- How have retrovirus pseudotypes contributed to our understanding of viral entry?
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Abstract

- Study of virus entry into host cells is important for understanding viral tropism and
- pathogenesis. Studying the entry of in vitro cultured viruses is not always practicable. Study
- of highly pathogenic viruses, viruses that do not grow in culture, and viruses that rapidly
- change phenotype in vitro can all benefit from alternative models of entry. Retrovirus
- particles can be engineered to display the envelope proteins of heterologous enveloped
- viruses. This approach, broadly termed 'pseudotyping', is an important technique for
- interrogating virus entry. In this perspective we consider how retrovirus pseudotypes have
- addressed these challenges and improved our understanding of the entry pathways of
- diverse virus species, including Ebolavirus, human immunodeficiency virus and hepatitis C
- virus.

Keywords:

- Pseudotype
- Pseudoparticle
- Entry pathway
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Executive Summary

- 20 The entry of a virion into a host cell is an essential step in viral life cycles. As such, entry is a potential target for clinical intervention. Viral entry pathways are also the target for neutralizing antibodies generated by immunization with vaccines possessing virion components.
- Enveloped viruses cause a range of diseases in humans and animals, from acute infections such as Ebola and Influenza, to chronic infections such as hepatitis B virus, hepatitis C virus and human immunodeficiency virus. The entry pathways of these enveloped viruses are complex and vary between virus species. Greater understanding of the steps of cell binding, internalisation and fusion are important for designing novel therapies for virus infections.
- 29 The ability to experimentally generate retroviruses pseudotyped with heterologous envelope proteins provides a flexible platform for investigating the entry pathways of a wide range of genetically diverse viruses. While culture models exist for some viruses that permit investigation of entry there are examples of viruses where investigating entry in isolation is beneficial.
- **Entry of highly pathogenic viruses such as Ebolavirus and Rabies virus can be studied using** retroviral pseudotypes as a surrogate entry model, without the safety concerns associated with working with full-length infectious viral genomes
- **•** In vitro culture of viruses with RNA genomes can lead to culture-associated adaptation and perturbation of the phenotype of the virus. The use of retroviral pseudotypes to study the entry pathways of these viruses can address this problem, using high-fidelity PCR to generate accurate representations of the envelope protein sequences from clinical specimens.
- Retroviral pseudotypes can be used to study the entry pathways of viruses that do not readily replicate in culture, such as hepatitis C virus.
- For those viruses that encode proteins in different overlapping open reading frames, the use of retroviral pseudotypes can facilitate specific investigation of the entry phenotype of mutants in isolation from the effects of coding changes in other virus-encoded proteins.
- 47 e Retroviral pseudotypes can also be used to identify host factors that act as restriction factors that act on the entry pathways of diverse viral species.
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Introduction

- Viruses possessing host-derived envelope membranes make up a large number of viral
- human pathogens, including members of the positive-stranded and negative stranded RNA
- viruses. The on-going challenges of global eradication of chronic virus infections such as
- human immunodeficiency virus 1 (HIV-1), hepatitis B virus (HBV) and hepatitis C virus (HCV)
- demonstrate that new therapeutic approaches are required for these infections. In addition,
- recent zoonotic outbreaks such as new influenza virus variants, Ebola virus and Zika virus
- have highlighted the need for improved surveillance and greater understanding of the
- pathogenicity of emerging viruses. Enveloped viruses important to human health are
- genetically diverse, for example members of the families *Flaviviridae*, *Retroviridae*,
- *Filoviridae*, *Arenaviridae*, *Orthomyxoviridae* and *Coronaviridae*. These virus families have
- distinct replication strategies and present unique challenges for therapy. However,
- expression of a virus-encoded trans-membrane protein that mediates the entry process is a
- common feature of all these virus species. As such, greater understanding of these distinct
- entry pathways may inform development of novel interventions for virus infections.

Entry pathways of enveloped viruses

- Understanding virus entry pathways is important for determining host range and tissue
- specificity of different virus infections. Enveloped viruses gain entry to a host cell by one of
- two main mechanisms, both of which require attachment of a virus particle to host-encoded
- receptors on the plasma membrane (reviewed in [1]). Viruses can utilise the host
- endocytosis pathway to internalise particles into endosomes, leading to pH-dependent
- fusion of the viral envelope with the host cell membrane in early endosomes (e.g. Nipah
- virus [2]) or at lower pH after endosome fusion with lysosomes (e.g. Dengue virus [3]).
- Alternatively, viral fusion and entry can take place at the cell surface in a pH-independent
- manner, with receptor engagement triggering membrane rearrangement to result in
- internalisation of the viral nucleocapsid at the plasma membrane. HIV-1 has long been cited
- as an example of pH-independent plasma membrane fusion [4, 5]. More recent analyses
- have demonstrated that HIV-1 fusion occurs in endosomes [6], although this has been
- disputed [7]. Irrespective of the mechanism of entry, virus-encoded membrane-spanning
- proteins mediate these receptor binding and membrane fusion events, resulting in
- internalisation of the capsid into the cell cytoplasm. As such, the envelope proteins are the
- main target of neutralizing antibodies, which block receptor interactions and aggregate virus
- particles. In addition, envelope proteins are often highly glycosylated, which may help
- protect the virus from host immune recognition [8]. Intimate knowledge of entry pathways
- can inform development of new therapies for virus infections, and has already led to the
- development of clinically approved drugs targeting entry of HIV-1 [9] and respiratory
- syncytial virus (RSV) [10].
- 89 It is important to note that the entry pathways of non-enveloped viruses is fundamentally
- different to that of enveloped viruses. Entry of these viruses does not involve membrane
- fusion and requires penetration of the virus particle through a host cell membrane
- (reviewed in [11]). This penetration is mediated by the virus' outer capsid antigens, which
- typically form highly ordered, symmetrical structures that contribute to virion structure. A
- good example of this is the Bluetongue virus (BTV) particle, which is formed of concentric
- layers of VP3, VP7 and VP5, with the outer-most virus protein VP2 embedded into this
- layered structure [12]. The surface proteins of enveloped and non-enveloped viruses may
- share some biochemical characteristics, but the characteristic trans-membrane domain of
- enveloped-virus surface proteins is lacking in these viruses.

 While laboratory investigation of the entry pathways of many viruses can be performed using cultured virus isolates, there are situations where this is not desirable: 1) Highly pathogenic viruses that require high level containment facilities; 2) Viruses that rapidly accumulate mutations and culture adaptations, and where investigation of accurate representations of virus populations is important; 3) Viruses that do not readily grow in culture, or where host restriction in cultured cells occurs at a post-entry step; 4) Viruses for which molecular clones are not available, or where reverse-genetics approaches are desired to manipulate the glycoproteins to investigate molecular determinants of entry pathways. This latter group includes viruses with overlapping reading frames, where mutations in the envelope proteins results in additional amino acid substitutions in other viral proteins. One possible approach to overcome the limitations of virus culture is the use of chimeric viruses. Because the envelope proteins are necessary and sufficient for entry, it is possible to study entry pathways using experimental models that reconstitute only the viral envelope protein binding to cell surfaces. This has led to the development of a range of experimental models with which to study viral tropism and entry, including infection with virus-like particles [13, 14], infectious pseudotypes [15-17], protein binding assays [18, 19] and liposome fusion assays [20, 21]. These models also facilitate assessment of inhibitors that specifically target

- viral entry pathways in isolation from other steps of the replication cycle [22, 23].
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Retrovirus-based pseudotypes

 Retroviruses are excellent platforms for creating chimeric virus particles with which to investigate viral entry. In contrast to many viruses, retroviruses are able to incorporate foreign proteins into their envelope membrane, including host-derived proteins and envelope proteins of other viruses [24-29]. This promiscuous protein incorporation naturally results in phenotype mixing in cells infected with different species of retrovirus [30, 31]. The wide range of viral surface proteins that have been successfully incorporated into retroviruses has recently been reviewed [32]. Following infection retroviruses deliver and integrate a DNA copy of their RNA genome into the host genome of an infected cell during replication. This facilitates introduction of reporter genes into retroviral genomes along with strong promoters, resulting in expression of the integrated reporter genes. This permits design of rapid, sensitive quantitative infection assays that can be easily re-purposed for studying the entry of a range of enveloped viruses (Figure 1). It should be noted that while we consider only retrovirus pseudotypes in this Perspective, heterologous viral glycoproteins have been successfully pseudotyped onto vesicular stomatitis virus (VSV) particles [33]. VSV pseudotypes, along with approaches using enveloped virus-like particles (VLPs) [34] have also made a significant contribution to our understanding of viral entry pathways. These experimental models are restricted to studying the surface proteins of

 enveloped viruses, as the surface capsid proteins of nonenveloped viruses cannot be readily incorporated into the chimeric retrovirus particles as they lack a trans-membrane domain. Retroviral pseudotyping provides an experimental model to investigate the early stages of entry of enveloped viruses without introducing sequence adaptations associated with *in vitro* culture and passage of viruses. In addition, this experimental system provides a robust model with which to rapidly investigate the phenotype of envelope proteins representing diverse viral variants. It is important to distinguish between pseudotypes generated with the two main groups of retrovirus packaging constructs: lentiviruses and gammaretroviruses. Pseudotypes based on lentiviruses such HIV-1 and simian immunodeficiency virus (SIV) are able to infect and integrate into terminally-differentiated cells. In contrast, those based on gammaretroviruses such as murine leukaemia virus (MLV) and gibbon ape leukaemia virus (GALV) generally require nuclear membrane degradation during mitosis to deliver and integrate their genome into that of the host cell, limiting investigations of virus entry pathways to actively dividing cells [35]. However, pseudotypes of the gammaretrovirus Friend MLV (F-MLV) may transduce non-dividing cells under certain conditions [36]. Despite these differences both HIV-1 and MLV have been popular choices for pseudotype virus production, with optimised protocols for both species [37, 38].

 Retroviral pseudotyping was originally applied to investigations into retrovirus entry using surface proteins encoded by the *env* gene (surface glycoprotein; SU and transmembrane protein; TM) [25, 27]. These retroviruses were also found to incorporate heterologous viral surface proteins [28, 39], permitting analysis of a wide range of variants [24]. Glycoproteins recovered from a range of retrovirus species have been pseudotyped onto heterotypic retrovirus backbones, including gammaretroviruses (ecotropic and amphotropic MLV and GALV), and lentiviruses. These studies revealed that different retrovirus species can possess pH-dependent or pH-independent entry pathways [40, 41], suggesting that different retroviruses use distinct entry pathways.

 The methodological approaches for the generating retroviral pseudotypes for studies of virus entry have been described and reviewed many times [42-44]. Here we discuss how retrovirus pseudotypes have contributed to the understanding of receptor usage, the cell

 biology of viral entry, and the consequences of genetic diversity on envelope protein function.

Application of retroviral pseudotypes to investigating entry of highly pathogenic viruses

- Filovirus infections, including Ebolavirus, are a major cause of haemorrhagic fevers and are
- increasingly studied after the unprecedented 2013-16 outbreak in West Africa. The entry
- pathways of haemorrhagic filoviruses are important targets for intervention, with a cocktail
- of therapeutic antibodies (Zmapp) being successfully used to protect against infection [45].
- While Ebolavirus (EBOV) and Marburg virus (MARV) have demonstrated the ability to rapidly
- emerge in resource-limited settings, other members of this family result in lower
- pathogenicity infections in humans. Their highly pathogenic nature makes investigations of
- Ebolaviruses restricted to containment level 4 laboratories. As such, retroviral pseudotypes
- 176 provide a safe, flexible platform for investigating the entry pathways of these viruses.
- 177 Filoviruses express two glycoproteins, GP_1 and GP_2 , cleaved from a single precursor [46],

 which mediate receptor binding and fusion, respectively. Retroviruses pseudotyped with the EBOV/MARV glycoproteins have been used to investigate the entry pathway in great detail 180 [15, 16]. The folate receptor α was initially proposed as a co-factor in the entry pathways of both viruses [47], but this has subsequently been queried [48]. T cell immunoglobulin and mucin domain 1 (TIM-1) was more recently identified as an entry factor for both viruses by assessing EBOV pseudotype entry in a panel of transformed cell lines with defined gene expression [49]. A similar approach identified members of the Tyro3 transmembrane tyrosine kinases (Axl, Dtk and Mer) as entry cofactors [50]. Differences in entry of EBOV and MARV were also revealed using pseudotypes. MARV pseudotype entry was resistant to glycosylation inhibitors in target cells, in contrast to the tunicamycin- and Endoglycosidase H-sensitive entry of EBOV [51]. This highlighted differences in cellular receptors for these two related viruses. Retrovirus pseudotypes have recently been applied to identifying the Niemann-Pick C1 (NPC1) protein as the major GP-binding receptor for Ebolavirus [52, 53]. They have also been used to identify molecular determinants of receptor tropism in different strains [54]. Together, these studies demonstrate that retrovirus pseudotypes are a powerful tool for investigating the receptor-mediated entry pathway of a range of filoviruses, which would otherwise require high-containment laboratories. These models have accelerated Ebolavirus research and recently assisted with defining the mode of action of antiviral antibodies that target the entry pathway [55].

- Rhabdovirus glycoproteins, including those of rabies virus, have been readily pseudotyped onto retrovirus backbones, including infectious equine anaemia virus and HIV-1 [56, 57]. These pseudotypes have been used to investigate the neuronal transport of virions mediated by the rabies virus glycoprotein [58]. Retrovirus pseudotypes of rabies, VSV and mokola viruses (as well as EBOV and lymphocytic choriomeningitis virus – LCMV) enabled investigation of neuronal cell tropism *in vivo*. Injection of these pseudotypes into the brains of mice facilitated post-mortem identification of permissive cell types by staining for the beta-galactosidase reporter enzyme packaged by the pseudotypes [59]. A similar technique has been used for skin cell tropism of VSV [60]. Furthermore, lentivirus pseudotyping of chimeric glycoproteins comprising domains of rabies G and VSV G enabled phenotyping of fusion glycoproteins possessing unique entry characteristics and neuronal cell tropism for use in retrograde transduction systems [61].
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Applications with highly diverse virus populations

An important use of retrovirus pseudotypes is accurate assessment of the phenotypes of

- envelope proteins recovered from viruses that undergo rapid mutation and selection *in vivo*.
- Passaging these viruses in cell culture can often result in rapid adaptation to culture, as the
- absence of the constraining environment of the natural host cell and immune responses
- 215 alters the selection pressures acting on a viral quasispecies. Isolating viral envelope protein
- gene sequences with high-fidelity approaches allows accurate assessment of the properties
- of circulating variants with pseudotypes [62, 63]. Studies of HIV-1 entry provide a good
- example of this approach. The error-prone nature of the virus-encoded reverse
- transcriptase facilitates rapid adaptation in tissue culture with mutations in the envelope
- glycoprotein gene *env* resulting in phenotypic changes [64, 65]. This hindered research into
- HIV entry, as receptor usage of laboratory-adapted, cultured strains was inconsistent with
- those naturally infecting human hosts. Retroviruses pseudotyped with HIV-1 envelope
- glycoproteins were used to identify host receptors as determinants of entry. HIV variants
- have differential usage of CD4 and the chemokine receptors CXCR4 and CCR5 [66-68], and it
- was recently demonstrated that CD4 usage by HIV-1 pseudotypes is linked to breadth of
- tropism [69].
- HIV-1 constructs pseudotyped with envelope glycoproteins that mediate pH-independent
- entry were also demonstrated to be enhanced by the action of the HIV-1 nef protein,
- revealing a role for nef in entry [70]. The entry properties of genetically diverse HIV-1 *env*
- clones were assessed using pseudotyped virus infection of peripheral blood mononuclear
- cells (PBMCs) [71]. The development of rapid HIV-1 pseudotype screening assays [72] has
- more recently facilitated assessment of entry of genetically diverse strains of HIV-1 for
- studies of entry inhibition [73].

Applications to viruses that do not grow in culture

 Study of the hepatitis C virus (HCV) entry pathway presents additional challenges to that of HIV-1, as HCV is not readily propagated *in vitro*. Only one strain of HCV (JFH-1) robustly replicates in tissue culture without the requirement for culture adaptations. Laboratory- generated chimeric viruses based on JFH-1 rapidly accumulate *in vitro* adaptations [74]. The error-prone replication of HCV coupled with the persistent, chronic nature of infection results in extreme levels of diversity between isolates identified in different host 241 backgrounds [75]. The diversity observed between different HCV genotypes far exceeds that observed for other chronic infections, such as HIV-1 [76]. HCV encodes two envelope proteins, E1 and E2, which are necessary and sufficient for mediating entry of the virus. However, these genes are able to tolerate extreme genetic diversity (reviewed in [75]), and are common sites of *in vitro* culture adaptations. Nucleotide sequence variation approaches 40% between individual viruses. Early studies of the entry pathway of HCV were severely limited by the lack of robust cell culture models in transformed hepatoma cultures. The introduction of retroviruses pseudotyped with the HCV E1 and E2 proteins (hepatitis C virus pseudoparticles; HCVpp) facilitated rapid developments in our understanding of the complex receptor cascade required for HCV entry (see Figure 1) [77, 78]. These studies initially identified CD81 and SR-BI as key receptors for entry [79], and later identified tight 252 junction proteins including claudins [80, 81] and occludin [82] as key entry factors that play a role in the entry cascade. HCVpp were also used to identify the sequential order of events in the entry cascade [83-85]. E2 is the major receptor binding protein, while E1 plays a role in chaperoning the folding of the E2 protein and contributes to envelope membrane fusion [86]. HCVpp were used to map the interactions between these glycoproteins and receptors to the amino acid level by studies using panels of individual point-mutation variants in controlled genetic backgrounds [87-90]. The fusion mechanism for HCV is yet to be completely resolved, but studies have located elements in both the E1 and E2 proteins that contribute to fusion [86, 91]. More recently, mutations that affect the pH-dependent nature

261 of HCV entry have been tested using pseudotypes, implicating the hypervariable 1 (HVR-1) in pH-dependent fusion [92].

 HCV tissue tropism has been investigated with HCVpp. These particles have been found to enter neuroblastoma cell lines [93] and neuroepithelioma cells [94]. Pseudotypes were also used to analyse the contribution of kinases in the HCV entry pathway [95], the process of endocytosis in the entry cascade [96] and the importance of cell polarity in HCV receptor association [97]. The discovery that human serum enhances infection of HCVpp into hepatocytes led to investigations of lipoprotein-mediated HCV infection. High-density lipoprotein (HDL) was discovered to enhance infection by accelerating endocytosis [98], through interactions with the SR-BI receptor [99] and the HVR-1 region of the HCV E2 glycoprotein [100].

- 272 The use of HCVpp to interrogate the properties of genetically diverse viral isolates has
- revealed that while all genotypes of HCV use conserved entry pathways [101], entry
- phenotypes vary considerably between isolates [102, 103]. Closely related members of an
- individual quasispecies can have significantly different entry efficiencies into host cells [38,
- 276 104]. These entry assays revealed different phenotypes of variants that are preferentially
- transmitted between hosts [63]. By comparing E1/E2 nucleotide sequences of genetically
- diverse functional HCVpp, discontinuous conserved receptor binding regions were proposed
- [87]. Interrogation of these regions with point mutations identified critical residues involved
- in the interaction with CD81. The success of this approach was revealed when core crystal structures of the E2 protein became available [105, 106], confirming the discontinuous
- nature of the conserved amino acids constituting the CD81 binding site.

Application to viruses with overlapping reading frames.

 The hepatitis B virus (HBV) life cycle has been studied in detail. However, the entry cascade has only recently been characterised at a molecular level [107]. HBV possesses a variable genome, with 8-10% difference observed on the nucleotide level between strains. HBV is categorised into at least 8 distinct genetic types, which differ in geographic distribution and pathogenicity [108]. The HBV envelope protein (HBV surface antigen - HBsAg) is expressed in three forms, with a common C-terminus but three separate initiation sites that encode the large (L), medium (M) and small (S) forms of the protein. The major sites of variability are in the surface antigen, particularly the antigenic region present in an external loop near the C-terminus. This provides the first challenge to investigating HBV entry, as culture models for rapidly assessing the phenotype of naturally occurring variants are not available [109]. In addition, the HBV genome possesses overlapping coding regions in all three translation frames. Mutations in the surface antigen can simultaneously introduce important mutations in the viral polymerase. As such, it is difficult to individually phenotype the properties of HBsAg variation in cell culture models. Retrovirus pseudotypes facilitated investigation of the HBV entry pathway [110, 111]. Initial studies identified that HBV infected primary human hepatocytes, but not hepatoma cell lines [110, 111], and illustrated the advantages of using lentivirus constructs for studies in terminally differentiated primary cells [110]. These studies also investigated the contributions of the large and small forms of the HBsAg in entry. With the discovery of the sodium taurocholate co-transporting

- polypeptide (NTCP) as a major entry factor [112] the models of HBV entry have rapidly
- advanced. Retroviruses pseudotyped with the HBsAg have recently been used to interrogate
- the steps in NTCP-mediated HBV entry [17], mapping NTCP binding sites to the N-terminus
- of the large surface antigen.

Application to identifying restriction factors

 Retrovirus pseudotypes have been extensively employed for serology screening and analysis of neutralising antibodies [32]. They have also been important for the investigation of host cell restriction factors, such as interferon-induced transmembrane proteins (IFITMs). IFITM proteins were originally identified during siRNA screening for inhibitors of highly-pathogenic avian influenza A virus (IAV) replication. However, IFITMs have also been shown to restrict a range of viruses at a replication-independent stage using retrovirus pseudotypes [113-115]. Transduction efficiency of pseudotypes bearing the glycoproteins of filo-, corona-, flavi-, rhabdo- or orthomyxo-viruses were shown to be variously inhibited at an entry or membrane-fusion stage by different IFITM proteins. Upregulation of homologous or orthologous IFITM isoforms using lentivirus transduction demonstrated the likelihood of a shared entry pathway or feature by these enveloped RNA viruses. Retrovirus pseudotyping is uniquely suited allow scrutiny of virus entry in isolation from replication enabling the rapid and direct comparative analysis of how this restriction mechanism effects different

viruses.

Limitations to using retroviral pseudotypes.

 While retroviral pseudotypes have proven to be a valuable tool to interrogate the entry pathways of a wide range of virus species, there are potential problems that must be taken into consideration when using this experimental model. The glycoproteins of some virus species are refractive to incorporation into functional retrovirus pseudotypes, leading to undetectable infection, even when using sensitive reporter assays (unpublished data). Even where viral envelope proteins can be successfully pseudotyped, single amino acid substitutions in the viral glycoprotein can have an impact on the conditions required to generate infectious particles [38]. Attempts to pseudotype flaviviruses such as West Nile virus (WNV) and Zika virus have proven difficult, despite related viruses such as HCV being amenable to pseudotype manufacture. This may be linked to the structural role of the envelope glycoproteins in virus species of the genera such as Flavivirus, while the glycoproteins of hepaciviruses such as HCV do not appear to play such a structural role in virions [116].

 Envelope glycoproteins can display different phenotypes when present on a retrovirus envelope rather than their natural virion. The neutralization phenotype of HIV-1 strains differs between primary virus isolates and their pseudotyped equivalents [73]. Point mutations in the HCV E1/E2 genes do not always result in the same phenotype when pseudotypes are compared to cell-cultured virus [90, 117]. Also, due to the nature of the producer cell lines, some post-translational modifications might not be accurately modelled by pseudotypes. An example of this is the complex of apolipoproteins that form when HCV is assembled in hepatocytes. Without producing the pseudotyped viruses in hepatocytes

- these essential modifications, particularly incorporation of Apolipoprotein E, do not occur
- [118, 119]. This could generate misleading data, especially if these virion components
- impact on the entry pathway of the virus.

 When preparing pseudotype entry assays for analysing virus entry pathways, it is important to optimise the assay for the envelope protein being tested (reviewed in [44]). Variables such as the type and source of producer cells, the amount and type of plasmids required to generate infectious particles, and the reporter gene all influence the outcome of infection experiments. Of particular importance is the selection of packaging plasmids. Differences in the assembly of murine leukemia virus and human immunodeficiency virus appear to influence incorporation of heterologous viral envelope proteins, and can determine if infection is successful [38]. The selection of appropriate target cells expressing necessary virus entry molecules also influences the ability to assay infection [78]. Together these considerations can impose practical limitations on assay setup, as glycoproteins from different virus species can behave very differently in these assays. Protocols for generation of particles can impact on the function of the expressed particles, with small changes being

- sufficient to alter function of a pseudotyped envelope protein [38].
- Finally, there have been concerns about potential contamination of cell lines used for these
- experiments with ecotropic retroviruses, which may affect results of infectivity assays with
- pseudotyped retroviruses. However, at least for HIV-1 infection assays, it has been
- demonstrated that these contaminants do not affect the results of infection assays [120].

Future applications for retroviral pseudotypes

 Pseudotypes have proven to be a robust experimental system to investigate the entry pathways of a wide range of genetically diverse virus species. The current interest in the potential for emerging (and re-emerging) viral pathogens means that retroviral pseudotypes could be deployed rapidly to identify receptor usage and tropism of newly-discovered pathogens. In addition, there is still plenty to learn about the entry pathways of established viral pathogens such as HCV and HBV. Retroviral pseudotypes will continue to be an

- essential experimental model for these studies.
- As viral entry has been highlighted as a potential target for clinical intervention in a wide
- variety of virus infections, the development of broadly-active inhibitors using these entry
- models may contribute to preparedness for viral epidemics. Retroviral pseudotypes
- facilitate high-throughput entry inhibitor screening without the requirement to understand
- the complexities of entry of a specific virus species. Characterising the interactions between
- entry inhibitors (such as neutralizing antibodies) and viral envelope proteins will also
- provide useful tools for investigating the molecular biology of virus entry.
- Retroviral pseudotypes have contributed to our knowledge of the entry pathways of a wide
- range of viruses. In addition to the examples given here, there are many studies of virus
- entry that utilise this model, including those of coronaviruses and Influenza viruses. While this Perspective article cannot hope to exhaustively cover each application, we hope that
- the examples provided here illustrate the versatility of pseudotypes as models of virus
- entry.

Figures

Figure 1. Generation of retroviruses pseudotyped with heterologous viral envelope

 proteins. A) In second-generation pseudotype models, retroviral pseudotypes are produced using genes encoded on three separate plasmids; a packaging vector possessing the entire retroviral gag/pol open reading frame of a specific retrovirus (usually HIV-1 or MLV; blue), a reporter vector possessing a reporter gene (usually either luciferase, green fluorescent protein, or β-galactosidase; green), the 3' and 5' LTRs of a retrovirus matched to the 393 packaging vector, along with a strong promoter and a retrovirus packaging signal (ψ) ; and a plasmid encoding the glycoprotein(s) of a heterologous virus of interest (yellow/orange). When transfected together into a suitable producer cell line (such as the human embryonic kidney cell line 293T), protein over-expression is driven by the strong CMV immediate-early promoter upstream of each gene. Retroviral particles are produced possessing the desired viral glycoprotein, and are released from the transfected cells into the surrounding media. **B)** In this way the envelope protein-encoding plasmid can be exchanged to produce particles mimicking different virus species.

 Figure 2. Retrovirus pseudotypes can be used to reveal many aspects of a virus entry cascade. The entry pathway of hepatitis C virus provides an excellent example of how retroviral pseudotypes can be used to dissect the complex series of events that result in internalisation of a virus into a host cell. **1.** Studies utilising retrovirus pseudotypes were used to identify essential molecular interactions between viral proteins and host cell receptors. CD81 and SR-B1 were identified as key receptors that initiate early events in the entry cascade [79]. **2.** The dynamic nature of interactions between receptor complexes can also be investigated. For HCV, tight junction proteins were identified as co-factors for entry, interacting dynamically with CD81 to traffic virus particles from the cell's apical surface to tight junctions [80, 81, 85]. **3.** Species-specific receptors can be identified. Another tight junction protein, occludin, was found to be species-specific requirement for HCV entry [82]. **4.** Pseudotypes can reveal the sequence of events that result in entry of the virus genome into the cell. HCV binding and entry involves sequential recruitment of host cell co-factors [83]. **5.** Host factors other than viral receptors can contribute to virus entry. For HCV, interaction between high density lipoprotein (HDL) and the SR-B1 receptor enhances infectivity [98-100]. **6.** Specific events leading to fusion of virus envelope and host membrane can be interrogated. pH-dependent membrane fusion was found to be mediated by specific regions of the HCV glycoproteins using retroviral pseudotype models [91]. **7.** In addition, specific conserved amino acids were found to be involved in E1-E2 interactions and receptor binding events [87, 88].

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Figure 1.

Figure 2.

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- This Perspective describes the principles of pseudotype generation on retrovirus backbones,
- discusses the merits of different systems and provides examples of the known methods for
- opotimising retrovirus PV production and infection assays