No false start for novel pseudotyped vectors
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Pseudotyped vectors can be used to introduce genes into cells or to study the entry process of the virus from which the outer shell of the recombinant virus is derived. Recently, several novel pseudotyped retroviruses and lentiviruses have been constructed. Virus vectors pseudotyped with an alphavirus glycoprotein hold special promise. The increasing diversity of the available pseudotyped vectors offers expanded opportunities for gene transfer to specific cells.

Abbreviations
AAV    adeno-associated virus
ASLV-A avian sarcoma and leukemia virus subgroup A
FIV    feline immunodeficiency virus
HBV    hepatitis B virus
HIV-1 human immunodeficiency virus type 1
HSV-1  herpes simplex virus type 1
LCMV   lymphocytic choriomeningitis virus
Mo-MuLV Moloney murine leukemia virus
RRV    Ross River virus
VSV-G  vesicular stomatitis virus G protein

Introduction
Pseudotyping, in its original sense, means that one or more of the structural proteins of a virus particle are not encoded by the nucleic acid carried by the virus. Using this broad definition, pseudotyped viruses include any recombinant viral gene transduction system that is dependent for genome packaging upon helper proteins expressed from defective genomes in the viral producer cells or upon infection by a helper virus. The common current usage in the gene-transfer field dictates that a pseudotyped virus is one in which the outer shell (the envelope glycoproteins of an enveloped virus or the capsid proteins of a nonenveloped virus) originates from a virus that differs from the source of the genome and the genome replication apparatus. In the case of enveloped viruses, the mechanism of genome packaging normally demands that the core (i.e. matrix, capsid, and/or nucleocapsid) proteins come from the same virus as the genome (Figure 1).

This review focuses mainly on pseudotyped viral vectors in which the genome and outer shell come from considerably different viruses (not pseudotypes between different murine leukemia viruses or between the various adeno-associated virus (AAV) serotypes, for example), although some description of recent results obtained with the latter type of pseudotyped viruses is presented. The potential role of pseudotype formation in the evolution of viruses is not explored here; an article on this issue with regard to insect viruses has recently been published [1*].

Why pseudotype?
Pseudotyped vectors have several experimental and clinical applications (Box 1). Firstly, the outer shell of a virus, through its interaction with cellular receptor molecules, plays a major role in determining the tropism of a virus, specifically at the entry step. Pseudotyping a viral vector can thereby provide it with an expanded ensemble of target cells or can restrict it to specific cells that are the object of experimental or therapeutic interest. Secondly, a pseudotyped vector can have an altered stability and/or interaction with the host immune system that increases its efficacy. One example is pseudotyped viral vectors that can be produced and/or concentrated to higher transduction titers than the viral vector with its native outer shell. Thirdly, comparison of transduction by a pseudotyped vector and by a vector bearing a native shell can be used to identify processes that are peculiar to entry mediated by the native proteins. Finally, the entry process of the virus from which the outer shell of the pseudotyped virus is derived can be illuminated.

The construction of novel recombinant pseudotyped retroviral vectors containing the glycoproteins of the Ross River virus (RRV; an alphavirus) illustrates several of these applications [2**]. Transduction by the RRV-glycoprotein-pseudotyped retrovirus was comparable to transduction by the commonly utilized vesicular stomatitis virus G protein (VSV-G) pseudotyped viruses. The retrovirus pseudotyped with RRV glycoprotein can transduce human and nonhuman cells from a number of tissues and can be efficiently concentrated. Stable cells producing RRV-pseudotyped retrovirus can be produced, and no pseudotransduction (transient transfer of the protein product of the gene that is carried by the vector in the absence of stable vector integration) is observed. The RRV-pseudotyped retrovirus possesses several advantages over the widely used VSV-G-pseudotyped viruses (Box 2). Transduction by the RRV-glycoprotein-pseudotyped virus could be specifically inhibited by antibodies that recognized the RRV E2 glycoprotein. Entry of the pseudotyped virus was also demonstrated to occur through acidified endosomes confirming most of the earlier data on alphaviral entry and helping to resolve a longstanding controversy.

Novel recombinant pseudotyped retroviral and lentiviral vectors bearing the glycoprotein of lymphocytic choriomeningitis virus (LCMV; an arenavirus) have some similar advantages to those of the RRV-glycoprotein-pseudotyped viruses. Pseudotyping by LCMV glycoproteins was first demonstrated through the recovery of transducing virus after LCMV infection of a Moloney murine leukemia virus (Mo-MuLV) packaging cell line [3]. The original attempts to produce pseudotypes containing LCMV glycoprotein from transfected glycoprotein cDNAs were unsuccessful,
presumably due to the lack of cell-surface expression of the LCMV glycoprotein. Cloning of a new LCMV glycoprotein cDNA from the virus yielded a construct that led to cell-surface expression of the glycoprotein [4] and pseudotype formation [5••]. A general lesson is that failure to generate a pseudotype with a particular expression construct for a glycoprotein from a given virus does not mean that pseudotype formation with glycoprotein from that virus is impossible. There might be a defect in the expression construct. In addition, glycoproteins from variant strains of a virus could have different capacities to form successful pseudotypes. One additional point concerns the confirmation of the widely reported stability advantage during concentration of some pseudotypes producing nonretroviral glycoproteins over those producing murine leukemia virus (MuLV) envelope proteins. The lability of the MuLV envelope proteins is the consequence of a thiol-disulfide exchange reaction that leads to the dissociation of its surface and transmembrane subunits [6,7]. It is possible that concentration of virus producing MuLV envelope proteins in medium at mildly acidic pH could prevent MuLV glycoprotein dissociation and the consequent reduction in transduction titers [7,8].

An important issue is that the subcellular distribution of a given glycoprotein might not be optimal for pseudotype formation. Glycoprotein expression at a particular membrane (e.g. plasma membrane, Golgi apparatus, endoplasmic reticulum) or site within a membrane (e.g. lipid rafts) might be critical for efficient pseudotype formation [9]. In the case of the RRV and LCMV glycoproteins, cell-surface expression was demonstrated (through acidic pH-dependent syncytia formation and flow cytometry, respectively). Conversely hepatitis B virus (HBV) assembles at post-endoplasmic reticulum/pre-Golgi membranes; however, cell-surface expression of the HBV large antigen can be obtained. Retroviral pseudotypes bearing the HBV surface antigens, which transduced solely human primary hepatocytes, could be produced, although with fairly low transduction titers [10]. It was demonstrated that the HBV large surface antigen was sufficient for the production of transducing viruses, but that the small surface antigen increased large surface antigen expression at the cell membrane and transduction titers.

A recent study of MuLV pseudotyped with envelope proteins of visna virus (a lentivirus) confirms the previously
recognized efficacy of removing bulky cytoplasmic domains of glycoproteins for successful pseudotype formation [11]. Conversely, lentivirus vectors pseudotyped with C-terminal truncated envelope proteins from avian sarcoma and leukemia virus subgroup A (ASLV-A; a retrovirus) have been reported [12], which make possible the efficient transduction of nondividing cells expressing a transgenic ASLV-A receptor (pseudotyped retroviruses generally transduce only dividing cells, whereas pseudotyped lentiviruses, such as the human immunodeficiency virus type 1 [HIV-1], are capable of transducing nondividing cells). An article on the construction of Mo-MuLV vectors pseudotyped with spleen necrosis virus envelope proteins with corrected glycoprotein cytoplasmic domain sequences has been published [13].

The list of enveloped viruses that can be pseudotyped is not restricted to the retroviruses. A vesicular stomatitis pseudotype bearing hepatitis C virus envelope glycoproteins may have utility for the study of hepatitis C virus entry [14], although this conclusion has been challenged [15]. Herpes simplex virus type 1 (HSV-1) vectors producing VSV-G and lacking the otherwise essential HSV-1 glycoprotein gB were capable of gene transfer, although at reduced transduction titers [16]. These examples demonstrate that there are many opportunities for moving beyond the limited set of pseudotyped viruses previously available.

### Tropism determination
Numerous recent publications address cellular transduction by pseudotyped lentiviruses in vivo. One example is the transduction of cynomolgus monkey retinal cells by VSV-G-pseudotyped feline immunodeficiency virus (FIV) [17]. Another study demonstrated that equine infectious anemia virus pseudotyped with the rabies G protein underwent retrograde axonal transport to distal neurons, whereas virus vectors with the VSV-G protein did not [18]. Recently it has been shown that when RRV-glycoprotein-pseudotyped FIV is injected intravenously into mice, this pseudotyped vector transduces mouse hepatocytes in vivo far more efficiently than VSV-G-pseudotyped FIV (Figure 2) and with no detectable liver toxicity [19]. By contrast, injection of the VSV-G-pseudotyped FIV resulted in a substantial increase in liver enzyme release into the serum. The effectiveness of the RRV-glycoprotein-pseudotyped FIV may result from enhanced stability in the bloodstream. In other experiments it was found that RRV-glycoprotein-pseudotyped FIV preferentially transduced neuroglial cells after injection into the brain, whereas VSV-G-pseudotyped FIV targeted predominantly neurons.

Pseudotyping of recombinant genomes from one AAV serotype with the capsids of other AAV serotypes has gained popularity recently. In one example, the dependence of entry upon heparan-sulfate-bearing cellular proteoglycans as well as transduction of several tissues by the various pseudotypes was compared [20,21].

### Engineering targeted-pseudotyped viruses
Targeting of specific cells through the introduction of ligands onto the shells of viral vectors that thereby bind to proteins expressed on particular cells has been a long-time goal of gene transfer researchers. Unfortunately, this field is a microcosm of the field of gene therapy in which early high-profile studies of questionable validity [22] convinced some people that the problem had been solved and discredited the discipline in the minds of others. Most of the careful studies utilizing modified enveloped viruses have not met with success, although in some systems alterations in tropism have been achieved. It appears that very

### Box 1
**Advantages of pseudotyped viruses for the study of viral entry.**

1. Study of the pseudotyped vector can have safety advantages.
2. The pseudotyped virus can be experimentally manipulated with greater ease. Mutations in genes in the outer shell can be introduced and tested more readily in the pseudotyped virus. The entry process of the pseudotyped virus might be more easily quantified.
3. Identification of cellular receptors or attachment factors for the virus is facilitated. For the purpose of this review the distinction is that attachment factors allow for accumulation of the virus on the cell surface so that they can bind to the receptors that actually facilitate viral entry.
4. Examination of the function of the shell proteins in a foreign context allows the experimenter to isolate the entry process from the other steps in viral replication.
5. Chemical and immunological reagents that specifically inhibit entry can be screened.

### Box 2
**Shortcomings of VSV-G-pseudotyped retroviruses and lentiviruses.**

1. Cells constitutively expressing VSV-G protein cannot be constructed [35,36]. Cells with inducible VSV-G expression frequently have detectable levels of protein produced, even when expression is not induced, and there appears to be selection for lower levels of production.
   - With passage of the cells.
2. Pseudotransduction (i.e., the transient transfer of the protein product of the gene that is carried by the pseudotyped virus in the absence of stable integration of the vector) by concentrated vector preparations has been observed [37,38].
3. VSV-G-pseudotyped viruses are neutralized by human serum [39].
4. Toxicity of the viral preparations to primary cells and in vivo has often been observed, although it is not commonly reported in the literature.
few of these approaches have been applied successfully to target gene transfer in vivo [23•], although some success with the targeting of modified naked viruses has been achieved [24]. Sometimes the best results are achieved through ‘negative’ or ‘inverse’ targeting where tropism for a given tissue is achieved by reducing the transduction of a competing tissue in the organism [25]. Recent publications appear to offer direction for future efforts. Pseudotyped retrovirus bearing Mo-MuLV envelope proteins into which small peptide ligands for human cell-surface proteins had been inserted were capable of transducing human cells, albeit with low efficiency, whereas those bearing the wild-type Mo-MuLV envelope proteins were not capable of transducing human cells [26•,27]. The issue still remains whether the entry into human cells is due to the usage of the cell-surface protein as a novel receptor or merely as an attachment factor (the distinction is described in Box 1). In the latter case it could be that interaction with the human cell-surface protein facilitates binding to the human Mo-MuLV receptor homologue, which normally has low affinity for the Mo-MuLV envelope proteins.

A different approach relies on the modular nature of the alphavirus glycoprotein complex, which consists of a trimer of a heterodimer of two polypeptides (E2 and E1), each of which possesses its own membrane-spanning domain. E2 is responsible for receptor binding, whereas E1 promotes membrane fusion. It had previously been shown that the tropism of Sindbis virus (an alphavirus) could be altered by inserting the IgG-binding domains of protein A (a cell wall protein of Staphylococcus aureus that binds the Fc portion of IgG) into the E2 glycoprotein and incubating the virus with antibodies against a cell-surface protein [28]. Infection could be targeted to cells expressing the antigen recognized by the antibodies. It was found that this redirection of alphaviral entry could be combined with the favorable properties of pseudotyped virus vectors containing alphavirus (RRV) glycoprotein (noted above). High-efficiency transduction by viruses bearing the modified Sindbis glycoproteins (expressed from genes constructed anew — presumably because of the lack of availability of the original published modified Sindbis genes, an example of another phenomenon that plagues the field) in the presence of specific antibodies could be targeted to cell-surface antigen-expressing cells [29•]. Pseudotyped viruses producing modified alphaviral glycoproteins could prove to be the vectors of choice for targeted gene delivery.

Receptor identification
Pseudotyped retroviruses producing a viral glycoprotein and carrying a selectable marker or drug resistance gene can be used to identify cellular receptors. One requirement is a cell line that is not susceptible to entry by the pseudotyped virus. For example, if hamster cells are not susceptible to entry, then panels of radiation hybrids between hamster cells and cells from a species that is susceptible to entry (the hybrids contain stably inherited fragments of the DNA from that species) can be screened for those that can be transduced by the pseudotyped virus. This allows functional mapping and eventually cloning of the receptor genes. Alternatively, cDNA libraries from susceptible cells can be transfected into nonsusceptible cells; those now containing the receptor genes can be transduced by the pseudotyped virus and selected. The Jaagsiekte sheep retrovirus receptor was identified by the radiation hybrid technique [30,31••]. The expression screening approach was used to identify a protein involved in the cellular entry of Marburg and Ebola viruses, although the exact role of this protein is unclear [32] (see also Update). It is likely that pseudotyped retroviruses will prove to be the probes of choice for examination of factors required for entry by a large variety of enveloped viruses.

Investigation of the cell biology of viral entry
Pseudotyped viruses offer several advantages for the study of the process of entry of the virus from which the outer
shell is derived (Box 1). As noted above, viruses pseudotyped with RRV glycoproteins were instrumental in confirming the low-pH dependence of alphaviral entry. HIV-1 particles produced in cells expressing the Nef protein are more infectious than those produced in cells lacking Nef [33]. It has been shown that transduction by HIV-1 pseudotyped with the glycoproteins of two viruses (VSV and Ebola virus) that enter cells in a low pH-dependent manner are not affected by Nef expression in the producer cell, whereas transduction by HIV-1 pseudotyped with the amphotropic MuLV envelope protein, which is capable of promoting membrane fusion at the cell surface at neutral pH, is increased when the virus is produced in cells expressing Nef [34]. Although it is clear that the path of ingress of the pseudotyped virus affects the dependence of transduction upon Nef, it cannot necessarily be concluded at this time that it is pH-dependence alone that determines Nef dependence. Pseudotyping experiments were also used to demonstrate that there is an effect of ubiquitination of capsid proteins upon transduction by AAV particles bearing either type 2 or type 5 capsids [20].

Nomenclature
Many of us are guilty of a lack of specificity in our descriptions of the components of pseudotyped viruses. The VSV-G protein that is commonly utilized is from the Indiana strain (no, this is not a plug for my current home state) and should be so indicated. Ecotropic or amphotropic MuLV is an insufficient description. There are numerous ecotropic MuLvs (e.g. Moloney, Friend, AKV); the amphotropic MuLV envelope protein is probably derived from 4070A. Investigators should give as complete a description of the viral source of the glycoproteins and cores as possible.

Conclusions
Pseudotyped viruses are more than interesting laboratory artifacts. In the past decade they have assumed their place at the forefront of gene transfer and therapy applications. Their increasing diversity seems to offer the greatest opportunities for successful transduction of specific cell types both in vitro and in vivo. They are also becoming more widely appreciated as tools for the investigation of viral entry pathways. Their integration into the laboratory and clinic offers many opportunities and challenges in the near future.

Update
Recent data obtained in experiments utilizing lentiviruses pseudotyped with the Ebola virus glycoprotein have shown that, although Jurkat cells are normally not transduced by such recombinant viruses, Jurkat cells expressing either of the lectins DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) or L-SIGN are susceptible to transduction [40]. These results bring into question the significance of the previously identified receptor in Ebola virus entry. In addition, the receptors for the human endogenous retrovirus type W (HERV-W) were identified through the use of HIV pseudotyped with the HERV-W envelope protein [41].

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest


Utilization of virus pseudotyped with modified alphaviral glycoproteins containing the IgG-binding domains of protein A for targeting cells expressing particular cell-surface antigens. The report provides support for the proposition that these glycoproteins have important advantages as framework for the outer shells of targeted retrovirus.


Identification of receptor through use of pseudotyped retrovirus. Pseudotyped virus also has potential for use in gene transduction of the lung.


