studied by researchers searching for enrichment of primitive hematopoeitic stem cells include i) $CD34^+/CD38^-$ or ii) $CD34^+/Lin^-$ (a cocktail of lineage markers). Two new subsets of $CD34^+$ cells have

recently been studied, $CD34^{+}/KDR^{+}$[167] and $CD34^{+}/AC133^{+}$[168-170]. Populations such as these have been isolated and transplanted in limiting numbers to SCID mice to verify the presence of SRC cells. Another isolation method for obtaining pluripotent stem cells from bone marrow as well as other tissue has been developed. This method identifies stem cell populations as a small sub-population that extrudes the Hoescht 33342 dye and has been referred to as the “Side Population” or “SP”. These cells have been shown to be $CD34^{-}$[171,172]. Recently, researchers have also begun to study the $CD34^{-}$ population of blood cells for the presence of SRCs. These various populations are commonly purified by fluorescence activated cell sorting (FACS).

The previous method of determining SRC potential is based on *in vivo* assays. Attempts to characterize hematopoietic stem cells by *in vitro* assays have also been developed[173]. The "Colony Assay"[174,175] involves culturing a small number of cells in a cytokine-supplemented media to allow the cells to expand. This is done in methylcellulose to keep proliferating cells together in a colony so that the daughter cells are in a confined area with the cell they originated from. After expansion for up to 2 weeks the colonies are scored according to the phenotype they represent, such as erythroid, granulocyte/monocyte, or a mix. The cell that originates each colony is termed a "Colony Forming Cell (CFC)". Another commonly used method, the "Long-Term Culture Initiating Cell (LTC-IC) Assay"[176,177] was developed to identify the presence of more primitive cells than the colony assay. The cells to be assayed are cultured in serial dilutions on a supportive feeder layer of irradiated bone marrow stromal cells. The cultures are maintained for 5 weeks. By this time, more committed progenitor colony forming cells have proliferated, terminally differentiated, and died. New cells and

colonies growing are from LTC-ICs. The cells are then harvested and plated to methylcellulose cultures. After 2-3 weeks, the colony numbers are assayed. It is important to realize that identifying and maintaining pluripotent stem cells (PSCs) in culture is difficult. They are typically quiescent in their natural environment and divide only when needed (signaled by local cues). Attempting to culture these cells without causing death or differentiation to a particular lineage is an important research area currently being explored[178-184]. This *ex vivo* expansion of hematopoietic stem cells so as to expand to all lineages of the blood, while self-renewing the primitive stem cells can be used to prepare stocks that aid bone marrow transplant (BMT) patients. *Ex vivo* expansion will save a great deal of time (several weeks) that is needed before total engraftment occurs in a body after a traditional BMT.

1.3.2 Endothelial Progenitor Cells

One example of using stem cell differentiation and homing for treatment of disorders is that of endothelial progenitor cells (EPCs). Several reports have demonstrated the presence and potential utility of EPCs in adult blood[168,185,186], including the treatment of ischemia by the enhancement of neovascularization[187-189] or the enhanced endothelialization of vascular grafts[190]. Due to the evidence that tumors cannot grow greater than 1-2 mm^3 without newly formed vascular networks feeding it, interest has grown in developing antiangiogenesis agents as a potential treatment[191-193]. This newly developed vascular network may be made up partly of EPCs from blood and/or marrow. A focus on EPCs as targets in anti-angiogenesis has been briefly examined. Ito et al.[194] isolated EPCs (adherent cells from human peripheral blood) and showed that they, as opposed to human umbilical vein endothelial cells (HUVECs), are

sensitive to angiostatin, which is used to treat cancer by inhibiting endothelial cell proliferation. This implies that angiostatin and possibly other related drugs control tumor development by inhibiting recruitment or development of EPCs for the developing vascular network. A potential therapeutic use for the differentiation and homing of EPCs is antiangiogenesis through delivery of a toxic gene to a tumor. This can be accomplished by harvesting $CD34^+$ cells from a patient with a solid tumor and transducing the cells with a vecor that contains the toxic gene. The cells, when implanted, would deliver this gene to the tumor, resulting in tumor death[195].

1.3.3 Gene Transfer to Stem Cells

Manipulation of the genetic make-up of stem cells could be used to treat disease by infusing the patient's own transduced (corrected) stem cells back into the afflicted individual, thus avoiding the rejection of foreign donor cells[143,196]. Subsequent cellular homing and/or differentiation and expression of the introduced transgene would correct the genetic deficit. This approach has been used to correct the SCID-X1 disorder in human patients by transducing $CD34^+$ bone marrow cells with a Moloney murine leukemia virus (MMLV) vector carrying the γc cytokine receptor gene[197]. Growth factor or morphogen gene therapy combined with stem cell transplantation may provide a method to direct the differentiation process, enhancing the recovery of specific cellular functions by stimulating the production of a specific lineage thereby replenishing the appropriate cell types. The ability to efficiently transduce unstimulated human stem cells with the vector of choice must precede experiments directed toward gene therapy applications.

Several vehicles and methodologies have emerged as potential gene delivery systems for therapeutic applications. Both viral and non-viral vectors have been employed for stem cell transduction with each having distinct benefits and disadvantages. For stem cells, issues relevant to the choice of vector include: (i) efficiency of transduction, (ii) mitotic state of the target cell (quiescent vs. dividing), (iii) maintenance of transduced DNA (integration vs. episomal), (iv) length of transgene expression (transient vs. long-term), (v) vector capacity, and (vi) unwanted host immune recognition of the transduced cells.

Initially, for stem cell transduction experiments, retroviral vectors were utilized for their ability to integrate a transgene into the host genome. Early *in vivo* experiments using murine hematopoietic stem cells compared ecotropic and amphotropic MMLVs carrying genes such as adenosine deaminase or glucoceribrocidase[32]. Donor cells were infected and transplanted into irradiated hosts. Long-term viral vector presence was seen, but at very low levels, in the amphotropic compared to the ecotropic vector. This may be partly explained by the differences in receptor expression levels in these cells. The amphotropic receptor GLVR2 was undetectable by RT-PCR. Several reports have shown that infection levels can be increased by the addition of cytokines to the cultures. This stimulation may be especially important for viral integration into non-dividing cells.

Initial attempts using the amphotropic MMLV to transduce unstimulated nonhuman primate and human hematopoietic stem cells have yielded poor transduction efficiency[32,198]. Emery, et al.[199] reported 9.6% of human $CD34^+$ stem cells were transduced by MMLV coding for neomycin resistance. The analysis was made on colony assays grown under selection. The transduction method involved culturing the cells in

Interleukin-3 (IL-3), IL-6 and stem cell factor (SCF) with two doses of vector at MOIs of up to 100 each. This could be increased slightly (1.6 fold) by culturing at 33°C. Cavazzana-Calvo, et al.[197] cultured human $CD34^+$ marrow cells on fibronectin coated plates in medium supplemented with calf serum, SCF, IL-3, and, FLT-3 Ligand (FL). MMLV vector carrying the γc cytokine receptor gene (SCID-X1 disease) was added daily for 3 days at an MOI of 1 each time. PCR analysis showed 20-40% of cells were expressing the gene and immunofluorescence showed 36% γc positive. The cells were transplanted after day three and 10 months later transgene expressing cells (T and natural killer-NK) were detected in two patients, and provided correction of the SCID mutation. Miyoshi et al.[200] compared VSV-G pseudotyped Lenti and MMLV vector transduction of $CD34^+$ human umbilical cord cells in SCF, IL-3, and IL-6. The cells were exposed for 5 hours to MOIs of 60 or 300 of each vector. Five days later transduction levels were comparable between vectors with 54% and 50% GFP positive at the higher MOI for Lenti and MMLV respectively. Samples of each were removed for colony assays to determine if CFC progenitors are transduced. The Lentivirus transduced cells were 8-fold higher in GFP^+ colonies than the MMLV infected cells. This may indicate that MMLV is not efficient at infecting more primitive progenitor cells. Dao, et al.[201] and Uchida, et al.[202] also reported that MMLV vectors are inefficient at transducing $CD34^+CD38^-$ cells. Evans, et al.[203] transduced human cord blood cells enriched for the $CD34^+CD38^-$ population with VSV-G Lentivirus. The cells were cultured in SCF, FLT-3 ligand (FL), IL-3, and IL-6 and two doses of MOI 20 were used. The average transduction efficiency was 59% while a comparative experiment with MMLV showed <4% GFP positive cells by flow cytometry. The Lentivirus transduced cells maintained this level of transduction

for at least 13 days in culture. These and other similar results indicate that Lentivirus is a more efficient vector than MMLV for the transduction of a variety of stem cell types and should be very useful as developments continue[204-206].

AAV vectors have recently been explored for the ability to transfer genes into human stem cells[207-209]. Nathwani, et al.[210] have developed AAV vector stocks deficient of helper and wild-type virus for gene transfer to human cord blood $CD34^+$ and $CD34^+/CD38^-$ cells. Using high MOIs of 1×10^6 and stimulating in high dose cytokine enriched medium for 48 hours, $CD34^+$ cells were 23% positive for GFP. In a second experiment, tumor necrosis factor (TNF) was also added to the culture and increased the transduction to 51%. Approximately 33% and 60% of the $CD38^-$ cells were GFP^+ in the absence or presence of TNF, respectively. The gene expression, however, was reported to be transient, indicating that possibly either the virus was episomal or the integrated vector was silenced.

Efficacy of gene transfer to human blood stem cells was examined using the nonintegrating Adenoviral vectors. Watanabe, et al.[211] transduced human BM mononuclear and $CD34^+$ cells at MOIs of 5, 50, and 500. The cells were cultured in IMDM with 10% FBS at 1×10^6/mL and virus was added. After 24 hours, the cells were harvested and assessed for transduction efficiency and colony assays were started with FACS positive cells. The $CD34^+$ cells averaged 7%, 12%, and 21% lacZ positive by fluorescein di-β-D-galactoside (FDG) staining at 5, 50 and 500 MOI respectively. Approximately 22% of the $CD34^+/CD38^-$ cells were FDG positive at the high MOI. Colony assays showed deleterious effects on colony numbers at the high MOI, but the low and mid MOIs were comparable to the uninfected colony numbers. Neering, et

al.[212] transduced human BM, mobilized PB, and UCB $CD34^+$ cells with Ad vectors that encode either a lacZ or alkaline phosphatase (AP) reporter gene. The cells were cultured in medium supplemented with 5% FBS, IL-3, IL-6, and SCF and an MOI of 500 was added 12-16 hours post plating. The cells were assayed 2 days later by flow cytometry. The BM cells were 45%, CB cells were 25%, and MPB were 23% AP positive. $CD34^+/CD38^-$ cells were 30% AP positive. Propidium Iodide (PI) is a viability dye that stains dead cells red. Cells at this MOI were on average 7.3% propidium iodide positive versus 5% in uninfected cells. Frey, et al.[213] demonstrated 19% GFP^+ cells infected with Ad at an MOI of 50 for PB $CD34^+$ cells 24 hours post infection. Higher levels of transduction were achieved when the cells were expanded for 10 days in various cytokine cocktails before infections. The level of adenoviral transduction appears to be limited by the presence of the major coxsackie and adenoviral receptor (CAR) on just 6-15% of $CD34^+$ cells[214,215].

Little research has been published on the infection of stem cells with HSV-1. This may be partly due to the fact that HSV does not integrate into the host cell chromosome, thus limiting its applications for gene therapy in $CD34^+$ cells. Dilloo, D, et al.[89] have shown that a disabled infectious single-cycle (DISC) HSV, an attenuated single replication cycle vector, can infect human bone marrow $CD34^+$ stem cells. The cells were cultured in stromal support cultures and exposed to an MOI of 2 for 2 hours and nearly 100% of the cells were reporter gene positive (LacZ gene driven by Simian Virus 40 promoter). This peak number occurred between 24-48 hours post-infection. Toxicity was low with 70-100% of the cells surviving the transduction. Long-term expression in culture was shown by a 27.5% $lacZ^+$ population at day 14, and 1-2% by day

21. It is important to note that this virus replicates once but does not seem to cause death, according to the report, even though similar vectors have been shown to be highly cytotoxic.

Coffin, et al.[216] have infected human peripheral blood $CD34^+$ cells with a replication attenuated HSV vector deficient in ICP34.5 and U_L43. The cells were concentrated to 1×10^7/mL and infected at an MOI of 10 for 2 hours. At 24 hpi, FACS analysis shows CMV-GFP^+ cells (10%). These cells were sorted for green and then stained for a second reporter gene's expression (LacZ under a Rouse Sarcoma Virus-RSV promoter in U_L43) with X-Gal (5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside). These sorted cells were found to be 100% positive for both genes. This report showed a marked decrease in transduction as compared to the Dilloo publication. Upon comparison several parameters that may be resulting in these differences include the tissue the cells are derived from, the viral adsorption step and the culture medium.

Gomez-Navarro, et al.[195] have infected $CD34^+$ human peripheral blood and monkey bone marrow cells with a non-replicative HSV-TOZ viral vector developed in the Glorioso lab. This vector is deficient in 3 immediate early genes, contains the lacZ reporter gene under control of the ICP0 promoter, and expresses thymidine kinase from the ICP4 promoter. The cells were incubated at 1×10^6/mL in 12 well plates. HSV-TOZ was directly added to the cultures at various MOI. At 12 hpi, FACS analysis for lacZ by FDG staining shows 99.9% positive cells at an MOI of 3. The infected cell culture showed no significant decrease in the number of viable cells at days 2, 4, or 7 post infection when compared to uninfected cells. *In vivo* experiments were performed on monkeys with healed skin autografts. Infected cells were transplanted and skin autograft

biopsies were taken and analyzed 5 days later. Results have shown infected cell targeting of the vascular walls by X-Gal staining and by in situ hybridization with both a LacZ gene probe and an HSV sequence probe. Mononuclear cells from the peripheral blood and bone marrow of the monkeys showed FDG positive cells for over 3 weeks. Ganciclovir was administered daily for 2 weeks to animals starting at 15 days post infected cell transplantation. The treatment induced a progressive necrotic process that was complete 5 days after the treatment ended such that the healed autograft lost blood supply and detached.

It is important to note that various detection methods were used to analyze the infected stem cells. Some reports analyzed the LacZ reporter gene by FDG loading. FDG is a substrate for β-galactosidase that is cleaved and results in fluorescein. This is sensitive and can be detected from <5 to >50,000 enzyme molecules per cell. A potential problem is that fluorescein can leak out of these cells and be taken up by other cells, giving false positives[217]. β-galactosidase is also present in viral preparations from the production and purification, thus adding an alternate source for the enzyme other than the infected cells being analyzed. X-Gal is a stain, similar to FDG, that can be used to visually determine β-galactosidase expressing cells but can be shady and thus subject to personal opinion. Another common reporter system used is GFP, which can be visually monitored by fluorescence microscopy or flow cytometry. Cells expressing GFP may not be detectable at the same low levels that β-galactosidase stained with FDG or X-Gal are, due to the multiple reactions that may occur per enzyme. These analytical differences may result in discrepancies when determining and comparing what percentage of cells, by reporter gene expression, have been transduced.

1.4 SUMMARY

The research performed for this thesis involves studies in three interrelated areas of replication-defective herpes simplex virus type-1 gene therapy vectors: application, production, and purification.

Hematopoietic stem cells have received much attention recently for the ability to "home" and differentiate into a variety of tissue, as well as repopulate a body's blood cell lineages. These unique properties make it an interesting tissue to study gene transfer into for potential tissue-specific therapeutic strategies. Current viral vectors have shown poor transduction results and require high levels of stimulating cytokines for achieving these results. In this thesis, studies on optimizing gene transfer to human umbilical cord blood $CD34^+$ cells are described. The presence and functionality of the herpesviral receptors were analyzed and new vectors were developed in multiple gene-deleted backbones in hopes of attaining decreased toxicity and tissue specific expression.

The $CD34^+$ cell transduction studies indicate a potential application for HSV-1 vectors. It was also learned that large amounts of vector will be required to make this a realistic treatment strategy. Therefore I next examined the effect of temperature and media conditions on vector stability. Applying these results to production scenarios demonstrated the ability to increase production levels of the vector through manipulation of culture conditions.

The FDA establishes guidelines on the amount of non-virion associated DNA and protein that can be injected into a human patient per dose of therapeutic. Thus the production of large amounts of vector for treatment of disease must be accompanied by a scalable purification system that meets these guidelines. In this work I studied the use of

filtration and chromatographic procedures that when combined provide a novel strategy aimed at concentrating and purifying high levels of produced vector.

2.0 MATERIALS AND METHODS

2.1 Gene Transfer to Human Umbilical Cord Blood $CD34^+$ Cells

2.1.1 Cell Culture

$CD34^+$ cells from human umbilical cord blood were subjected to positive selection by magnetic bead column chromatography according to the manufacturer's protocol (Miltenyi, Auburn, CA). Briefly, mononuclear cells obtained by Ficoll-Paque centrifugation are labeled with a hapten conjugated anti-CD34 antibody and purified with an anti-hapten secondary antibody attached to a magnetic bead. The recovered cells are washed and resuspended in BIT9500 (StemCell Technologies, Vancouver, BC) diluted with Iscove's Modified Dulbecco's Medium (Life Technologies Inc., Gaithersburg, MD) as directed and supplemented with 50 ng/mL stem cell factor (SCF) and 10 ng/mL thrombopoietin (StemCell Technologies). A sample of both the unlabeled mononuclear and $CD34^+$ enriched cells are then removed and labeled with a CD34 antibody recognizing a different epitope and stem cell purification assessed by flow cytometry.

Vero cells obtained from ATCC (CCL81) are cultured in Minimum Essential Medium (Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL Penicillin and Streptomycin (Life Technologies Inc.). The ICP4/ICP27 IE gene complementing cell line, $7b^{(75,218)}$, is cultured identical to Vero cells. CHO cells expressing Herpesvirus entry mediator receptors A or C (HveA or HveC) designated as M1A or $M3A^{(100)}$ respectively, were a gift from Dr. Gary Cohen and Dr. Roselyn Eisenberg (University of Pennsylvania). CHO lines are cultured in F-12K medium supplemented with 10% FBS, 100 U/mL Penicillin and Streptomycin, 250 μg/mL G418, and 150 μg/mL Puromycin (Life Technologies Inc.).

2.1.2 Virus Vector Construction

The construction of the multiple IE gene deletion mutant QOZHG has been described previously[219]. This viral recombinant has deletions of the essential IE genes ICP27 and ICP4. The TAATGARAT sequences within the ICP22 and ICP47 promoters have been mutated[77,138] so they are no longer recognized and activated by VP16, and hence are confined to expression as early, or β genes in complementing cell lines. The reporter gene cassette consisting of the human cytomegalovirus IE promoter driving enhanced green fluorescence protein (HCMV IEp-GFP) is present in the ICP27 locus. The virion host shutoff gene U_L41 is disrupted by the insertion of an HSV-1 ICP0 IE promoter-*lac*Z expression cassette. This vector was created by a genetic cross of the vector TOZ.1 (ICP4$^-$, ICP27$^-$, ICP22$^-$, U_L24^-::ICP4 IEp-thymidine kinase, U_L41^-::ICP0 IEp-*lac*Z[218] and d106 (ICP4$^-$, ICP27$^-$::HCMV IEp-GFP, βICP22, βICP47)[77,138] and was propagated on complementing 7b cells. Vector stocks were purified using OptiPrep (Life Technologies Inc., Cat. No. 103-0061) gradients and titered on 7b cells.

TOZ.1, a triple IE gene deletion mutant virus with the deletion of three IE genes (ICP4, ICP27, and ICP22), contains the *lac*Z gene inserted under control of the ICP0 promoter[218] in the UL41 locus. In addition, the normal early gene promoter for thymidine kinase (UL23) has been altered by the substitution of the ICP4 IE gene promoter which also affects the downstream reading frame encoding UL24. TOZHG is identical to TOZ.1 except that it also carries a reporter gene cassette consisting of the human cytomegalovirus IE promoter driving enhanced green fluorescence protein (HCMV IEp-GFP) within the ICP27 locus. The GFP transgene was obtained from the pEGFP-N1 plasmid (Clontech). Vectors expressing a transgene from the HSV latency

active promoter (LAP2), an endothelial specific promoter (vWF), or a combination of these have been similarly engineered. Breifly, recombination plasmids were developed that contain the desired promoter with GFP flanked at both ends by ICP27 gene sequences. Recombinant vectors are developed by first infecting 7b cells at MOI=3 with the background vector (TOZ.1). The cells are then transfected with the desired recombination plasmid. The infection/transfection culture is harvested when cytopathic effects are visually present. The harvested vector is screened by infections of limiting dilutions and visually monitoring developing plaques for GFP expression.

The human erythropoietin expressing vector, DHEPO, has deletions of the essential IE genes ICP27 and ICP4. The expression cassette consisting of the human cytomegalovirus IE promoter driving human erythropoietin (HCMV IEp-EPO) was recombined into the U_L41 locus of the vecotr DHZ.5 that contains an HCMV IEp-lacZ reporter cassette in U_L41. $LacZ^-$ recombinants were identified, subjected to three rounds of limiting dilution and the presence of the EPO expression cassette in the U_L41 locus confirmed by Southern blot analysis.

2.1.3 Flow Cytometry Analysis of GFP

QOZHG HSV-1 vector transduced cells were washed twice and resuspended at $0.25x10^6$/mL in 1% FBS and 1% paraformaldehyde in PBS. Minimally 1×10^5 total cells per sample were added to 5 mL round bottom polystyrene tubes (Falcon/Becton-Dickinson, San Diego, CA). The cells were analyzed by flow cytometry (FACSCalibur or FACSTAR, Becton-Dickinson) using a mock sample as the forward and side scatter control to set cell population gating and as the autofluorescence control to set

fluorescence gating boundaries. Cell data was typically collected for 10,000 gated events per sample.

2.1.4 Flow Cytometry Analysis of HveA and HveC

CHO-HveA (M1A), CHO-HveC (M3A), or $CD34^+$ cells were concentrated to 1×10^6/mL in 1% FBS/1×PBS. Aliquots of $2x10^5$ cells were incubated with either anti-HveA (1:300 dilution in PBS/1%FBS), anti-HveC (5 μg/mL) or both antibodies for 30 minutes on ice. Both anti-HveA (R140, polyclonal)[97] and anti-HveC (CK41, monoclonal)[100] were gifts of Drs. Cohen and Eisenberg (U. of Penn.). For $CD34^+$ cell analysis, CK41 was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). Cells were diluted, washed twice, and resuspended at 1×10^6/mL and incubated with either FITC-conjugated anti-rabbit secondary antibody (1:150, Sigma, St. Louis, MO), PE-conjugated anti-mouse secondary antibody (1:150, Sigma), or strepavidin-PE conjugated secondary antibody (Sigma) for $CD34^+$ cells, or both secondary antibodies for 30 minutes on ice. Cells were again washed twice, and resuspended at $0.25x10^6$/mL in 1% paraformaldehyde, 1% FBS in PBS. The cells were analyzed by flow cytometry as described above for GFP. For analysis of GFP and either receptor, anti-HveA was labeled with Cy3-conjugated anti-rabbit secondary antibody and anti-HveC was labeled with either strepavidin-PE (Sigma) or strepavidin-APC conjugated secondary (Sigma) as indicated. This analysis was performed for two separate cell harvests.

2.1.5 Immunohistochemistry for ICP0

$CD34^+$ cells (1×10^6/mL) were either mock-infected or infected with vector at an MOI of 30 immediately after purification and directly incubated in a 12-well tissue culture plate at 37°C/5% CO_2. Sixteen hours after vector addition the cells were removed

from culture, washed, and resuspended in PBS containing 1% FBS. 1×10^5 cells were removed from each sample, fixed in 1% paraformaldehyde, and analyzed for GFP expression by flow cytometry. Cytospins of 1×10^5 cells per slide (superfrost/plus; Fisher, Pittsburgh, PA) were prepared by centrifugation at 450 rpm for 5 minutes at room temperature, fixed with 100% cold methanol for 2 minutes, and allowed to air dry. Slides were blocked with 10% horse serum in PBS for 1 hour at room temperature, incubated with primary ICP0 monoclonal antibody (1:2000 dilution: Virusys; East Coast Biologics, Inc., North Berwick, ME) at 4°C overnight. The cells were washed twice in PBS/1% FBS and incubated with PE-conjugated anti-mouse secondary antibody (1:500, Sigma) for 2 hours at room temperature in the dark. The slides were then washed twice with PBS, overlayed with Gel/mount (Biomedia, Foster City, CA), and a coverslip applied. Slides were maintained at 4°C in the dark and images collected by fluorescence microscopy using a Xillix digital camera attached to a personal computer-based image analysis system (MCID, St. Catharines, Ontario, Canada).

2.1.6 Immunohistochemistry for PML

Uninfected $CD34^+$ cells were prepared as described for ICP0 immunohistochemistry except that cells were stained with monoclonal anti-PML antibody (0.25 μg/mL or 0.8 μg/mL dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-conjugated anti-mouse secondary (1:500, Sigma).

2.1.7 Immunohistochemistry for HveA and HveC

Cells were prepared as described in the Flow Cytometry Analysis methods except Cy3-conjugated anti-rabbit secondary antibody was used in place of FITC-conjugated

secondary antibody for HveA analysis. Cytospins were prepared as above except cells were fixed with 1% paraformaldehyde prior to cytospin.

2.1.8 Dextran Sulfate Analysis

$CD34^+$ cells were harvested, resuspended at 1×10^6/mL, and plated in a 96-well plate at 1×10^5 per well. Various amounts of dextran sulfate (Amersham Pharmacia Biotech, Piscataway, NJ) were mixed thoroughly with the cells and incubated at room temperature for 10 minutes. QOZHG vector was then added (MOI=5 or 10), mixed thoroughly, and incubated at 37°C. Sixteen hours post infection the cultures were washed twice with medium and resuspended at $0.25x10^6$/mL in 1% paraformaldehyde and 1% FBS in PBS and GFP expression analyzed by flow cytometry. Transduction with MOI 5 or 10 were performed on separate cell harvests.

2.1.9 Neutralization Assay

CHO-HveA (M1A), CHO-HveC (M3A), or $CD34^+$ cells (1×10^6/mL) in their respective mediums and anti-HveA, anti-HveC, or both antibodies were added at the indicated concentrations and incubated at 4°C for 1 hour. QOZHG vector was added at the indicated MOI and mixed. CHO-HveA and CHO-HveC cells were rocked for 1 hour at 37°C, washed twice with medium, plated, and incubated in the appropriate medium at 37°C. $CD34^+$ cells were plated without washing, and incubated at 37°C. All cultures were harvested 16 hours post viral addition, washed twice, resuspended at $0.25x10^6$/mL in 1% paraformaldehyde and 1% FBS in PBS, and analyzed by flow cytometry for GFP expression. The experiment was performed in duplicate using cells obtained from one harvest.

2.1.10 Erythropoietin ELISA

Isolated $CD34^+$ cells (1×10^6/mL) were either mock infected or infected at MOI=3, 10, or 30 with DHEPO by the optimized transduction method determined for QOZHG. The cells were cultured in BIT9500 with SCF and TPO. Samples of culture supernatant were removed daily and analyzed for EPO production by ELISA, as per manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN). The experiment was performed on one cell harvest and samples were analyzed in duplicate.

2.2 Vector Production

2.2.1 Cell and Virus

The replication defective mutant vector TOZ.1 is deleted for the IE genes ICP4, ICP27 and ICP22, as well as the virion host shut-off (vhs) protein (U_L41) and U_L24[218]. In addition it contains a reporter gene cassette consisting of the lacZ gene under the control of the ICP0 promoter inserted within the U_L41 locus. The 7b cell line is a Vero cell derivative designed to express the essential IE genes ICP4 and ICP27 upon infection[75,218]. The 7b cells were maintained in 150 cm^2 vented cap flasks (Becton Dickinson, MD) with 25 mL of Minimum Essential Medium (MEM) w/ Earle's salts, 1500 mg/ml sodium bicarbonate, 2 mM L-glutamine, 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 10% Fetal Bovine Serum (Life Technologies, Gaithersburg, MD). MEM, with supplements, was used for all experiments except where noted. The cells were maintained in a 37°C incubator at 5% CO_2 with a humidifying water pan. The cells were subcultured by seeding at 0.3×10^5 cells/cm^2 and removed by trypsinization when reaching about 1.2×10^5 cells/cm^2 (100% confluency).

2.2.2 Cell Growth Experiment

Cells grown at 37°C were subcultured into 25 cm^2 flasks (Becton Dickinson, San Diego, CA) with 2.25×10^5 cells in 3.6 mL fresh serum medium and incubated at 37°C or 33°C. Duplicate flasks were harvested for each temperature and time, and viable cell counts performed using Trypan Blue (Sigma, St. Louis, MO) non-viable cell stain.

2.2.3 Virus Titer

Viral titers were determined by performing standard viral plaque assays. Serial dilutions of virus samples were incubated with 1×10^6/mL freshly trypsinized 7b cells in 1.5 mL safe-lock eppendorf tubes on a Nutator rocker (Becton Dickinson) at 37°C for 1 hour. Infected cells were plated on 12-well plates (Becton Dickinson) at 3.2×10^5 cells/well (80% confluency) in a final volume of 800 μL. After cell adhesion (12 hours), the medium was removed and replaced with a solution of 0.5% methyl-cellulose (Aldrich, Milwaukee, WI) in supplemented MEM. Cultures were stained after 4 days with 1% crystal violet (Sigma) in MeOH/water (50%/50%) and the number of plaques counted. All assays were performed in duplicate and results are presented either as the number of plaque forming units per volume (PFU/mL) or per cell (PFU/cell). Titers less than 10^3 were not recorded.

2.2.4 Synchronous Infections

Freshly trypsinized 7b cells grown at 37°C were suspended at 1×10^6/mL in fresh serum medium in 1.5 mL eppendorf tubes and stock vector was added corresponding to 5 PFU per cell (high MOI) to ensure all cells are simultaneously infected. Titers of viral stocks were determined by plaque assays of aliquots of the stocks prior to and again concurrently with initiation of the experiment. The infection solutions were rocked for 1

hour, centrifuged (514×g), the supernatant removed, and the cells resuspended in fresh serum medium. The cells were then plated in duplicate in 96-well plates (Becton-Dickinson) at 3.3×10^4 cells/well in a total volume of 100 μL and incubated at 37ºC or 33ºC. Wells were harvested at various times post infection by first removing the supernatant then harvesting the cells by adding 100 μL of fresh serum-free medium and scraping the well with a pipette tip. The samples were stored at –70ºC and titered simultaneously at the end of the sampling period.

2.2.5 Production Infections

Infections were performed similar to synchronous infections. Viral stock was added to cells of passage 30, 45, or 80, corresponding to 0.05 PFU per cell (low MOI) and infections were plated into 6-well plates (Becton Dickinson) at 8×10^5 cells in a total of 2 mL fresh serum medium per well and incubated at 37ºC or 33ºC immediately or shifted to 33ºC 24 hours post plating. Samples of 30-40 μL of supernatant were removed at various times following infection and the medium replaced with fresh serum medium to maintain a constant volume. The experiments were performed on two cell populations that were separated after drug selection.

2.2.6 Virus Decay Experiments in Media with or without Serum

A stock of virus was prepared by harvesting viral supernatant from 10-150 cm^2 tissue culture plate infections and centrifuging (514×g) to remove cell debris. Supernatant was then centrifuged (40,000×g) to pellet the virus, which was subsequently resuspended in 1 mL of medium without serum, aliquotted, and stored at -70°C. Stocks were typically $1\text{-}2\times10^8$ pfu/mL. An aliquot was thawed and diluted with the fresh serum-containing medium or medium without serum to approximately $1\text{-}2\times10^6$ PFU/mL to

mimic the viral concentration in the supernatant of infected cells. Duplicate 6-well plates of this virus solution were incubated at 37°C and 33°C in a total volume of 2 mL per well. Samples were removed at time points, frozen, and titered as above. The numbers of infectious viral particles were determined over time, plotted, and an exponential trend-line was determined by Excel.

2.2.7 Virus Decay Experiments in Culture Conditioned Medium

To obtain conditioned medium, infections were performed in 6-well plates and the culture medium was removed (60 hours post-infection) and centrifuged (514×g) to remove cellular debris while retaining the released virus in the medium. This supernatant was re-plated to empty plates (no cells) and incubated at 37°C or 33°C to mimic released virus in the conditioned medium. The numbers of infectious viral particles were determined over time, plotted, and an exponential trend-line was determined by Excel.

2.2.8 Glucose and Lactate Assays

Samples were obtained from duplicate infected cell cultures (performed as described previously in 'Production Infections' section) conducted at 37ºC and 33ºC and stored at -70°C until analyzed with enzymatic assay kits (Sigma) to measure glucose concentration (Cat. No. 16-50) and lactate concentration (Cat. No. 735-10).

2.3 Vector Purification

2.3.1 Vector Centrifugation

Low Speed: Vector infection cultures were harvested and centrifuged (Beckman, Model GPC, Rotor No. GH-3.7) at 1000×g for 10 minutes at 4°C. Clarified supernatants were removed from pelleted debris and processed as indicated. High Speed: Vector supernatant was centrifuged at either 13,000×g, 27,000×g, or 48,000×g for 10, 20, or 30

minutes at 4°C (Beckman, Model No. J2-21M, Rotor No. JA-20). Supernatants were removed and the pellets were resuspended to a 50× concentration in serum-free medium. Centrifugation experiments were performed in duplicate and titers were performed on both supernatant and pellet to analyse vector presence.

2.3.2 Dead-End Filtration of Vector

Syringe Filtration: Centrifuge-clarified virus supernatant (1 mL) was filtered separately or serially through a 1.2 μm, 0.8 μm (Schleicher and Schuell, Cat. Nos. 10465206 and 10465204 respectively), 0.65 μm (Osmonics, Cat. No. SO6SP02500), 0.45 μm, or 0.22 μm (Millipore, Cat. Nos. SCHA0250S and SLGVR25LS respectively) syringe filter using luer lock 3 mL sterile syringes. The experiments were performed in duplicate and flow-through vector samples were titered to analyze vector recovery. Centrifugal Filtration: Infection culture harvests were clarified by centrifugation and either unfiltered (10 mL or 20 mL) or filtered through a 1.2 μm or 0.65 μm syringe filter (10 mL) and loaded onto 300,000 molecular weight cut-off, 20 mL capacity Centricon centrifugal filters (Millipore, Cat. No. SE3P001L99). The vector was centrifuged at 1000×g and 4°C. The process was stopped periodically to monitor retained volume. When desired concentration was reached, the retentate was recovered according to manufacturers instructions. Experiments were performed in duplicate and titers were performed on vector before centrifugal filtration and on recovered vector.

2.3.3 Electron Microscopy

Transmission electron microscopy analysis was performed on a sample obtained from the vector production harvest. Harvest supernatant with cell debris was pelleted by centrifugation at 20,000×g for one hour. The pellet was fixed with glutaraldehyde,

stained with osmium tetroxide and dehydrated by a series of increasing concentration ethanol washes. The pellet is then coated in Epon, embedded, and cured. Slices of 90 nm were collected on copper grids and coated with methanol and uranyl acetate, washed and treated with lead citrate. After drying, the samples were viewed by electron microscopy and photographed at various magnifications.

2.3.4 Heparin Chromatography

Vector prepared by centrifugation was purified using a 1 mL heparin chromatography column (Amersham Pharmacia Biotech, Cat. No. 17-0406-01). Heparin columns are prepacked with 6% agarose beads of 34 μm diameter. The beads are loaded with 10 mg heparin per mL of gel. The bead pores have a 4×10^6 molecular weight exclusion limit.

Centrifuged vector was resuspended in 1× phosphate buffered saline (PBS) and loaded at 0.7 mL/minute using a Pharmacia LKB Pump P-1. The column was washed with a series of 5 column volumes of PBS containing 0.133 M, 0.5 M, 1.5 M, and 3.0 M NaCl. Elutes were analyzed for vector presence by titration and total protein by Bio-Rad colorimetric assay (Bio-Rad, Hercules, CA, Cat. No. 500-0006).

Vector purified by tangential-flow filtration was purified using 5 mL heparin chromatography columns (Amersham, Cat. No. 17-0407-01). Vector was loaded at 1.4 mL/minute and washed with 7 column volumes of PBS containing 0.133 M, 0.25 M, 0.75 M, and 1.5 M NaCl. Each elute was collected as two separate 3.5 column volume fractions. Elutes were analyzed for vector presence by titration, total protein by Bio-Rad Protein Assay, and total double-stranded DNA by PicoGreen (Molecular Probes Inc., Eugene, OR).

2.3.5 Tangential-Flow Filtration

The filters and system used in this work were provided by or purchased from A/G Technology, Inc (Needham, MA). The three filters used are 0.8 μm (Cat. No. CFP-8-D-4MA), 0.65 μm (Cat. No. CFP-6-D-4MA), and 0.1 μm (Cat. No. CFP-1-D-4MA) nominal pore size. The surface areas of the filter cartridges were each approximately 0.046 m^2 and were packed with 75 hollow-fiber tubes of 0.75 mm internal diameter each. The pump and stand system used is the Quixstand (Cat. No. QSM-03S) with a Watson/Marlow 313S 400 rpm pump. Initial running parameters were determined by conferring with the manufacturer. A feed flow of near maximal pump capacity was used in order to minimize gel layer formation. This corresponded to a flow rate of 1 L/min or a shear rate of 5,300 sec^{-1} based on clean water flux studies. The permeate collection flow rates were maintained at 25-30 mL/min as a compromise to prevent pore plugging due to high flux rates yet still provide a reasonable process time, considering process volumes of 1-2 liters. This flow rate was achieved by the use of a permeate control pump (Cole-Parmer, Cat. No. 7553-30) for the 0.8, 0.65, and 0.1 μm filters.

To analyze the large-pore filter systems, the 0.8 and 0.65 μm filter systems were each loaded with 1 liter of total vector infection harvest. The system was run as described until the retained solution was at the system hold-up volume of 100 mL. Permeate was collected and analyzed for clarified vector. High recovery of vector (up to 90%) in the permeate was achieved by washing the cell debris with PBS, which increased the total permeate volume to 1.5 L. The 0.1 μm filter systems were run using the parameters described and additionally washing the retained vector with PBS to perform a

buffer exchange prior to chromatography. Retentate was harvested when the volume was reduced to approximately 200 mL. Permeate was also collected and analyzed for vector passage.

3.0 GENE TRANSFER TO HUMAN UMBILICAL CORD BLOOD CD34$^+$ CELLS

3.1 Introduction

The CD34$^+$ population of cells derived from bone marrow, cord blood or peripheral blood contain a small population of stem cells capable of reconstituting the hematopoietic system in bone marrow ablated animals and humans. Evidence is accumulating that a similar stem cell population can differentiate into a variety of tissue types suggesting their broad utility for tissue regeneration. Alternative hematopoietic stem cell populations have been used to repopulate damaged liver[220,221] and muscle[149,153,222]. Published work reported the use of an HSV recombinant vector to demonstrate homing of infected CD34$^+$ monkey bone marrow cells to sites of angiogenesis in skin-grafted monkeys[195]. This work indicated that recombinant herpes simplex virus type 1 (HSV-1) vectors are capable of transduction of stimulated CD34$^+$ cells derived from the bone marrow of primates and mobilized peripheral blood from humans.

HSV was shown to provide a useful vector system for gene transfer applications where modified stem cells may be therapeutic, however, neither the receptors used for virus entry into hematopoietic stem cells nor the susceptibility of unstimulated human cells to HSV-mediated gene transfer have been determined. In addition, the optimal MOI and minimal time of infection have not been established. In this study, I evaluated the ability of a replication defective HSV vector for marker gene transfer to unstimulated

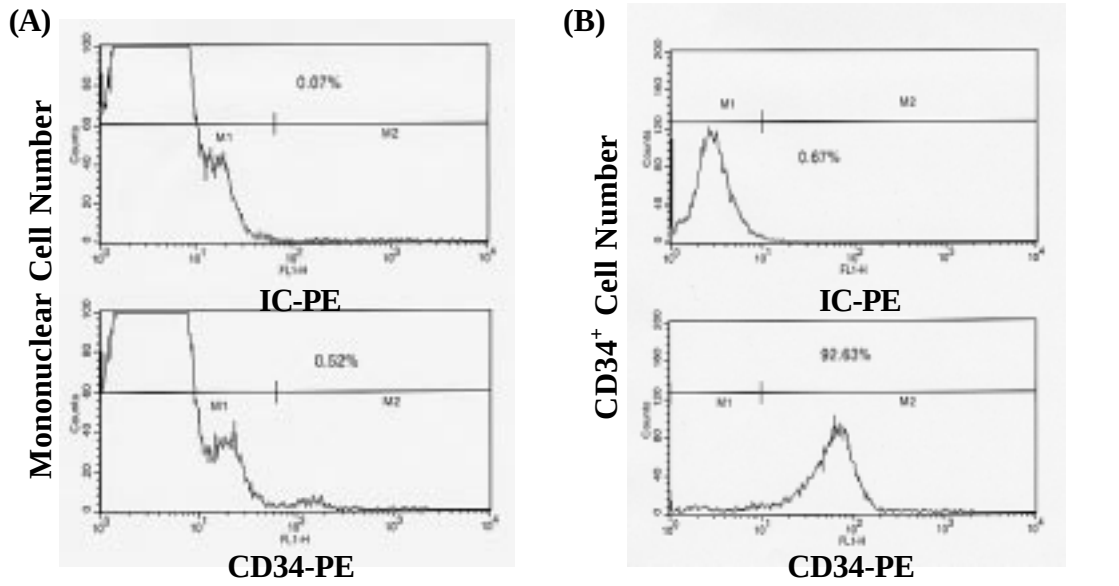
human $CD34^+$ stem cells derived from human umbilical cord blood. One or both of the HSV receptors HveA and HveC were detected on 95% of $CD34^+$ stem cells and infection was inhibited by combined receptor specific antibodies. The virus receptor interactions were novel in as much as virus-exposed cells resulted in the loss of cell surface HveC, an effect not observed for HveA. Under optimized conditions, we observed transduction efficiencies of 55%-75% with minimal adsorption times of 10 hours, confirming that HSV vectors may provide an important tool for advancing stem cell biology and the use of unstimulated $CD34^+$ stem cells for regenerative therapies. Specifically, I have demonstrated this by employing a replication defective HSV vector that was capable of secreting the therapeutic product erythropoietin.

3.2 Results

CD34-expressing cells were selected by antibody-magnetic bead column chromatography on mononuclear cells isolated from human umbilical cord blood obtained at cesarean-section births. These stem cell enriched populations, typically greater than 90% $CD34^+$ (Figures 1A & B), were used for all subsequent experiments.

3.2.1 Conditions for HSV Infection of Unstimulated $CD34^+$ Cells

Experiments were performed to establish optimal conditions for infection of unstimulated $CD34^+$ cells in culture using a replication-defective ($ICP4^-$, $ICP27^-$) recombinant HSV vector, $QOZHG^{(219)}$, which carries a GFP reporter expression cassette in the ICP27 locus and a lacZ cassette in the U_L41/vhs locus gene (Figure 1C). ICP0 is the only unaltered viral IE gene expressed from QOZHG.



(C) QOZHG Viral Vector

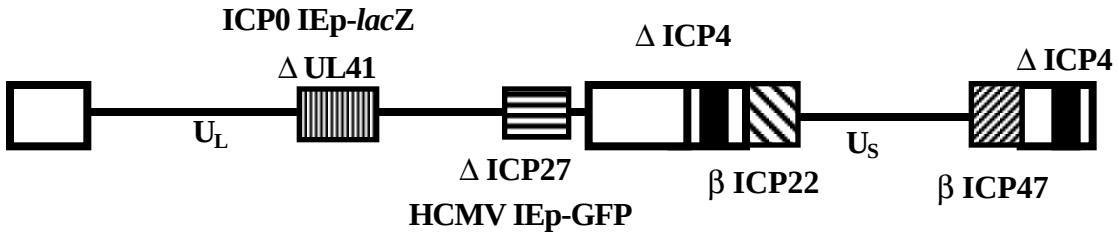


Figure 1. Flow Cytometry analysis of human umbilical cord blood cells and schematic of the HSV-1 vector QOZHG.

Initial transduction experiments performed on the $CD34^+$ cells employed standard methods used to transduce Vero cells, which are highly susceptible to HSV-1. The standard method of infection is to concentrate the cells at 1×10^6/mL in a tube, add virus, rock for 1 hour at 37ºC, wash, and then plate the cells. This protocol resulted in 96% of Vero cells transduced at MOI=5. In contrast, relatively low transduction levels (9%) of $CD34^+$ cells were observed, even at MOI=50, using flow cytometry analysis of GFP^+ cells (Figures 2A & B).

Systematic variation of cell densities, viral adsorption times, and vector doses (MOIs) revealed maximum transduction efficiencies in the range of 60-75% at an MOI of 30. Extension of virus adsorption times had marked positive effects on transduction at optimum cell densities of $1\text{-}5\times10^6$/mL (Figure 3A). As expected, transduction efficiencies increased with increased MOIs, but the differences faded at the highest MOIs (Figure 3B). Analysis of the flow cytometry histogram for an infection at MOI=30 and 16 hour adsorption shows a range of GFP expression including a population of highly fluorescent cells at the far right of the plot (Figure 3C). From these results, infection conditions were standardized to virus incubation with 1×10^6 cells/mL in tissue culture wells at an MOI of 10-30 for 16-18 h after pipettor mixing and before virus removal by washing and subsequent plating or analysis. Routine transduction efficiencies under these conditions were 35-45% (MOI=10) and 60-70% (MOI=30).

The QOZHG vector is replication defective, and the only genes expressed are the two transgenes (GFP and *lac*Z) and ICP0, the sole IE gene product remaining. ICP0 protein has been shown to accumulate in the nucleus and co-localize with nuclear domains

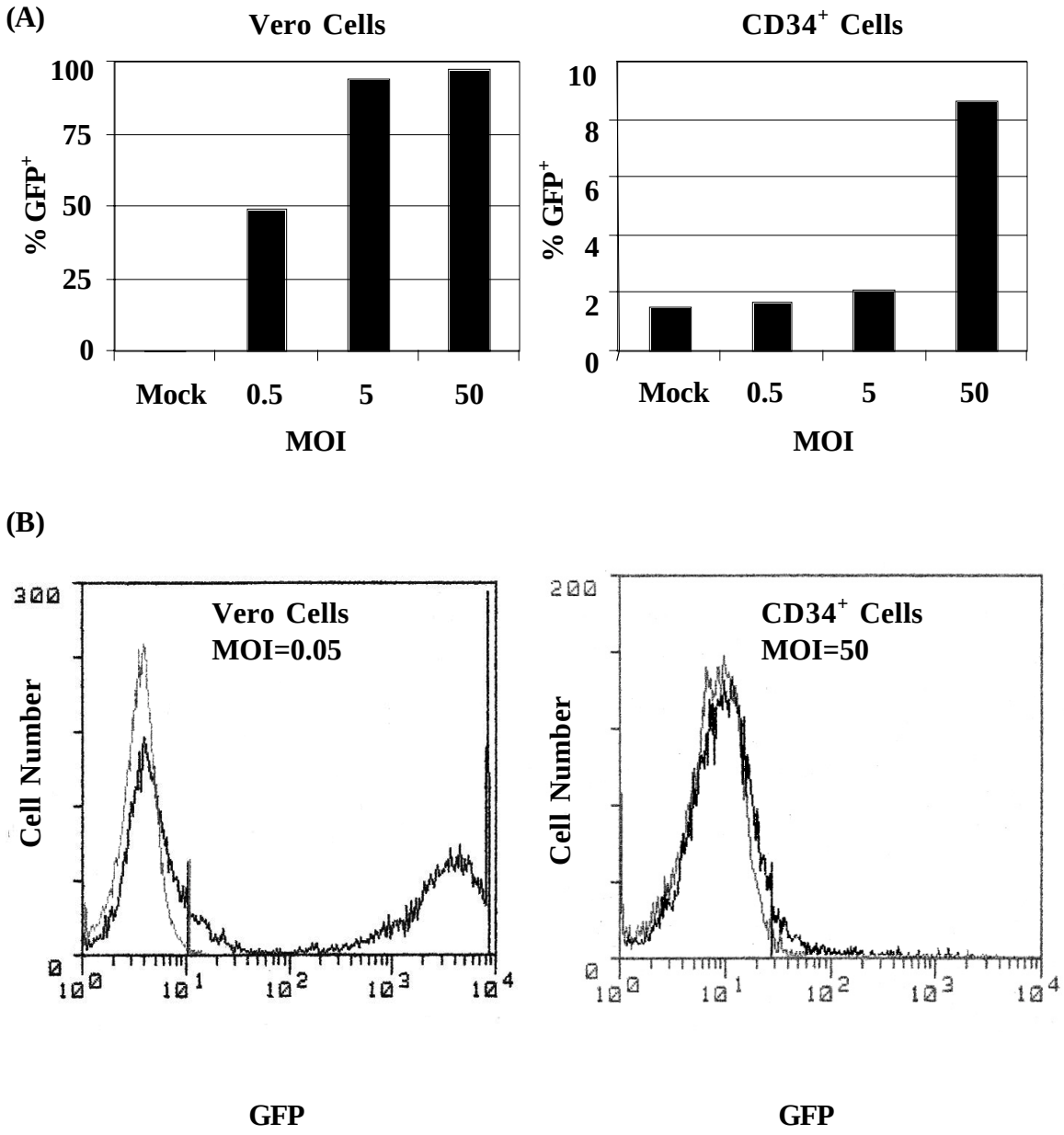


Figure 2. Flow cytometry analysis of QOZHG transduced Vero and $CD34^+$ cells.

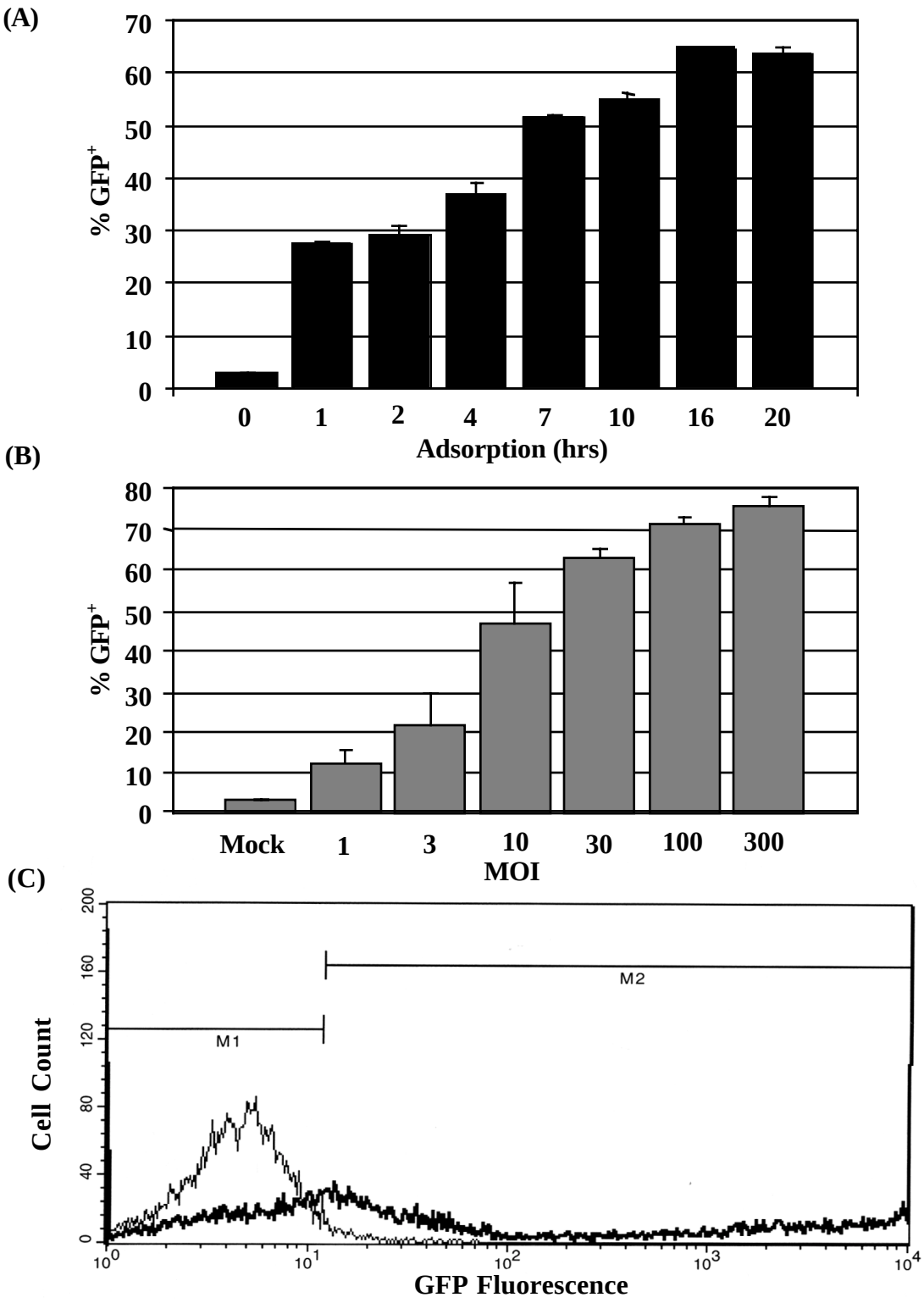


Figure 3. Transduction of $CD34^+$ cells with QOZHG vector.

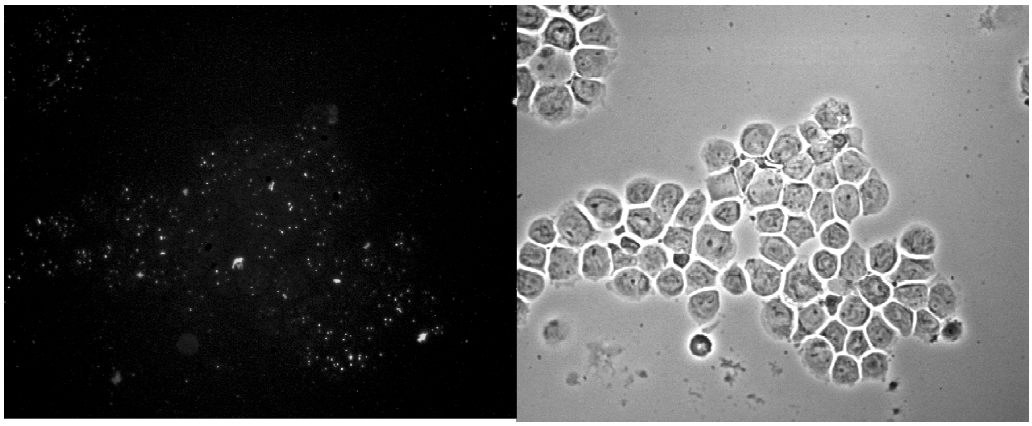
(ND10s)[223-225] also called PML (promyelocitic leukemia) bodies. By immunohistochemistry, PML bodies were visible in approximately 95% uninfected $CD34^+$ cells (Figure 4A) while nuclear ICP0 accumulation (Figure 4B) was seen in >50% of infected (MOI 30) cells. Various intensities of ICP0 were observed suggesting that some cells may express low levels of ICP0 beyond the detection limits of immunofluorescence.

Based on these results, two issues were further explored. First, we examined whether the extended adsorption times required for maximal transduction was indicative of a deficiency in glycosaminoglycans (GAGs) on the surface of $CD34^+$ cells limiting the rate of initial virus attachment. Second, we examined the distribution of the HSV gD receptors HveA and HveC on $CD34^+$ cell populations as a possible explanation for the incomplete transduction of these populations observed even at very high MOI (Figure 3B).

3.2.2 Involvement of Cell Surface GAGs

Dextran sulfate promotes HSV infection of cells such as sog9 that are deficient for GAGs, the receptors for initial HSV attachment via gB and gC[226,227]. In contrast, dextran sulfate inhibits infection of GAG-positive cells. Infections of unstimulated $CD34^+$ stem cells were carried out in the presence of increasing amounts of dextran sulfate and transduction efficiencies determined by flow cytometry for GFP expression. Inhibition of infection was observed at dextran sulfate concentrations of 0.1 μg/ml or higher with no effect at 0.03 μg/ml (Figures 5A & B). This result indirectly indicates that

(A)



(B)

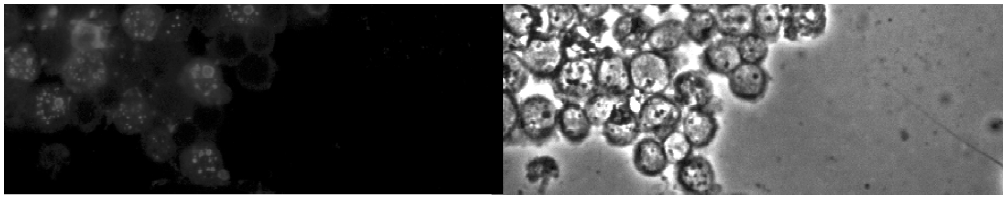


Figure 4. Immunohistochemical analysis of $CD34^{+}$ cells for (A) PML and (B) ICP0.

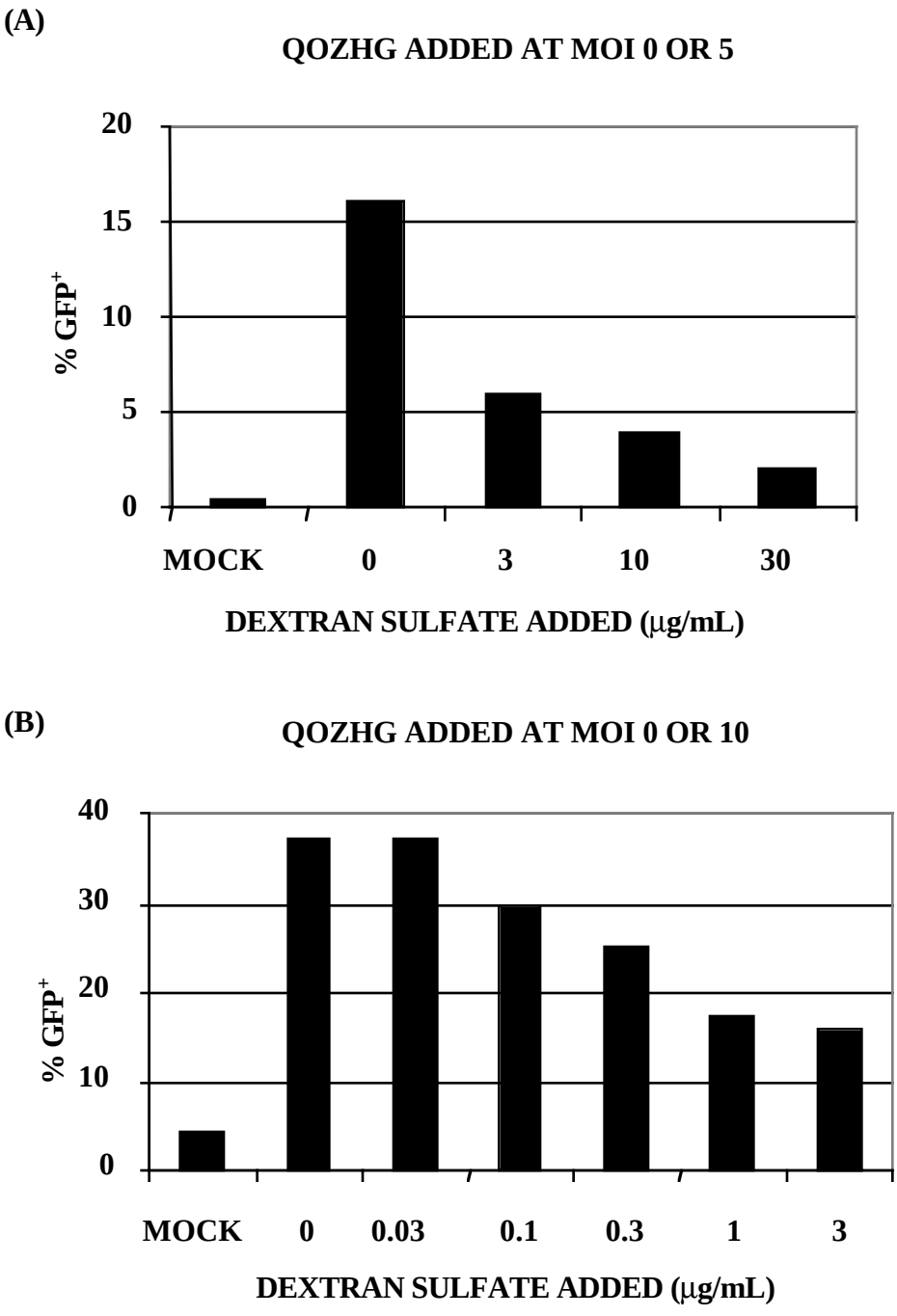


Figure 5. Effect of dextran sulfate on transduction of $CD34^+$ cells.

$CD34^+$ cells carry GAGs on their surface which contribute to HSV infection, suggesting that GAG deficiency was not the cause of the slow kinetics of $CD34^+$ cell infection.

3.2.3 Detection of gD Receptors on Unstimulated $CD34^+$ Cells

Flow cytometry analysis was used to determine the presence of the HSV gD receptors HveA and HveC on two $CD34^+$ stem cell populations isolated from several umbilical cords. In the absence of stimulation, HveA and HveC were detected on 75% and 85%, respectively, of the cells in one $CD34^+$ cell preparation (Figure 6A). Dual-label analysis revealed four distinct subpopulations with HveA (6%), HveC (21%), both receptors (68%), or neither (5%) present on the cell surface. Analysis of the second cord revealed similar subpopulations (Figure 6B), although the percentages were somewhat different (HveA, 30%; HveC, 7%; both, 57%; neither, 6%). Immunofluorescent analysis confirmed the surface expression of HveA and HveC in $CD34^+$ cell populations in comparison with control CHO cell lines expressing either receptor alone (Figure 7). Since more cells carried one or both receptors (94-95%) than could be infected under optimal conditions at high MOI (70-80%, Figure 2B), the question arose whether both receptors were functional and whether different receptor-positive subpopulations are susceptible to infection.

3.2.4 Functionality of gD Receptors on CD34+ Cells

The functional activities of the gD receptors HveA and HveC on $CD34^+$ stem cell populations were evaluated by virus neutralization assays using antibodies to the individual receptors. Antibody specificities and effective blocking dosages were determined on CHO cell lines expressing HveA (CHO-HveA cells) or HveC (CHO-HveC

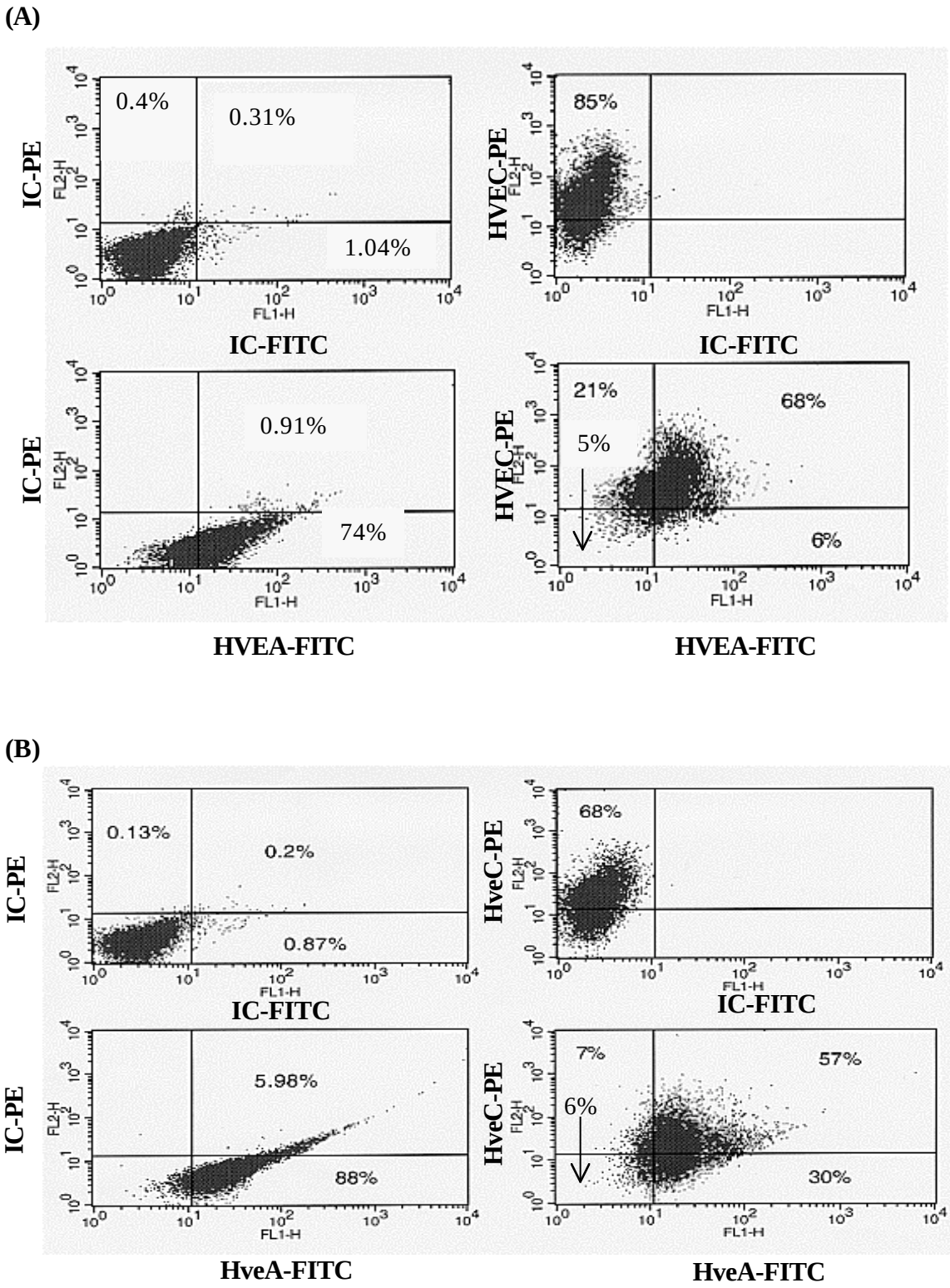


Figure 6. Dual-label flow cytometry analysis of HveA and HveC on $CD34^{+}$ cells.

cells) from transduced genes (data not shown); untransduced CHO cells are refractory to HSV infection. Incubation of $CD34^+$ cells with antibodies to HveA for 1 h prior to infection with QOZHG had a small effect on transduction efficiency (20% reduction) while pre-incubation with anti-HveC antibodies showed no significant effect (Figure 8). However, pre-incubation with both antibodies reduced the number of GFP positive cells by 55%, indicating that either receptor can be utilized for HSV-1 infection of stem cells. Consistent with this interpretation, the reductions accomplished by the antibodies separately and together correlated reasonably well with the representations of the different receptor-bearing subpopulations in the second of the $CD34^+$ cultures studied above by dual-label flow cytometry (Figure 6B). Alternatively, it is possible that other gD receptors were involved in addition or instead of Hve A and HveC, such as 3-OST modified heparan sulfate[104].

To strengthen the evidence that both HveA and HveC on the surface of $CD34^+$ cells could support infection, dual-label flow cytometry was performed for GFP expression and each receptor separately after infection. The results showed that 50% of the cells in one culture were positive for HveA and GFP while 15% were HveA-positive but negative for GFP (Figure 9A). Remarkably, only 6% of the cells in this experiment were positive for HveC, which compares with a range of 68-85% HveC-positive cells detected in uninfected cultures (see Figure 6). To confirm this observation, the HveC portion of the experiment was repeated using a lower MOI (MOI=10) and a different fluorochrome for HveC detection, allophycocyanin (APC) which is not affected by GFP expression, unlike PE. In this repeat experiment, 84% of the cells in a mock-infected

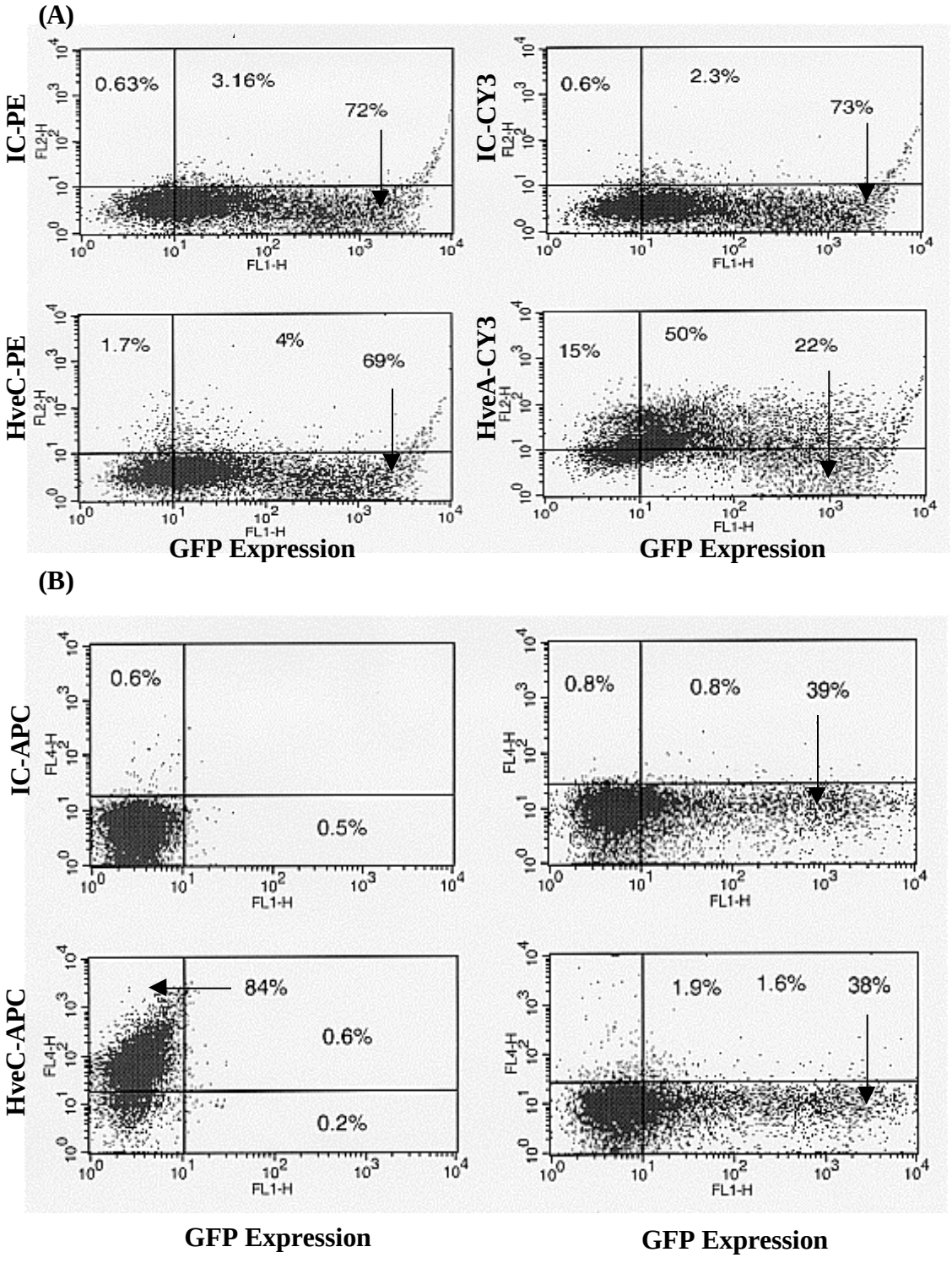


Figure 9. HveA and HveC receptor analysis on infected $CD34^{+}$ cells.

population were positive for HveC, but only 4% in a virus-infected culture (Figure 9B). This dramatic reduction suggests that the receptor was either internalized or down regulated by virus binding. Although the numbers indicate that 80% of the cells changed from $HveC^+$ to $HveC^-$ in the presence of virus, GFP expression as evidence of transduction was observed in only 40% of the cells. Different interpretations of this result will be considered in the Discussion.

3.2.5 Erythropoietin Gene Transfer to CD34+ Cells

The glycoprotein erythropoietin (EPO) is the primary cytokine involved in regulating red blood cell production. Patients suffering from low levels of EPO are currently treated by injection of recombinant human EPO (rhEPO) on a daily basis to balance their red blood cell production. The level of purity and amounts of recombinant human protein required has led to the employment of alternative strategies. An alternate treatment for this disorder would involve transducing the EPO gene to cells that would reside in the marrow and continually express the cytokine at relevant levels, thus replacing the continual need for rhEPO injections. A subfraction of $CD34^+$ cells may return to the marrow upon reimplantation and reside there long-term, thus making these cells a potential target for gene delivery. In order to test this hypothesis in culture, isolated $CD34^+$ cells were either mock transduced or transduced with a replication defective HSV vector containing the HCMV:EPO expression cassette, DHEPO, at MOI=3, 10, or 30. Samples of supernatant were removed daily for 7 days and analyzed by ELISA (Figure 10). Although EPO was detectable at both MOI=3 (16-38 mIU/mL) and 10 (56-112 mIU/mL), maximal levels (130-630 mIU/mL) were achieved at the higher

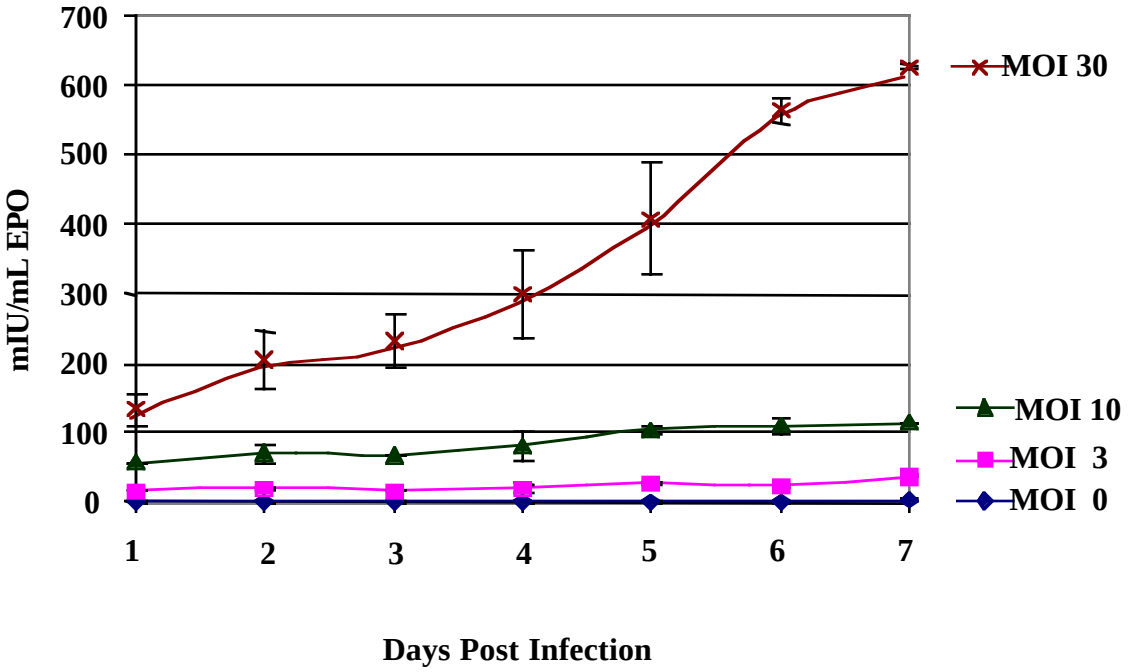


Figure 10. Analysis of rhEPO production from $CD34^{+}$ cells transduced with HSV-1 vector DHEPO at various MOI.

MOI (30). Together, these results demonstrate expression of a therapeutic gene product from HSV vector transduced $CD34^+$ stem cells and that biologically relevant levels of that product were secreted into the culture medium.

3.2.6 Analysis of $CD34^+$ Cells in Culture Post Infection

Since reasonable levels of transduction have been attained with the QOZHG vector in the minimal medium, the next goal was to analyze infected and uninfected cells for up to 1 week in culture. This involved examining infected cell toxicity, GFP expression, and characterization of continued surface marker presence or loss thereof such as CD34. The day one infected cell analysis showed high levels of transduction with HSV-1. Significant toxicity was observed in the high MOI of 30 between 16-40 hpi. This is seen by flow cytometry analyses and viable cell number counts, which are summarized in Figure 11 A, B, and C. Visually after ~24 hours the highly fluorescent cells were shrunken and pignotic in comparison to the lower and non-fluorescent cells. The flow cytometry results confirm that virtually no highly fluorescent cells remain on day 2. The surface marker CD34 remained on mock cells at high level for 3 days, whereas the infected cells were dramatically reduced after day 2 (Figure 11 D).

In the next experiment, FACS was performed on infected and uninfected cells at 16 hpi. The infected cells were sorted and collected by three categories: negative, mid-, and high fluorescence (Figure 12). They were each plated into separate wells and visually monitored. The results showed virtually all high-level GFP^+ cells were dead in 1-3 days post sorting. The mid-level GFP cells showed 30-40% death over 1 to 2 days and were

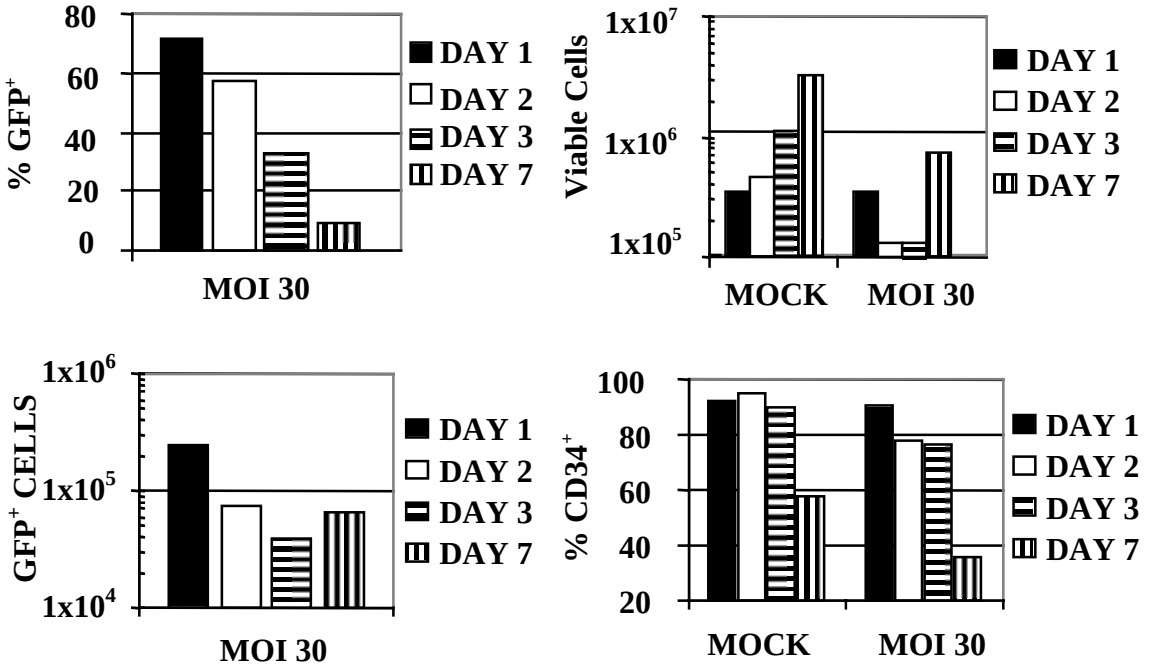


Figure 11. Analysis of QOZHG transduced $CD34^+$ cells for one week in culture.

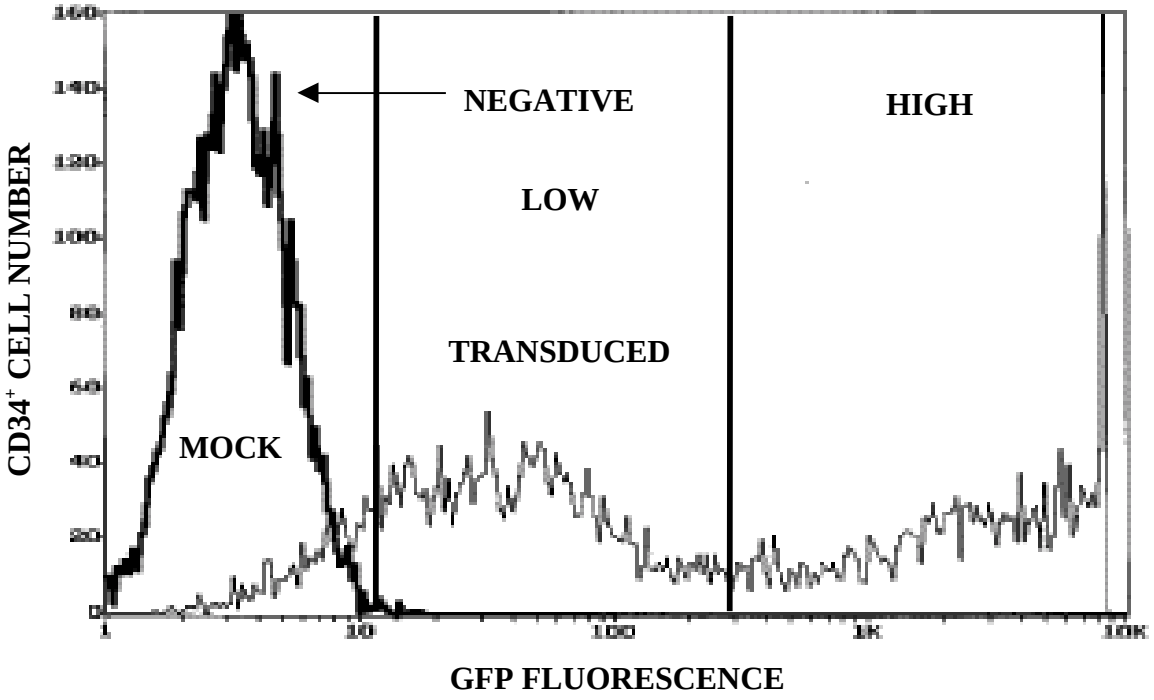


Figure 12. FACS analyzed regions of QOZHG infected $CD34^+$ cells.

not visually fluorescent after this time. After 2 days, cell growth became dominant in this culture, with the remaining cells surviving. The negative population showed high proliferation and no GFP expression from time of plating. It may be important to note that for the *in vivo* animal models these cells would be infected and infused into the animal. Based on adsorption experiments, that time frame would be less than 16 hours post infection. Cell survival upon implantation may dramatically increase due to the body being the natural environment, providing trophic factors to counter any cytotoxic effects.

The probable cause of *in vitro* toxicity is that the expressed levels of ICP0 are very toxic to the cells (Figure 4). In order to examine this further, a vector, d109 of Dr. Neil DeLuca's, is deficient in the ICP0 gene, does possess U_L41, and does not express LacZ, but otherwise is identical to QOZHG. $CD34^+$ stem cells were transduced with either d109 or QOZHG and monitored visually. The d109 infected cells showed no GFP expression but also no toxicity even at very high MOIs of 100-1000 for at least 3 days (data not shown). From this observation, it may be inferred that ICP0 protein has an effect on HCMV promoter activity in the HSV background and also can have a toxic effect on infected cells.

The possibility of ICP22 expression in infected cells was explored in addition to ICP0. The expression of ICP22 can result in several effects on other genes present in the virus. Northern blot analyses performed in the Glorioso lab demonstrate that ICP22 is expressed in QOZHG infected Vero cells, although at greatly reduced levels compared to a vector with no manipulation of the ICP22 gene or promoter (d120). No ICP22 expression was found in a vector deleted for that gene, TOZ. Greater levels of ICP0

expression were also seen by Northern blot with QOZHG than TOZ, most likely due to ICP22 expression. An early gene, gB, which should not be expressed without ICP22, was also seen with QOZHG but not TOZ. These viral gene transcription results were enough to obviate the development of a GFP expressing TOZ vector, named TOZHG.

3.2.7 HSV-1 Vector Development

The two major problems encountered by the use of QOZHG were found to be toxicity and length of transgene expression. The results of gene expression analysis suggest a TOZ background vector (Figure 13) is an appropriate choice to reduce vector-associated toxicity while allowing the HCMV promoter to be active upon transduction. The TOZ background vector is completely deficient for ICP4, ICP22, ICP27, U_L41, and U_L24. The disruption of U_L24 is the result of a promoter change for U_L23, thymidine kinase-TK. The original early promoter of TK has been replaced with the IE promoter of ICP4. TOZ expresses the lacZ reporter gene in the identical locus as QOZHG does.

The possibility of increased length of transgene expression may be possible through the use of the herpesviral latency promoter. The Glorioso lab has previously developed and shown that the latency associated promoter 2 (LAP2):transgene system works well in culture and also *in vivo*. In neurons, LAP2 and chimeric LAP2:HCMV promoter systems have demonstrated long-term transgene expression[228,229]. In order to make a systematic comparison, TOZ vectors were developed that contain either the LAP2:GFP, HCMV:GFP, or LAP2:HCMV:GFP expression constructs in the ICP27 locus (Figure 13), the same location that QOZHG contains HCMV:GFP.

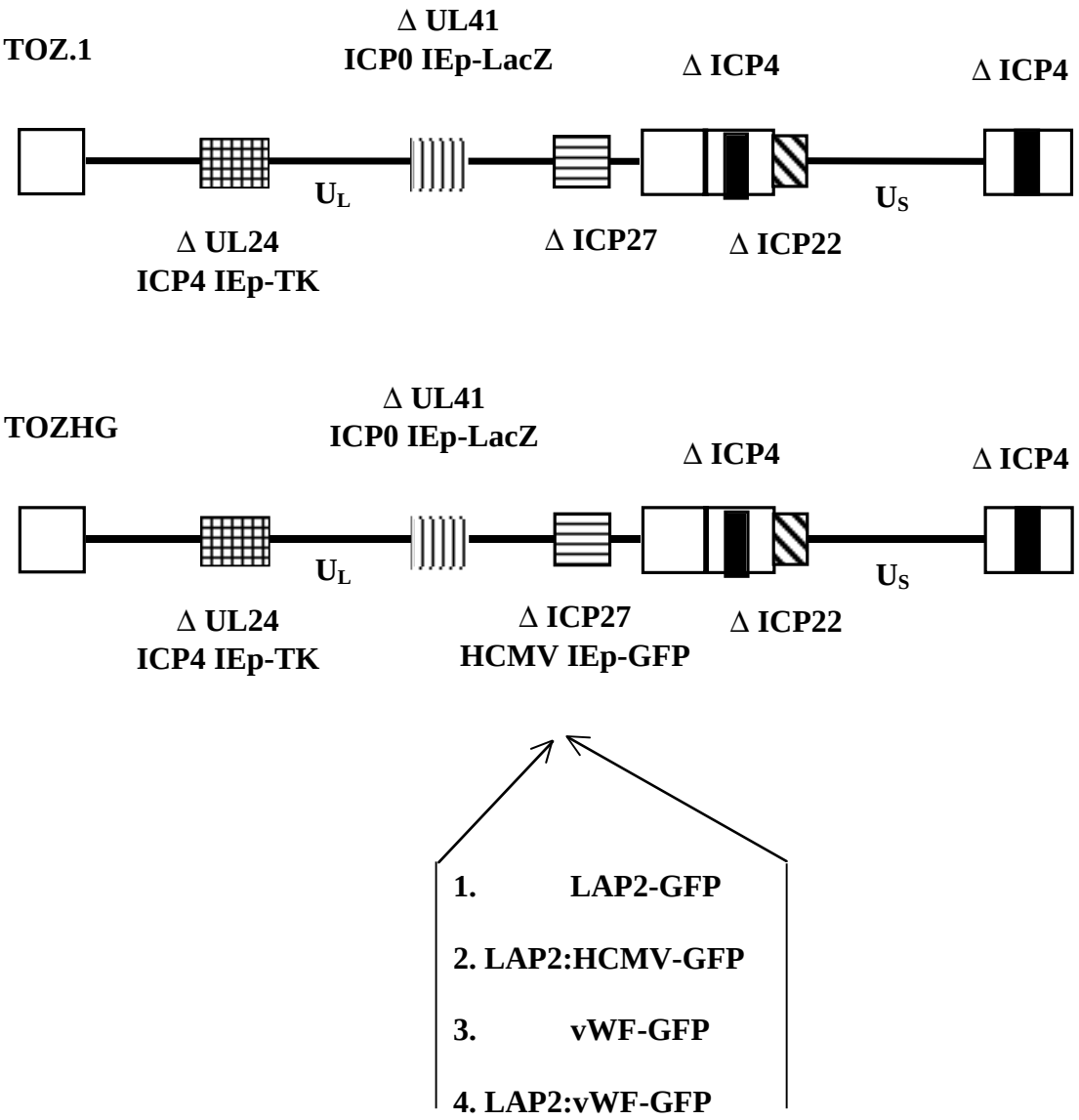


Figure 13. Diagram of promoter construct panels developed in the HSV-1 TOZ.1 background vector.

One of the ultimate goals of the lab is to transduce $CD34^+$ cells that will deliver the vector to tumor sites through homing and differentiation into angiogenic endothelium. Since the best vector currently available, considering both toxicity and expression, is TOZ, a vector with that background was used for introducing the endothelial specific promoter, von Willebrand factor. The new vectors contain vWF:GFP and LAP2:vWF:GFP expression cassettes in the ICP27 locus (Figure 13). Comparisons can now be made with this panel of vectors by transducing, Vero, HUVEC, and $CD34^+$ cells to determine toxicity, length of transgene expression, and tissue specific expression.

3.3 Discussion

The delivery of therapeutic genes to human stem cells has considerable potential for the treatment of a variety of genetic and acquired diseases. An ability to efficiently transfer genes to stem cells *ex vivo* without extensive cell manipulation or stimulation and to direct the expression of therapeutic gene products upon re-implantation into the patient is essential for successful therapies. Transduction of stem cells using viral vectors has met with varying levels of success, with limitations apparently related to both the biology of stem cells and the properties of the vectors used. In this context, we have determined the best conditions for transduction of unstimulated, minimally manipulated $CD34^+$ stem cells derived from human umbilical cord blood by a replication-defective HSV-1 vector and have examined the involvement of cellular receptors for the HSV glycoproteins gB, gC, and gD.

A range of transduction efficiencies has previously been reported for human stem cells exposed to different HSV vectors. For example, nearly 100% transduction without vector-associated toxicity was reported for bone-marrow derived $CD34^+$ cells using an HSV-2 single-cycle replicating vector at MOIs of 2-20[89]. These experiments involved culturing of the stem cells on a stromal support supplemented with serum and exposure to virus for 2 hours. Likewise, another study reported 99% transduction of IL-3 and IL-6 stimulated $CD34^+$-mobilized peripheral blood stem cells following incubation with a replication-defective HSV-1 vector at an MOI of 3[195]. However, only 10% of a similarly selected population of $CD34^+$ stem cells cultured in serum with or without cytokine stimulation were transduced in a study using a replication competent HSV-1 vector and a 2 h period of virus adsorption[216]. The variations in these results may be attributable to differences in infection protocols and detection procedures as well as the handling and conditioning of the cells, including treatment with cytokines. This study was conducted to standardize conditions for infection of unstimulated cells for maximum preservation of stem cell characteristics. A vector was used for these experiments that is replication defective and displays minimal cytotoxicity on a variety of cell lines due to a combination of impaired immediate-early synthesis of the IE products ICP22 and ICP47 and deletion of the ICP4 and ICP27 IE genes.

We observed that increasing the virus adsorption time from 1 h, commonly used for infection of cell lines used to propagate HSV (e.g.Vero cells), to 16 h more than doubled the number of transduced $CD34^+$ cells at an MOI of 30 (Figure 3A). These conditions routinely yielded 65% transduction or more for individual cord blood stem cell preparations, with levels as high as 85% recorded. Since the cell isolation and infection protocols were standardized and the virus lot the same in each instance, this variability may reflect differences in the quality of primary human cell sources and perhaps genetic differences among the cell donors. It is likely that the differences in relative abundance of the four gD receptor-bearing subpopulations noted between individual stem cell preparations may also play a role, whether these are rooted in environmental or genetic variations.

The observations of slow infection kinetics and incomplete transduction of stem cell populations were further explored by experiments examining the presence of HSV receptors on these cells. Infection was inhibited by dextran sulfate, indicating that the cells were not substantially defective in the synthesis of GAGs, the targets of initial virus attachment via gB and gC. Following primary attachment, HSV infection requires interaction of gD with cell-surface receptors which initiates fusion of the virus envelope with cell membranes resulting in virus entry. One or both of two common gD receptors, HveA and HveC, were identified on the surface of a great majority (94-95%) of the cells

in independent stem cell preparations, suggesting that most of the cells had both primary and secondary HSV receptors. However, since the cell populations were not homogeneous with regard to HveA and HveC expression, it is possible that the different receptor-bearing subpopulations differed in their susceptibility to HSV infection.

We observed that 57% and 68% of the cells expressed both gD receptors while 5%-6% lacked both and the remainder carried one or the other. The maximum infection figure of 85% was in the range of the total fraction of gD receptor-bearing cells (94-95%) although the routine figure of 65% infection suggested that a portion of the infected cells may have escaped detection due to inadequate expression of the reporter GFP gene; alternatively, a portion of the receptor-positive cells was HSV resistant. A report comparing expression of GFP in $CD34^{+}$ cells from adenoviral vectors containing different promoters and polyadenylation signals found the PGK-1 promoter increased both GFP expression and transduction levels when compared to a similar vector utilizing the HCMV promoter[(230)].

Infection reached nearly 30% after a 1 h adsorption period, but barely increased when the adsorption time was doubled. Infection appeared to accelerate again when the virus was left with the cells for 2-5 hrs, but gains in infected cell numbers per additional hour of adsorption time were modest after this period although relatively stable to at least 16 hours. This result raises the possibility that different subpopulations of cells were

infected at different rates. For example, a nearly half-maximal level of infection was reached with just 1 h of virus incubation, which could correspond to the fraction of cells displaying both HveA and HveC; likewise, it is possible that HveA-positive cells were not infected at the same rate as HveC-positive cells. It is also possible that one of the subpopulations was largely resistant to infection, as suggested by the observations that (i) antibodies to HveC did not noticeably inhibit infection, (ii) only 40% of the cells became infected in an experiment where 80% of the cells lost detectable surface expression of HveC upon incubation with virus, and (iii), as already mentioned, the percentage of gD receptor-positive cells was generally larger than the fraction of cells that could be detectably infected. It may be hypothesized from these results that contact between the virus and HveC on stem cells generally results in internalization of the virus:HveC complex into a non-productive intracellular compartment rendering the HveC-positive subpopulation largely resistant to HSV. This hypothesis is consistent with our previous results demonstrating internalization and degradation of a virus targeted to a non-traditional HSV receptor such the erythropoietin[231] or VSV-G[232] receptor.

An alternate explanation for this result is that binding of virus to HveC initiates receptor cycling in both infected and non-transduced cells. Similar results have been reported in other virus systems where binding of the viral glycoproteins results in the cycling of one receptor but not another[233-235]. The lab is currently exploring the effects of

gD binding to HveC with respect to activation of cellular functions that may ultimately aid in priming of the host cell for virus replication.

Due to toxicity and short-term transgene expression *in vitro* by the QOZHG vector, it was needed to develop new vectors in order to further study gene transfer to $CD34^+$ cells. Vectors were developed within the exisiting TOZ background in which HCMV, LAP2, or LAP2:HCMV promoter:GFP constructs were introduced. These constructs will be compared by transducing $CD34^+$ cells, examining cell survival, and monitoring length of GFP expression in order to determine the appropriate vector for future work. Similarly, vectors were developed containing the endothelial specific promoter vWF or the chimeric LAP2:vWF promoters with GFP. These constructs were developed to determine if endothelial specific transgene expression is possible in the HSV background and could prolonged expression be gained by the chimeric promoter.

Lastly, this study has shown that $CD34^+$ cells transduced with a vector containing an HCMV:EPO expression cassette (MOI=30) produced high levels of EPO in culture supernatant (600 mIU/mL) at 6-7 days post infection. This model represents another potential clinically relevant application for HSV transduced $CD34^+$ cells.

3.4 Conclusions and Future Work

This work has demonstrated that 93%-95% of $CD34^+$ cells possess one or both herpesviral fusion receptors, that they are functional, and that 65% of the cells were transduced at MOI=30 by the vector QOZHG after 16 hours of adsorption. Although these results are promising, further studies indicated that the vector was highly toxic to the cells and resulted in limited in length of transgene expression *in vitro*. The ability to

develop a vector that is able to efficiently infect $CD34^+$ cells with low toxicity and extended or tissue specific transgene expression is crucial in the future applications of this project. A panel of vectors has been developed that represent the best current herpesviral vector technology for achieving these requirements.

Once the appropriate vector is determined, transduced stem cells may then be investigated for the potential to induce anti-angiogenesis in an animal tumor model. Infected and uninfected stem cells can be infused into mice to verify cell homing to angiogenic sites, differentiation into endothelial cells, and expression of herpes thymidine kinase (TK). If the infected cells are found to be present at the tumor site, GCV killing will be attempted with vWF:TK and/or ICP4:TK vectors.

4.0 PRODUCTION OF HSV-1 VECTORS

4.1 Introduction

Now that it is established that $CD34^+$ cells can be transduced with HSV-1 vectors, many questions regarding the infection process, gene expression, and cell differentiation should be further examined. However, for all these studies, significant amounts of highly purified vectors are needed, particularly if one notes that it is more difficult to produce high titers and maintain low contaminant protein levels of TOZ background vectors due to its more disabled genome, in comparison to QOZHG. Thus the optimization and scale-up of vector manufacturing processes is vital for further research and eventual application of gene therapy using HSV-1 vectors. Because of the importance of production and purification of the vectors, I examined several issues for developing efficient production and purification schemes.

Replication-defective HSV-based viral vectors deleted for one or several essential immediate-early (IE) regulatory gene products can be propagated *in vitro* in complementing cell lines engineered to express the missing essential functions resulting in virus replication[109] and ultimately lysis of the host cell. Vector-associated cytotoxicity in non-complementing cells is minimized through deactivating the cytotoxic immediate early genes in the HSV-1 vector[75-78,138,218,236]. The progressive deactivation of the HSV-1 regulatory genes not only progressively reduces the cytotoxicity of the virus, but it also reduces vector yield. For example, the yield achieved with replication defective vectors deleted for multiple IE genes is only about 0.1% of that obtained using wild-type virus. Hence, it is difficult to obtain sufficient amounts of high quality vector stocks.

The HSV-1 vectors currently employed in clinical trials[237,238] are replication competent and minimally deleted for the gene γ34.5, which is involved in neurovirulence[239-242], and hence do not face such production issues. This is also the case for DISC-HSV-2 vaccine vectors[89,243,244] that are deficient only in the binding and/or entry protein, glycoprotein H (gH). However, even these minor alterations to the HSV genome result in viral recombinants that are more difficult to grow and purify compared to wild-type virus. Thus, obtaining highly purified high titer vector stocks is one of the major obstacles remaining in the use of viral vectors for human clinical trials.

In a previous publication[245], another graduate student and I examined the interative infection parameters of cell number, multiplicity of infection, and metabolism with the goal being to optimize these culture conditions. The results were used for scale-up production using a Corning CellCube bioreactor. It was found that allowing cell growth to reach near or over confluent conditions, infecting at MOI=0.02, and perfusion of medium at 24 hours post infection result in efficient production of vector. Additional parameters that may affect vector harvest due to viral stability and production were studied and will be presented here.

To further improve the vector yield, I examined the effect of temperature and media conditions on the stability of an HSV-1 vector, and the resulting production of the vector by cells cultured at lower temperatures. The results reveal that HSV stability is 2.5-fold greater at 33°C than at 37°C and is further stabilized at 4°C. Additionally, a significantly higher half-life was measured for the vector in infection culture conditioned serum medium compared to fresh medium with or without serum. Synchronous infections incubated at 33°C produced 2-fold higher amounts of vector than infected cells

incubated at 37°C, but with a lag of 16-24 hours. Vector yield was 3-fold higher and remained stable at peak levels for a longer period of time in cultures incubated at 33°C than 37°C. A pronounced negative effect of increased cell passage number on vector yield was also observed. Vector production at 33°C yielded similar levels regardless of passage number but was reduced at 37°C as passage number increased. Together, these results contribute to improved methods for high titer HSV vector production.

4.2 Results

4.2.1 Comparison of Temperature, Medium, and pH on Vector Stability

Infectivity and/or stability of enveloped virions has been shown to be affected by various conditions such as medium components, pH, and temperature[246-253]. It has been shown that thermal or pH inactivation of HSV results in particles that have a slightly increased density compared to untreated virus[254,255]. Inactivated particles were found to retain the ability to adsorb to the surface of cells but were greatly reduced in productive entry[254,256].

Figure 14 shows the effect of temperature on stability of the vector at 37°C, 33°C, and 4°C in various media. An average half-life for each temperature and medium condition was calculated assuming first order kinetics using the trend-line equation determined using Excel. The calculated half-lives of 21.8 hours, 8.8 hours, and 6.7 hours for infection culture conditioned serum medium, fresh serum containing medium (10%), or fresh medium without serum, respectively, were determined at 37°C (Figure 14A). The virion half-life was extended ~2.5-fold (59.2, 22.4, and 15.1 hours in infection culture conditioned medium, fresh medium with serum, or fresh medium without serum,

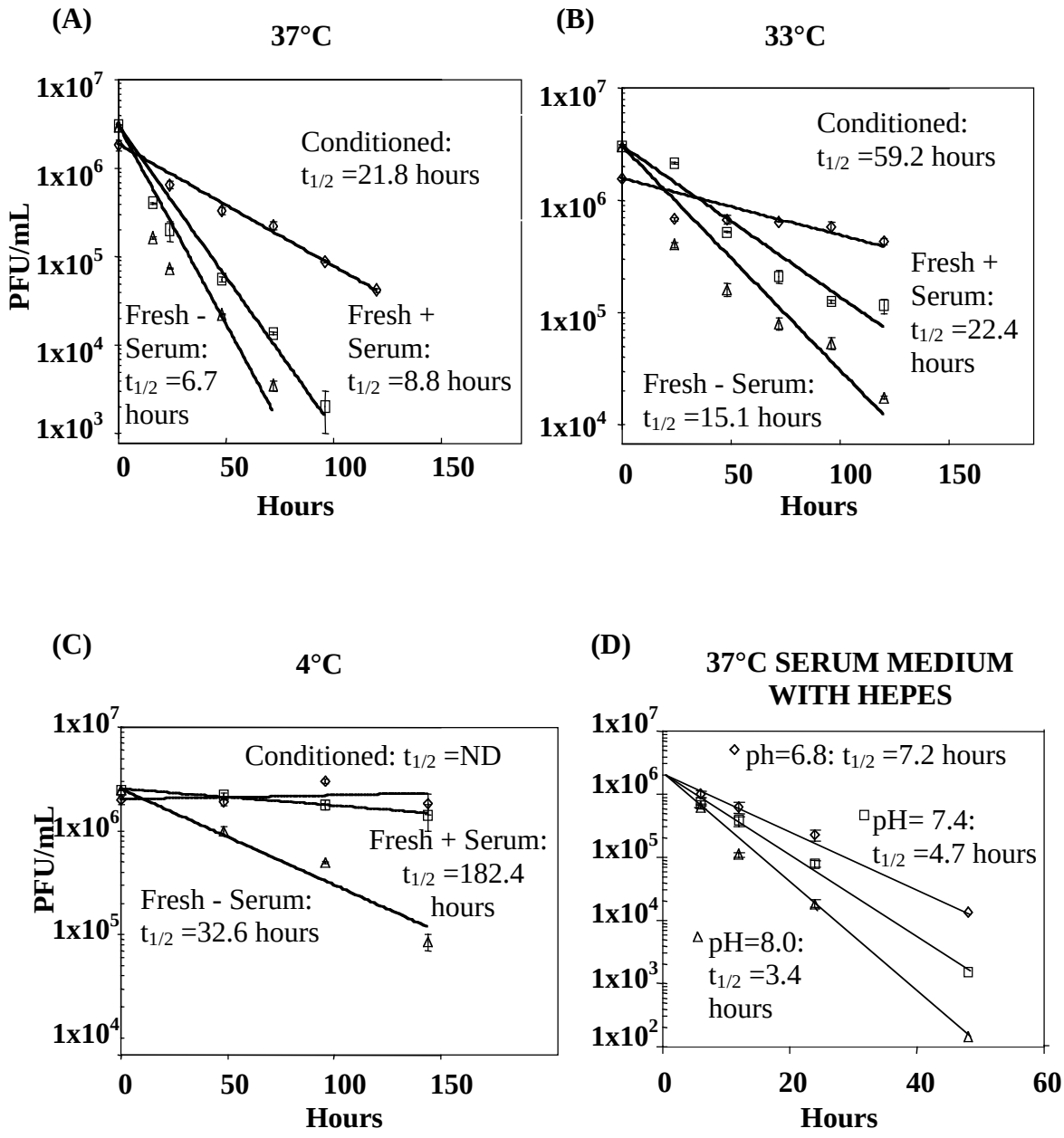


Figure 14. Effect of temperature, medium, and pH on vector stability.

respectively) when incubated at 33ºC (Figure 14B) compared to incubation at 37°C. Viral stability was further increased at 4°C (Figure 14C) with half-lives of 182.4 and 32.6 hours in fresh serum medium and fresh medium without serum, respectively. The decay rate was insignificant for vector in infection culture conditioned serum medium, at 4°C. Since pH has been also proven to affect vector stability, this was next examined. Pelleted vector was resuspended in serum medium buffered with HEPES and adjusted to pH values of 6.8, 7.4, or 8.0. These values were chosen as representative of the range which can be found in infection cultures at various times of the process. Figure 14D shows that HSV vector is most stable in pH 6.8 with decreases in stability as pH increases. Together the combined results of Figure 14 demonstrate that HSV-1 has increased stability at lower temperatures, in serum medium that has been conditioned by productive infection, and at lower pH values.

4.2.2 Effect of Lower Temperature on Complementing Cell Growth

Since vector stability increased ~2.5-fold when the incubation temperature was reduced to 33°C, regardless of incubation medium, we next examined the effects of temperature on cell growth. For this, cells passaged at 37°C were subcultured and incubated at 37°C or at 33°C. Duplicate flasks were harvested for each temperature over time and viable cell counts performed. The growth curves (Figure 15) yielded doubling times of 22 hours and 32 hours for cells growing at 37°C and 33°C, respectively, calculated using the data points from 24 to 96 hours post plating and assuming first order kinetics. In addition to a slower growth rate, the cells cultured at 33°C plateaued at a lower final cell density (2.0×10^6 vs. 3.5×10^6).

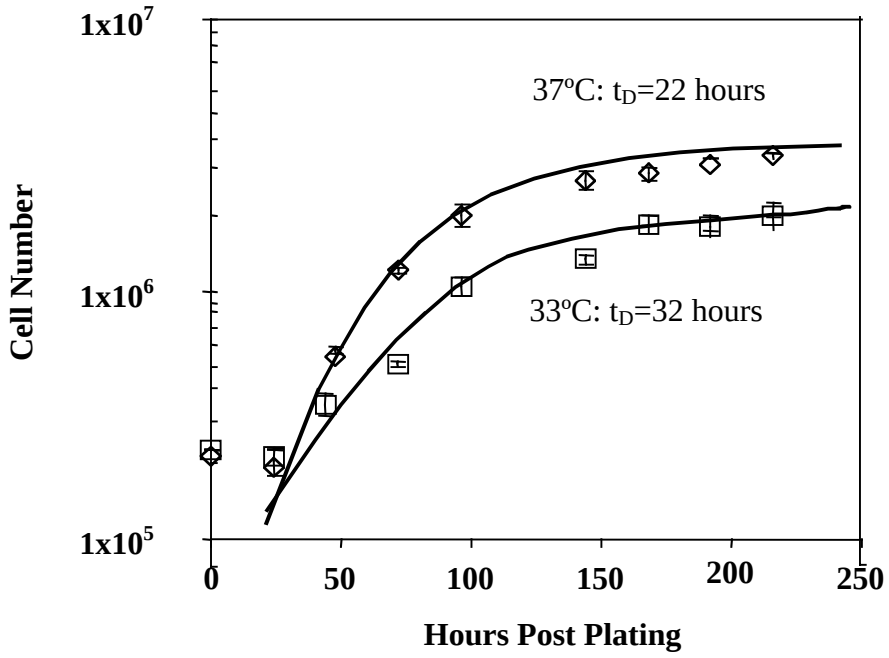


Figure 15. Effect of temperature on cell growth.

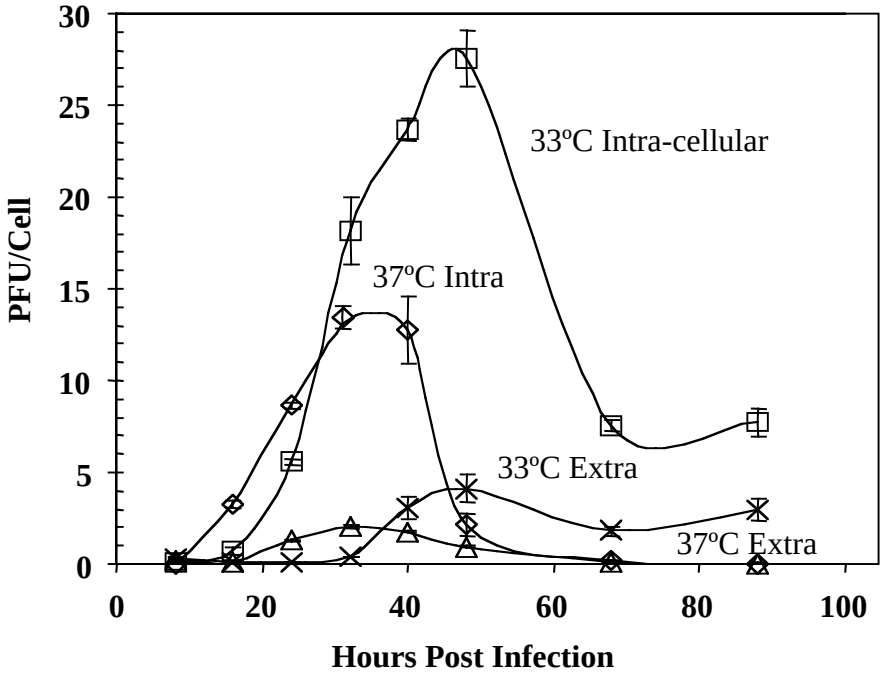


Figure 16. Intracellular and extracellular virus production for synchronous infections incubated at 33°C and 37°C.

4.2.3 Comparison of Vector Production from Synchronous Infection at Lower Temperature

Since the lower temperature did not dramatically alter cell growth, vector production was also examined at this lower temperature (33°C). Initial infections were conducted at a high multiplicity of infection (MOI=5.0) to ensure synchronous production, although actual vector production is normally conducted at a much lower MOI (0.01-0.05). This should result in minimal impact from secondary infections and thus enable us to assess the time it takes for the virus to be both produced and released into the culture as well as the number of vector particles produced per cell (burst size). Cells cultured at 37°C were sub-cultured, mixed with virus, and incubated at 37°C or 33°C and the number of virus particles produced determined by standard plaque assays. We observed a 2-fold increase in virus production, both intracellular and extracellular, at 33°C compared to the standard temperature of 37°C (Figure 16). The peak of intracellular virus production and the release of extracellular virus occurred 16-24 hours earlier at 37°C than at 33°C. The vector decay in the supernatant appears to be faster at 37°C, which was not unexpected based on the decreased half-life at that temperature (Figure 14). Since the burst size, or PFU per cell, was delayed but significantly higher at 33ºC, production schemes using lower MOI involving a temperature shift were examined.

4.2.4 Effect of Temperature and Cell Passage Number on Viral Production

We set out to investigate the ability to obtain high titer virus stocks by shifting temperature between the cell growth phase (37°C) and that of virus harvest (33°C) based on our previous results (Figures 14 and 15). These experiments were conducted at an MOI of 0.05, which is normally used for the propagation of this vector. Cells previously

cultured at 37°C were infected and incubated at 37°C for the first 24 hours, after which time some plates were shifted to 33°C. The supernatants were sampled over time for determination of the number of infectious extracellular viral progeny produced (Figure 17). The reason for the delay in temperature shift was to maximize secondary infection occurring during the first 24 hours at 37°C in combination with increased vector release and stability at 33°C.

Complementing cells are continuously passaged usually in the absence of drug selection due to expense and reduced growth rate. We had previously observed that cell passage number played some role in vector production. In order to determine the effect of cell passage on the production of vector, infections were performed on high passage cells (Figure 17). The transfer of infected cultures to 33°C resulted in a 3-fold increase in vector concentration in the supernatant for low passage (30) cells (Figure 17A). As the passage number increased, the difference in vector levels became 4- and 10-fold higher at passages of 45 (Figure 17B) and 80 (Figure 17C), respectively. The increased difference in vector concentration came at the expense of the infection cultures that remained at 37°C, as the production levels remained near constant at 33°C regardless of passage. Similar results were also obtained if the shift to 33°C was made immediately after infection instead of at 24 hours post infection, except that the peak in virus production exhibited about a 24-hour lag (data not shown). It was observed that when titering the same vector stock, developing plaques are smaller and ~50% less in number for high passage cells compared to low passage cells.

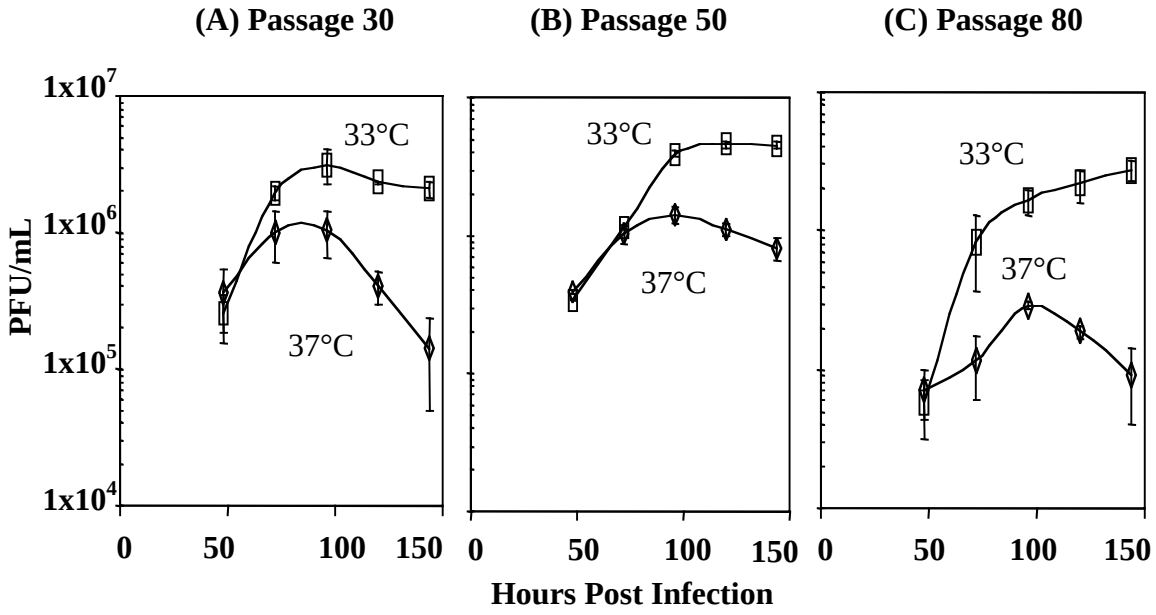


Figure 17. Effects of temperature and cell passage number on production of HSV vector.

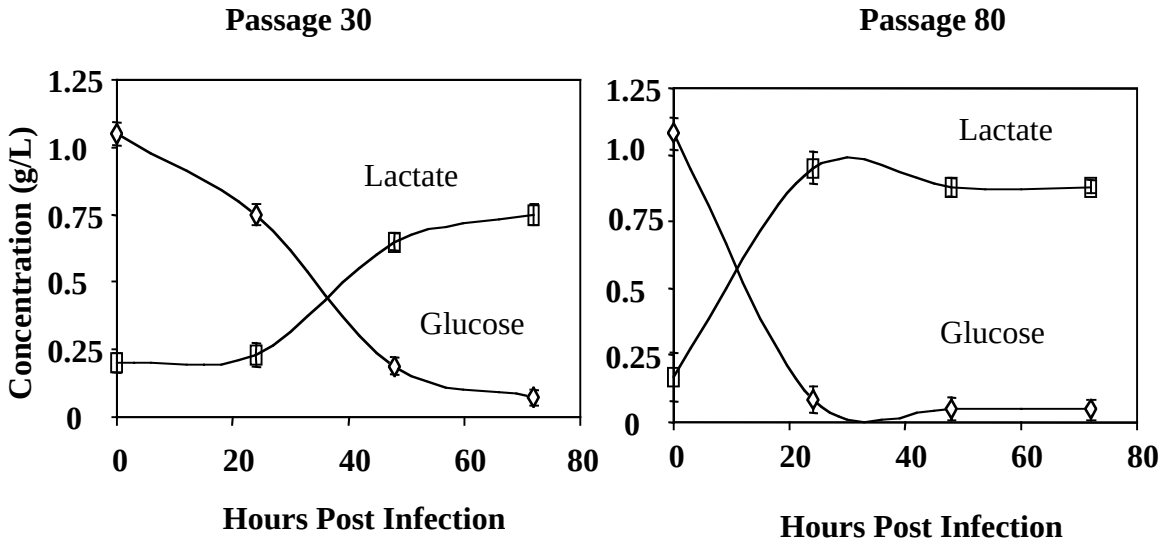


Figure 18. Effect of virus infection on extracellular glucose and lactate levels.

4.2.5 Effect of Passage Number on Cell Metabolism

A possible explanation for the difference incurred by higher passage cells may be due to variation in primary glucose metabolism. To see if there are substantial variations in cell metabolism as a function of passage number, glucose consumption and lactate formation were measured for cells of 30 and 80-passage numbers. Cells were infected at MOI=0.05, as in the previous production experiments, and supernatant samples removed over time. A substantially higher rate of glucose utilization and lactate formation was observed for the longer-passaged cells (Figure 18).

4.3 Discussion

The ability to produce and purify high levels of replication-defective viral vectors is a major limiting factor for the advancement of human gene therapy clinical trials. In prelude to use of a bioreactor, we examined the effects of varying two infection parameters, cell number and initial vector amount added (MOI), as well as examining the infection culture metabolism. These results were used to develop a scheme for use with the Corning CellCube bioreactor for production of two vectors, QOZHG and TOZ. The results showed that vector produced/surface area, compared to that obtained from small-scale tissue culture flasks, can be attained in the bioreactor system. There are several additional parameters that, when optimized, may result in increased levels of vector production.

One important viral property that can have a significant impact on vector harvest is stability, which is affected by several conditions. A study of the effect of temperature and medium on HSV vector stability revealed increased half-life at lower temperatures (33°C and 4°C) and in conditioned serum containing medium. From a scale-up

standpoint, the increased stability at 33°C may translate into increased vector yield during production by a thermal shift from 37°C to 33°C. The lack of virion decay at 4°C in conditioned medium can be beneficial once the virus is harvested from the production infection during large-scale vector production. Since purification schemes often have lengthy processing times, significant vector loss resulting from medium or temperature related instabilities can be minimized by using conditioned medium and placing the samples at 4°C. The increased stability of conditioned serum medium compared to fresh serum medium may be partly explained by pH. The pH of the conditioned medium was about 0.4 pH units lower than that of fresh medium (6.9 vs. 7.3). Since the virus tends to be more stable at slightly acidic pH in culture medium[249], the half-life differences we have observed in the two media may be partly related to these variations. The presence of serum (10%) in the medium was found to increase the stability in comparison to medium without serum. In addition, we have previously reported experiments in which individual protease inhibitors or a protease inhibitor cocktail added to the virus culture did not aid in vector stability in infection culture conditioned medium[257].

In order to determine the effect of reduced temperature on complementing cells, initially cell growth was monitored at 33°C and 37°C. Cells cultured at the reduced temperature displayed a 50% longer doubling time and achieve a 40% lower final density. Viral production on a per cell basis at both 37°C and 33°C using high MOI synchronous infections showed a 2-fold increase in vector concentration at 33°C with a delay of 16-24 hours.

When applying this observation to typical vector production conditions (MOI=0.05), the transfer of infected cell cultures from 37°C to 33°C demonstrated

significantly increased vector production. The reduced temperature resulted in 3-fold higher levels of vector yield with cells of low passage (p30). This difference increased (4- and 10-fold) as passage number increased (p45 and p80). Another important observation is that the high levels of extracellular vector produced remained constant over a longer period of time and at each passage when the culture temperature was reduced to 33°C. This is most likely a coordinated effect between the higher production level (Figure 16) and the increased virus half-life (Figure 14). This observation will have a dramatic impact upon scale-up production by making the selection of harvest time less critical than it would at 37°C, where the failure to harvest at the proper time results in dramatically decreased yields.

The higher HSV vector production levels achieved at the lower temperature of 33°C are in accord with the reports on retrovirus vectors[247,248,252]. To our knowledge, this is the first report on the effect of passage number on vector yield at different temperatures, and only limited data exist on the effect of long-term culturing on cell growth, metabolic, and genetics changes. However, a recent work with insect cells revealed some insights into cell growth and recombinant protein production in the insect-baculovirus expression system[258]. Early passage cells grew to a 2- to 3-fold higher cell density and displayed higher levels of recombinant protein production. In addition, the older-passaged cells grew to a lower density, although the cells showed a shorter lag phase and about 20% faster growth rate initially. Hence, higher rates of glucose consumption and lactate formation may indeed lead to a lower final cell density, higher initial growth rate, and perhaps even a shorter lag time. Thus, it is possible that the higher glycolytic activities displayed by the Vero cell cultures in Figure 18, are the

common characteristics of longer-passaged cells of different origins and may contribute to decreased vector production at 37°C.

4.4 Conclusions and Future Work

In order to meet increased demands of high titer HSV vector stocks, we have efficiently scaled-up production from roller bottle flasks to a 21,250 cm^2 Corning CellCube bioreactor[245]. Additional studies aimed at increasing vector harvest demonstrate a significantly longer virus half-life at 4°C or 33°C than at 37°C, as well as increased vector stability in conditioned culture medium compared to the fresh medium with or without serum. The application of these parameters can be tested in the bioreactor for additional increases in vector producton levels. A second biorector system, the New Brunswick CelliGen$^+$, is an alternate configuration that utilizes microcarriers to increase surface area while requiring low volumes of media. This is an interesting system that may prove to be a better configuration.

5.0 PURIFICATION OF HSV-1 VECTORS

5.1 Introduction

The U.S. Food and Drug Administration (FDA) sets limits on the amounts of contaminant protein and DNA present in biologically derived drugs. These amounts vary depending on the type and application of drug as well as the required dosage. For example, current clinical vaccine preparations are required to contain less than 100 μg complementing cell protein and 10 ng DNA per dose[(259)]. These limits have not yet been established for replication-defective herpes vectors and thus will need to be set by our lab when we approach the clinically relevant manufacturing stage and require FDA approval of our product. The limits set for vaccines are a reasonable guideline in which to compare developed purification schemes.

In order to study and design a vector separation system, it is imperative to understand the production process. The productive infection of HSV results in lysis of the cells and release from the surface they were adhered to. In addition, the culture medium contains 10% fetal bovine serum that cells require in order to produce high levels of vector. Thus the vector production process results in a slightly viscous slurry of cell debris, virus, DNA/RNA, and proteins. Since HSV is an enveloped virus, its ability to infect cells is compromised by damage to this lipid membrane, so vector stability issues must also be considered in developing a purification process.

In current lab purification techniques, the entire infection culture is harvested and centrifuged at low speeds (1,000×g) to clarify the virus from cell debris. This supernatant is then centrifuged at high speeds (13,000-48,000×g) to pellet virus from small debris, protein, and DNA molecules. The viral pellet is resuspended in a small volume and

loaded onto a density gradient which separates infectious virions from remaining debris such as cell organelles, defective viral particles, and empty capsids. The fraction containing the majority of infectious vector is removed and centrifuged at high speed to pellet the virus again in order to perform a buffer exchange out of the density gradient solvent and into a phosphate buffered saline solution. The benefits of this protocol are that concentration is high and it is technically easy to perform. The disadvantages of this process include significant vector losses and clumped vector particles due to forces of centrifugation, and requirements for extensive personnel handling, which increases the chances for contamination. In addition, this process may not prove to be efficiently scalable. These disadvantages motivated us to develop new processes that would be more efficient, require less handling, and be scalable. Efficiency and scalability are critical factors as highly purified vectors are needed for assessing their efficacy in clinical trials. Certainly, if gene therapy becomes a widespread treatment or preventative tool in medicine, the amounts of vector reqired will increase dramatically.

Several reports describe the purification of vectors, including retrovirus, adenovirus, adeno-associated virus, and herpesvirus by vector size, density, and/or affinity[259-268]. Based on these works, and with our goal being to attain contaminant levels near that of vaccines while maintaining high levels of vector recovery, current lab purification procedures were examined and alternate down-stream strategies were investigated.

A two-stage filtration process is proposed. Vector preparation is first separated from larger particles such as cell debris by a larger filter size, followed by a smaller size filter for removal of smaller particles. Tangential-flow filtration was selected to

minimize membrane fouling. These two filtration steps should partially purify and concentrate the vector. Heparin ion exchange/affinity chromatography[259] was adapted for the next step following filtration.

Initially upon infection, HSV glycoproteins gB or gC bind to cell surface heparan sulfate glycosaminoglycans through a charge interaction. Trybala, et al.[260] have demonstrated that the presence of sodium chloride or heparin negatively affects the ability of HSV to bind to cells in culture. Thus heparin chromatography columns can be used to bind the virus and sodium chloride can be used to elute the vector.

In this chapter, results of my work using the standard centrifugation process are first presented. These results are then compared with the new process of filtration and chromatography that I developed. The results demonstrated that the combined filtration and chromatography steps proved superior to the standard centrifugation protocol. The virus recovery was high in this process and greater than 99% of DNA and proteins were also removed.

5.2 Results and Discussion

5.2.1 Analysis of Vector Centrifugation

First the recovery yield using a standard centrifugation protocol was examined. In the centrifugation protocol, after infection and lysis of the cells, the entire infection culture was harvested and pooled. The cell debris was separated from virus by centrifugation at low forces of 1000×g. This initial step resulted in the loss of 50% of the virus (data not shown). This dilute vector solution is then subjected to high force centrifugation to pellet the virus. In order to examine the efficiencies of vector pelleting, this step was repeated using various centrifugation forces and lengths of time. Vector

supernatant was centrifuged at either 13,000×g, 27,000×g, or 48,000×g for 10, 20, or 30 minutes, at 4ºC. The presence of virus was then examined in both the supernatant and the resuspended pellet. Figure 19 shows the results of the best recoveries for each centrifugal force (all 30 minute times). The highest recovery of vector in the pellet, 50%, was found in the preparation treated at highest force. For all three cases, 40% of the virus was unrecoverable after examining both the pellet and the supernatant. Considering that the process is performed twice, once before and once after the gradient, this dictates that 25% (0.5×0.5) is the maximal recovered virus from these steps. The density gradient procedure, which has been previously optimized by the Glorioso lab, results in recoveries of up to 80% of infectious virus (data not shown). Thus the overall centrifugation process can result in optimal recoveries of $0.5 \times 0.8 \times 0.5$, or 20%, with volume concentration factors greater than 50×. However, if one also considers the very first centrifugation step for settling the cells (50% loss), then the overall yield of recovery would reduce to 10%. Recoveries of new purification strategies were next studied with the goal being to increase recoveries but maintain comparable concentration factors.

5.2.2 Initial Experiments Using Various Filter Sizes

Our lab and others have shown that by electron microscopy analysis individual virions are approximately 200 nm in diameter. During virus replication the cells lyse and some virions may remain associated with cell membrane or debris, or other virions. This may result in discrepancies when attempting to filter the virus by various pore sizes. In order to determine actual production vector size, infections were initially harvested and separated from cell debris by centrifugation. Vector preparations were filtered through syringe filter membranes using pore sizes ranging from 1.2 μm down to 0.22 μm. Figure

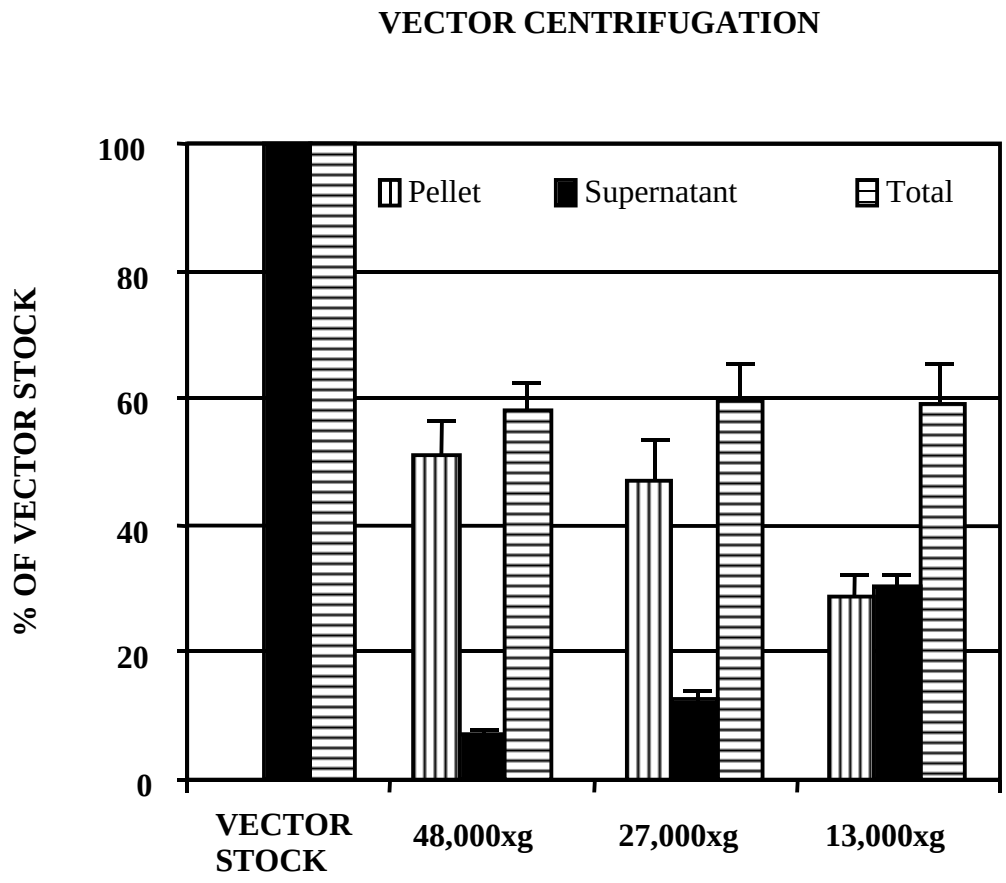


Figure 19. Effect of centrifugation force on vector purification.

20A shows that greater than 85% of the virus passes through filter sizes of 1.2, 0.8, or 0.65 μm, but only 25% and 1% pass through a 0.45 or a 0.22 μm pore, respectively.

Based on the virus size of about 200 nm we expected a higher passage of vector through the filters, in particular for 0.45 μm. It was felt that it is likely vector aggregation may lead to formation of larger particles which are excluded from the filter. Electron microscopy experiments revealed (Figure 20B) that indeed there is some aggregation of vector present in our sample. Thus at least some loss or exclusion of vectors from the filter may be attributed to their aggregation.

To remove proteins and other macromolecules, filtrate from large pore-size filters were loaded onto a 300,000 molecular weight cut-off Centricon centrifugal filter system. Figure 20C shows that 60%-85% of the virus was recovered. These initial dead-end filtration techniques demonstrate the potential to purify and concentrate virus by filtration. The use of a tangential-flow filtration system may minimize vector losses associated from gel layer formation or pore plugging that occurred in this system.

5.2.3 Initial Heparin Chromatography for Vector Purification

Initial studies were performed to examine the ability of HSV-1 to bind to the heparin column and verify what NaCl concentration elutes the vector. Vector was loaded to the column and the vector concentration in the effluent was determined. The vector was eluted with 5 column volumes each of 0.5 M, 1.5 M, and 3.0 M NaCl in PBS, pH 7.0. Nearly 70% of the bound vector eluted in the 0.5 M NaCl, 30% in the 1.5 M NaCl, and 2% in the 3.0 M NaCl as shown in Figure 21A. Additional analyses indicate that 90% of total protein was eliminated during vector loading (Figure 21B).

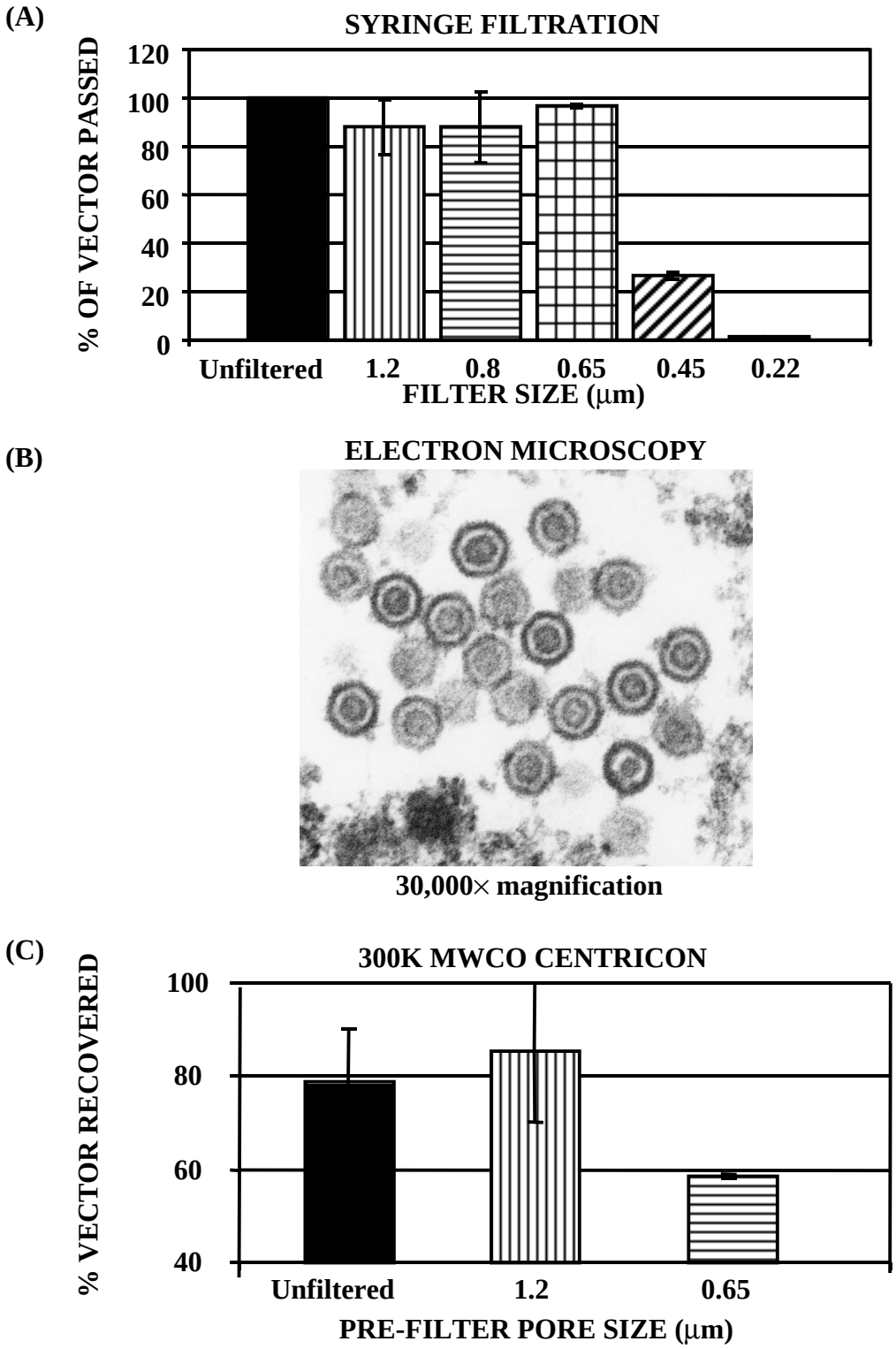


Figure 20. Effect of filter pore size on vector passage and retention.

5.2.4 Combined Tangential-Flow Filtration and Heparin Chromatography Vector Purification

I next examined the combination of filtration and chromatography steps for the purification and concentration of HSV-1 vectors. A tangential-flow filtration system (Quixstand system from A/G Technology) shown in Figure 22 was used in the experiments. The system has a feed reservoir, a recirculation pump, a filter cartridge, inlet and outled pressure gages, a port for permeate collection, and a recirculation line. Pump speed and direction can be controlled by the system, along with the permeate collection rate by adjusting filter outlet pressure or attaching a second pump for permeate control. Retained product can be harvested by use of a retentate port located at the base of the feed reservoir. Three different pore size filtration cartridges were studied with this system. The larger two sizes 0.8 μm and 0.65 μm are designed to allow vector passage but retain cell debris. The smaller size of 0.1 μm is designed to retain and concentrate the vector while passing smaller debris. Vector was produced in roller bottles and after cell lysis the harvests, 2 liters, were used as the initial virus preparation. One liter of the harvest was processed initially by either a 0.8 μm or a 0.65 μm filter as described in Materials and Methods. The permeate of each system was then processed separately by a 0.1 μm filter. The vector stocks were concentrated to 200 mL and washed with PBS to perform a buffer exchange in preparation for heparin chromatography. Both filter purified vector stocks were separately loaded onto heparin columns as described in Materials and Methods. Weakly bound protein was removed by washing with 0.25 M NaCl and vector was eluted in 0.75 M NaCl.

Analyses of both systems were made by examining vector recovery, total protein, and total DNA from samples taken during the processes and displayed in Table 1. Analysis of the 0.8 μm system shows that the two filtration steps recover 77% (0.9 × 0.86) of the initial vector. The 0.65 μm system recovers 61% (0.7 × 0.87) of the loaded vector. The chromatography step recovered 75% and 81% of the bound vector after purification by the 0.8 μm and 0.65 μm filtration systems, respectively. The overall vector recoveries were 58% and 49% for these systems. The vector was concentrated 59× using both filter sizes and final contaminant levels were near identical with removal of greater than 99% of both total protein and DNA. It is important to note that the recovery yield of about 50% in our system is substantially higher than that of the centrifugation protocol (10%) noted earlier.

Table 1. Comparison of combined filtration and chromatography purification systems.

	% VIRUS RECOVERED	TOTAL PROTEIN	TOTAL DNA	TOTAL VOLUME
STOCK	**100**	**4.3 g**	**13 mg**	**1 L**
EXPERIMENT #1				
0.8 μm FILTER	**90**	**4.0**	**0.7**	**1.5**
0.1 μm FILTER	**86**	**0.6**	**0.28**	**0.21**
CHROMATOGRAPHY	**75**	**0.030**	**0.055**	**0.0175**
OVERALL RECOVERY	**58**			
EXPERIMENT #2				
0.65 μm FILTER	**70**	**3.8 g**	**0.7 mg**	**1.5 L**
0.1 μm FILTER	**87**	**0.4**	**0.25**	**0.24**
CHROMATOGRAPHY	**81**	**0.02**	**0.05**	**0.0175**
OVERALL RECOVERY	**49**			

5.3 Conclusions and Future Work

The combined filtration and chromatography system is a highly efficient process for concentration and purification of HSV-1 vectors. The overall vector recovery of 50% is substantially higher than that of the centrifugation based process (10%). This high recovery yield is not attained by compromising the concentration factor. About 60-fold concentration was achieved for both the centrifugation-based and the filtration-chromatography process. Additional benefits of the combined filtration and chromatography system include minimal personnel handling, which reduces the chances for errors and contamination while increasing the overall safety by minimizing vector exposure to technicians, and scalability of the process.

BIBLIOGRAPHY

1. Hoogerbrugge, P. M., van-Beusechem, V. W., Fischer, A., De-bree, M., le-Deist, F., Perignon, J. L., Morgan, G., Gaspar, B., Faribanks, L. D., Skeoch, C. H., Moseley, A., Harvey, M., Levinsky, R. J., and Valerio, D., "Bone Marrow Gene Transfer in Three Patients with Adenosine Deaminase Deficiency," Gene Therapy, Vol. 3 (1996), pp. 179-183.

2. Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Breenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A., and Anderson, W. F., "T Lymphocyte-Directed Gene Therapy for ADA- SCID: Initial Trial Results after 4 Years.," Science, Vol. 270 (1995), pp. 475-480.

3. Hartigan-O'Conner, D., and Chamberlain, J. S., "Developments in Gene Therapy for Muscular Dystrophy," Microscopic Research Technology, Vol. 48 (2000), pp. 223-238.

4. Tawil, R., "Outlook for Therapy in the Muscular Dystrophies," Seminar in Neurology, Vol. 19 (1999), pp. 81-86.

5. vanDeutekom, J. C., Hoffman, E. P., and Huard, J., "Muscle Maturation: Implications for Gene Therapy," Molecular Medicine Today, Vol. 4 (1998), pp. 214-220.

6. Fassati, A., Murphy, S., and Dickson, G., "Gene Therapy of Duchenne Muscular Dystrophy," Advanced Genetics, Vol. 35 (1997), pp. 117-153.

7. Boucher, R. C., "Status of Gene therapy for Cystic Fibrosis Lung Disease," Journal of Clinical Investigation, Vol. 103 (1999), pp. 441-445.

8. Welsh, M. J., "Gene Transfer for Cystic Fibrosis," Journal of Clinical Investigation, Vol. 104 (1999), pp. 1165-1166.

9. Wyman, T., Rohrer, D., Kirigiti, P., Nichols, H., Pilcher, K., Nilaver, G., and Machida, C., "Promoter-Activated Expression of Nerve Growth Factor For Treatment of Neurodegenerative Diseases," Gene Therapy, Vol. 6 (1999), pp. 1648-1660.

10. Fink, D. J., DeLuca, N. A., Goins, W. F., and Glorioso, J. C., "Gene Transfer to Neurons Using Herpes Simplex Virus-Based Vectors," Annual Review in Neuroscience, Vol. 19 (1996), pp. 265-287.

11. Karpati, G., Lochmuller, H., Nalbantoglu, J., and Durham, H., "The Principles of Gene Therapy for the Nervous System," Trends in Neuroscience, Vol. 19 (1996), pp. 49-54.

12. Latchman, D. S., and Coffin, R. S., "Viral Vectors in the Treatment of Parkinson's Disease," Movement Disorders, Vol. 15 (2000), pp. 9-17.

13. Freese A., “Restorative Gene Therapy Approaches to Parkinson’s Disease,” Medical Clinicians of North America, Vol. 83 (1999), pp. 537-548.

14. Fink, D. J., Poliani, P. L., Oligino, T., Krisky, D. M., Goins, W. F., and Glorioso, J. C., “Development of an HSV-Based Vector for the Treatment of Parkinson’s Disease,” Experimental Neurology, Vol. 144 (1997), pp. 103-121.

15. Kennedy, P. G., “Potential Use of Herpes Simplex Virus (HSV) Vectors for Gene Therapy of Neurological Disorders,” Brain, Vol. 120 (1997), pp. 1245-1259.

16. Nielsen, P. E., “Peptide Nucleic Acids: On the Road to New Gene Therapeutic Drugs,” Pharmacology and Toxicology, Vol. 86 (2000), pp. 3-7.

17. Galderisi, U., Cascino, A., and Giordano, A., “Antisense Oligonucleotides as Therapeutic Agents,” Journal of Cell Physiology, Vol. 181 (1999), pp. 251-257.

18. Soomets, U., Hallbrink, M., and Langel, U., “Antisense Properties of Peptide Nucleic Acids,” Frontiers in Bioscience, Vol. 4 (1999), pp. D782-786.

19. McIvor, R. S., "Gene Therapy of Genetic Diseases and Cancer," Pediatric Transplantation, Vol. 3 suppl. 1 (1999), pp. 116-121.

20. Roth, J. A., and Cristiano, R. J., “Gene Therapy for Cancer: What Have We Done and Where Are We Going?,” Journal of the National Cancer Institute, Vol. 89 (1997), pp. 21-39.

21. Wei, M. X., Tamiya, T., Hurford, R. K., Boviatsis, E. J., Tepper, K. I., and Chiocca, E. A., “Enhancement of Interleukin 4-Mediated Tumor Regression in Athymic Mice by In Situ Retroviral Gene Transfer,” Human Gene Therapy, Vol. 6 (1995), pp. 439-445.

22. Anderson, W. F., “Human Gene Therapy,” Nature, Vol. 392 (1998), pp. 25-30.

23. Templeton, N. S., and Lasic, D. D., “New Directions in Liposome Gene Delivery,” Molecular Biotechnology, Vol. 11 (1999), pp. 175-180.

24. Cristiano, R. J., “Targeted, Non-Viral Gene Delivery for Cancer Gene Therapy,” Frontiers in Bioscience, Vol. 3 (1998), pp. 1161-1170.

25. Mahvi, D. M., Sheehy, M. J., and Yang, N. S., “DNA Cancer Vaccines: A Gene Gun Approach,” Immunology and Cell Biology, Vol. 75 (1997), pp. 456-460.

26. Treco, D. A., and Selden, R. F., “Non-Viral Gene Therapy,” Molecular Medicine Today, Vol. 1 (1995), pp. 314-321.

27. Meager, A., ed., Gene Therapy Technologies, Applications and Regulations, "DNA Condensation and Receptor-Mediated Gene Transfer, by A. Ziady and T. Ferkol" (New York: Wiley and Sons LTD, 1999), Chapter 2.

28. Meager, A., ed., Gene Therapy Technologies, Applications and Regulations, "Cationic Liposomes for Gene Therapy Applications, by J. P. Clancy and E. S. Sorscher" (New York: Wiley and Sons LTD, 1999), Chapter 1.

29. Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds., Fields Virology, 2nd Ed., "Retroviridae and Their Replication, by Coffin, J. M." (Raven Press New York, 1990), Ch. 51. pp. 1437-1500.

30. Shimotohno, K., and Temin, H. M., "Loss of Intervening Sequences in Genomic Mouse Alpha-Globin DNA Inserted in an Infectious Retrovirus Vector," Nature, Vol. 299 (1982), pp. 265-268.

31. Tabin, C. J., Hoffman, J. W., Goff, S. P., and Weinberg, R. A., "Adaptation of a Retrovirus as a Eucaryotic Vector Transmitting the Herpes Simplex Virus Thymidine Kinase Gene," Molecular and Cellular Biology, Vol. 2 (1982), pp. 426-436.

32. Havenga, M., Hoogerbrugge, P., Valerio, D., and van Es, H. H. G., "Retroviral Stem Cell Gene Therapy," Stem Cells, Vol. 15 (1997), pp. 162-179.

33. Miller, D. G., and Miller, A. D., "A Family of Retroviruses that Utilize Related Phosphate Transporters for Cell Entry," Journal of Virology, Vol. 68 (1994), pp. 8270-8276.

34. Kim, J. W., Closs, E. I., Albritton, L. M., and Cunningham, J. M., "Transport of Cationic Amino Acids by the Mouse Ecotropic Retrovirus Receptor," Nature, Vol. 352 (1991), pp. 725-728.

35. Vos, Jean-Michel H., ed., Viruses in Human Gene Therapy, "Retroviral Vectors in Human Gene Therapy, by Morgan, R. A." (Durham, North Carolina: Carolina Academic Press, 1995), Chapter 4.

36. Kaptein, L. C. M., Greijer, A. E., Valerio, D. and van Beusechem, V. W., "Optimized conditions for the Production of Recombinant Amphotropic Retroviral Vector Preparations," Gene Therapy, Vol. 4 (1997), pp. 172-176.

37. Kotani, H., Newton, P. B., Zhang, S., Chiang, Y. L., Otto, E., Weaver, L., Blaese, R. M., Anderson, W. F., and McGarrity, G. J., "Improved Methods of Retroviral Vector Transduction and Production for Gene Therapy," Human Gene Therapy, Vol. 5 (1994), pp. 19-28.

38. Miller, D. G., Adam, M. A., Miller, A. D., "Gene Transfer by Retrovirus Vectors Occurs Only in Cells that are Actively Replicating at the Time of Infection," Molecular and Cellular Biology, Vol. 10 (1990), pp. 4239-4242.

39. Lund, A. H., Duch, M., and Pedersen, F. S., "Transcriptional Silencing of Retroviral Vectors," Journal of Biomedical Science, Vol. 3 (1996), pp. 365-378.

40. Federico, M., "Lentiviruses as Gene Delivery Vectors," Current Opinions in Biotechnology, Vol. 10 (1999), pp. 448-453.

41. Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds., Fields Virology, 2nd Ed., "Human Immunodeficiency Viruses and Their Replication, by Wong-Staal, F.," (Raven Press New York, 1990), Ch. 53. pp. 1529-1544.

42. Ono, A., Orenstein, J. M., and Freed, E. O., "Role of the Gag Matrix Domain in Targeting Human Immunodeficiency Virus Type 1 Assembly," Journal of Virology, Vol. 74 (2000), pp. 2855-2866.

43. Dupont, S., Sharova, N., DeHoratius, C., Virbasius, C., Zhu, X., Bukrinskaya, A., Stevenson, M., and Green, M. R., "A Novel Nuclear Export Activity in HIV-1 Matrix Protein Required for Viral Replication," Nature, Vol. 402 (1999), pp. 681-685.

44. Meager, A., ed., Gene Therapy Technologies, Applications and Regulations, "Lentiviral Vectors, by A. M. L. Lever" (New York: Wiley and Sons LTD, 1999), Chapter 4.

45. Akkina, R. K., Walton, R. M., Chen, M. L., Li, X. Q., Planelles, V., and Chen, I. S. Y., "High-Efficiency Gene Transfer into $CD34^+$ Cells with a Human Immunodeficiency Virus Type 1 Based Retroviral Vector Pseudotyped with Vesicular Stomatitis Virus Envelope Glycoprotein G," Journal of Virology, Vol. 70 (1996), pp. 2581-2585.

46. Blomer, U., Naldini, L., Kafri, T., Verma, I. M., and Gage, F. H., "Highly Efficient and Sustained Gene Transfer in Adult Neurons with a Lentivirus Vector," Journal of Virology, Vol. 71 (1997), pp. 6641-6649.

47. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M., "Sustained Expression of Genes Delivered Directly into Liver and Muscle by Lentiviral Vectors," Nature Genetics, Vol. 17 (1997), pp. 314-317.

48. Sakoda, T., Kasahara, N., Hamamori, Y., and Kedes, L., "A High-Titer Lentiviral Production System Mediates Efficient Transduction of Differentiated Cells Including Beating Cardiac Myocytes," Journal of Molecular Cell Cardiology, Vol. 31 (1999), pp. 2037-2047.

49. Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds., Fields Virology, 2nd Ed., "Adenoviridae and Their Replication, by Horwitz, M. S.," (Raven Press New York, 1990), Ch. 60. pp. 1679-1722.

50. Vos, Jean-Michel H., ed., Viruses in Human Gene Therapy, "Adenovirus-Mediated *In vivo* Gene Therapy, by M. Perricaudet and L. D. Stratford-Perricaudet" (Durham, North Carolina: Carolina Academic Press, 1995), Chapter 1.

51. Stratford-Perricaudet, L. D., Levrero, M., Chasse, J., Perricaudet, M. and Briand, P., "Evaluation of the Transfer and Expression in Mice of an Enzyme-Encoding Gene Using a Human Adenovirus Vector," Human Gene Therapy, Vol. 1 (1990), pp. 241-256

52. Herz, J., and Gerard, R. D., "Adenovirus-Mediated Transfer of Low Density Lipoprotein Receptor Gene Acutely Accelerates Cholesterol Clearance in Normal Mice," Proceedings of the National Academy of Science, Vol. 90 (1993), pp. 2812-2816.

53. Suzuki, M., Singh, R. N., and Crystal, R. G., "Regulatable Promoters for Use in Gene Therapy Applications: Modification of the 5'-Flanking Region of the CFTR Gene with Multiple cAMP Response Elements to Support Basal, Low-Level Gene Expression that can be Upregulated by Exogenous Agents that Raise Intracellular Levels of cAMP," Human Gene Therapy, Vol. 7 (1996), pp. 1883-1893.

54. Connelly, S., Smith, T. A. G., Dhir, G., Gardner, J. M., Mehaffey, M. G., Zaret, K. S., McClelland, A., and Kaleko, M., "*In vivo* Gene Delivery and Expression of Physiological Levels of Functional Human Factor VIII in Mice," Human Gene Therapy, Vol. 6 (1995), pp. 185-193.

55. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R., "Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5," Journal of General Virology, Vol. 36 (1977), pp. 59-74.

56. Smith, A. E., "Viral Vectors in Gene Therapy," Annual Review of Microbiology, Vol. 49 (1995), pp. 807-838.

57. Kanagea, Y., Makimura, M., and Saito, I., "A Simple and Efficient Method for Purification of Infectious Recombinant Adenovirus," Japanese Journal of Medical Science Biology, Vol. 47 (1994), pp. 157-166.

58. Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L., and Verma, I., "Cellular Immune Response to Adenoviral Vector Infected Cells Does Not Require *De Novo* Viral Gene Expression: Implications for Gene Therapy," Proceedings of the National Academy of Science, Vol. 95 (1998), pp. 11377-11382.

59. Worgall, S., Wolff, G., Falck-Pedersen, E., and Crystal, R., "Innate Immune Mechanisms Dominate Elimination of Adenoviral Vectors Following *In vivo* Administration," Human Gene Therapy, Vol. 8 (1997), pp. 37-44.

60. Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds., Fields Virology, 2nd Ed., "Parvoviridae and Their Replication, by Berns, K. I.," (Raven Press New York, 1990), Ch. 62. pp. 1743-1764.

61. Flotte, T. R., Solow, R., Owens, R. A., "Gene Expression from Adeno-Associated Virus Vectors in Airway Epithelial Cells," American Journal of Respiratory Cell Molecular Biology, Vol. 70, (1992), pp. 349-356.

62. Samulski, R. J., Chang, L., and Shenk, T., "Helper-Free Stocks of Recombinant Adeno-Associated Viruses: Normal Integration Does Not Require Viral Gene Expression," Journal of Virology, Vol. 63 (1989), pp. 3822-3828.

63. Flotte, T. R., Barrazza-Ortiz, X., Solow, R., "An Improved System for Packaging Recombinant Adeno-Associated Virus Vectors Capable of *In vivo* Transduction," Gene Therapy, Vol. 2 (1995), pp. 39-47.

64. Clark, K. R., Voulgaropoulou, F., Fraley, D. M., and Johnson, P. R., "Cell Lines for the Production of Recombinant Adeno-Associated Virus," Human Gene Therapy, Vol. 6 (1995), pp. 1329-1341.

65. Kearns, W. G., Afione, S., Fulmer, S. B., Caruso, J., Pearson, P., Flotte, T., and Cutting, G. R., "Recombinant AAV Vectors Demonstrate Episomal Expression in Immortalized Human Bronchial Epithelial Cells," American Journal of Human Genetics, Vol. 55 (1994), p. A225.

66. Affione, S. A., Conrad, C. K., Adams, R., Renolds, T. C., Guggino, W. B., Carter, B. J., and Flotte, T. R., "*In vivo* Model of Adeno-Associated Virus Vector Persistence and Rescue," Journal of Virology, Vol. 70 (1994), p. 3235.

67. Frenkel, N., Locker, H., and Vlazny, D. A., "Studies of Defective Herpes Simplex Viruses," New York Academy of Science, Vol. 354 (1980), pp. 347-370.

68. Geller, A., and Freese, A., "A Defective HSV-1 Vector Expresses Escherichia Coli _-Galactosidase in Cultured Peripheral Neurons," Science, Vol. 241 (1988), pp. 1667-1669.

69. Dash, R., Lawrence, M., Ho, D., and Sapolsky, "A Herpes Simplex Virus Vector Overexpressing the Glucose Transporter Gene Protects the Rat Dentate Gyrus from an Antimetabolite Toxin," Experimental Neurology, Vol. 137 (1996), pp. 43-48.

70. During, M., Naegele, J., O'Malley, K., and Geller, A., "Long-Term Behavioral Recovery in Parkinsonian Rats by an HSV Vector Expressing Tyrosine Hydroxylase," Science, Vol. 266 (1994), pp. 1399-1403.

71. Geller, A., Keyomarsi, K., Bryan, J., and Pardee, A., "An Efficient Deletion Mutant Packaging System for Defective Herpes Simplex Virus Vectors: Potential Applications to Human Gene Therapy and Neuronal Physiology," Proceedings of the National Academy of Science, Vol. 87 (1990), pp. 8950-8954.

72. Roizman, B., and Markovitz, N., "Herpes Simplex Virus Virulence: The Functions of the Gamma (1) 34.5 Gene," Journal of Neurovirology, Vol. 3 (1997), pp. S1-S2.

73. Goldstein, D. J., and Weller, S. K., "Herpes Simplex Virus Type-1 Induced Ribonucleotide Reductase Activity is Dispensible for Virus Growth and DNA Synthesis: Isolation and Characterization of an ICP6 lacZ Insertion Mutant," Journal of Virology, Vol. 62 (1988), pp. 196-205.

74. Krisky, D., Wolfe, D., Goins, W. F., Marconi, P., Ramakrishnan, R., Mata, M., Rouse, R. J. D., Fink, D. J., and Glorioso, J. C., "Deletion of Multiple Immediate Early Genes From Herpes Simplex Virus Reduces Cytotoxicity and Permits Long-Term Gene Expression in Neurons," Gene Therapy, Vol. 5 (1999), pp. 1593-1603.

75. Marconi, P., Krisky, D., Oligino, T., Poliani, P. L., Ramakrishnan, R., Goins, W. F., Fink, D. J., and Glorioso, J. C., "Replication-Defective HSV Vectors for Gene Transfer *In Vivo*," Proceedings of the National Academy of Science, Vol. 93 (1996), pp. 11319-11320.

76. Samaniego, L., Webb, A., and DeLuca, N., "Functional Interaction Between Herpes Simplex Virus Immediate-Early Proteins During Infection: Gene Expression as a Consequence of ICP27 and Different Domains of ICP4, Journal of Virology, Vol. 69 (1995), pp. 5705-5715.

77. Samaniego, L., Wu, N., and DeLuca, N., "The Herpes Simplex Virus Immediate-Early Protein ICP0 Affects Transcription from the Viral Genome and Infected Cell Survival in the Absence of ICP4 and ICP 27," Journal of Virology, Vol. 71 (1997), pp. 4614-4625.

78. Wu, N., Watkins, S. C., Schaffer, P. A., and DeLuca, N., "Prolonged Gene Expression and Cell Survival After Infection by a Herpes Simplex Virus Mutant Defective in the Immediate-Early Genes Encoding ICP4, ICP27, and ICP22," Journal of Virology, Vol. 70 (1996), pp. 6358-6368.

79. Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds., Fields Virology, 3nd Ed., "Herpes Simplex Viruses and Their Replication, by Roizman, B.," (Lippincott-Raven Press Philadelphia, 1996), Ch. 72. pp. 2231-2295.

80. Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G., "Entry of Alphaherpesviruses Mediated by Poliovirus Receptor-Related Protein 1 and Poliovirus Receptor," Science, Vol. 280 (1998), pp. 1618-1620.

81. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G., "Herpes Simplex Virus-1 Entry into Cells Mediated by a Novel Member of the TNF/NGF Receptor Family," Cell, Vol. 87 (1996), pp. 427-436.

82. Mellerick, D. M., and Fraser, N., "Physical State of the Latent Herpes Simplex Virus Genome in a Mouse Model System: Evidence Suggesting an Episomal State," Virology, Vol. 158 (1987), pp. 265-275.

83. Efstathiou, S., Minson, A., Field, H., Anderson, J., and Wildy, P., "Detection of Herpes Simplex Virus-Specific DNA Sequences in Latently Infected Mice and in Humans," Journal of Virology, Vol. 57 (1986), pp. 446-455.

84. Ecob-Prince, M., Hassan, K., Denheen, M., and Preston, C., "Expression of β-Galactosidase in Neurons of Dorsal Root Ganglia Which are Latently Infected with Herpes Simplex Virus Type 1," Journal of General Virology, Vol. 76 (1995), pp. 1527-1532.

85. Huang, Q., Vonsattel, J.P., Schaffer, P., Martuza, R., Breakefield, X., and DiFiglia, M., "Introduction of a Foreign Gene (Escherichia coli LacZ) into Rat Neostriatal Neurons Using Herpes Simplex Virus Mutants: a Light and Electron Microscopic Study," Experimental Neurology, Vol. 115 (1992), pp. 303-316.

86. Goins, W. F., Lee, K. A., Cavalcoli, J. D., O'Malley, M. E., DeKosky, S. T., Fink, D. J., and Glorioso, J. C., "Herpes Simplex Virus Type 1 Vector-Mediated Expression of Nerve Growth Factor Protects Dorsal Root Ganglion Neuron form Peroxide Toxicity," Journal of Virology, Vol. 73 (1999), pp. 519-532.

87. Chen, X., Schmidt, M. C., Goins, and Glorioso, J. C., "Two Herpes Simplex Virus Type 1 Latency-Active Promoters Differ in Their Contributions to Latency-Associated Transcript Expression During Lytic and Latent Infections," Journal of Virology, Vol. 69 (1995), pp. 7899-7908.

88. Roizman, B., Whitley, R. J., and Lopez, C., eds., The Human Herpesviruses, "The Family Herpesviridae, by Roizman, B.," (Raven Press, Ltd., New York, 1993), Ch. 1., pp. 1-9.

89. Dilloo, D., Rill, D., Entwistle, C., Boursnell, M., Zhong, W., Holden, W., Holladay, M., Inglis, S., and Brenner, M., "A Novel Herpes Vector for the High-Efficiency Transduction of Normal and Malignant Human Hematopoietic Cells," Blood, Vol. 89 (1997), pp. 119-127.

90. Efstathiou, S., and Minson, A. C., "Herpes Virus-Based Vectors," British Medical Bulletin, Vol. 51 (1995), pp. 45-55.

91. Gruenheid, S., Gatzke, L., Meadows, H., and Tufaro, F., “Herpes Simplex Virus Infection and Propagation in a Mouse L Cell Mutant Lacking Heparan Sulfate Proteoglycans,” Journal of Virology, Vol. 67 (1993), pp. 93-100.

92. Shieh, M. T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G., “Cell Surface Receptors for Herpes Simplex Virus are Heparan Sulfate Proteoglycans,” Journal of Cell Biology, Vol. 116 (1992), pp. 1273-1281.

93. Herold, B. C., WuDunn, D., Soltys, N., and Spear, P. G., “Glycoprotein C of Herpes Simplex Virus Type 1 Plays a Principal Role in the Adsorption of Virus to Cells and in Infectivity,” Journal of Virology, Vol. 65 (1991), pp. 1090-1098.

94. Foster, T. P., Chouljenko, V. N., and Kousoulas, K. G., "Functional Characterization of the HveA Homolog Specified by African Green Monkey Kidney Cells with a Herpes Simplex Virus Expression the Green Fluorescence Protein," Virology, Vol. 258 (1999), pp. 365-374.

95. Krummenacher C., Nicola, A.V., Whitbeck, J.C., et al., “Herpes Simplex Virus Glycoprotein D Can Bind to Poliovirus Receptor-Related Protein 1 or Herpesvirus Entry Mediator, Two Structurally Unrelated Mediators of Virus Entry,” Journal of Virology, Vol. 72 (1998), pp. 7064-7074.

96. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G., “Herpes simplex virus 1 entry into cells mediated by a novel member of the TNF/NGF receptor family,” Cell, Vol. 87 (1996), pp. 427-436.

97. Terry-Allison, T., Montgomery, R. I., Whitbeck, J. C., Xu, R., Cohen, G. H., Eisenberg, R. J., and Spear, P. G., "HveA (Herpesvirus Entry Mediator A), a Coreceptor for Herpes Simplex Virus Entry, also Participates in Virus-Induced Cell Fusion," Journal of Virology, Vol. 72 (1998), pp. 5802-5810.

98. Rooney, I. A., Butrovich, K. D., Glass, A. A., et al., “The Lymphotoxin-Beta Receptor is Necessary and Sufficient for LIGHT-Mediated Apoptosis of Tumor Cells,” Journal of Biological Chemistry, Vol. 275 (2000), pp. 14307-14315.

99. Sarrias, M. R., Whitbeck, J. C., Rooney, I., et al. “The Three HveA Receptor Ligands, gD, LT-Alpha And LIGHT Bind To Distinct Sites On HveA,” Molecular Immunology, Vol. 37 (2000), pp. 665-673.

100. Krummenacher, C., Baribaud, I., Ponce de Leon, M., et al., “Localization Of A Binding Site For Herpes Simplex Virus Glycoprotein D On Herpesvirus Entry Mediator C By Using Antireceptor Monoclonal Antibodies,” Journal of Virology, Vol. 74 (2000), pp. 10863-10872.

101. Shukla, D., Dal Canto, M. C., Rowe, C. L., Spear, P. G., "Striking Similarity Of Murine Nectin-1alpha To Human Nectin-1alpha (HveC) In Sequence And Activity As A Glycoprotein D Receptor For Alphaherpesvirus Entry," Journal of Virology, Vol. 74 (2000), pp. 11773-11781.

102. Cocchi, F., Lopez, M., Menotti, L., et al., "The V Domain Of Herpesvirus Ig-Like Receptor (HIgR) Contains A Major Functional Region In Herpes Simplex Virus-1 Entry Into Cells And Interacts Physically With The Viral Glycoprotein D," Proceedings of the National Academy of Science, USA, Vol. 95 (1998), pp. 15700-15705.

103. Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G., "Entry Of Alphaherpesviruses Mediated By Poliovirus Receptor-Related Protein 1 And Poliovirus Receptor," Science, Vol. 280 (1998), pp. 1618-1620.

104. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G., "A Novel Role for 3-O-Sulfated Heparan Sulfate in Herpes Simplex Virus 1 Entry," Cell, Vol. 99 (1999), pp. 13-22.

105. Kristie, T. M., and Roizman, B., "Host Cell Proteins Bind to the Cis-Acting Site Required for Virion-Mediated Induction of Herpes Simplex Virus 1 Alpha Genes," Proceedings of the National Academy of Science, Vol. 84 (1987), pp. 71-75.

106. McKnight, J. L. C., Kristie, T. M., and Roizman, B., "Binding of the Virion Protein Mediating α Gene Induction in Herpes Simplex Virus 1-Infected Cells to its Cis Site Requires Cellular Proteins," Proceedings of the National Academy of Science, Vol. 84 (1987), pp. 7061-7065.

107. Campbell, M. E. M., Palfeyman, J. W., and Preson, C. M., "Identification of Herpes Simplex Virus DNA Sequences Which Encode a Trans-Acting Polypeptide Responsible for Stimulation of Immediate Early Transcription," Journal of Molecular Biology, Vol. 180 (1984), pp. 1-19.

108. Batterson, W., and Roizman, B., "Characterization of the Herpes Simplex Virion-Associated Factor Responsible for the Induction of Alpha-Genes," Journal of Virology, Vol. 46 (1983), pp. 371-377.

109. DeLuca, N. A., McCarthy, A. M., and Schaffer, P. A., "Isolation and Characterization of Deletion Mutant of Herpes Simplex Virus Type 1 in the Gene Encoding Immediate-Early Regulatory Protein ICP4," Journal of Virology, Vol. 56 (1985), pp.558-570.

110. DeLuca, N. A., and Schaffer, P. A., "Activation of Immediate-Early, Early, and Late Promoters by Temperature-Sensitive and Wild-Type Forms of Herpes Simplex Virus Type 1 Protein ICP4," Molecular and Cellular Biology, Vol. 5 (1985), pp. 1997-2008.

111. Uprichard, S. L., and Knipe, D. M., "Herpes Simplex ICP27 Mutant Viruses Exhibit Reduced Expression of Specific DNA Replication Genes," Journal of Virology, Vol. 70 (1996), pp. 1969-1980.

112. McCarthy, A. M., McMahan, L., and Schaffer, P. A., "Herpes Simplex Virus Type 1 ICP27 Deletion Mutants Exhibit Altered Patterns of Transcription and are DNA Deficient," Journal of Virology, Vol. 63 (1989), pp. 18-27.

113. Sacks, W. R., Greene, C. C., Aschman, D. P., and Schaffer, P. A., "Herpes Simplex Virus Type 1 ICP27 is an Essential Regulatory Protein," Journal of Virology, Vol. 55 (1985), pp. 796-805.

114. Gelman, I. H., and Silverstein, S., "Identification of Immediate Early Genes From Herpes Simplex Virus That Transactivate the Virus Thymidine Kinase Gene," Proceedings of the National Academy of Science, Vol. 82 (1985), pp. 5265-5269.

115. O'Hare, P., and Hayward, G. S., "Evidence for a Direct Role for Both the 175,000- and 110,000-Molecular-Weight Immediate-Early Proteins of Herpes Simplex Virus in the Transactivation of Delayed-Early Promoters," Journal of Virology, Vol. 53 (1985), pp.751-760.

116. Sacks, W. R., and Schaffer, P. A., "Deletion Mutants in the Gene Encoding the Herpes Simplex Virus Type 1 Immediate-Early Protein ICP0 Exhibit Impaired Growth in Cell Culture," Journal of Virology, Vol. 61 (1987), pp. 829-839.

117. Stow, N. D., and Stow, E. C., "Isolation and Characterization of a Herpes Simplex Virus Type 1 Mutant Containing a Deletion Within the Gene Encoding the Immediate-Early Polypeptide Vmw110," Journal of General Virology, Vol. 67 (1986), pp. 2571-2585.

118. Sears, A. E., Halliburton, I. W., Meignier, B., Silver, S., and Roizman, B., "Herpes Simplex Virus 1 Mutant Deleted in the α22 Genes Growth and Gene Expression in Permissive and Restrictive Cells and Establishment of Latency in Mice," Journal of Virology, Vol. 55 (1985), pp. 338-346.

119. Carter, K. L., and Roizman, B., "Alternatively Spliced mRNAs Predicted to Yield Frame-Shift Proteins and Stable Intron 1 RNAs of the Herpes Simplex Virus 1 Regulatory Gene α0 Accumulate in the Cytoplasm of Infected Cells," Proceedings of the National Academy of Science, Vol. 93 (1996), pp. 12535-12540.

120. York, A. I., Roop, C., Andrews, D. W., Ridell, S. R., Graham, F. L., and Johnson, D. C., "A Cytosolic Herpes Simplex Virus Protein Inhibits Antigen Presentation to $CD8^+$ T Lymphocytes," Cell, Vol. 77 (1994), pp. 525-535.

121. Prod'hon, C., Machuca, I., Berthomme, H., Epstein, A., and Jacquemont, B., "Characterization of Regulatory Functions of the HSV-1 Immediate-Early Protein ICP22," Virology, Vol. 226 (1996), pp. 393-402.

122. Watson, R., and Clements, J., "A Herpes Simplex Virus Type 1 Function Continuously Required for Early and Late Virus RNA Synthesis," Nature, Vol. 285 (1980), pp. 329-330.

123. Mavromara-Nazos, P., and Roizman, B., "Activation of Herpes Simplex Virus 1 _2 Genes by Viral DNA Replication," Virology, Vol. 161 (1987), pp. 593-598.

124. Holland, L. E., Anderson, K. P., Shipman, C., and Wagner, E. K., "Viral DNA Synthesis is Required for Efficient Expression of Specific Herpes Simplex Virus Type 1 mRNA," Virology, Vol. 101 (1980), pp. 10-24.

125. Stevens, J. G., "Human Herpesviruses: A Consideration of the Latent State," Microbiology Review, Vol. 53 (1989), pp. 318-332.

126. Cook, M. L., Stevens, J. G., "Pathogenesis of Herpetic Neuritis and Ganglionitis in Mice: Evidence of Intra-Axonal Transport of Infection," Infection Immunology, Vol. 7 (1973), pp. 272-288.

127. Ecob-Prince, M., Rixon, F., Preston, C., Hassan, K., and Kennedy, P., "Reactivation *In vivo* and *In vitro* of Herpes Simplex Virus from Mouse Dorsal Root Ganglia Which Contain Different Levels of Latency-Associated Transcripts," Journal of General Virology, Vol. 74 (1993), pp. 995-1002.

128. Rodahl, E., and Stevens, J., "Differential Accumulation of Herpes Simplex Virus Type 1 Latency-Associated Transcripts in Sensory and Autonomic Ganglia," Virology, Vol. 189 (1992), pp. 385-388.

129. Speck, P. G., and Simmons, A., "Divergent Molecular Pathways of Productive and Latent Infection with a Virulent Strain of Herpes Simplex Virus Type 1," Journal of Virology, Vol. 65 (1991), pp. 4004-4005.

130. Spivack, J. G., Woods, G. M., and Frazer, N. W., "Identification of a Novel Latency-Specific Splice Donor Signal Within HSV Type 1 2.0-Kilobase Latency-Associated Transcript (LAT): Translation Inhibition of LAT Open Reading Frames by the Intron Within the 2.0-Kilobase LAT," Journal of Virology, Vol. 65 (1991), pp. 6800-6810.

131. Deatly, A. M., Spivack, J. G., Lavi, E., O'Boyle, D., and Fraser, N. W., "Latent Herpes Simplex Virus Type 1 Transcripts in Peripheral and Central Nervous Systems Tissues of Mice Map to Similar Regions of the Viral Genome," Journal of Virology, Vol. 62 (1988), pp. 749-756.

132. Croen, K. D., Ostrove, J. M., Dragovic, L. J., Smialek, J. E.,and Straus, S. E., "Latent Herpes Simplex Virus in Human Trigeminal Ganglia. Detection of an Immediate Early Gene 'Antisense' Transcript by In Situ Hybridization," New England Journal of Medicine, Vol. 317 (1987), pp. 1427-1432.

133. Rodahl, E., and Haarr, L., "Analysis of the 2-Kilobase Latency-Associated Transcript Expressed in PC12 Cells Productively Infected with Herpes Simplex Virus Type 1: Evidence for a Stable, Nonlinear Structure," Journal of Virology, Vol. 71 (1997), pp. 1703-1707.

134. Zabolotny, J., Krummenacher, C., and Fraser, N. W., "The Herpes Simplex Virus Type 1 2.0-Kilobase Latency-Associated Transcript is a Stable Intron Which Branches at a Guanosine," Journal of Virology, Vol. 71 (1997), pp. 4199-4208.

135. Hill, J. M., Maggioncalda, J. B., Garza, J., Su, Y.-H., Fraser, N. W., and Block, T. M., "*In vivo* Epinephrine Reactivation of Ocular Herpes Simplex Virus Type 1 in the Rabbit is Correlated to a 370-Base-Pair Region Located Between the Promoter and the 5' End of the 2.0-Kilobase Latency-Associated Transcript," Journal of Virology, Vol. 70 (1996), pp. 7270-7274.

136. Leib, D. A., Bogard, C. L., Kosz-Vnenchak, M., Hicks, K. A., Coen, D. M., Knipe, D. M., and Schagger, P. A., "A Deletion Mutant of the Latency-Associated Transcript of Herpes Simplex Virus Type 1 Reactivates from the Latent Infection," Journal of Virology, Vol. 63 (1989), pp. 2893-2900.

137. Steiner, I., Spivack, J. G., Lirette, R. P., Brown, S. M., MacLean, A. R., Subak-Sharpe, J., and Fraser, N. W., "Herpes Simplex Virus Type 1 Latency-Associated Transcripts are Evidently Not Essential for Latent Infection," European Molecular Biology Organization, Vol. 8, pp. 505-511.

138. Samaniego, L. A., Neiderhiser, L., and DeLuca, N. A., "Persistence and Expression of the Herpes Simplex Virus Genome in the Absence of Immediate-Early Proteins," Journal of Virology, Vol. 72 (1998), pp. 3307-3320.

139. Smibert, C. A., Johnson, D. C., and Smiley, J. R., "Identification and Characterization of the Virion-Induced Host Shutoff Product of Herpes Simplex Virus Gene U_L 41," Journal of General Virology, Vol. 73 (1992), pp. 467-470.

140. Kwong, A. D., and Frenkel, N., "The Herpes Simplex Virus Virion Host Shutoff Function," Journal of Virology, Vol. 63 (1989), pp. 4834-4839.

141. Oroskar, A., and Read, G., "Control of mRNA Stability by the Virion Host Shutoff Function of Herpes Simplex Virus," Journal of Virology, Vol. 63 (1989), pp. 1897-1906.

142. Gage, G. H., "Cell Therapy," Nature, Vol. 392 suppl. (1998), pp. 18-24.

143. Asahara, T., Kalka, C., and Isner, J. M., “Stem Cell Therapy and Gene Transfer for Regeneration,” Gene Therapy, Vol. 7 (2000), pp. 451-457.

144. Fuchs, E., and Segre, J. A., “Stem Cells: A New Lease on Life,” Cell, Vol. 100 (2000), pp. 143-155.

145. Lemischka, I., “The Power of Stem Cells Reconsidered?,” Proceedings of the National Academy of Science, Vol. 96 (1999), pp. 14193-14195.

146. Weissman, I. L., “Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution,” Cell, Vol. 100 (2000), pp. 157-168.

147. Jones, P. H., Harper, S., and Watt, F. M., “Stem Cell Patterning and Fate in Human Epidermis,” Cell, Vol. 80 (1995), pp. 83-93.

148. Potten, C. S., and Morris, R. J., "Epithelial Stem Cells *In vivo*," Journal of Cell Science, Vol. 10 supp. (1988), pp. 45-62.

149. Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M., and Mulligan, R. C., “Dystrophin Expression in the MDX Mouse Restored by Stem Cell Transplantation,” Science, Vol. 401 (1999), pp. 390-394.

150. Jackson, K. A., Mi, T., and Goodell, M. A., “Hematopoietic Potential of Stem Cells Isolated from Murine Skeletal Muscle,” Proceedings of the National Academy of Science, Vol. 96 (1999), pp. 14482-14486.

151. Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C., and Vescovi, A. L., “Turning Brain into Blood: a Hematiopoietic Fate Adopted by Adult Neural Stem Cells *In vivo*,” Science, Vol. 283 (1999), pp. 534-537.

152. Prockop, D. J., “Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues,” Science, Vol. 276 (1997), pp. 71-74.

153. Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F., “Muscle Regeneratio by Bone Marrow-Derived Myogenic Progenitors,” Science, Vol. 279 (1998) pp. 1528-1530.

154. Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M., “Isolation of Putative Progenitor Endothelial Cells for Angiogenesis,” Science, Vol. 275 (1997), 964-967.

155. Piacibello, W., Sanavio, F., Severino, A., Dane, A., Gammaitoni, L., Fagioli, F., Perissinotto, E., Cavalloni, G., Kollet, O., Lapidot, T., and Aglietta, M., “Engraftment in Nonobese Diabetic Severe Combined Immunodeficient Mice of Human $CD34^+$ Cord Blood Cells After *Ex vivo* Expansion: Evidence for the Amplification and Self-Renewal of Repopulating Stem Cells,” Blood, Vol. 93 (1999), pp. 3736-3749.

156. Greiner, D. L., Hesselton, R. A., and Shultz, L. D., “SCID Mouse Models of Human Stem Cell Engraftment,” Stem Cells, Vol. 16 (1998), pp. 166-177.

157. Bhatia, M., Wang, J. C. Y., Kapp, U., Bonnett, D., and Dick, J. E., “Purification of Primitave Human Hematopoietic Cells Capable of Repopulating Immune-Deficient Mice,” Proceedings of the National Academy of Science, Vol. 94 (1997), pp. 5320-5325.

158. Cashman, J. D., Lapidot, T., Wang, J. C. Y., Doedens, M., Shultz, L. D., Lansdorp, P., Dick, J. E., and Eaves, C. J., “Kinetic Evidence of the Regenration of Multilineage Hematopoiesis From Primitive Cells in Normal Human Bone Marrow Transplanted Into Immunodeficient Mice,” Blood, Vol. 89 (1997), pp. 4307-4316.

159. Hogan, C. J., Shpall, E. J., McNulty, O., McNiece, I., Dick, J. E., Shultz, L. D., and Keller, G., “Engraftment and Development of Human $CD34^+$ Enriched Cells From Umbilical Cord Blood in NOD/LtSz-scid/scid Mice,” Blood, Vol. 90 (1997), pp. 85-96.

160. Wang, J. C. Y., Doedens, M., and Dick, J. E., “Primitive Human Hematopoietic Cells Are Enriched in Cord Blood Compared With Adult Bone Marrow or Mobilized Peripheral Blood as Measured by the Quantitative *In vivo* SCID-Repopulating Assay,” Blood, Vol. 89 (1997), pp. 3919-3924.

161. Dick, J. E., “Normal and Leukemic Human Stem Cells Assayed in SCID Mice,” Seminars in Immunology, Vol. 8 (1996), pp. 197-206.

162. Burt, R. K., “Clinical Utility in Maximizing $CD34^+$ Cell Count in Stem Cell Grafts,” Stem Cells, Vol. 17 (1999), pp. 373-376.

163. Buhring, H., Seiffert, M., Bock, T. A., Scheding, S., Thiel, A., Scheffold, A., Kanz, L., and Brugger, W., “Expression of Novel Surface Antigens on Early Hematopoietic Cells,” Annals New York Academy of Sciences, Vol. 872 (1999), pp. 25-38.

164. Craig, W., Kay, R., Cutler, R. L., and Lansdorp, P. M., "Expression of Thy-1 on Human Hematopoietic Progenitor Cells," Journal of Experimental Medicine, Vol. 177 (1993), pp. 1331-1342.

165. Baum, C. M., Weissman, I. L., Tsukamoto, A. S., Buckle, A. M., and Peault, B., "Isolation of a Candidate Human Hematopoietic Stem Cell Population," Proceedings of the National Academy of Science, Vol. 89 (1992), pp. 2804-2808.

166. Terstappen, L. W., Huang, S., Safford, M., Lansdorp, P. M., and Loken, M. R., "Sequential Generations of Hematopoietic Colonies Derived from Single Nonlineage-Committed $CD34^+CD38$- Progenitor Cells," Blood, Vol. 77 (1991), pp. 1218-1227.,

167. Ziegler, B. L., Valtieri, M., Porada, G. A., De Maria, R., Muller, R., Masella, B., Gabbianelli, M., Cassela, I., Pelosi, E., Bock, T., Zanjani, E. D., and Peschle, C., “KDR Receptor: A Key Marker Defining Hematopoietic Stem Cells,” Science, Vol. 285 (1999), pp. 1553-1558.

168. Gehling, U. M., Ergun, S., Schumacher, U., Wagener, C., Pantel, K., Otte, M., Schuch, G., Schafhausen, P., Mende, T., Kilic, N., Kluge, K., Schafer, B., Hossfeld, D. K., and Fiedler, W., "*In vitro* Differentiation of Endothelial Cells from AC133-Positive Progenitor Cells," Blood, Vol. 95 (2000), pp. 3106-3112.

169. De Wynter, E. A., Buck, D., Hart, C., Heywood, R., Coutinho, L. H., Clayton, A., Rafferty, J. A., Burt, D., Guenechea, G., Bueren, J. A., Gagen, D., Fairbairn, L. J., Lord, B. I., Testa, N. G., "$CD34^{+}AC133^{+}$ Cells Isolated from Cord Blood are Highly Enriched in Long-Term Culture Initiating Cells, NOD/SCID-Repopulating Cells and Dendritic Cell Progenitors, Stem Cells, Vol. 16 (1998), pp. 387-396.

170. Yin, A., Miraglia, S., Zanjani, E. D., Almeida-Porada, G., Ogawa, M., Leary, A. G., Olweus, J., Kearney, J., and Buck, D. W., "AC133, a Novel Marker for Human Hematopoietic Stem and Progenitor Cells," Blood, Vol. 90 (1997), pp. 5002-5012.

171. Goodell, M. A., Rosenzweig, M., Kim, H., Marks, D. F., DeMaria, M., Paradis, G., Grupp, S. A., Sieff, C. A., Mulligan, R. C., and Johnson, R. P., "Dye Efflux Studies Suggest that Hematopoietic Stem Cells Expressing Low or Undetectable Levels of CD34 Antigen Exist in Multiple Species," Nature Medicine, Vol. 3 (1997), pp. 1337-1345.

172. Goodell, M., Brose, K., Paradis, G., Conner, A., and Mulligan, R., "Isolation and Functional Properties of Murine Hematopoietic Stem Cells That are Replicating *In vivo*," Journal of Experimental Medicine, Vol. 183 (1996), pp. 1797-1806.

173. Eaves, C. J., Sutherland, H. J., Udomsakdi, C., Lansdorp, P. M., Szilvassy, S. J., Fraser, C. C., Humphries, R. K., Barnett, M. J., Phillips, G. L., and Eaves, A. C., "The Human Hematopoietic Stem Cell *In vitro* and *In vivo*," Blood Cells, Vol. 18 (1992), pp. 301-307.

174. Ogawa, M., "Differentiation and Proliferation of Hematopoietic Stem Cells," Blood, Vol. 81 (1993), pp. 2844-2853.

175. McNiece, I. K., Stewart, F. M., Deacon, D. M., Temeles, D. S., Zsebo, K. M., Clark, S. C., and Quesenberry, P.J., "Detection of a Human CFC with a High Proliferative Potenetial," Blood, Vol. 74 (1989), pp. 609-612.

176. Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W., and Lansdorp, P. M., "Characterization and Partial Purification of Human Marrow Cells Capable of Initiating Long-Term Hematopoiesis *In vitro*," Blood, Vol. 74 (1989), pp. 1563-1570.

177. Nakahata, T., Ogawa, M., "Hemopoietic Colony-Forming Cells in Umbilical Cord Blood with Extensive Capability to Generate Mono- and Multipotential Hemopoietic Progenitors," Journal of Clinical Investigation, Vol. 70 (1982), pp. 1324-1328.

178. Albella, B., Segovia, J. C., Guenechea, G., Pragnell, I. B., and Bueren, J. A., "Preserved Long-Term Re-Population of Hematopoietic Grafts Subjected to *Ex vivo* Expansion with Stem Cell Factor and Interleukin 11," Transplantation, Vol. 67 (1999), pp. 1348-1357.

179. Guenechea, G., Segovia, J. C., Albella, B., Lamana, M., Ramirez, M., Regidor, C., Fernandez, M. N., and Bueren, J. A., "Delayed Engraftment of Nonobese Diabetic/Severe Combined Immunodeficient Mice Transplanted with *Ex vivo*-Expanded Human $CD34^+$ Cord Blood Cells," Blood, Vol. 93 (1999), pp. 1098-1105.

180. Kawada, H., Ando, K., Tsuji, T., Shimakura, Y., Nakamura, Y., Chargui, J., Hagihara, M., Itagaki, H., Shimizu, T., Inokuchi, S., Kato, S, and Hotta, T., "Rapid *Ex vivo* Expansion of Human Umbilical Cord Hematopoietic Progenitors Using a Novel Culture System," Experimental Hematology, Vol. 27 (1999), pp. 904-915.

181. Yagi, M., Ritchie, K. A., Sitnicka, C., Roth, G. J., and Bartelmez, S., "Sustained *Ex vivo* Expansion of Hematopoietic Stem Cells Mediated by Trombopoietin," Proceedings of the National Academy of Science, Vol. 96 (1999), pp. 8126-8131.

182. Collins, P. C., Neilson, L. K., Patel, S. D., Papoutsakis, E. T., and Miller, W. M., "Characteriztion of Hematopoietic Cell Expansion, Oxygen Uptake, and Glycolysis in a Controlled, Stirred-Tank Bioreactor System," Biotechnology Progress, Vol. 14 (1998), pp. 466-472.

183. Collins, P. C., Miller, W. M., and Papoutsakis, E. T., "Stirred Culture of Peripheral and Cord Blood Hematopoietic Cells Offers Advantages over Traditional Static Systems for Clinically Relevant Applications," Biotechnology and Bioengineering, Vol. 59, (1998), pp. 534-543.

184. Tisdale, J. F., Hanazono, Y., Sellers, S. E., Agricola, B. A., Metzger, M. E., Donahue, R. E., and Dunbar, C. E., "*Ex vivo* Expansion of Genetically Marked Rhesus Peripheral Blood Progenitor Cells Results in Diminished Long-Term Repopulating Ability," Blood, Vol. 92 (1998), pp. 1131-1141.

185. Nieda, M., Nicol, A., Denning-Kendall, P., Sweetenham, J., Bradley, B., and Hows, J., "Endothelial Cell Precursors are Normal Components of Human Umbilical Cord Blood," British Journal of Haematology, Vol. 98 (1997), pp. 775-777.

186. Shi, Q., Rafii, S., Wu, M. H., Wijelath, E. S., Yu, C., Ishida, A., Fujita, Y., Kothari, S., Mohle, R., Sauvage, L. R., Moore, M. A. S., Storb, R. F., and Hammond, W. P., "Evidence for Circulating Bone Marrow-Derived Endothelial Cells," Blood, Vol. 92 (1998), pp. 362-367.

187. Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W. M., Silver, M., Kearne, M., Li, T., Isner, J. M., and Asahara, T., "Transplantation of *Ex vivo* Expanded Endothelial Progenitor Cells for Therapeutic Neovascularization," Proceedings of the National Academy of Science, Vol. 97 (2000), pp. 3422-3427.

188. Isner, J. M., and Asahara, T., "Angiogenesis and Vasculogenesis as Therapeutic Strategies for Postnatal Neovascularization," Journal of Clinical Investigation, Vol. 103 (1999), pp. 1231-1236.

189. Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J. M., and Asahara, T., "Ischemia- and Cytokine-Induced Mobilization of Bone Marrow-Derived Endothelial Progenitor Cells for Neovascularization," Nature Medicine, Vol. 5 (1999), pp. 434-438.

190. Bhattacharya, V., McSweeney, P., Shi, Q., Bruno, B., Ishida, A., Nash, R., Storb, R. F., Sauvage, L. R., Hammond, W. P., and Wu, M. H., "Enhanced Endothelialization and Microvessel Formation in Polyester Grafts Seeded with $CD34^+$ Bone Marrow Cells," Blood, Vol. 95 (2000), pp. 581-585.

191. Folkman, J., "Antiangiogenic Gene Therapy," Proceedings of the National Academy of Science, Vol. 95 (1998), pp. 9064-9066.

192. Kong, H., and Crystal, R. G., "Gene Therapy Strategies for Tumor Antiangiogenesis," Journal of the National Cancer Institute, Vol. 90 (1998), pp. 273-286.

193. Folkman, J., "Fighting Cancer by Attacking its Blood Supply," Scientific American, Vol. 275 (1996), pp. 150-154.

194. Ito, H., Rovira, I. I., Bloom, M. L., Takeda, K., Ferrans, V. J., Quyyumi, A. A., and Finkel, T., "Endothelial Progenitor Cells as Putative Targets for Angiostatin," Cancer Research, Vol. 59 (1999), pp. 5875-5877.

195. Gomez-Navarro, J., Contreras, J. L., Arafat, W., Jiang, X. L., Krisky, D., Oligino, T., Marconi, P., Hubbard, B., Glorioso, J. C., Curiel, D. T., and Thomas, J. M., "Genetically Modified $CD34^+$ Cells as Cellular Vehicles for Gene Delivery into Areas of Angiogenesis in a Rhesus Model," Gene Therapy, Vol. 7 (2000), pp. 43-52.

196. Halene, S., and Kohn, D. B., "Gene Therapy Using Hematopoietic Stem Cells: Sisyphus Approaches the Crest," Human Gene Therapy, Vol. 11 (2000), pp. 1259-1267.

197. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J., Bousso, P., Le Deist, F., and Fischer, A., "Gene Therapy of Human Severe Combined Immunodeficiency (SCID)-X1 Disease," Science, Vol. 288 (2000), pp. 669-672.

198. Kay, M. A., Liu, D., and Hoogerbrugge, P. M., “Gene Therapy,” Proceedings of the National Academy of Science, Vol. 94 (1997), pp. 12744-12746.

199. Emery, D. W., Andrews, R. G., and Papayannopoulou, T., “Differences Among Nonhuman Primates In Susceptibility to Bone Marrow Progenitor Transduction with Retrovirus Vectors,” Gene Therapy, Vol. 7 (2000), pp. 359-367.

200. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E., “Transduction of Human $CD34^+$ Cells That Mediate Long-Term Engraftment of NOD/SCID Mice by HIV Vectors,” Science, Vol. 283 (1999), pp. 682-686.

201. Dao, M. A., Shah, A. J., Crooks, G. M., and Nolta, J. A., "Engraftment and Retroviral Marking of $CD34^+$ and $CD34^+CD38^-$ Human Hematopoietic Progenitors Assessed in Immune-Deficient Mice," Blood, Vol. 91 (1998), pp. 1243-1255.

202. Uchida, N., Sutton, R. E., Friera, A. M., He, D., Reitsma, M. J., Chang, W. C., Veres, G., Scollay, R., and Weissman, I. L., "HIV, but not Murine Leukemia Virus, Vectors Mediate High Efficiency Gene Transfer into Freshly Isolated G0/G1 Human Hematopoietic Stem Cells," Proceedings of the National Academy of Science, U S A, Vol. 95 (1998), pp. 11939-11944.

203. Evans, J. T., Kelly, P. F., O’Neill, E., and Garcia, J. V., “Human Cord Blood $CD34^+CD38^-$ Cell Transduction via Lentivirus-Based Gene Transfer Vectors,” Human Gene Therapy, Vol. 10 (1999), pp. 1479-1489.

204. Case, S. S., Price, M. A., Jordan, C.T., et al., “Stable Transduction Of Quiescent CD34(+)CD38(-) Human Hematopoietic Cells By HIV-1-Based Lentiviral Vectors,” Proceedings of the National Academy of Science, U S A, Vol. 96 (1999), pp. 2988-2993.

205. Haas, D. L., Case, S. S., Crooks, G. M., Kohn, D.B., “Critical Factors Influencing Stable Transduction Of Human CD34(+) Cells With HIV-1-Derived Lentiviral Vectors,” Molecular Therapy, Vol. 2 (2000), pp. 71-80.

206. Sirven, A., Ravet, E., Charneau, P., et al., “Enhanced Transgene Expression In Cord Blood CD34(+)-Derived Hematopoietic Cells, Including Developing T Cells And NOD/SCID Mouse Repopulating Cells, Following Transduction With Modified Trip Lentiviral Vectors,” Molecular Therapy, Vol. 3 (2001), pp. 438-448.

207. Fisher-Adams, G., Wong, K. K. Jr., Podsakoff, G., Forman, S. J., and Chatterjee, S., “Integration of Adeno-Associated Virus Vectors in $CD34^+$ Human Hematopoietic Progenitor Cells after Transduction,” Blood, Vol. 88 (1996), pp. 492-504.

208. Goodman, S., Xiao, X., Donahue, R. E. , Moulton, A., Miller, J., Walsh, C., Young, N. S., Samulski, R. J., and Nienhuis, A. W., “Recombinant Adeno-Associated Virus-Mediated Gene Transfer into Hematopoietic Progenitor Cells,” Blood, Vol. 84 (1994), pp. 1492-1500.

209. Zhou, S. Z., Cooper, S., Kang, L. Y., Ruggieri, L., Heimfeld, S., Srivastava, A., and Broxmeyer, H. E., “Adeno-Associated Virus 2-Mediated High Efficiency Gene Transfer into Immature and Mature Subsets of Hematopoietic Progenitor Cells in Human Umbilical Cord Blood,” Journal of Experimental Medicine, Vol. 179 (1994), pp. 1867-1875.

210. Nathwani, A. C., Hanawa, H., Vandergriff, J., Kelly, P., Vanin, E. F., and Nienhuis, A. W., “Efficient Gene Transfer Into Human Cord Blood $CD34^+$ Cells and the $CD34^+$ $CD38^-$ Subset Using Highly Purified Recombinant Adeno-Associated Viral Vector Preparations That Are Free Of Helper Virus and Wild-Type AAV,” Gene Therapy, Vol. 7 (2000), pp. 183-195.

211. Watanabe, T., Kuszynski, C., Ino, K., Heimann, D. G., Shephard, H. M., Yasui, Y., Maneval, D. C., and Talmadge, J. E., "Gene Transfer into Human Bone Marrow Hematopoietic Cells Mediated by Adenovirus Vectors," Blood, Vol. 87 (1996), pp. 5032-5039.

212. Neering, S. J., Hardy, S. F., Minamoto, D., Spratt, S. K., and Jordan, C. T., "Transduction of Primitive Human Hematopoietic Cells with Recombinant Adenovirus Vectors," Blood, Vol. 88 (1996), pp. 1147-1155.

213. Frey, B. M., Hackett, N. R., Bergelson, J. M., Finberg, R., Crystal, R. G., Moore, M. A. S., and Rafii, S., "High-Efficiency Gene Transfer into *Ex vivo* Expanded Human Hematopoietic Progenitors and Precursor Cells by Adenovirus Vectors," Blood, Vol. 92 (1998), pp. 2781-2792.

214. Rebel, V. I., Hartnett, S., Denham, J., et al., “Maturation And Lineage-Specific Expression Of The Coxsackie And Adenovirus Receptor In Hematopoietic Cells,” Stem Cells, Vol. 18 (2000), pp. 176-182.

215. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A., “Efficient Gene Transfer Into Human CD34(+) Cells By A Retargeted Adenovirus Vector,” Journal of Virology, Vol. 74 (2000), pp. 2567-2583.

216. Coffin, R. S., Thomas, S. K., Thomas, N. S. B., Lilley, C. E., Pizzey, A. R., Griffiths, C. H., Gibb, B. J., Wagstaff, M. J. D., Inges, S. J., Binks, M. H., Chain, B. M., Thrasher, A. J., Rutault, K., and Latchman, D. S., "Pure Populations of Transduced Primary Human Cells can be Produced Using GFP Expressing Herpes Virus Vectors and Flow Cytometry," Gene Therapy, Vol. 5 (1998), pp. 718-722.

217. Fiering, S. N., Roederer, M., Nolan, G. P., Micklem, D. R., Parks, D. R., and Herzenber, L. A., “Improved FACS-Gal: Flow Cytometric Analysis and Sorting of Viable Eukaryotic Cells Expressing Reporter Gene Constructs,” Cytometry, Vol. 12 (1991), pp. 291-301.

218. Krisky, D. M., Marconi, P. C., Oligino, T. J., Rouse, R. J. D, Fink, D. J., Cohen, J. B., Watkins, S. C., et al., "Development Of Herpes Simplex Virus Replication Defective Multigene Vectors For Combination Gene Therapy Applications," Gene Therapy, Vol. 5 (1998), pp.1517-1530.

219. Chen, X., Li, J., Mata, M., et al., "Herpes Simplex Virus Type 1 ICP0 Protein Does Not Accumulate In The Nucleus Of Primary Neurons In Culture," Journal of Virology, Vol. 74 (2000), pp. 10132-10141.

220. Lagasse, E., Connors, H., Al Dhalimy, M., et al., "Purified Hematopoietic Stem Cells Can Differentiate Into Hepatocytes In Vivo," Nature Medicine, Vol. 6 (2000), pp. 1229-1234.

221. Petersen, B. E., Bowen, W. C., Patrene, K. D., et al., "Bone Marrow As A Potential Source Of Hepatic Oval Cells," Science, Vol. 284 (1999), pp. 1168-1170.

222. Prockop, D. J., "Marrow Stromal Cells As Stem Cells For Nonhematopoietic Tissues, Science, Vol. 276 (1997), pp. 71-74.

223. Everett, R., and Maul, G., "HSV-1 IE Protein Vmw110 Causes Redistribution of PML," European Molecular Biology Organization Journal, Vol. 13 (1994), pp. 5062-5069.

224. Maul, G. G., Guldner, H. H., and Spivack, J. G., "Modification Of Discrete Nuclear Domains Induced By Herpes Simplex Virus Type1 Immediate Early Gene 1 Product (ICP0)," Journal of General Virology, Vol. 74 (1993), pp. 2679-2690.

225. Maul, G. G., Ishov, A. M., and Everett, R. D., "Nuclear Domain 10 As Preexisting Potential Replication Start Sites Of Herpes Simplex Virus Type-1," Virology, Vol. 217 (1996), pp. 67-75.

226. Yeung, S., Bockhold, K., and Tufaro, F., "Efficient Infection of Mature Skeletal Muscle with Herpes Simplex Virus Vectors by Using Dextran Sulfate as a Co-Receptor," Gene Therapy, Vol. 6 (1999), pp. 1536-1544.

227. Dyer, A. P., Banfield, B. W., Martindale, D., Spannier, D., and Tufaro, F., "Dextran Sulfate Can Act as an Artificial Receptor to Mediate a Type-Specific Herpes Simplex Virus Infection Via Glycoprotein B," Journal of Virology, Vol. 71 (1997), pp. 191-198.

228. Goins, W. F., Lee, K. A., Cavalcoli, J. D., O'Malley, M. E., DeKosky, S. T., Fink, D. J., and Glorioso, J. C., "Herpes Simplex Virus Type 1 Vector-Mediated Expression of Nerve Growth Factor Protects Dorsal Root Ganglion Neurons from Peroxide Toxicity," Journal of Virology, Vol. 73 (1999), pp. 519-532.

229. Chen, X., Schmidt, M. C., Goins, W. F., and Glorioso, J.C., "Two Herpes Simplex Virus Type-1 Latency Active Promoters Differ In Their Contribution To Latency-Associated Transcript Expression During Lytic And Latent Infection," Journal of Virology, Vol. 69 (1995), pp. 7899-7908.

230. Fan, X., Brun, A., and Karlsson, S., "Adenoviral Vector Design For High-Level Transgene Expression In Primitive Human Hematopoietic Progenitors," Gene Therapy, Vol. 7 (2000), pp. 2132-2138.

231. Laquerre, S., Anderson, D. B., Stolz, D.B., and Glorioso, J. C., "Recombinant Herpes Simplex Virus Type 1 Engineered For Targeted Binding To Erythropoietin Receptor-Bearing Cells," Journal of Virology, Vol. 72 (1998), pp. 9683-9697.

232. Goins, W. F., Anderson, D. B., Laquerre, S., Watkins, S.C., and Glorioso, J. C., "Vesicular Stomatitis Virus Glycoprotein G (VSV-G) Can Substitute for Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein D (gD) in Attachment and Entry of a Virus Deleted for Us3-8," In Preparation

233. Misse, D., Cerutti, M., Noraz, N., et al., "A CD4-Independent Interaction of Human Immunodeficiency Virus-1 gp120 with CXCR4 Induces their Cointernalization, Cell Signaling, and T-cell Chemotaxis, Blood, Vol. 93 (1999), pp. 2454-2462.

234. Wang, J. M., Ueda, H., Howard, O. M., et al., "HIV-1 Envelope gp120 Inhibits the Monocyte Response to Chemokines Through CD4 Signal-Dependent Chemokine Receptor Down-Regulation," Journal of Immunology, Vol. 161 (1998), pp. 4309-4317.

235. Amara, A., Gall, S.L., Schwartz, O., et al., "HIV Coreceptor Downregulation As Antiviral Principle: SDF-1alpha-Dependent Internalization Of The Chemokine Receptor CXCR4 Contributes To Inhibition Of HIV Replication," Journal of Experimental Medicine, Vol. 186 (1997), pp. 139-146.

236. Johnson, P., Wang, M., and Friedmann, T., "Improved Cell Survival By The Reduction Of Immediate-Early Gene Expression In Replication-Defective Mutants Of Herpes Simplex Virus Type 1 But Not By Mutation Of The Viron Host Shutoff Function," Journal of Virology, Vol. 68 (1997), pp. 6347-6362.

237. Markert, J., Medlock, M., Rabkin, S., Gillespie, G., Todo, T., Hunter, W., Palmer, C., et al., "Conditionally Replicating Herpes Simplex Virus Mutant, G207 For The Treatment Of Malignant Glioma: Results Of A Phase I Trial," Gene Therapy, Vol. 7 (2000), pp. 867-874.

238. Rampling, R., Cruickshank, G., Papanastassiou, V., Nicoll, J., Hadley, D., Brennan, D., Petty, R., et al., "Toxicity Evaluation Of Replication-Competent Herpes Simplex Virus (ICP 34.5 Null Mutant 1716) In Patients With Recurrent Malignant Glioma. Gene Therapy, Vol. 7 (2000), pp. 859-866.

239. Chambers, R., Gillespie, G. Y., Soroceanu, L., Andreansky, S., Chatterjee, S., Chou, J., Roizman, B., et al., "Comparison Of Genetically Engineered Herpes Simplex Viruses For The Treatment Of Brain Tumors In A Scid Mouse Model Of Human Malignant Glioma," Proceedings of the National Academy of Science, USA, Vol. 92 (1995), pp. 1411-1415.

240. MacLean, A., ul-Fareed, M., Robertson, L., Harland, J., and Brown, S., 1991. "Herpes Simplex Virus Type 1 Deletion Variants 1714 And 1716 Pinpoint Neurovirulenece-Related Sequences In Glasgow Strain 17+ Between Immediate Early Gene 1 And The 'a' Sequence," Journal of General Virology, Vol. 72 (1991), pp. 631-639.

241. Martuza, R., Malick, A., Markert, J., Ruffner, K., and Coen, D., "Experimental Therapy Of Human Glioma By Means Of A Genetically Engineered Virus Mutant," Science, Vol. 252 (1991), pp. 854-856.

242. Whitley, R., Kern, E., Chatterjee, S., Chou, J., and Roizman, B., "Replication Establishment Of Latency, And Induced Reactivation Of Herpes Simplex Virus γ_1 34.5 Deletion Mutants In Rodent Models," Journal of Clinical Investigation, Vol. 91 (1993), pp. 2837-2843.

243. Kim, S., Carew, J., Kooby, D., Shields, J., Entwisle, C., Patel, S., Shah, J., et al., "Combination Gene Therapy Using Multiple Immunomodulatory Genes Transferred By A Defective Infectious Single-Cycle Herpes Virus In Squamous Cell Cancer," Cancer Gene Therapy, Vol. 7 (2000), pp. 1279-1285.

244. O'Keeffe, R., Johnston, M. D., and Slater, N. K., "The Primary Production Of An Infectious Recombinant Herpes Simplex Virus Vaccine," Biotechnology and Bioengineering, Vol. 57 (1998), pp. 262-271.

245. Ozuer, A., Wechuck, J. B., Goins, W. F., Wolfe, D., Glorioso, J. C., and Ataai, M. M., "Effect of Genetic Background and Culture Conditions on Production of Herpesvirus-based Gene Therapy Vectors," Biotechnology and Bioengineering, In Press.

246. Higashikawa, F., and Chang, L., "Kinetic Analyses Of Stability Of Simple And Complex Retroviral Vectors," Virology, Vol. 280 (2001), pp.124-131.

247. Kaptein, L. C., Greijer, A. E., Valerio, D., and van Beusechem, V. W., "Optimized Conditions For The Production Of Recombinant Amphotropic Retroviral Vector Preparations," Gene Therapy, Vol. 4 (1997), pp. 172-176.

248. Kotani, H., Newton, P. B., Zhang, S., Chiang, Y. L., Otto, E., Weaver, L., Blaese, R. M., et al., "Improved Methods Of Retroviral Vector Transduction And Production For Gene Therapy," Human Gene Therapy, Vol. 5 (1994), pp. 19-28.

249. Lancz, G., "Effect Of pH On The Kinetics Of Herpes Simplex Virus Inactivation At 36 Degrees," Virology, Vol. 75 (1976), pp. 488-491.

250. Lancz, G., "Influence Of Environmental pH On The Preservation And Inactivation Of Herpes Simplex Virus," Proceedings of the Society of Experimental Biological Medicine, Vol.162 (1979), pp. 238-240.

251. Lancz, G., and Sample, J., "Thermal-pH Inactivation Of Herpes Simplex Virus: Interdependence Of The Medium Composition And The pH On The Rate Of Virus Inactivation," Archives of Virology, Vol. 84 (1985), pp. 141-146.

252. Lee, S. G., Kim, S., Robbins, P. D., and Kim, B. G., "Optimization Of Environmental Factors For The Production And Handling Of Recombinant Retrovirus," Applied Microbiology and Biotechnology, Vol. 45 (1996), pp. 477-483.

253. Wallis, C., and Melnick, J., "Thermostabilization And Thermosensitization Of Herpesvirus," Journal of Bacteriology, Vol. 90 (1965), pp. 1632-1637.

254. Lancz, G., "Physical Integrity Of Herpes Simplex Virus Following Thermal Inactivation," Archives of Virology, Vol. 64 (1980), pp. 375-381.

255. Yanagi, K., "Irreversible Conversion Of The Physical State Of Herpes Simplex Virus Preceding Inactivation By Thermal Or Antibody Treatment," Journal of Virology, Vol. 38 (1981), pp. 737-748.

256. Matis, J., Kudelova, M., and Rajcani, J., "Interference Of The Low-Ph Inactivated Herpes Simplex Virus Type 1 (HSV-1) Strain HSZP With The Early Shutoff Function Of Superinfecting HSV-1 Strain KOS," Virus Research, Vol. 60 (1999), pp. 81-86.

257. Wechuck, J. B., Goins, W. F., Glorioso, J. C., and Ataai, M. M., "Effect Of Protease Inhibitors On Yield Of HSV-1-Based Viral Vectors," Biotechnology Progress, Vol. 16 (2000), pp. 493-496.

258. Donaldson, M. S., and Shuler, M. L., "Effects Of Long-Term Passaging Of BTI-Tn5B1-4 Insect Cells On Growth And Recombinant Protein Production," Biotechnology Progress, Vol. 14 (1998), pp. 543-547.

259. O'Keeffee, R. S., Johnston, M. D., and Slater, N. K. H., "The Affinity Adsorptive Recovery of an Infectious Herpes Simplex Virus Vaccine," Biotechnology and Bioengineering, Vol. 62 (1999), pp. 537-545.

260. Trybala, E., Liljeqvist, J., Svennerholm, B., and Bergstrom, T., "Herpes Simplex Virus Types 1 and 2 Differ in Their Interaction with Heparan Sulfate," Journal of Virology, Vol. 74 (2000), pp. 9106-9114.

261. Dyer, A. P., Banfield, B. W., Martindale, D., Spannier, D., and Tufaro, F., "Dextran Sulfate Can Act as an Artificial Receptor to Mediate a Type-Specific Herpes Simplex Virus Infection Via Glycoprotein B," Journal of Virology, Vol. 71 (1997), pp. 191-198.

262. Lyddiatt, A., and O'Sullivan, D. A., "Biochemical Recovery and Purification of Gene Therapy Vectors," Current Opinion in Biotechnology, Vol. 9 (1998), pp. 177-185.

263. O'Neil, P. F., and Balkovic, E. S., "Virus Harvesting and Affinity-Based Liquid Chromatography," Bio/Technology, Vol. 11 (1993), pp. 173-178.

264. Clark, K. R., Liu, X., McGrath, J. P., and Johnson, P. R., "Highly Purified Recombinant Adeno-Associated Virus Vectors Are Biologically Active and Free of Detectable Helper and Wild-Type Viruses," Human Gene Therapy, Vol. 10 (1999), pp. 1031-1039.

265. Reiser, J., "Production and Concentration of Pseudotyped HIV-1-Based Gene Transfer Vectors," Gene Therapy, Vol. 7 (2000), pp. 910-913.

266. Todo, T., Feigenbaum, F., Rabkin, S. D., Lakeman, F., Newsome, J. T., Johnson, P. A., Mitchell, E., Belliveau, D., Ostrove, J. M., and Martuza, R. L., "Viral Shedding and Biodistribution of G207, a Multimutated, Conditionally Replicating Herpes Simplex Virus Type 1, after Intracerebral Inoculation in Aotus," Molecular Therapy, Vol. 2 (2000), pp. 588-595.

267. Reeves, L., and Cornetta, K., "Clinical Retroviral Vector Production: Step Filtration Using Clinically Approved Filters Improves Titers," Gene Therapy, Vol. 7 (2000), pp. 1993-1998.

268. Zolotukhin, S., Byrne, B. J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R. J., and Muzycka, N., "Recombinant Adeno-Associated Virus Purification Using Novel Methods Improves Infectious Titer and Yield," Gene Therapy, Vol. 6 (1999), pp. 973-985.