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Retargeting of Herpes Simplex Virus (HSV) Vectors

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Abstract

Gene therapy applications depend on vector delivery and gene expression in the appropriate target cell. Vector infection relies on the distribution of natural virus receptors that may either not be present on the desired target cell or distributed in a manner to give off-target gene expression. Some viruses display a very limited host range, while others, including herpes simplex virus (HSV), can infect almost every cell within the human body. It is often an advantage to retarget virus infectivity to achieve selective target cell infection. Retargeting can be achieved by (i) the inclusion of glycoproteins from other viruses that have a different host-range, (ii) modification of existing viral glycoproteins or coat proteins to incorporate peptide ligands or single-chain antibodies (scFvs) that bind to the desired receptor, or (iii) employing soluble adapters that recognize both the virus and a specific receptor on the target cell. This review summarizes efforts to target HSV using these three strategies.

Introduction

With the approval of Glybera (alipogene tiparvovec) in Europe [1,2] for the treatment of lipoprotein lipase deficiency and IMLYGIC (T-VEC, talimogene laherparepvec) [3,4] in the US [5] for the treatment of malignant melanoma, gene therapy is beginning to show promise as an approved alternative to drug and radiation therapies in the treatment of human disease. However, further progress will require the refinement of gene therapy approaches before it becomes a true everyday therapeutic. Current gene therapy applications, such as Glybera, often rely on tissue-specific promoters to limit therapeutic gene expression to specific cells. While this strategy helps minimize off-target effects, it does not ensure delivery of the viral vector to the intended cell or tissue. One strategy being employed to refine gene therapy is the use of transductional targeting to limit viral vector infection to only the desired target cell, a strategy called “retargeting”.

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Competing Financial Interests

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Limiting vector transduction to specific cell types can benefit many gene therapy applications, including those for chronic pain and cancer. For example, in pain gene therapy, most standard approaches fail to limit infection to specific peripheral nerve fiber subtypes. Ideally, one only wants to silence the activity of C-fiber neurons that are the nociceptors involved in pain sensation, while avoiding nerve fibers that regulate proprioception, pressure or itch so that they will function normally. Cancer gene therapy approaches have recently drawn interest due to the success of T-VEC. However, even that oncolytic herpesvirus (oHSV) is not restricted to which type of cells it can transduce, but is designed to selectively replicate in rapidly dividing tumor cells based on the absence of the viral *ICP34.5* gene. As an alternative approach to limiting oHSV infection and lysis to tumor cells, our group and the groups of Campadelli-Fiume and Roizman have designed retargeted oHSV that preferentially infect breast or brain tumors. In this strategy, viral envelope glycoproteins that bind to widespread cellular receptors for HSV, are modified to ablate natural receptor binding and incorporate ligands or single-chain antibodies (scFv) that recognize the human epidermal growth factor (EGF) receptor 2 (HER2) [6,7], the interleukin-13 receptor IL-13R α 2 [8], or the EGF receptor (EGFR) [9,10] that are often over-expressed in these and other tumors.

All viruses display a natural tropism for specific cell types, tissues and organs within the body. Tropism-determining factors include how the virus (i) encounters the host, (ii) attaches to host cell receptors that enable entry, (iii) establishes itself within the host, (iv) influences pathogenesis and disease, and (v) counteracts the host immune response. Retargeting can be performed to either expand the tropism of viruses that infect only a very limited number of cell types or, more commonly, to restrict the tropism of viruses that infect many cells of the host. Non-enveloped viruses employ one or more viral capsid proteins to interact with the host cell, while enveloped viruses use one or more glycoproteins to bind to and enter host cells. While many enveloped viruses, such as measles, influenza or HIV, employ a single or two glycoproteins to achieve cell binding and entry, members of the herpesvirus family rely on an array of glycoproteins to enter cells and spread from infected to uninfected cells. Herpes simplex virus (HSV) encodes 12 different glycoproteins (Figure 1A) and uses glycoproteins B, C, D, E, H, I, K and L for entry and cell-to-cell spread within the host (reviewed in [11–13]). Most of these glycoproteins contribute to viral tropism, making retargeting of HSV a distinct challenge. HSV retargeting is a worthwhile pursuit, however, given the attractive features HSV offers as a gene therapy platform, including a capacity to accommodate very large or multiple transgenes and infection without integration into host chromosomes.

Years ago we performed some of the first retargeting studies with HSV by genetic fusion of full-length erythropoietin (EPO) to HSV glycoprotein C (gC) that had been N-terminally truncated to eliminate attachment of the virus to cellular heparan sulfate proteoglycans (HS or HSPG) in the background of a virus lacking the HS-binding region of gB [14]. Although this resulted in entry of the gC-EPO virus into EPO receptor-bearing cells, the virus was still able to enter cells that possess the natural receptors for HSV entry. Thus, we had expanded HSV's tropism rather than restricting it to EPO receptor-bearing cells. In order to achieve full retargeting of HSV, virus interaction with the canonical HSV entry receptors must be blocked or eliminated and functionally replaced with alternate ligand-receptor interactions.

The choice of alternate receptors is limited to candidates that are markers for the target cell and are recognized by peptide ligands or single-chain antibodies (scFvs) that preferentially do not activate the normal physiological function of the receptor. As summarized below, studies by a number of laboratories over the past 20 years have greatly enhanced our understanding of HSV entry, ultimately allowing the development of a first generation of fully retargeted HSV vectors.

HSV Attachment and Entry

The HSV particle (Figure 1B) is composed of an icosahedral-shaped nucleocapsid containing the 152-kb double-stranded DNA genome (Figure 1A), an amorphous tegument layer consisting of viral and cellular proteins, and a lipid envelope acquired from the host cell that contains the 12 viral glycoproteins. The glycoproteins are grouped according to whether they are essential or non-essential for HSV entry [15–18]. Another group of important players in the HSV entry process are the cellular receptors that HSV normally engages to achieve attachment and entry. Initially, HSV gB and gC bind via arginine- and lysine-rich regions to negatively-charged HSPG present on almost all cells of the body (Figure 2A) [19–21]. This interaction enables virus surfing of the cell surface membrane [22] until HSV gD encounters one of its cognate receptors that are more restricted in their expression than HSPG, yet are present on numerous cell types (Figure 2A).

HSV gD can bind to either (i) HVEM (TNFRSF14, HveA, CD270), a member of the TNF/NGF superfamily of receptors whose natural ligands include LIGHT, LT α , BTLA and CD160 [23], (ii) nectin-1 (PVRL1, HveC, CD111), a member of the immunoglobulin superfamily of receptors whose natural ligands include afadin and PVRL3 (nectin-3) [24], and (iii) 3-O-sulfated heparan sulfate (3-OS HS) generated by specific sulfotransferase family members, including 3-OST-3A/B [25,26] (Figure 2B). The region of gD containing the binding sites for these receptors has been mapped to the first ~230 residues of the protein and mutants with alterations in this region have enabled the identification of residues that are crucial to receptor binding [27–30]. In addition, mutation and deletion studies have defined a profusion domain beyond residue 260 that is required for virus entry [31,32], supporting the idea that gD represents the fusion trigger [33]. Refined knowledge of the interactions between gD and nectin-1 [34] or HVEM [35] has come from crystallography studies compared to un-liganded gD [36] and are consistent with the model that the gD-receptor interaction results in a conformational change that displaces the N-terminus of gD and exposes the gD pro-fusion domain [37,38]. The next step in the entry process involves transmission of the fusion signal from the fusion trigger (gD) to the fusion regulator (Figure 2A), the gH/gL heterodimer [12,33,39], whose structure has been defined by crystallography [40]. Additionally, it has been shown that gH can bind to integrins like $\alpha v\beta 3$ [41] and $\alpha v\beta 6/\alpha v\beta 8$ [42], aiding in the formation of the fusion complex (Figure 2A). During the process of fusion complex formation, gL is displaced from the gH/gL heterodimer [33,39]. The final member of the fusion complex is gB (Figure 2A), which has been shown to bind to PILR α [71]. The crystal structure of gB [43,44] confirms that it is the true HSV fusogen, being the only HSV glycoprotein with sequence similarity to viral class III fusion proteins, in particular the vesicular stomatitis virus (VSV) G glycoprotein [45,46]. These final

interactions within the fusion complex lead to exposure of the gB fusion loops [47], which enable fusion of the HSV envelope with the cell surface or endosomal membrane [33,39].

Retargeting of HSV Attachment/Entry

Because of the complex involvement of various HSV glycoproteins in the attachment and entry process, it has been a difficult task to completely retarget HSV, including the elimination of entry via the native HSV gD receptors while achieving levels of infection similar to those obtained with wild-type virus on susceptible cells. Multiple strategies have been employed (Figure 3 and Table 1) including (a) the use of pseudotyped virus replacing one or more HSV glycoproteins with those of another virus that has a different natural tropism, (b) the incorporation of peptide ligands into HSV gD or gC, (c) the incorporation of scFvs into gD, gC or gH, and (d) the use of adapter molecules that are capable of binding to both HSV and the desired target receptor.

Two early studies employed the VSV-G glycoprotein for “pseudotyping” HSV (Figure 3a and Table 1a), replacing HSV-1 gD [48] or gB [49]. Interestingly, when VSV-G was substituted for HSV gB, entry was seen at levels approaching that of wild-type HSV in rat striatum. These data are consistent with the structural similarity between VSV-G and HSV gB, apparently allowing VSV-G to act as the fusogen for HSV-1 entry. However, it is unknown whether the presence of gD in these particles still allows virus entry via the canonical gD receptors. In the gD replacement study, the pseudotyped viruses showed reduced entry efficiency and appeared to enter cells solely via the endocytic pathway, with the majority of virions being trapped in and degraded by the low pH of the endosomal pathway. Importantly, these studies illustrated that the HSV-1 entry machinery could be manipulated, replacing individual glycoproteins with functional alternatives, and infectious virions could be produced with the alternate proteins mediating entry into cells bearing the appropriate receptors.

Our group was the first to explore the use of adapter molecules to enable HSV retargeting (Figure 3d). The adapter protein consists of a portion that can bind to an HSV glycoprotein such as gD and a second component that is able to bind to a suitable target receptor. This adapter, or bridging protein, can be produced in bacteria or mammalian cells, purified using protein tags, and then added to virus just prior to infection of the target cells. Although this approach produced reasonable efficiency of retargeted cell transduction *in vitro* [50,51], it required meticulous purification of the adapter and precise determination of the timing of adapter addition to the virus prior to infection. A recent study by Baek and colleagues [52] employed a nectin-1:scFv anti-CEA adapter that was shown to increase the transduction efficiency of MKN45 flank tumors in nude mice compared to the no adapter control animals and produced a 3-fold reduction in tumor volume. While adapters that rely on intact nectin-1- or HVEM-binding sites in gD can mediate targeting to alternate receptors, detargeting from nectin-1 or HVEM can be sub-optimal due to incomplete occupancy of these binding sites by the adapter, allowing background entry through nectin-1 or HVEM. Furthermore, spread from initially infected cells requires either the presence of natural HSV-1 receptors on the uninfected acceptor cells or continued availability of the adapter.

The retargeting methods that have proven most efficacious have been the introduction of peptide ligands (Figure 3b and Table 1b) or scFvs (Figure 3c and Table 1c) into gD, or more recently gH. The majority of these studies have been performed to limit oHSV infection to only specific tumor cell types. The Roizman group has shown success in rendering virus entry uniquely dependent on the expression of IL-13R α 2 or the urokinase plasminogen activator receptor (uPAR) on target cells [8,53,54] by genetic incorporation of peptide ligands into both gC and gD. Retargeting of gC was aimed at increasing the selectivity of virus attachment while gD retargeting was expected to limit entry to target receptor-expressing cells. Despite promising results *in vitro*, the potential increased ability of these viruses to infect and kill glial tumor targets in animals has yet to be confirmed. The Campadelli-Fiume group has employed recombinant oHSV containing an anti-HER2 scFv in gD to target HER2-expressing tumor cells [7,55–60] in culture and *in vivo*. Early studies employed gD recombinants that could still enter cells by binding to the canonical gD receptor nectin-1 [55–57,60] and although they displayed good efficacies in ovarian and glioma tumor models in mice [55,57,60], exclusive targeting of the HER2 tumor receptor was not achieved. Subsequent reports from this group [7,58,59] employed oHSV that were mutationally inactivated for binding to nectin-1 and HVEM, resulting in sole entry into HER2-bearing tumor cells. These vectors displayed reduced toxicity when injected into the brains of nude mice, suggesting decreased off-target infection, and entry could be completely blocked using the Herceptin HER2 antibody, supporting the conclusion that these oHSV gained entry into tumor cells exclusively by retargeted gD interaction with cell-surface HER2.

Recent work from the Campadelli-Fiume group described an alternative approach to vector retargeting by the introduction of the anti-HER2 scFv into gH [6], either in a virus that retained the ability to enter via the canonical gD receptors or one lacking this ability. The results not only demonstrated HER2-specific entry of the anti-HER2 scFv-gH virus, but also established the possibility of virus entry via receptor engagement by a gH-based ligand in the absence of functional gD. This finding steps away from the established view of HSV entry, where gD binding to its receptor triggers signaling through gH to the fusogen gB to achieve fusion between the virus envelope and the cell membrane. Instead, it suggests that modifying gH to incorporate an alternative receptor binding function can circumvent the requirement for gD-receptor interactions. This finding opens up a range of possibilities in HSV targeting, allowing modification of not only gD, but also gH as complete retargeting strategies.

Studies in our own lab [10] and with collaborators [9] have succeeded in scFv-mediated oHSV retargeting to EGFR and its uniquely tumor-specific variant EGFRvIII [10], the carcinoembryonic antigen (CEA) [10] present on gastric cancer tumor cells and to epithelial cell adhesion molecule (EpCAM) found in abundance on colon cancer cells [9]. In these instances, the targeting scFv was introduced into gD modified to disrupt the binding sites for its natural receptors. Both recombinants included modifications in gB (gB:N/T) previously shown in our lab to dramatically increase the rate of HSV entry [61,62]. The inclusion of these mutations enabled target receptor-dependent entry of the EGFR, CEA and the EpCAM retargeted oHSV at levels approaching those of virus bearing wild-type gD into cells bearing a canonical gD receptor. The EGFR/EVFRvIII retargeted vector was shown to be effective in

the treatment of an orthotopic glioblastoma multiforme (GBM) model employing primary human GBM cells [10].

Conclusions

Over the past two decades, the field of HSV retargeting has made tremendous progress. Much of our current ability to target infection to different receptors and to prevent infection of nectin-1 and HVEM expressing cells has come from a greater understanding of the contributions of individual amino acids and functional domains of gD, gC, gB, and gH/gL to the processes of attachment and entry. This knowledge has been substantially refined by resolution of the crystal structures of gD, nectin-1-bound gD, HVEM-bound gD, gB, and the gH/gL heterodimer. The field has also benefited from studies defining novel receptor-ligand pairs as well as the availability of scFvs that do not activate the target receptor, a concern that is most significant when using HSV as a vector for therapeutic gene delivery. While all completely retargeted HSV vectors reported to date are replication-competent oHSVs, the development of complementing cell lines expressing suitable target receptors will make this technology transferable to replication-defective HSV vectors that offer a large payload capacity for gene therapy. In recent years, the use of HSV BACs to rapidly generate novel recombinant viruses has dramatically affected HSV vector design, and CRISPR technology will further facilitate the creation and propagation of future generations of retargeted HSV vectors. Such progress will allow for more rapid evaluation of retargeting receptor-ligand pairs, including scFvs and similar targeting molecules, facilitate the identification of glycoprotein mutations that further enhance retargeted virus entry, and open new approaches toward optimization of HSV cell-to-cell spread. In the coming years, these and other efforts can be expected to bring retargeted HSV vectors closer to clinical applicability.

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dependence on a gD-receptor interaction to provide the fusion signal to the gB-gH/gL complex can be compensated by alterations of the amino acid sequences of gB and gH.

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Highlights

- Understanding the contributions of HSV gD, gC, gB, and gH/gL in attachment and entry
- Complete elimination of HSV attachment and entry via natural receptors
- Selection of glycoprotein mutations increasing entry of retargeted HSV
- Efficient retargeting using single chain antibodies or ligands in HSV gD and gH

(A) HSV Glycoproteins

(B) HSV Virus Particle

Figure 1. HSV glycoproteins

(a) The location of the HSV-1 encoded viral glycoproteins within the HSV-1 genome is depicted. Those listed below the viral genome are essential for virus replication, while those above it are non-essential and their deletion does not block virus replication in culture or *in vivo*. However, these dispensable glycoproteins can contribute to virus host range, pathogenesis and the host response to viral infection *in vivo*. Glycoprotein K (gK) is not found in the envelope of mature virus particles. Glycoproteins in boxes have been altered in efforts to retarget HSV. (b) The HSV particle is composed of an icosahedral-shaped capsid containing a 152-kb double-stranded linear DNA genome. The viral nucleocapsid is surrounded by an amorphous tegument layer consisting of both viral and cellular proteins. The virus envelope contains 12 viral glycoproteins, those essential for entry (gD, gB, gH/gL) and modified for vector retargeting (gD, gB, gC, gH) are depicted.

(A) HSV Attachment and Membrane Fusion

(B) HSV gD Receptors

- **HVEM (Herpesvirus entry mediator, HveA, TNFRSF14, CD270)**
 - Binds to gD of HSV-1 and HSV-2 strains
 - Binds to natural ligands LIGHT, LT α , BTLA, CD160
- **Nectin-1 α (poliovirus receptor-related protein 1 α PVRL1, HveC, CD111)**
 - Binds to gD of all HSV-1, HSV-2, PRV, and BHV-1 strains
 - Binds to natural ligand afadin and PVRL3
- **Nectin-2 α (poliovirus receptor-related protein 2 α , HveB)**
 - Binds to gD of HSV-2, HSV-1 (rid1 and ANG), and PRV
- **3-OS HS (3-O-sulfated heparan sulfate)**
 - Non-protein receptor binds to HSV-1 gD only

Figure 2. HSV-1 attachment and entry into host cells and the glycoprotein D cell surface receptors

(a) Virus attachment to susceptible cells involves the movement of a virus particle along the host cell membrane surface until gC and gB encounter and bind to heparan sulfate proteoglycan (HSPG) moieties present on various glycosylated proteins on the cell surface that brings the virus into close proximity with the cell membrane in order for the virus to initiate binding to specific entry receptors. Diffusion of the virus along the cell surface results in contact of gD with either the TNF receptor superfamily member HVEM (HveA, TNFRSF14, CD270), the immunoglobulin superfamily member nectin-1 (PVRL1, HveC, CD111 receptor), or 3-O sulfated heparan sulfate. At this point gB can bind to PILR α while the gH/gL heterodimer engages integrins $\alpha v \beta 3$, $\alpha v \beta 6$ or $\alpha v \beta 6$. Binding of gD to its cognate receptors leads to a conformational change in gD resulting in exposure of the C-terminal profusion or fusion-activating domain of gD. The exposed profusion domain of gD initiates fusion of the virus envelope with the cell membrane through signal transfer via gH to gB with concomitant gL release from the gH/gL heterodimer. Fusion results in release of the virus capsid into the cytoplasm of the cell. (b) Host cell receptors involved in the entry of alpha herpesviruses HSV-1, HSV-2, bovine herpes virus-1 (BHV-1), pseudorabies virus (PRV) as well as the HSV-1 rid1 and ANG mutants. HVEM, nectin-1 and -2 are present on

almost all cell types of the human body as well as on most vertebrate cells except those of the testes and ovaries. The alpha herpesviruses that bind these receptors are indicated, as well as the natural ligands for these receptors.

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(A) Other Viral Glycoproteins

(B) Peptide Ligand

(C) Single-chain Antibody

(D) Soluble Adapter

Figure 3. Strategies employed for HSV vector retargeting

(a) Introduction of glycoproteins from other viruses in place of HSV glycoproteins. The G-glycoprotein from Vesicular stomatitis virus (VSV-G) has been used to pseudotype various viruses and has been introduced into genomic and amplicon-based vectors lacking either gD or gB. VSV-G retargeted viruses could be neutralized by antibodies to VSV-G or VSV. (b) Peptide ligands for naturally occurring receptors have been introduced into HSV gD or gC, in most instances replacing canonical receptor-binding sequences of those glycoproteins. (c) Similar to the introduction of ligands, sequences encoding single-chain antibodies (scFvs)

have been introduced into the HSV gD and recently gH genes to achieve retargeting. Introduction of either peptide ligands or scFvs into HSV gD with a combination of specific deletions and/or mutations have enabled complete virus retargeting producing entry of cells solely via the novel receptor target. **(d)** Soluble adapter molecules have also been employed and are composed of a dual interactive component, one capable of binding to HSV gD and the other to the cell surface receptor target. Virus must be mixed with the adapter prior to infection in order to attain entry via the specific target receptor.

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Table 1

HSV Retargeting Studies

(A) Other Virus Glycoproteins							
Virus	gD	gB	gC	gH/L	Ligand-Location	References	
Us3-8	HveA ⁻ /HveC ⁻ *	Wt	Wt	Wt	VSV-G (gD)	[48]	
Amplicon VSV-G	Wt	gB	Wt	Wt	VSV-G (gB)	[49]	

(B) Peptide Ligands							
Virus	gD	gB	gC	gH/L	Ligand-Location	References	
KgBpK ⁻ -gC-EPO	Wt	HS ⁻	HS ⁻ *	Wt	EPO (gC)	[14]	
KgBpK ⁻ -gC-preS1	Wt	HS ⁻	HS ⁻ *	Wt	HBV- sAg (gC)	[65]	
R5111	HveA ⁺ /HveC ⁺ *	HS ⁻	HS ⁻ *	Wt	IL--13 (gD)	[8,53,54]	
R5141	HveA ⁻ /HveC ⁻ *	HS ⁻	HS ⁻ *	Wt	IL--13 (gD)	[8]	
R5181	HveA ⁺ /HveC ⁺ *	HS ⁻	HS ⁻ *	Wt	IL--13 (gC); uPA (gD)	[53]	
Amplicon pCONGA-H	Wt	Wt	HS ⁻ *	Wt	HIS-tag (gC)	[66]	
Amplicon pCONGA-MG11	Wt	Wt	HS ⁻ *	Wt	MG11 peptide (gC)	[67]	
Amplicon gC-BDNF	Wt	HS ⁻	HS ⁻ *	Wt	pre-pro BDNF (gC)	[63,64]	
Amplicon gC-GDNF	Wt	HS ⁻	HS ⁻ *	Wt	pre-pro GDNF (gC)	[63,64]	
Amplicon NMDA NR2A/2B	Wt	HS ⁻	HS ⁻ *	Wt	NMDA NR2A/B Ab (gC)	[68,69]	

(C) Single Chain Antibodies (scFvs)							
Virus	gD	gB	gC	gH/L	Ligand-Location	References	
KGNEp	HveA ⁻ /HveC ⁻ *	N/T	Wt	Wt	scFv EpCAM (gD)	[9]	
KGNE	HveA ⁻ /HveC ⁻ *	N/T	Wt	Wt	scFv EGFR (gD)	[9,10]	
KGNC	HveA ⁻ /HveC ⁻ *	Wt	Wt	Wt	scFv CEA (gD)	[10]	
R-LM113	HveA ⁻ /HveC ⁺ *	Wt	Wt	Wt	scFv HER2 (gD)	[55,57,58,60]	
R-LM249	HveA ⁻ /HveC ⁻ *	Wt	Wt	Wt	scFv HER2 (gD)	[7,58,59]	
R-VG809	HveA ⁻ /HveC ⁻	Wt	Wt	Wt [*]	scFv HER2 (gH)	[6]	

(C) Single Chain Antibodies (scFvs)						
Virus	gD	gB	gC	gH/L	Ligand-Location	References
R-LM31	Wt *	Wt	Wt	Wt	scFv HER2 (gD)	[57]
R-LM11	HveA ⁻ /HveC ⁺ *	Wt	Wt	Wt	scFv HER2 (gD)	[56]
R-LM39	HveA ⁻ /HveC ⁺ *	Wt	Wt	Wt	scFv HER2 (gD)	[57]
HSV1716 scFv CD55	HveA ⁻ /HveC ⁻ *	Wt	Wt	Wt	scFv CD55 (gD)	[70]
Amplicon pCONGA-MR1-1	Wt	HS ⁻	HS ⁻ *	Wt	scFv EGFR MR1-1 (gC)	[67]

(D) Adapters						
Virus	gD	gB	gC	gH/L	Ligand-Location	References
HVEM:CEA Adapter	Wt	Wt	Wt	Wt	scFv CEA	[52]
Nectin1:EGFR Adapter	Wt	Wt	Wt	Wt	scFv EGFR	[51]
Nectin1 Adapter	Wt	Wt	Wt	Wt	nectin1-HveC	[50]

* denotes the presence of the retargeting molecule within the virus