A small peptide derived from the HIV-1 gp120 glycoprotein forms positively charged fibrils that enhance transduction efficiencies of retro- and lentiviral vectors

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Maral Yolamanova
Ashgabat, Turkmenistan

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1st reviewer: Prof. Dr. Jan Münch
2nd reviewer: Prof. Dr. Barbara Spellerberg

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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<tr>
<td>β-gal</td>
<td>Beta-galactosidase</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>bp</td>
<td>Basepair</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>DRK</td>
<td>German: Deutsches Rotes Kreuz</td>
</tr>
<tr>
<td>ds</td>
<td>Double strand</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-A</td>
<td>Enhancing factor A</td>
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<td>EF-C</td>
<td>Enhancing factor C</td>
</tr>
<tr>
<td>env</td>
<td>Envelope</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<td>Fig.</td>
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<td>g</td>
<td><em>gravitational force</em></td>
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<td>g</td>
<td>Gram</td>
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<td>gag</td>
<td>Group specific antigen</td>
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<td>Gal</td>
<td>Galactosidase</td>
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<td>GALV</td>
<td>Gibbon ape Leukemia Virus</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>gp120</td>
<td>Envelope glycoprotein of HIV, 120 kDa</td>
</tr>
<tr>
<td>gp41</td>
<td>Envelope glycoprotein of HIV, 41 kDa</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td><em>i.e.</em></td>
<td><em>id est</em></td>
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<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>l</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MgCl2</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine Leukemia Virus</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
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<tr>
<td><em>nef</em></td>
<td>Negative factor</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>p24</td>
<td>Capsid protein of HIV, 24 kDa</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td><em>Potentia hydrogenii</em></td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td><em>pol</em></td>
<td>Polymerase</td>
</tr>
<tr>
<td>RD114</td>
<td>Envelope glycoprotein of Feline Leukemia Virus</td>
</tr>
<tr>
<td><em>rev</em></td>
<td>Regulator of expression of virion proteins</td>
</tr>
<tr>
<td>rhIL-6</td>
<td>Recombinant human interleukin-6</td>
</tr>
<tr>
<td>rhSCF</td>
<td>Recombinant human Stem Cell Factor</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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LIST OF ABBREVIATION

RRE  
Rev responsive element

RT  
Room temperature (21°C - 25°C)

sec  
Second(s)

SEVI  
Semen-derived Enhancer of Viral Infection

SIN  
Self-inactivating vector

S.O.C  
Super Optimal broth with Catabolite repression

ss  
Single strand

TAE  
Tris-acetate-EDTA

tat  
Transactivator of transcription

TEM  
Transmission electron microscopy

ThT  
Thioflavin T

Tris  
Tris(hydroxymethyl)aminomethane

U3  
Unique 3

U5  
Unique 5

V3  
Variable loop 3

v/v  
Volume per volume

vif  
Viral infectivity factor

vpr  
Viral protein R

vpu  
Viral protein U

VSV  
Vesicular Stomatitis Virus

w/v  
Weight per volume

YFP  
Yellow Fluorescent Protein

Amino acid code

A  Alanine  N  Asparagine
C  Cysteine  P  Proline
D  Aspartic acid  Q  Glutamine
E  Glutamic acid  R  Arginine
F  Phenylalanine  S  Serine
G  Glycine  T  Threonine
H  Histidine  U  Selenocysteine
I  Isoleucine  V  Valine
K  Lysine  W  Tryptophan
L  Leucine  Y  Tyrosine
M  Methionine
1 INTRODUCTION

Introduction of genetic material into the cells offers many prospects for basic research, e.g. for constitutive or regulated gene expression, cDNA library construction, siRNA delivery, insertional mutagenesis or generation of transgenic animals (Matrai et al., 2010; Tiscornia et al., 2002). Furthermore the opportunity to treat disease by either modifying the gene expression or correction of abnormal genes has been used in approaches for therapeutic application (Bunnell et al., 1995; McTaggart et al., 2002; Frecha et al., 2010; Matrai et al., 2010). Gene therapy holds great promise for the treatment of inherited metabolic disorders such as adenosine deaminase deficiency and cystic fibrosis as well as acquired diseases such as cancer, cardiovascular and some infectious diseases (Candotti et al., 1012; Isner, 2002; Bunnell BA and Morgan RA, 1998). Current methods for transferring therapeutic genes can be divided into two large groups: viral and non-viral methods. To the non-viral delivery systems belong naked DNA, liposomes and DNA-protein complexes (Ramamoorth M and Narvekar A, 2015). The main problems with non-viral delivery are (i) relatively low transduction efficiency; (ii) transiency of gene expression; (iii) lack of specific targeting. Therefore, the application of non-viral systems in gene therapy is low (Journal of Gene Medicine, © 2015 John Wiley and Sons Ltd). Viral vectors are the most efficient vehicles for gene transfer in vivo. The major advantages of viral vectors are high transduction efficiency and specificity of transgene delivery using the cell’s own biosynthetic machinery. Viral gene delivery can be classified into two general groups: the viral vectors which integrate into the host cell genome, such as, retroviral, lentiviral and adeno-associated viral vectors and non-integrating vectors like herpes simplex virus type 1 and adenoviral vectors. Moreover, some of viral vectors; e.g. lentiviruses, have the unique ability to efficiently replicate in non-dividing cells as they target the nucleus without the requirement of mitosis (von Schwedler et al., 1994). Nowadays viral vectors are employed in more than 70% of all clinical gene therapy trials (Journal of Gene Medicine, © 2015 John Wiley and Sons Ltd).

1.1 Retroviruses

Retroviruses are classified into three genera: the oncoretroviruses (e.g. MLV, murine leukemia virus), the lentiviruses (e.g. HIV, Human Immunodeficiency Virus), and the spumaviruses including the Human Foamy Virus (HFV) (Coffin et al., 1997; Heneine et al., 1998). Retroviruses are lipid-enveloped viruses carrying two copies of single-stranded
positive RNA, which range between 7 to 13 kb in length. The virus enters the cell through the specific interactions between viral envelope glycoproteins and the cellular receptors leading to fusion between the viral envelope and the host membrane. The viral RNA enters the cytoplasm and is reversely transcribed into double-stranded DNA. The viral DNA is permanently integrated into the host cell chromosomal DNA and called a provirus (Coffin et al., 1997; Turner and Summers, 1999).

Basic genomic features are common to all retroviruses. Two copies of the long terminal repeat (LTR) flank the ends of the viral genome (Fig. 1) and contain all of the requisite signals for gene expression: enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. Each LTR can be divided into three elements: U3, R and U5. While the promoter region with the regulatory sequences is contained in the U3 region at the 5’-extremity of the LTR, the polyadenylation signal is located in the U5 region at the 3’-extremity of the LTR. The sequence identity of the LTR is important in the process of reverse transcription, because the polymerase jumps from one end of the template to the other. A sequence named packaging signal (psi, Ψ) is found near the 5’end of the viral genome (Fig. 1) and is required for specific packaging of the viral RNA into newly forming virions (Coffin et al., 1997; Turner and Summers, 1999).

Retrovirus genomes contain three essential genes: gag (group-specific antigen), pol (polymerase), and env (envelope glycoprotein) (Fig. 1). The gag sequence encodes the three main structural proteins capsid, matrix and nucleocapsid, which are necessary for the assembly and release of virus and virus-like particles. The pol sequence encodes the enzymes protease, reverse transcriptase and integrase, which are responsible for the reverse transcription of the viral genome from RNA to DNA during the infection process and for
the integration of the proviral DNA into the host cell genome. The env sequence encodes the glycoproteins, necessary for interaction with host receptors and virus entry (Coffin et al., 1997; Turner and Summers, 1999).

Moreover, lentiviruses possesses several additional genes, i.e. tat and rev encoding regulatory proteins and vif, vpr, vpu and nef encoding accessory proteins (Turner and Summers, 1999). The Tat (transactivator of transcription) protein stimulates viral gene expression during transcription initiation and elongation (Mahlknecht et al., 2008). The Rev (regulator of expression of viral proteins) protein is required for efficient cytoplasmic accumulation of viral RNA (Malim and Emerman, 2008). The Vif (virion infectivity factor) protein counteracts a cellular restriction factor, APOBEC3G, that inhibits HIV-1 (Sheehy et al., 2002). The Vpu (viral protein unknown) degrades CD4 and promotes virus release by counteracting the cellular restriction factor tetherin (Neil et al., 2006). The Vpr (viral protein regulatory) arrests cellular proliferation in the G₂ phase of the cell cycle, promotes cellular differentiation and interacts with cellular proteins involved in DNA repair (Malim and Emerman, 2008). The Nef (negative factor) performs various activities that promote viral immune evasion and replication (Kirchhoff et al., 2004; Münch et al., 2005; Schindler et al., 2006).

1.2 Retroviral vectors

Retroviruses provide a well suited platform for the stable transfer of genetic information into target cells (McTaggart et al., 2002). The 1-st generation of retroviral vectors contains a single packaging plasmid encoding the all HIV-1 genes besides envelope protein which was encoded on a separate plasmid (Wei et al., 1981). In the 2-nd generation of retroviral vector, the HIV-derived packaging component was reduced to the gag, pol, tat and rev genes. The transfer plasmid contains the viral LTRs and Ψ packaging signal and plasmid encoded env gene were separated into additional plasmids (Naldini et al., 1996). For reasons of biosafety, the 3-d generation plasmid systems were developed (Markowitz et al., 1988). Currently used systems comprise a transfer construct, a packaging construct and an envelope construct. In the case of lentiviral vectors a fourth plasmid which codes rev gene is also necessary because it was shown that Rev facilitates export of the RNA from the nucleus. The transfer construct carrying the transgene of interest contains the LTR sequences necessary for the genomic integration of the virus into the target cells, the Ψ signal for packaging the genomic viral RNA into the viral capsid and Rev Response Element (RRE), a binding site for the Rev protein, needed for lentiviral vectors.
The gag and pol (gag/pol) genes, which are required for particle production and transduction of target cells, env gene, that encodes the envelope glycoproteins, and rev gene, are separated onto three different helper plasmids, in which the Ψ signal and LTRs are removed (Fig. 2). The tat is eliminated from the 3-d generation system through the addition of a chimeric 5′ LTR fused to a heterologous promoter on the transfer plasmid. Expression of the transgene from this promoter is no longer dependent on Tat transactivation.

Figure 2: Schematic representation of the retroviral vectors and workflow. The gene of interest is cloned in a plasmid (transfer vector) that contains the retroviral regulatory elements (LTR, long terminal repeats) as well as elements to regulate the expression of the transgene. For gammaretrovirus based packaging systems, the transfer vector is co-transfected in HEK293T cells with two additional vectors carrying gag/pol and env genes, respectively. For lentivirus based packaging systems, the transfer vector is co-transfected in HEK293T cells with three additional vectors carrying gag/pol, env and rev, respectively. In the HEK293T cells the viral particles are formed, bud from the cell surface and accumulate in the culture medium (supernatant).

These three or four plasmids are co-transfected into producer cells, usually the human embryonic kidney HEK293 cell line. The helper plasmids include all the structural proteins needed in order to package a new virus. Due to the Ψ deletion within the helper plasmid, the transcribed helper RNA does not get incorporated into the new recombinant virus. Thus, progeny virions are replicative incompetent. They are able to infect cells and promote integration of the transgenes into the cellular genome, but no viral particles can be further generated. This system avoids the possibility of homologous recombination between the three plasmids, which could give rise to replication-competent retroviruses.
The process by which DNA is transferred by a retroviral vector is termed transduction.

Furthermore, self-inactivating retroviral vectors were developed. These vectors are constructed by deleting the enhancer and/or the promoter within the U3 region of the 3’ LTR. After reverse transcription, the deletion is transferred to the 5’ LTR of the proviral DNA, resulting in an inactive provirus (Miyoshi et al., 1998; Yu et al., 1986).

1.3 Pseudotyping

One interesting property of retroviral vectors is their ability to be enveloped by different glycoproteins (GP). The incorporation of heterologous GP into retroviral particles is termed pseudotyping and is used to alter the target cell tropism or to increase the stability and infectivity of retroviral vectors (Sanders, 2002). Nowadays the following GPs are extensively used for pre-clinical and clinical studies due to their attractive cell tropism: the envelope protein of Gibbon-ape Leukemia Virus (GaLV), Feline Endogenous Virus (RD114), amphotropic Mirine Leukemia Virus (MLV) and Vesicular Stomatitis Virus (VSV-G) (Akkina et al., 1998; Neff et al., 2004; Cronin et al., 2005). The most widely used GPs for pseudotyping retroviral vectors is VSV-G GP (Burns et al., 1993), which confers a broad tropism and generates stable particles that can be purified and cryopreserved. GaLV and RD114 pseudotyping have shown excellent gene transfer efficiencies for primary T lymphocytes and human hematopoietic cells (CD34+) (Bauer et al., 1995; Bunnell et al., 1995; Uckert et al., 2000, Kelly et al., 2001). However, pseudotyping of lentiviral vectors with RD114 and GaLV GP showed reduced infectivity. To overcome this problem, chimeric GPs were developed by replacing the cytoplasmatic tail of the RD114 and GaLV envelope proteins (designated TR) with that of MLV GP to increase their incorporation into viral particles (Stitz et al., 2000; Sandrin et al., 2002; Sandrin et al., 2004). Lentiviral vectors pseudotyped with GaLV/TR and RD114/TR GP have shown high titer, stability of viral particle and resistance to inactivation in the presence of human sera.

1.4 Limitations for efficient retroviral gene transfer

Major obstacles associated with retroviral vectors include the production of low-titer viral stocks and inefficient transduction rates, particularly into specific primary human cells, such as macrophages and CD34+ hematopoietic stem cells. In the best cases, between $10^6$
to $10^7$ infective viral particles per mL of cell culture supernatant are produced by commonly used producer systems (Pear et al., 1993; Dull et al., 1998). These concentrations are enough for certain *in vitro* applications. However, high concentrations of viral stocks are required for gene therapy applications in order to improve transduction efficiencies (Andreadis et al., 1999). Several techniques to concentrate virus stocks like optimizing transfection protocols, use of new designed retroviral vectors and/or ultracentrifugation have been developed (Kotani et al., 1994; Reiser, 2000; Bajaj et al., 2001; Gatlin et al., 2001; Zhang et al., 2001).

Usually, virus attachment requires the interaction of the viral envelope GP with specific receptors on the cell surface. The densities of GP on the virions and the appropriate entry receptors on the targets cells are often low and in the majority of encounters the viral particle will be repelled by the repulsion between the negatively charged viral and cellular membranes. Furthermore, the slow diffusion and rapid decay of retrovirus particles are important reasons for the low transduction efficiencies (Andreadis et al., 2000; Chuck et al., 1996; Le Doux et al., 1999). Various approaches have been developed to increase the efficiency of virion attachment and thus gene delivery but they all have significant limitations and improved methods are still urgently needed.

### 1.5 Approaches to enhancing retroviral transduction efficiency

Frequently used strategies to increase attachment rates include low speed centrifugation of virions onto their target cells (Ho et al., 1993; Bahnson et al., 1995; Quintas-Cardama et al., 2007) and/or treatment with cationic polymers (e.g., hexadimethrine bromide (polybrene), diethylaminoethyl (DEAD)-dextran) (Hodgson and Solaiman, 1996; Vogt, 1967), and cationic peptides (e.g., protamine sulfate) (Cornetta and Anderson, 1989; Hodgson and Solaiman, 1996). Positively charged compounds may enhance transduction efficiency via charge shielding, in which the compounds reduce electrostatic repulsion by neutralizing virus and cell surface charge (Davis et al., 2004). Moreover, cationic compounds could allow the virus to overcome electrostatic repulsion by aggregation of the virus, thereby enhancing the rate of sedimentation of the virus onto the cell surface (Davis et al., 2002). These methods are often inconvenient, associated with cytotoxicity, cell type dependent and/or poorly effective. Furthermore, most adjuvants and agents that facilitate virion attachment are not suitable for transduction of highly sensitive primary human cells because they affect their viability and functionality.
The most commonly used and most effective technique to promote viral gene delivery is coating of cell culture plates, flasks or bags with RetroNectin (Williams et al., 1991; Hanenberg et al., 1996; Moritz et al., 1996; Hanenberg et al., 1997). RetroNectin is a chimeric protein that comprises 574 amino acids and contains a cell-binding domain, a heparin-interacting region and a CS-1 site (Fig. 3). The fibronectin cell-binding and CS-1 sites interact with the very late antigens (VLA) 4 and 5 on the cell surface and the heparin domain binds the virions, thereby bringing them into close proximity and promoting transduction (Williams et al., 1991; Hanenberg et al., 1996; Moritz et al., 1996). However, RetroNectin has to be coated onto plates to capture virions and several washing steps have to be applied. Thus, the use of this relatively expensive compound is time consuming and laborious.

Figure 3. Mechanism of RetroNectin mediated increase of transduction efficiencies.
It has been previously shown, that semen-derived peptides form amyloid fibrils, termed Semen-derived Enhancer of Virus Infection (SEVI), promote HIV-1 infection (Münch et al., 2007) (Fig. 4). Under conditions of limiting viral dilutions SEVI boost the HIV infection by up to $10^5$-fold (Münch et al., 2007). It was hypothesized, that positively charged fibrils bind to negatively charged surface of virions and target cells. This reduces the charge-charge repulsions between them and promotes virion attachment and fusion. (Roan et al., 2009, Brender et al., 2009). Last studies showed that SEVI improve the efficiency of retroviral gene transfer (Wurm et al., 2010; Wurm et al., 2011). However, SEVI has some drawbacks for usage as an enhancer of viral gene transfer. For example, the amyloidogenic peptides that form SEVI are relatively large (38 amino acids) and thus relatively expensive to produce. Furthermore, it takes several hours to generate the active amyloid fibrils (Münch et al, 2007). Finally, sometimes very large amyloid aggregates are obtained particularly after longer storage and repeated freeze-thaw cycles that are poorly effective in boosting retroviral transduction.

1.6 Peptide derived from HIV-1 envelope glycoprotein boost HIV-1 infection

To better characterize the interaction of HIV-1 with its cellular receptors, we screened a library of peptides derived from different regions of the surface envelope glycoprotein gp120 of HIV-1 (group M, subtype B, strain HxB2) (Gurgo et al., 1988). HIV-1 group M is the ‘major’ group and is responsible for the majority of the global HIV epidemic (Hemelaar, 2012). This GP together with gp41 produces by the proteolytic cleavage of the precursor protein gp160, which is encoded by the env gene of HIV-1. gp120 is responsible for virus binding to the receptor (CD4) and co-receptors (CCR5 and CXCR4) expressed by target cells and triggers the subsequent fusion between viral envelope and cell membrane

Figure 4. Putative mechanism of SEVI action. Abundant semen protein prostatic acid phosphatase (PAP) form amyloid fibrils termed SEVI. The fibrils capture HIV virions and promote their attachment to target cells. (Source: N. Roan and W. Greene, 2007, Cell, 131(6):1044-1046).
(Kwong et al., 1998). gp120 is composed of five regions called C1–C5 and conserved among different HIV-1 strains, five highly glycosylated and hypervariable regions called V1–V5 and contains 30 β-strands and 6 α-helices (Fig. 5). One of peptides derived from HIV-1 gp120 efficiently enhanced HIV-1 infection. This peptide corresponds to amino acids 413 to 431 of the HIV-1 gp120. This peptide partially covers the β19 and β20 strands which proposed to be involved in co-receptor interaction (Kwong et al., 1998). We hypothesised that this peptide might be used as an effective means to increase retroviral gene transfer in basic research and clinical applications.

Figure 5. Structure of the HIV-1 gp120. Strands β19 and β20 overlapping the amino acids 413 to 431. (Source: Kwong et al., 1998, Nature, 393:648-59).
1.7 Aim of present study

Retroviral and lentiviral gene transfer is the method of choice for the stable introduction of genetic material into cells and offers many prospects for basic research and for the treatment of genetic disorders, malignancies and infection diseases. Application of retro- and lentiviral gene transfer systems is often hampered by low transduction efficiencies, particularly into specific primary human cell types, such as macrophages and CD34+ hematopoietic stem cells. Various approaches have been developed to increase the efficiency of gene delivery but they all have significant limitations and improved methods are urgently needed. Therefore, the aim of the present study was to develop convenient and broadly applicable tools for effective and safe retroviral gene transfer, based on a peptide derived from HIV-1 external gp120. To achieve this goal, the following points were investigated:

- Identification of most effective peptide fragment required for infection enhancement
- Determination of effect of peptide on cell viability
- Investigation of interaction between peptide and virus particles
- Investigation of interaction between peptide and cells surface
- Determination of effect of peptide on retroviral transduction efficacies and cell viability in comparison to other enhancer in vitro
- Analysis of peptide in vivo studies using a mouse model
2 MATERIAL AND METHODS

2.1 Material

2.1.1 Eukaryotic cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293T</td>
<td>Human renal epithelial cell line which was transformed with adenovirus type 5 and expresses SV40 (simian virus 40) large T-antigen (Graham et al., 1977). The cells were obtained from the American Type Culture Collection (ATCC).</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human epithelial carcinoma cell line (Gey, 1952). The cells were obtained from ATCC.</td>
</tr>
<tr>
<td>TZM-bl</td>
<td>CXCR4-positive HeLa cell clone that was engineered to express CD4 and CCR5. TZM-bl cells contain HIV LTR-driven β-galactosidase and luciferase reporter cassettes that are activated by HIV tat expression. (Wei, 2002). The cells were obtained from the NIH AIDS Reagent Program.</td>
</tr>
<tr>
<td>CEM-M7</td>
<td>Human T lymphoid cell line, which expresses luciferase and GFP under control of the LTR promoter (Hsu et al., 2003). The cells were kindly provided by N. Landau (New York, NY, USA).</td>
</tr>
<tr>
<td>KG-1</td>
<td>Human acute myeloid leukemia cell line which express transmembrane and soluble forms of CD34. (Koeffler, 1978; Fernández M, 2000). The cells were obtained from ATCC.</td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin fibroblasts cell line. The cells were kindly provided by J. von Einem (Institute of Virology, Ulm, Germany.).</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human acute monocytic leukemia cell line (Tsuchiya et al., 1980). The cells were obtained from the American Type Culture Collection. The cells were obtained from ATCC.</td>
</tr>
<tr>
<td>K562</td>
<td>Human erythroleukemic cell line (Anderson LC et al., 1979). The cells were obtained from ATCC.</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>Human astroglioma cell line with epithelial-like morphology (Ponten and Macintyre, 1968). The cells were obtained from the NIH AIDS Reagent Program.</td>
</tr>
<tr>
<td>BON</td>
<td>Human pancreatic carcinoid cell line (Evers, 1994). The cells were obtained from ATCC.</td>
</tr>
</tbody>
</table>
2.1.2 Primary cells

Human Peripheral Blood Lymphocytes (PBL)  Human Peripheral Blood Mononuclear Cells (PBMCs) were separated from buffy coat using a Ficoll gradient. PBL were enriched from the PBMC fraction by an overnight plastic adherence at 37°C to remove adherent monocytes. Lymphocytes were activated for 3 days with 20 U/ml IL2 and 2 µg/ml PHA.

Human Macrophages  Monocytes were separated using adherence on plastic. After 45 min, non-adherent cells were removed by rinsing in PBS. Monocytes were differentiated to macrophages by incubation for 7 days with 10 ng/ml GM-CSF.

Human CD34+ cells  Cells were received from the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, and collected by apheresis of G-CSF (granulocyte colony stimulating factor) treated individuals. Cells were maintained in CellGro® SCGM medium (CellGenix Germany) supplemented with 10% FCS, 100 ng/ml rhSCF, 20 ng/ml rhIL-3, 25 ng/ml rhIL-6.

2.1.3 Bacteria

*E. coli* XL-2 blue™ recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F’ |proAB lacZΔM15 Tn10 (Tetr) Amy Camr] Agilent Technologies, Santa Clara, CA, USA

2.1.4 Nucleic acids

Plasmid DNAs

pBRHIV-1NL4-3_92Th014  The plasmid encodes the HIV-1 NL4-3 provirus. The V3-loop region has been exchanged by the V3-loop of the R5-tropic 92th014.12 isolate (Papkalla et al., 2002).

pBRNL4-3_92Th014.12(R5)nef* IRES_g-Luc  The plasmid encodes the pBRNL4-3_92Th014.12 provirus and a Gaussia-luciferase cassette in place of nef.

pSRS11 SF GFPpre  Self-inactivating (SIN) retrovirus transfer vector, driving by the Rous sarcoma virus (RSV) promoter in the producing cell. Expression of the green fluorescent protein (GFP) is directed by an internal spleen-focus forming virus promoter (SF) (Schambach et al., 2006b). The plasmid was kindly provided by J. Bohne.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M57-DAW</td>
<td>MLV gag/pol expression plasmid utilizing an SV promotor (Schambach et al., 2006b). The plasmid</td>
</tr>
<tr>
<td></td>
<td>was kindly provided by J. Bohne.</td>
</tr>
<tr>
<td>phCMV-RD114</td>
<td>Construct expressing endogenous feline virus (RD114) glycoprotein. The plasmid was kindly</td>
</tr>
<tr>
<td></td>
<td>provided by F.L. Cosset.</td>
</tr>
<tr>
<td>phCMV-GaLV</td>
<td>Construct expressing Gibbon Ape Leukemia Virus (GaLV) glycoprotein. The plasmid was kindly</td>
</tr>
<tr>
<td></td>
<td>provided by F.L. Cosset.</td>
</tr>
<tr>
<td>phCMV-MLV</td>
<td>Construct expressing amphotropic Murine Leukemia Virus (MLV4070A) glycoprotein. The plasmid</td>
</tr>
<tr>
<td></td>
<td>was kindly provided by F.L. Cosset.</td>
</tr>
<tr>
<td>pRRL.cPPT.SFeGFP.pre</td>
<td>Self-inactivating (SIN) lentiviral vector, contains the spleen focus-forming virus LTR (SF), the</td>
</tr>
<tr>
<td></td>
<td>central polypurine tract (PPT), which increase the efficiency of reverse transcription and the</td>
</tr>
<tr>
<td></td>
<td>posttranscriptional regulatory element (PRE) of woodchuck hepatitis virus (increase infectious</td>
</tr>
<tr>
<td></td>
<td>titer). (Zufferey et al., 1999; Schambach et al., 2006a). The plasmid was kindly provided by</td>
</tr>
<tr>
<td></td>
<td>L. Naldini</td>
</tr>
<tr>
<td>pMDLg/pRRE</td>
<td>CMV-driven expression plasmid that contains gag, pol and Rev-responsible element (RRE) from HIV-1,</td>
</tr>
<tr>
<td></td>
<td>a binding site for the Rev protein which facilitates export of the TNA from the nucleus (Dull et</td>
</tr>
<tr>
<td></td>
<td>al., 1998). The plasmid was kindly provided by J. Bohne.</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>Rev cDNA-expressing plasmid in which the joined second and third exons of HIV-1 rev under the</td>
</tr>
<tr>
<td></td>
<td>transcriptional control of RSV U3 (Dull et al., 1998). The plasmid was kindly provided by D.</td>
</tr>
<tr>
<td></td>
<td>Trono.</td>
</tr>
<tr>
<td>phCMV-RD/TR</td>
<td>Chimeric envelope gp carrying the MLV gp cytoplasmic tail. Relatively short cytoplasmic tail of</td>
</tr>
<tr>
<td></td>
<td>MLV increases incorporation of RD gp on lentiviral core (Sandrin et al., 2002). The plasmid was</td>
</tr>
<tr>
<td></td>
<td>kindly provided by F.L. Cosset.</td>
</tr>
<tr>
<td>phCMV-GaLV/TR</td>
<td>Chimeric envelope gp carrying the MLV gp cytoplasmic tail. Relatively short cytoplasmic tail of</td>
</tr>
<tr>
<td></td>
<td>MLV increases incorporation of GaLV gp on lentiviral core (Sandrin et al., 2002). The plasmid was</td>
</tr>
<tr>
<td></td>
<td>kindly provided by F.L. Cosset.</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vector which expresses the envelope protein of the vesicular stomatitis virus (VSV-G) (Schindler</td>
</tr>
<tr>
<td></td>
<td>et al., 2003)</td>
</tr>
<tr>
<td>MLV GAG YFP</td>
<td>The plasmid encodes MLV gag proteins fused to YFP. The particles are non-infectious (kindly</td>
</tr>
<tr>
<td></td>
<td>provided by W. Mothes).</td>
</tr>
</tbody>
</table>
Molecular weight size marker

1 kb Plus DNA Ladder  Life Technologies GmbH, Darmstadt

2.1.5 Proteins

Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction endonucleases</td>
<td>New England Biolab, Ipswich, MA, USA</td>
</tr>
<tr>
<td>EDTA-Trypsin</td>
<td>Invitrogen/Gibco (Karlsruhe)</td>
</tr>
</tbody>
</table>

RetroNectin

RetroNectin was obtained from TAKARA (TAK T100B) and coated on “Non-Tissue Culture Treated 96 well plates” (BD 351172) as recommended by the manufacturer.

2.1.6 Peptides

HIV-1 p12  Abcam, Cambridge, UK

PAP248-286  GIHKQKEKSRLQGGVNLNEILNMKRATQIPSYKKLIMY
EF-A      NITLQCKIKQIQIMWQEVG
EF-B      NITLQCKIK
EF-C      QCKIKQIINMWQ
EF-D      KIKQIINMWQ
EF-E      QCKIKQ
EF-F      QCKIKQIINMW
EF-G      KIK
EF-H      QIINMWQEVG
EFcon     TITLPCRIKQIQINMWQGVG

The peptides were synthesized by Celtek Bioscience (Nashville, TN, USA) and ViroPharmaceutical GmbH & Co KG (Hannover, Germany) using standard Fmoc solid-
phase peptide synthesis, purified by preparative RP HPLC and analyzed by HPLC and MS. Peptides derived from HIV-1 gp120 were dissolved in DMSO (Merck, 1029521000) to 10 mM or 10 mg/ml stocks and stored at -20°C. PAP248-286 was dissolved in PBS at a concentration of 10 mg/ml and agitated overnight at 37°C and 179 g in a thermomixer to promote amyloid fibril formation.

### 2.1.7 Chemical reagent

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Carl Roth GmbH + Co. KG, Karlsruhe</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Ratiopharm GmbH, Ulm</td>
</tr>
<tr>
<td>Bacto tryptone</td>
<td>Becton Dickinson, Franklin Lakes, NJ, USA</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>Becton Dickinson, Franklin Lakes, NJ, USA</td>
</tr>
<tr>
<td>Buffers for restriction digestion</td>
<td>New England Biolabs, Ipswich, MA, USA</td>
</tr>
<tr>
<td>Calcium chloride dihydrate (CaCl2 x 2H2O)</td>
<td>AppliChem GmbH, Darmstadt</td>
</tr>
<tr>
<td>CellGro® SCGM medium</td>
<td>CellGenix, Germany</td>
</tr>
<tr>
<td>CellMask™ Deep Red</td>
<td>Thermo Fisher Scientific, Marietta, USA</td>
</tr>
<tr>
<td>Chloroquine diphosphate salt</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Congo Red</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>DEAE-dextran</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate dihydrate (Na2HPO4 x 2H2O)</td>
<td>AppliChem GmbH, Darmstadt</td>
</tr>
<tr>
<td>DMEM</td>
<td>Life Technologies GmbH, Darmstadt</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>AppliChem GmbH, Darmstadt</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Life Technologies GmbH, Darmstadt</td>
</tr>
<tr>
<td>Ficoll separation solution</td>
<td>Merck KGaA, Darmstadt</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Life Technologies GmbH, Darmstadt</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sigma Aldrich, St. Louis, MO, USA</td>
</tr>
</tbody>
</table>
2 MATERIAL AND METHODS

L-Glutamine Life Technologies GmbH, Darmstadt
Hepes (C8H18N2O4S) Sigma Aldrich, St. Louis, MO, USA
HPLC water VWR International GmbH, Darmstadt
Hydrogen chloride (HCl) VWR International GmbH, Darmstadt
Isopropanol Merck KGaA, Darmstadt
Methanol Merck KGaA, Darmstadt
MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide Sigma Aldrich, St. Louis, MO, USA
Penicillin/Streptomycin (Pen/S) Life Technologies GmbH, Darmstadt
Phosphate buffered saline (PBS) Life Technologies GmbH, Darmstadt
PHA (Phytohaemagglutinin) Thermo Fisher Scientific, Marietta, OH, USA
Protamine sulfate Sigma Aldrich, St. Louis, MO, USA
Potassium chloride (KCl) Merck KGaA, Darmstadt
Roti®-Load DNA Carl Roth GmbH + Co. KG, Karlsruhe
RPMI-1640 medium Life Technologies GmbH, Darmstadt
Recombinant Human Interleukin-2 (rhIL-2) Miltenyi Biotec GmbH, Bergisch Gladbach
Recombinant Human Interleukin-3 (rhIL-3) R&D Systems, Wiesbaden-Nordenstadt
Recombinant Human Interleukin-6 (rhIL-6) R&D Systems, Wiesbaden-Nordenstadt
Recombinant Human Stem Cell Factor (rhSCF) R&D Systems, Wiesbaden-Nordenstadt
SOC medium Life Technologies GmbH, Darmstadt
Sodium ascorbate (C6H7NaO6) Sigma Aldrich, St. Louis, MO, USA
Sodium chloride (NaCl) Merck KGaA, Darmstadt
Sodium hydroxide (NaOH) Merck KGaA, Darmstadt
Sulfuric acid (H2SO4) Sigma Aldrich, St. Louis, MO, USA
SureBlue™ TMB Microwell Peroxidase Substrate KPL, Gaithersburg, MD, USA
TAE buffer (50x) 5 PRIME, Gaithersburg, MD, USA
Thioflavin T (ThT) stock solution Sigma Aldrich, St. Louis, MO, USA
2 MATERIAL AND METHODS

Tris VWR International GmbH, Darmstadt
Tryptone Becton Dickinson, Franklin Lakes, NJ, USA
Trypsin / EDTA, 0.05% Life Technologies GmbH, Darmstadt
Triton X-100 Sigma Aldrich, St. Louis, MO, USA
Tween 20 Sigma Aldrich, St. Louis, MO, USA

2.1.8 Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-channel manifold for QuikSip™ BT-Aspirator</td>
<td>BRAND GMBH + CO KG, Wertheim</td>
</tr>
<tr>
<td>Cell culture flasks</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>Cell culture well plates</td>
<td>Greiner Bio-One GmbH, Frickenhausen</td>
</tr>
<tr>
<td>Falcons (15 ml and 50 ml)</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>Micro tubes, 1.5 and 2.0 ml, PP, with attached PP cap</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>Millex-HA Filter Unit 0.45 μm</td>
<td>Merck KGaA, Darmstadt</td>
</tr>
<tr>
<td>Polystyrene microplates</td>
<td>Corning, Corning, NY, USA</td>
</tr>
<tr>
<td>MaxiSorp™</td>
<td>Thermo Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>White plates</td>
<td>Thermo Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Serological pipettes (5 ml, 10 ml and 25 ml)</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>μ-Slide VI0.4</td>
<td>Ibidi, Planegg / Martinsried</td>
</tr>
<tr>
<td>Tips without filter, Type A/B/E (10, 200, 1,000 μl)</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
</tbody>
</table>

2.1.9 Technical equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge 5417 R (Rotor: F-45-30-11)</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Centrifuge 5810 R (Rotor: A-4-81)</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Multipette plus</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
<tr>
<td>Thermomixer comfort</td>
<td>Eppendorf AG, Hamburg</td>
</tr>
</tbody>
</table>
### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR Hei-Standard (stirrer)</td>
<td>Heidolph Instruments GmbH &amp; Co. KG, Schwabach</td>
</tr>
<tr>
<td>Titramax 100 (plate shaker)</td>
<td>Heidolph Instruments GmbH &amp; Co. KG, Schwabach</td>
</tr>
<tr>
<td>Pipetus®</td>
<td>Hirschmann Laborgeräte GmbH &amp; Co. KG, Eberstadt</td>
</tr>
<tr>
<td>DISCOVERY Comfort Multichannel</td>
<td>HTL Lab Solutions Warsaw, Poland</td>
</tr>
<tr>
<td>Pipette</td>
<td></td>
</tr>
<tr>
<td>FE20–FiveEasy™ pH-meter</td>
<td>Mettler-Toledo, Columbus, OH, USA</td>
</tr>
<tr>
<td>VMax Kinetic ELISA Microplate</td>
<td>Molecular Devices, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Reader SoftMax Pro 5.3</td>
<td></td>
</tr>
<tr>
<td>HERAsafe® KSP 18</td>
<td>Thermo Fisher Scientific, Marietta, OH, USA</td>
</tr>
<tr>
<td>Steri-Cult CO2-Inkubator Modell 3311</td>
<td>Thermo Fisher Scientific, Marietta, OH, USA</td>
</tr>
<tr>
<td>Vortex-Genie®</td>
<td>VWR International GmbH, Darmstadt</td>
</tr>
<tr>
<td>Orion II Microplate Luminometer</td>
<td>Zylux Corporation, Pforzheim</td>
</tr>
<tr>
<td>Software Simplicity</td>
<td></td>
</tr>
<tr>
<td>Thermomax microplate reader</td>
<td>Molecular devices, UK</td>
</tr>
</tbody>
</table>

#### 2.1.10 Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropix® Gal-Screen® assay</td>
<td>Applied Biosystems, Foster City, CA, USA</td>
</tr>
<tr>
<td>CellTiter-Glo®Luminescent Cell Viability Assay</td>
<td>Promega, Madison, WI, USA</td>
</tr>
<tr>
<td>Luciferase Assay System (E1501)</td>
<td>Promega, Madison, WI, USA</td>
</tr>
<tr>
<td>Wizard® Plus Midipreps DNA Purification System</td>
<td>Promega, Madison, WI, USA</td>
</tr>
</tbody>
</table>
2.1.11 Buffers and solutions

2 M CaCl2

*Gaussia* substrate dilution buffer: 0.1 M Tris, 0.3 M sodium ascorbate, adjust pH to 7.4

10x HBS

8.18 g NaCl
5.94 g Heps
0.25 g Na2HPO4 x 2 H2O
dissolved in 100 ml H2O, adjusted to pH 7.12

2.1.12 Media

Bacteria culture media

LB medium

10 g/l bacto tryptone, 5 g/l bacto yeast extract, 8 g/l NaCl, 1 g/l glucose, add 100 mg/l ampicillin prior to use

LB/AMP agar

add 15 g/l agar and 100 mg/l ampicillin to LB medium

S.O.C. medium

LifeTechnologies/Invitrogen (Carlsbad)

Cell culture media

DMEM supplemented with

350 μg/ml L-glutamine
120 μg/ml penicillin
120 μg/ml streptomycin sulfate
10% (v/v) heat-inactivated FCS

used for TZM-bl, HEK293T, HeLa, HFF cells

RPMI 1640 supplemented with

350 μg/ml L-Glutamine
120 μg/ml penicillin
120 μg/ml streptomycin sulfate
10% (v/v) heat-inactivated FCS
(add 10 ng/ml IL-2, 1 μg/ml PHA to stimulate PBMCs)

used for CEMx-M7, KG-1, K562, Jurkat T, BON and PBLs
EMEM supplemented with 350 μg/ml Glutamine
120 μg/ml penicillin
120 μg/ml streptomycin sulfate
1% Non-Essential Amino Acids (NEAA)
1mM Sodium Pyruvate (NaP)
10% (v/v) heat-inactivated FCS

used for U-87 MG cells

CellGro® SCGM medium supplemented with

100 ng/ml rhSCF
20 ng/ml rhIL-3
25 ng/ml rhIL-6
10% (v/v) heat-inactivated FCS

used for human CD34+ cells which were obtained from the Institut für Klinische Transfusionsmedizin und Immungenetik, Ulm

2.2 Methods

2.2.1 DNA methods

2.2.1.1 Plasmid DNA preparation
Plasmid DNA for transfection was prepared using the Wizard™ Plus Midiprep Kit according to the manufacturer’s protocol. The DNA concentration and quality was determined using a Nano Drop 2000 spectrophotometer.

2.2.1.2 Restriction digest
Plasmid DNA was checked by incubating 1 µg of DNA with 0.5 µl of appropriate restriction enzymes and 2 µl of the recommended 10 x NEB restriction buffer at 37 °C for 1 h on a thermomixer. Sterile water was added to a final volume of 20 µl.

2.2.1.3 Agarose gel electrophoresis
The restricted plasmid DNA was analysed by agarose gel electrophoresis. 20 µl of the digested DNA was mixed with 5 µl loading dye and separated on 0.7% agarose gels (containing 0.05 µg/ml ethidium bromide) in 1 x TAE buffer at 120 V for 30 min using the Voltage PowerPAC Basic Power Supply. The 1kb+ ladder (Life Technologies GmbH, Darmstadt) was used as a standard. DNA fragments were visualized with the Gel Doc™ XR system (Bio-Rad).
2.2.2 Bacterial methods

2.2.2.1 Bacterial culture
The used plasmids contained a gene coding for ampicillin resistance. Therefore, transformed bacteria were grown in LB medium or on LB agar plates containing 50 µg/ml ampicillin. The bacteria were grown in LB medium for 14-16 h at 37 °C on a shaker and the inoculated LB agar plates were incubated at 37 °C.

2.2.2.2 Bacterial transformation
1 µg of plasmid DNA was incubated with 5 µl of *Escherichia coli* XL2 Blue™ cells on ice for 20 min. After the cells were heat-shocked for 30 sec at 42 °C they were incubated on ice for 2 min, followed by the addition of 200 µl SOC medium. The transformed cells were incubated at 37 °C on a shaker for 30 min and plated on LB agar plates containing 50µg/ml ampicillin. The plates were incubated at 37 °C in a compartment drier overnight.

2.2.3 Cell culture

2.2.3.1 Adherent and suspension cell culture
The adherent and suspension cells were maintained at 37 °C in an incubator in 5 % CO₂ and 95 % air humidity. The cells were grown in 25 cm² or 75 cm² cell culture flasks (Sarstedt, Nümbrecht) depending on the requirement of cells, in DMEM or RPMI-1640, respectively. The adherent cells were split by trypsinization twice a week. Trypsin treatment of cells was performed by removal of the culture medium from the cells. The cells were washed with PBS and 1-3 ml trypsin was applied to the cells. The cells were incubated at 37 °C until they became detached. The cells were collected, centrifuged at 1,300 rpm for 3 minutes, resuspended in the fresh medium and re-seeded at a lower density in new flasks. Suspension cells were collected and afterwards centrifuged at 1,300 rpm for 3 min and passaged 1:5 in fresh culture medium.

2.2.3.2 Isolation of primary blood cells
Buffy-coat (lymphocyte concentrate from 500 ml whole blood), obtained from the Transfusion Center of the Ulm University Hospital, was diluted 1:3 with PBS containing 2% FCS. Ficoll separating solution was overlayed with the diluted blood and centrifuged at 515 g for 20 min without brakes. The white interphase layer formed by peripheral blood
mononuclear cells (PBMCs) was transferred in a fresh tube and washed twice with PBS containing 2 % FCS.

**Peripheral blood lymphocytes (PBL)** were enriched from the PBMC fraction by overnight plastic adherence at 37 °C to remove adherent monocytes. After separation lymphocytes were cultured in supplemented RPMI culture medium and activated for 3 days with 20 ng/ml IL-2 and 2 µg/ml PHA prior to transduction.

**Monocytes-derived macrophages:** Monocytes were separated from lymphocytes by adherence to tissue culture plastic (Nunc™ Cell Culture Treated Flasks, Thermo Scientific, #136196) for 45 min. Non-adherent cells were removed by rinsing with RPMI medium. Monocytes were stimulated with 10 ng/ml GM-CSF in RPMI cell culture medium. At day 3 the medium with growth factor was replaced and the cells were used for experiments at day 7.

### 2.2.4 Viral methods

#### 2.2.4.1 Virus stocks generation via calcium phosphate transfection of HEK293T cells

R5-tropic HIV-1 was produced by transient transfection of HEK293T cells with the pBRHIV-1NL4-3_92Th014 construct using calcium phosphate precipitation. The calcium phosphate transfection is a method for introduction of DNA into mammalian cells based on the formation of a calcium phosphate-DNA precipitate (Graham and van der Eb, 1973; Wigler et al., 1977). One day before transfection, 293T cells were seeded in 6-well plates. At a confluence of 60-70 % the cells were used for transfection. For this 5 µg DNA per well was mixed with 13 µl 2 M CaCl₂ and the total volume was made up to 100 µl with water. This solution was added dropwise to 100 µl of 2xHBS. The transfection cocktail was vortexed for 5 sec and added dropwise to the cells. The transfected cells were incubated for 8-16 h before the medium was replaced by fresh supplemented DMEM. 48 h post transfection, virus stocks were prepared by collecting the supernatant and centrifuging it at 805 g for 3 min. Virus stocks were quantified by p24 Elisa (AIDS Reagent and Reference Programme, SAIC Frederick, USA) and stored at -80 °C.

Retroviral vectors were produced by transient transfection of HEK293T cells with calcium phosphate using GFP encoding pSRS11 SF GFPpre, M57-DAW (gag/pol plasmid) and glycoprotein expression plasmid. In the present study retroviral particles were pseudotyped with Gibbon Ape Leukemia virus (GaLV), endogenous feline leukemia virus (RD114), Vesicular Stomatitis Virus (VSV-G) or amphotropic Murine Leukemia Virus (MLV) envelope glycoproteins. 24 hours prior to transfection, HEK293T cells were seed at 2 x 10⁶
cells per 100 mm cell culture dish in DMEM 10 % FCS to achieve 70-80 % confluence at the time of transfection. At the day of transfection DMEM medium was replaced with 10 ml medium containing chloroquine (final concentration of 25 μM). Chloroquine decreases the intracellular degradation of plasmid DNA in the lysosomes, thus increasing the amount of plasmid DNA that reaches the nucleus (Walker et al., 1996). Thereafter, 5 μg transfer vector, 13 μg M57-DAW and 2 μg envelope plasmid were mixed directly with 0.25 mM CaCl$_2$ in dH2O (total volume - 500 µl). This is then added dropwise to the same volume of 2xHBS buffer where a precipitation of calcium phosphate occurs. The transfection cocktail was vortexed for 5 sec and added dropwise to the cells. The medium was changed after 16 hours to avoid cell toxic effects of the chloroquine treatment. Supernatant containing the viral particles was collected 48 hours after transfection, centrifuged at 805 g for 3 min and used immediately for transduction or stored at -80ºC until required.

Luciferases expressing retroviral vectors were generated by co-transfecting a luciferase encoding mouse leukaemia virus vector with M57-DAW and GaLV, RD114, VSV-G or MLV glycoprotein expression plasmids using calcium phosphate precipitation as described above.

Lentiviral vectors encoding GFP were generated by transient transfection of 293T cells as described above. 4 μg pRRL.cPPT.SFeGFP.pre, 8 μg pRSV-Rev, 4 μg pMDLg/pRRE and 2-4 μg appropriate chimeric envelope glycoprotein with the cytoplasmic tail derived from the MLV gp: phCMV-RD114/TR, phCMV-GaLV/TR, Ampho MLV (kindly provided by F.L. Cosset) or VSV-G were used.

Luciferase encoding lentiviral particles were generated by cotransfection of an env-deleted pBRHIV-1 NL4_3 derivative encoding luciferase instead of nef and constructs expressing VSV-G, MLV or chimeric envelope glycoprotein derived from GaLV and RD114.

### 2.2.4.2 Determination of titer of retro- and lentiviral supernatants

Titers (transducing units/ml) of the viral supernatants were determined by transducing 10$^5$ HeLa cells with serial dilutions of viral supernatant. After 3 days post-transduction the percentage of GFP positive cells was determined by FACS analyses.

\[
\text{Titer (HeLa-transducing units/ml)} = \frac{10^5 \times \left( \frac{\text{% of GFP-positive cells}}{100} \right) \times \text{(target HeLa cells)}}{\text{volume of supernatant (in ml)}}
\]
2.2.4.3 Effect of EF-C on infection of TZM-bl reporter cell line
One day before infection, TZM-bl cells were seeded (10,000 cells/well) in F-96-well plates (Greiner Bio-one, Frickenhausen). Virus stocks were normalized based on their amounts of HIV-1 p24 capsid antigen. Infections were performed with 0.1 or 1 ng p24. 40 µl viral stocks were treated with the 40 µl various dilutions of peptides, vortexed and incubated for 5 min room temperature. Concentrations of peptide during preincubation with virus were 100, 10, 1, 0.1 and 0.0 µg/ml. Subsequently, 20 µl of these virus stocks were used to infect 180 µl cell cultures. Infection rates were determined 2 or 3 days post infection by quantifying β-galactosidase in cellular lysates.

2.2.4.4 Effect of EF-C on transduction efficiencies of different cell line
One day before transduction, adherent cells were seeded (10,000 cells/well) in F-96-well plates (Greiner Bio-one, Frickenhausen). 200,000 suspension cells were seeded on the day of experiment. Virus stocks were normalized based on their HeLa-transducing units content. Transduction experiments were performed with viral vector stocks at a MOI of 1. 40 µl viral stocks were treated with the 40 µl various dilutions of peptides, mixed and incubated for 5 min at room temperature. Concentrations of peptide during pre-incubation with virus were 100, 10, 1, 0.1 and 0.0 µg/ml. Subsequently, 20µl of these virus stocks were used to infect 180 µl cell cultures. After overnight incubation, supernatants were removed and cells were further cultivated in 200 µl fresh media. Infection rates were determined 2 or 3 days post infection by quantifying β-galactosidase or luciferase activities in cellular lysates. After 3 days post transduction, cells were used for FACS analyses or analysed by detecting luciferase activity in the cell supernatant.

2.2.4.5 Effect of EF-C on transduction efficiencies of primary cells
Transduction experiments with primary cells were performed with viral vector stocks at a MOI of 1 and 10. Primary cells (PBL, macrophages, CD34+) were resuspended in virus stocks containing 50, 25 and 0 µg of EF-C. After 4 hours incubation at 37 °C, cells were washed and further cultivated in their own specific growth media. GFP positive cells were determined 3 or 4 days later by using flow cytometry analysis.

2.2.4.6 EF-C mediated pull down assay
900 µl of R5-tropic HIV were mixed with 100 µl of EF-C (final concentration: 50 µg/ml) or PBS. After 10 min incubation at the room temperature, stocks were centrifuged for 5
min at 10,000 g. **Fig. 16a, b:** The supernatants were collected and the pellets resuspended to the original volume in PBS and cell culture medium DMEM. Both, pellets and supernatants were used to infect TZM-bl cells. Infection rates were measured at day 2 post-infection using β-galactosidase screen assay. **Fig. 16c:** The supernatant was collected and the resulting pellet resuspended in 100 µl DMEM. The amount of HIV-1 p24 capsid antigen in EF-C precipitated pellet and in the original virus stock was determined by a HIV-1 p24 ELISA.

### 2.2.4.7 Comparison of infection enhancing activity of EF-C and others enhancers

**Fig. 21a:** 40 µl of freshly prepared EF-C, polybrene, protamine sulphate, DEAD dextran and SEVI fibrils (obtained after overnight agitation) were serially diluted in PBS and subsequently incubated with equal volumes of HIV-1 containing 0.1 ng p24 antigen. Peptide concentrations during preincubation with virus were 50, 25, 12.5, 6.25, 3.1, 1.56, 0.78, 0.0 µg/ml. After 5 min incubation at room temperature 20 µl of these mixtures were added to 180 µl TZM-bl cells (1 x 10⁴/well) seeded the day before. After overnight incubation, supernatants were removed and cells were further cultivated in fresh media. Infection rates were determined 2 days later by quantifying β-galactosidase activities in cellular lysates.

**Fig. 22:** 120,000 KG-1 cells were plated in 96-well plate in 100µl RPMI. Transduction experiments were performed with viral vector stocks at a MOI of 1. 10µl of different dilution of EF-C (1,000; 500; 250 µg/ml) or polybrene/protamine sulphate (320 µg/ml; 160 µg/ml) were added to 90µl VS, mixed and incubated for 5 min at room temperature. 100 µl KG-1 cells were transduced with 100 µl of these mixtures. After overnight incubation, supernatants were removed and cells were further cultivated in fresh RPMI media. Transduction rates were measured 5 day later by FACS.

### 2.2.4.8 Coating experiments using EF-C and RetroNectin

RetroNectin was obtained from TAKARA (TAK T100B) and coated on “Non-Tissue Culture Treated 96 well plates” (BD Biosciences #351172) as recommended by the manufacturer.

**Fig. 23b:** 50, 10, 1 and 0 µg of EF-C per well were used to coat Costar 96 well plates over night at 4°C. The next day, peptide was removed and plates were incubated with HIV-1 for 1 or 4 hrs. Wells were then washed once in PBS and bound virus was determined by p24 ELISA.
Fig. 24a: Four different microtiter plates (A: Thermo Scientific NuncTM Nunc F96 MicroWell™ Plates Polystyrene Clear No.:167008; B: Thermo Scientific NuncTM Nunc F96 MicroWell™ Plates Polystyrene Clear No.: 442404; C: CORNING (96 well) EIA/RIA Flat Bottom, High Bind, Nonsterile No.: 3590; D: CORNING (96-Well) Multiple Well Cluster Plate TC Treated No.: 3596) were coated overnight at 4 °C with 10, 1, 0.1, and 0.0 µg freshly diluted EF-C (50 µl each). Thereafter, the peptide solutions were removed, wells washed and 50 µl HIV-1 R5 (0.1 ng p24 antigen) was added. After 4 hrs incubation at 37 °C, virus was removed and 1 x 10^5 TZM-bl cells (200 µl) per well were added, and 2 days later infection rates were determined by Gaussia-luciferase assay.

Fig. 24b: 50, 10, 1 and 0 µg of EF-C per well were added to Costar 96 well plates and incubated overnight at 4 °C. Peptide was then removed and 50 µl of a lentiviral vector containing the GaLV env was added to each well. 4 hrs later, the inoculum was removed and wells were either washed once in PBS or left untreated. Thereafter, 1 x 10^6 KG-1 cells were added. After 5 days, transduction rates were determined using FACS analysis.

2.2.4.9 Fusion assay.

This sensitive flow cytometry-based HIV-1 virion-fusion assay was conducted essentially as described (Cavrois et al., 2002; Cavrois et al., 2004). VSV-G HIV-1 NL4.3 Env- GFP containing BlaM-Vpr chimeric proteins were produced by triple-transfection of 293T cells. Medium only and VSV-G HIV-1 NL4.3 Env- GFP BlaM-Vpr virions were incubated with EF-C at the indicated concentrations and then added to TZM-bl cells. After 4 hours, cells were washed and loaded with CCF2/AM dye overnight at room temperature. Fusion was monitored by BD FACSCanto II Flow Cytometer and the corresponding software BD FACS Diva. Since EF-C caused some auto-fluorescence in this assay, samples containing EF-C in the absence of virus were used as negative control and subtracted from all values.

2.2.4.10 ß-galactosidase assay

HIV-1 infection rates in TZM-bl cells were quantified by removing the cell culture medium 2 or 3 days post infection, adding 50 µl of 1:1 dilution of Gal-sceen® substrate (Applied Biosystems, T1027) in PBS to each well. After 30 min incubation at room temperature, 40µl of the cell lysates were transferred into a F-96-Nunclon-delta white micro-well plate (Nunc™, Langenselbold) and the light emission was monitored with an Orion Microplate Luminometer (Berthold Detection systems, Pforzheim). The enzyme
activity was measured as relative light units/second (RLU/sec) using the computer program Simplicity 4.02 (Berthold Detection systems, Pforzheim).

2.2.4.11 Luciferase Assay
To detect transduction rates of luciferase encoding lentiviral and retroviral vectors, suspension cells were pelleted by centrifugation at 1,300 rpm for 3 min. The supernatant was then removed and cells were lysed in 40 µl 5-fold diluted Luciferase Cell Culture Lysis Reagent (Promega, E1531). When adherent cells were used, the supernatant was removed, and 40 µl Luciferase Lysis Buffer added. After 5 min, cells were resuspended and 30 µl of the lysates were transferred into white microtiter plates. Thereafter, 70µl luciferase substrate reconstituted in luciferase buffer (Luciferase Assay system, Promega, E1501) was added, and luminescence was measured using a luminometer (Orion microplate luminometer).

2.2.4.12 Cell Viability
To determine the effect of the peptides on cellular metabolic activity, different cell lines were incubated with various concentrations of peptides under experimental conditions corresponding to those used in the respective infection and transduction assays in the absence of virus. After 2 or 3 days of incubation, CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571) was used as recommended by the manufacturer. Cells were treated for three days with the indicated final concentrations of fibrils. Thereafter, CellTiter-Glo® Buffer was thawed, equilibrated to room temperature, and mixed with CellTiter-Glo® Substrate. The cell culture medium was removed, 100 µl fresh medium and 100 µl CellTiter-Glo® Reagent were added, agitated for 2 minutes to induce cell lysis, pipetted into white lumiplates, and incubated for 10 min to stabilize the luminescence signal. Luminescence intensities were the recorded in a luminometer (Orion microplate luminometer).

2.2.4.13 Flow cytometric analysis
Adherent cells were washed once in PBS, trypsinized, resuspended in DMEM + 10% FCS, transferred into FACS tubes, and pelleted for 3 min at 1,300 rpm. The supernatant was removed, and after an additional washing step in PBS and subsequent centrifugation, cells were fixed with 200 µl FACS buffer containing 2% paraformaldehyde and incubated for 30 min at 4 °C. Suspension cells were resuspended, transferred into FACS tubes, washed one
time in PBS and fixed as described above. FACS analysis was performed using the BD FACSCanto II Flow Cytometer and the corresponding software BD FACS Diva.

2.2.5 Imaging methods

2.2.5.1 Fluorescence microscopy
To detect the interaction of EF-C fibrils with virion, fibrils were labelled with a Rhodamine B dye (Rho-EF-C). 10 µl of Rho-EF-C (250 µg/ml) were mixed with 90 µl of a MLV GAG YFP virus-like particles and 30 µl of this mixture were transferred to an IBIDI µ-slide VI 0.4 chamber (Ibidi) for visualization. To investigate the interaction of EF-C with cells, 5 × 10^4 HeLa cells were seeded in 8 well slides and incubated overnight. Prior to imaging, cell membranes were stained with CellMask™ Deep Red according to the manufacturer's instruction (Invitrogen) and virus or Rho-EF-C/virus mixtures were added to cells. For EF-C coating experiments on plastic slides and 3D reconstitution, EF-C (50 µg/ml) was stained with ProteoStat® Amyloid Plaque Detection Kit (Lörrach, Germany) as per manufacturer’s instructions. EF-C was incubated for 10-12 hours on IBIDI slide. Afterwards, EF-C was thoroughly washed with PBS and further incubated with YFP labelled MLV-Gag virions for 1 hour. EF-C/virion complex was washed with PBS to remove unattached virions. Fluorescence images were taken with the AxioVision software ZEN 2009 using Zeiss LSM 710 confocal scanning equipment with a 63 × 1.4 NA immersion oil objective.

2.2.5.2 Atomic force microscopy (AFM)
A 10 µg/ml infection-enhancing EF-C stock solution, prepared by diluting the peptide dissolved in DMSO into Mili-Q water was further diluted to give a final concentration of 5 µg/ml. Next, 10 µL were added to a freshly cleaved mica surface and dried at 65 °C overnight to remove DMSO. AFM measurements were performed on a Multimode III AFM (Bruker) Images were analysed with the SPIP software version 5.1.5 (Image Metrology A/S, DK). More than 50 individual fibrils were extracted and their geometrical parameters were then analyzed to calculate the corresponding fibril diameters. Dr. Christoph Meier (Institute for Organic Chemistry III/Macromolecular Chemistry, University Ulm, Germany) performed the AFM analysis of the samples.
2.2.6 Proteomic methods

2.2.6.1 Thioflavin T (ThT) binding.
ThT stock solution (Sigma) was prepared by adding 8mg ThT to 10ml phosphate buffer (10mM phosphate, 150mM NaCl, pH 7.0). This solution was filtered through a 0.2 µm syringe filter, and further diluted 50-fold in PBS. 1.5 µl of a 1 mg/ml peptide stock solutions were added to 148.5 µl Thioflavin T solution. Mixtures were vortexed and transferred into Corning® CellBIND® 96 Well Flat Clear Bottom Black Polystyrene Microplates (Corning). Fluorescence emission intensities were measured from 470 to 590 nm (band-pass 10 nm) using a spectrofluorometer (SAFAS flix Xenius n°5473, SAFAS).

2.2.6.2 CongoRed interaction.
Peptide solutions (10 µl) were placed into 1.5 ml tubes and 200 µl CongoRed (CR, Sigma Aldrich, HT60-1KT) was added. The solutions were vortexed and incubated for 2 min at room temperature. Stained amyloid was pelleted by centrifugation at 14,000 rpm for 5 min. The supernatant was removed and the red pellet dissolved in DMSO (100 µl). This solution was transferred into a 96-well plate and the optical density was measured at 490/650 nm using Thermomax microplate reader (Molecular Devices Vmax).

2.2.6.3 ζ-potential measurement
For analyzing ζ-potentials of the fibrils, 50 µl of freshly prepared EF-C (1 mg/ml) were diluted to 1 ml of an aqueous solution of KCl (10^{-3} M). The ζ-potential was derived from the electrophoretic mobility of the peptides and measured using a Zeta Nanosizer (Malvern Instruments, UK) and the corresponding DLS Nano software. Each measurement was performed in triplicate. ζ-potentials of the buffers were measured and the values of each buffer were subtracted from all corresponding samples.
3. RESULTS

3.1 Identification of gp120 fragments that promote HIV-1 infection

To analyze the interaction of HIV-1 with its cellular receptors, synthetic peptides corresponding to different regions of the external viral glycoprotein gp120 of HIV-1 strain HxB2 were tested. For this purpose, a CCR5 tropic HIV-1 (R5 HIV-1) virus stock was generated by transient transfection of HEK293T cells with proviral DNA. The viral titer was determined by p24 ELISA and virus dilution containing 1 ng p24 antigen was treated with different concentrations of freshly prepared gp120 fragments. After 5 minutes of incubation, these mixtures were then used to infect the TZM-bl reporter cell line. Infection rates were determined 3 days later by β-galactosidase assay. Surprisingly, these rates showed that one of the tested gp120 fragments (Enhancing Factor A; EF-A) efficiently enhanced HIV-1 infection. In the presence of 12.5 µg/ml of EF-A infection rates of R5 HIV-1 were increased by 34 fold relative to control infections containing no peptide (0 µg/ml) (Fig. 6a). This peptide with the sequence NITLQCKIKQINMWQEVG corresponds to amino acids (aa) residues 413 to 431 of the HIV-1 gp120.

Figure 6. Identification of gp120 fragments that enhance HIV-1 infection. (a) The EF-A gp120 fragment enhances HIV-1 infection. Virus was treated with indicated concentrations of the peptide and mixtures were then used to infect TZM-bl reporter cells. Infection rates were determined 3 days after infection using β-galactosidase assay. The numbers above the bars give the n-fold enhancement of infection relative to that measured in the absence of EF-A. RLU/s, relative light units per second; the asterisk indicates over-infection. Data represent mean values ±SD obtained from triplicate infection. (b) Overview of analyzed gp120 fragments. The localization in the HIV-1 Env precursor is indicated schematically. Segments of gp120 are designated as follows: black boxes, conserved regions C1–C5; white boxes, variable regions V1–V5. Numbers correspond to the amino acid position in the HIV-1 strain HxB2 gp120 sequence. Active peptides are indicated in red. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
Treatment of virus with 25 µg/ml of EF-A and subsequent infection of TZM-bl cells caused massive virus induced cytopathic effect (CPE) which was observed by light microscopy (data not shown). The reason for this phenomenon is overinfection of cells (Fig. 6a). The cells became rounded, fused with adjacent cells and form a giant, multinucleate cells called syncytia.

To confirm this effect of EF-A and to determine the minimal peptide fragment required for infection enhancement, next seven length variants (B to H) of EF-A and another peptide (EFcon) representing the consensus sequence of all strains of HIV-1 group M subtype were synthesized (Fig. 6b). These peptides were analysed using the infectivity assay described above. The peptides corresponding to C- (EF-B) and N-terminal (EF-H) halves of EF-A did not enhance HIV-1 infection (Fig. 7), whereas peptides that contain the core region of EF-A (aa residues 419 to 427 of gp120) promoted HIV-1 infection (Fig. 7). Moreover, the size of peptide has played a decisive role, because too small peptides (EF-E and EF-G) did not augment infection. In detail, treatment of 0.1 ng virus with 100 µg/ml of EF-C, EF-D, EF-F and EFcon boosted HIV infection between 17 to 27 fold, while EF-B and EF-H displayed no or slight effects (Fig. 7). The small 12-aa peptide (EF-C) with the sequence QCKIKQIINMWQ was identified as the most effective enhancer (Fig. 7). This peptide has been named Enhancing Factor C (EF-C).

Figure 7. Activity of various synthetic gp120 fragments in promoting HIV-1 infection. 0.1 ng R5 HIV-1 virions were treated with equal amount of different concentrations of freshly prepared gp120 fragments. After 5 min incubation, these mixtures were used to infect TZM-bl cells. Infection rates determined 2 days later by β-galactosidase assay. Shown are average values derived from triplicate measurements +/- SD. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
3.2 gp120 fragments are not cytotoxic.

To analyse possible cytotoxic effects, freshly dissolved peptides, which enhanced HIV-1 infection, were incubated with TZM-bl cells for three days and then the metabolic activity in these samples was assessed by CellTiter-Glo® Luminescent Cell Viability Assay (Promega). This method is based on the use of the luciferase reaction to measure the amount of ATP from metabolically active (viable) cells. Quantification of intracellular ATP levels showed that none of the tested peptides affected cell growth and viability (Fig. 8a). Next, the effect of the most potent HIV enhancing peptide, EF-C, on the viability of different cell lines and primary cells was tested using the same bioluminescence assay. The TZM-bl cell line, a Jurkat leukaemic T-cell line, a progenitor like cell line (KG-1), a B/T cell hybrid cell line (CEM-M7) and primary peripheral blood mononuclear cells (PBMC) were incubated with various concentrations of EF-C peptide for 3 days. The cell viability assay showed that EF-C has no toxic effects for the indicated cells (Fig. 8b), a high metabolic activity was determined for all tested cells even in the presence of 100µg/ml of EF-C.

Figure 8. gp120 derived fragments are not cytotoxic. (a) Indicated peptide solutions were incubated with 1 x 10^5 TZM-bl cells for 3 days at 37°C. Metabolic activity was determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Shown are average values derived from triplicate measurements +/- SD. Concentrations shown are final cell culture concentrations. (b) Indicated cells were incubated with EF-C peptide for three days and cellular ATP concentrations were measured by the Cell Viability Assay. (Reprinted with friendly permission from Yolamanova, Meier *et al.*, 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)

3.3 gp120 peptides that enhance HIV infection form amyloid fibrils

Dissolving the peptide in Dimethylsulfoxid (DMSO) (10 mg/ml) prior to introduction into the aqueous solution (1 mg/ml) promoted the formation of a turbid solution. This property of EF-C seemed similar to earlier observations with SEVI. freshly dissolved solution of
chemically synthesized PAP248-286 is unable to increase infection. Upon overnight agitation of PAP248-286 at 37 °C the solution became turbid and the enhancement activity was restored (Münch et al., 2007). Biochemical and biophysical analyses showed that positively charged amyloid fibrils were thereby formed. It was hypothesized that these fibrils decrease the electrostatic repulsion between negatively charged viral and cell membranes (Münch et al., 2007; Roan et al., 2007; Ye et al., 2009).

To determine whether the gp120 fragments may also form amyloid aggregates, the reactivity of a freshly prepared peptide solutions to the amyloid specific dyes Thioflavin T (ThT) (Biancalana et al., 2009) and Congo Red (Puchtler and Sweat, 1965; Elghetany and Saleem, 1988) was analysed as described for SEVI (Münch et al., 2007). ThT is a benzothiazole dye that exhibit enhanced fluorescence upon binding to β-sheet structure of amyloid proteins and is commonly used to diagnose amyloid fibrils. ThT fluorescence scans showed that solutions containing the peptides EF-C, EF-A, EFcon, EF-D and EF-F displayed increased fluorescence intensities (Fig. 9a). Further evidence that gp120 derived peptides form amyloid aggregates was derived by a Congo Red (CR) staining assay. Congo red is a hydrophilic chemical agent that binds to amyloid fibrils and causes an apple green birefringence under polarized light. In contrast to the PBS control, the EF-C, EF-A, EFcon, EF-D and EF-F solutions formed a red coloured pellet after centrifugation, indicating the dye specifically intercalated into the amyloid (Fig. 9b).

Figure 9. Formation of amyloid material by the indicated gp120 fragments monitored by (a) Thioflavin T fluorescence and (b) Congo Red staining. OD, optical density; CPS, counts per second. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
3. RESULTS

Notably, ThT fluorescence intensities and results from Congo Red staining correlated with the activity of gp120 fragments to increase virus infection and EF-C showed the highest values (Fig. 10). In addition, EF-C, which was direct dissolved in PBS, contained only minor amounts of Congo Red stainable material and was largely inactive. This indicates that the activity of the gp120 fragments to enhance infection emerges from the presence of amyloid aggregates.

For further clarification of the mechanism underlying infectivity enhancement, structure and morphology of EF-C was analysed using atomic force microscopy (AFM) and $\zeta$ (Zeta) potential measurement. AFM is a powerful technique that enables to visualize nanoscale structures (e.g., amyloid fibrils) in an aqueous environment and measure their properties (e.g., fibril size) (Stine et al., 1996; Mostaert et al., 2006). AFM analyses of an aqueous EF-C peptide solution obtained immediately after diluting the peptide DMSO stock (10 mg/ml) tenfold in water (1 mg/ml) revealed the formation of fibrillary aggregates with a diameter of $3.4 \pm 0.1$ nm (Fig. 11). This is significantly smaller than the diameter of SEVI fibrils derived after overnight agitation ($5.3 \pm 0.1$ nm) (Fig. 11), therefor I referred to EF-C fibrils also as nanofibrils herein after.

Figure 10. Amyloid characteristics correlate with enhancement of HIV-1 infection. Values were derived from experiments shown in Fig. 7 and 9 a, b. Correlation analyses were performed with GraphPad Prism 5 software. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
3. RESULTS

3.1 To determine the surface charge of the fibrils, the ζ-potential of the EF-C fibrils was analysed. ζ-potential measurements showed that EF-C fibrils exhibit positive charges of +17.7 ± 1.7 mV, similar to that of SEVI (+17.8 ± 1.1 mV). Summarized, this results show that the EF-C peptide adopts a fibrillar structure with a β-sheet arrangement and a positive surface charge.

3.4 Formation of EF-C fibrils can be achieved in several solvents and their virus enhancing activity is stable

For certain experimental setups it might be beneficial to dilute EF-C stocks (10 mg/ml in DMSO) in other buffers than PBS to form fibrils. To test this, I diluted a 10 mg/ml DMSO stock of EF-C in PBS, H₂O or the cell culture media RPMI 1640 (Gibco) and DMEM (Gibco). The resulting 1 mg/ml solutions were further diluted 3-fold and mixed with 1 ng p24 antigen of R5 HIV-1. 10⁵ TZM-bl cells in 180 µl were then inoculated with 20 µl of these mixtures and infection rates were determined by luciferase assays 2 days later. EF-C preparation in all tested solvents showed similar results and enhanced HIV-1 infection from 12 x 10⁴ RLU/s measured in the absence of peptide to 9.5-10 x 10⁶ RLU/s if virions were exposed to 100 µg/ml EF-C (13-fold enhancement) (Fig. 12a). Thus, amyloid fibrils can be formed in different solvents that might be advantageous for several approaches, e.g. in experiments with cells depending on specific media.

To find out how long the dilutions peptide in PBS retain their virus enhancing activity, a 10 mg/ml DMSO stock of EF-C was diluted twice in PBS (final concentration: 100 µg/ml). Dilutions were then stored at 4°C or -20°C. After 2 weeks, both samples and a freshly prepared EF-C solution were further diluted with 0.1 ng p24 antigen of HIV-1 and analysed for their HIV enhancing activity 2 days later. Infection assays revealed that the stored peptide solutions increased virus infection with almost the same activity as the
freshly prepared peptide (Fig. 12b). Thus, EF-C peptide could be stored for at least two weeks without losing its enhancing activity.

Figure 12. The virus enhancing activity in EF-C dilutions is active in various solvents and media and is stable. (a) A 10 mg/ml DMSO stock of EF-C was diluted in either PBS, H2O or cell culture media RPMI1640 (Gibco) and DMEM (Gibco). Indicated concentrations of solutions were mixed with 1 ng R5 HIV-1. These mixtures were added to TZM-bl cells. Infection rates were determined 2 days later by luciferase assay. Shown are average values derived from triplicate measurements +/- SD. Concentrations given on the x-axes are EF-C concentrations during virion treatment. (b) Freshly prepared EF-C solution and samples, which were diluted and stored at 4°C or -20°C for two weeks, were analyzed for their HIV enhancing activity in TZM-bl cells as described above. Shown are average values derived from triplicate measurements +/-SD. Concentrations given on the x-axes are EF-C concentrations during virion treatment. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)

3.5 EF-C fibrils increase viral attachment and fusion

Like SEVI, EF-C fibrils are derived from a peptide with a net positive charge, suggesting that the respective fibrils enhance infection by neutralizing the repulsion between the negatively charged viral and cellular membranes (Münch et al., 2007). To directly visualize the interaction of EF-C nanofibrils with virions, I labelled the nanofibrils with a Rhodamine B dye (Rho-EF-C) and controlled their enhancing activity. Rho-EF-C fibrils increase virus infection as efficiently as the unlabelled fibrils (Fig. 13a). After that, I mixed labelled fibrils with CFP-labelled retroviral particles (MLV-CFP) and monitored their interaction using fluorescence confocal microscopy (Fig. 13b). All virions (green) associated with the fibrils (red) within seconds. In the presence of EF-C, no individual viral particles could be detected, highlighting efficient and stable complex formation between virions and fibrils (Fig. 13b).
To further clarify the mechanism underlying EF-C-mediated infectivity enhancement, I studied whether the fibrils increase virion attachment to the cell surface and subsequent fusion into the target cells. 10µl of Rho-EF-C (250 µg/ml) were added to 90µl CFP labelled MLV virus, and the mixtures were then added to HeLa cells. Fluorescence microscopy after 2 and 4 hours of incubation revealed that the amount of viral particles attached to the cell surface in the presence of fibrils is drastically increased (Fig. 14a). The viral uptake and internalization of fibrils was evident from the loss of fluorescence intensities after 24 hours (Fig. 14a). In parallel, the HeLa cells were inoculated with MLV-CFP alone. Images obtained after 24 hours of incubation showed that only few viral particles were entry into the cells (Fig. 14b).
To confirm the idea that EF-C efficiently delivers virions to the cell membrane and enhances viral fusion, a flow cytometry-based HIV-1 virion-fusion assay was performed (Cavrois et al., 2002). Therefore I produced VSV-G pseudotyped lentivirus particles carrying a β-lactamase reporter protein (BlaM) which fused to the amino terminus of the virion protein Vpr (BlaM-Vpr). During the fusion, BlaM-Vpr protein transfer into the target cell and this transmission can be monitored by the enzymatic cleavage of CCF2, a fluorescent dye substrate of β-lactamase, loaded into the target cells. Cleavage of the β-lactam ring changes the fluorescence emission spectrum and this change can be detected by flow cytometry. I inoculated TZM-bl cells with either PBS-treated or EF-C-treated virus. After 4 hours, cells were washed and loaded with CCF2/AM dye overnight. Next
day, the cells were washed and fusion was monitored with a BD FACSCanto II Flow Cytometer (Becton Dickinson, San Jose, CA) (Fig. 15). Since EF-C caused some auto-fluorescence in this assay, samples containing EF-C in the absence of virus were used as negative control and subtracted from all values. Treatment of BlaM-Vpr with 50 µg/ml EF-C resulted in 47% fused cells, whereas only 2.8% were fused in the absence of the enhancer (Fig. 15). Thus, addition of EF-C fibrils increased attachment and subsequent fusion of the viral particle to the target cells.

**Figure 15. EF-C fibrils enhance virion fusion.** TZM-bl cells were transduced with a VSV-G pseudotyped BlaM-Vpr lentiviral vector in the presence of the indicated concentrations of EF-C and virion fusion was determined by FACS. The numbers in panels indicate the percentages of fused cells. Percentages from lower panel were subtracted from upper panel.

### 3.6 EF-C fibrils allow to concentrate viral particles

Nowadays, time-consuming ultracentrifugation is the most commonly used method to concentrate retroviral stock solutions (Reiser, 2000; Bajaj et al., 2001; Gatlin et al., 2001; Zhang et al., 2001). The efficient formation of macroscopic complexes between virions and EF-C fibrils (Fig. 13b) prompted me to test whether this allows precipitation and concentration of virions in a bench-top centrifuge. For this purpose R5 tropic HIV was mixed with EF-C or PBS and centrifuged for 5 min at 10,000 g. The supernatants (used-up medium) were taken and the pellets resuspended to the original volume in PBS and cell culture medium DMEM (Fig. 16a). Both, pellets and supernatants were used to infect TZM-bl cells. Beta-galactosidase screen assay performed 2 days later showed that only the pelleted fraction of EF-C increased reporter gene expression whereas the respective supernatants lacked any virus enhancing activity demonstrating that the infectivity
enhancing fibrils of EF-C are pelletable (Fig. 16a, b). The PBS treated virus sample much less infectious than fibril-treated virus. Thus, centrifugation of HIV-1 with EF-C fibrils allowed precipitation of essentially all virions. One advantage of this approach is that metabolized old medium of the virus stock can be easily exchanged by fresh medium of choice. A second advantage is the possibility to easily concentrate virus by reconstituting pelleted virus in a lower volume than the original one. To test this, 1,000 µl of EF-C treated HIV stock was centrifuged at 10,000 g for 5 min. Afterwards, the supernatant was collected and the resulting pellet resuspended in 100 µl DMEM. The amount of HIV-1 in each fraction was determined by an HIV-1 p24 ELISA. The EF-C precipitated pellet contained almost 1.2 µg capsid antigen whereas the original virus stock contained less than 100 ng (Fig. 16c). Thus, EF-C fibrils do not only allow to precipitate virus from a virus solution but also to increase virus concentration by one single centrifugation step.

Figure 16. Nanofibrils bind viral particles and allow their precipitation and concentration. (a) Schematic drawing of the experimental outline to pellet virions. 10 ml of a virus stock preparation containing used-up medium was treated with PBS (1) or 50 µg/ml EF-C (2) and subjected to low speed centrifugation (5 min; 10,000 g; RT) After removal of used-up medium (3), the pelleted virions were resuspended in fresh medium (4) or buffer (5). Reducing the volume of resuspension medium allows the virus to be concentrated (6). (b) Infectivity of the resulting solutions. (c) p24 ELISA of the original virus stock (1) and EF-C-treated virus that was pelleted and resuspended in 1/10th of the volume of the medium used as the original stock (6). (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
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These results are of great importance because this fast and convenient fibril-based approach allows to replace time-consuming (60-120 min) ultra-centrifugation (50,000 g at 4°C) that is normally used to enrich virus. The convenient exchange of old and perhaps toxic medium by EF-C along with the simultaneous increase of transduction efficiencies could be particularly attractive for effective gene delivery into primary cells that require special media. In sum, EF-C allows to precipitate and to concentrate virions by brief centrifugation and simultaneously increases infection/transduction efficiency.

3.7 EF-C fibrils are potent enhancer of lenti- and retroviral transduction

Since low transduction rates represent a significant problem in retroviral gene transfer, the effect of EF-C on transduction rates of relevant target cells by retro- and lentiviral vectors was tested. I generated gamma-retroviral (MLV-derived) and lentiviral (HIV-derived) particles expressing luciferase or GFP as transgene. These vectors were pseudotyped with the Gibbon Ape Leukemia Virus (GaLV), the endogenous feline leukemia virus (RD114), the Vesicular Stomatitis Virus (VSV-G) or the amphotrophic Murine Leukemia Virus (MLV) envelope proteins. For the lentiviral vector truncated variants of GaLV and RD114 containing mutation in the cytoplasmic domain of Env were used. These Env proteins were selected because they are commonly used for gene transfer and have already been tested in animal models and/or in clinical trials (Burns et al., 1993; Dybing et al., 1997; Lee et al., 2001; Relander et al., 2005).

At first, I analyzed the impact of EF-C on transduction of HEK 293T cells by luciferase encoding lenti- and retroviral vectors pseudotyped with different glycoproteins. Luciferase assays performed 2 days after transduction demonstrated that brief exposure to EF-C enhanced transduction rates of all analysed vectors (Fig. 17a, b). For example luciferase activities in cellular lysates transduced with untreated retrovirus particles resulted in values below 5.0 x 10^5 RLU/s, whereas infection rates of nanofibril-treated virus were increased up to 6.5 x 10^6 (RD114) or even 1.4 x 10^7 RLU/s (MLV) (Fig. 17b). Similar results were obtained for lentiviral particles with the exception of the VSV-G pseudotype. Reporter gene activities after infection with untreated vectors were below 3 x 10^4 RLU/s, but were markedly increased after transduction with nanofibril-treated virions (1.6 x 10^5) (Fig. 17a). The enhancing effect was dose-dependent and largely independent of the viral vector or envelope. Notably, EF-C did not allow virus transduction in the absence of a functional
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Env protein (Fig. 17a, b). This indicates that EF-C fibrils do not allow unspecific entry into target cells.

To further examine whether fibrils are suitable for therapeutic application, I examined their capability to promote retro- and lentiviral transduction in a variety of cell types. GFP encoding viral vectors commonly used in gene transfer settings (Naldini, 1997; Dull et al., 1998; Zufferey et al., 1998; Schambach et al., 2006) were produced by transient transfection of HEK 293T cells. Cell culture supernatants containing retro- or lentiviral vectors harvested two days later were used for transduction. Treatment of GaLV pseudotyped retroviral vector (RV-GaLV) with EF-C efficiently enhanced transduction of the human epithelial cervical cancer HeLa cell line from 5% in the control to 98% after 100 µg/ml EF-C treatment (Fig. 18a). Gene transfer into the skin fibroblast is a promising approach to treat inherited or acquired dermatological diseases (Wei et al., 1999). Therefore, I tested whether EF-C nanofibrils enhance gene transfer into foreskin fibroblasts (HFF) cell line. Treatment of RV-GaLV with EF-C resulted in 40.8% GFP positive cells 3 days post transduction whereas only 1.8% were transduced in the absence of the enhancer (Fig. 18b), corresponding to a 23-fold enhancement of transduction rates.
I also performed experiments in non-adherent KG-1 cells, a human acute myeloid leukemia cell line that represents an early stage of hematopoietic differentiation and that is often used in colony formation assays instead of human bone marrow cells (Koeffler et al., 1978). The rate of GFP expressing cells was 1.5% in the control whereas treatment with 50 µg/ml EF-C led to GFP expression in 65.8% of the cells (Fig. 19a). Thus, EF-C does not only favour transduction of adherent cells but also of those growing non-adherent. Furthermore, I transduced BON, a human pancreatic carcinoid cell line, and U87MG cell line, a glioblastoma, astrocytoma cell line with RD114 pseudotyped lentiviral vector (LV-RD114). Those cell lines have been widely used as models for translational study against dangerous types of cancers, human pancreatic tumors and human malignant glioma (Yao et al., 2007; Schmidt et al., 2013). In the presence of 50 µg/ml of EF-C, 83.5% of BON cells and 90.7% of U87MG cells were GFP positive, whereas inoculation with untreated virus showed transduction rates of 5% and 4.4% respectively (Fig. 19b). In sum these results show that EF-C nanofibrils generally facilitate transduction of different cell lines.
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To analyse the transduction enhancing activity of EF-C fibrils in primary cells, I transduced PHA/IL-2 activated human peripheral blood lymphocytes (PBL) with LV-RD114 in the presence or absence of nanofibrils. FACS analyses revealed that EF-C nanofibrils increased the percentage of GFP positive cells after infection with a multiplicity of infection (MOI) of 1 from 2.3 % to more than 24 % and after infection with a MOI of 10 to a maximum of 44% (Fig. 20a). To test, whether EF-C might also favour transduction of macrophages that are considered as hard to transduce cells (Leyva et al., 2011), monocytes-derived macrophages were transduced with a LV-RD114 stocks that were either treated with EF-C fibrils or PBS. Flow cytometry and fluorescence microscopy 3 days after transduction revealed that 50 µg/ml of EF-C fibrils enhanced lentiviral gene delivery into macrophages about 13-fold and allowed viral gene expression in almost half of them (Fig. 20b).

Figure 19. EF-C fibrils potently enhance lentiviral transduction in different cell types. (a) FACS analysis of KG-1 cell line transduced with LV-RD114 that were untreated or treated with the indicated concentrations of EF-C fibrils. The numbers in panel indicate the percentages of transduced (GFP+) cells. (b) Effect of nanofibrils on lentiviral gene transfer into human glioblastoma U87MG cells and human endocrine pancreatic tumour cell line BON. The numbers above the bars indicate n-fold enhancement compared to the titer measured in the absence of peptide. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
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Next, I analysed whether EF-C enhances transduction of stem cells. Human CD34+ hematopoietic stem cells (HSC), which represent a major target in gene therapy studies, were incubated with a LV-RD114 that has been treated with EF-C or PBS prior to transduction. FACS analyses performed 4 days later showed that transduction of fibrils-treated vector resulted in more than 25.6% of transduced and GFP positive cells, whereas inoculation with untreated virus showed transduction rates of only 0.2% (Fig. 20c). These results demonstrate that EF-C treatment of a vector preparation markedly enhanced gene transfer into primary cells including HSCs.

3.8 Comparison between EF-C fibrils and other agents used to promote retroviral transduction

Next, I compared the efficiency of EF-C mediated enhancement with commercially available enhancers like cationic compounds. Positively charged polymers such as polybrene and (DEAE)-dextran enhance the attachment of the virus and subsequent infection by bridging interactions between negatively charged viral and cell membranes. Protamine sulphate is a highly cationic polypeptide derived from the sperm of salmon that enhances retroviral gene transfer in a similar manner as cationic polymers. HIV enhancing
activity of EF-C was also compared with SEVI (Semen-derived Enhancer of Viral Infection), amyloid fibrils that are generated from the fragments of prostatic acidic phosphatase (PAP248-286) in semen (Münch et al., 2007). In detail, 0.1 ng R5 HIV-1 viral stocks were treated with equal concentrations of freshly prepared EF-C, polybrene, protamine sulfate, DEAE dextran and SEVI fibrils obtained after overnight agitation. After 5 min incubation, these mixtures were used to infect TZM-bl cells. Infection rates determined 2 days later showed that EF-C fibrils enhanced HIV-1 infection more efficiently than polybrene, protamine sulfate and DEAE dextran and was also slightly more effective than amyloid SEVI fibrils obtained after overnight agitation (Fig. 21a).

Next, I tested the effect of EF-C, protamine sulfate, polybrene, DEAD dextran on the metabolic activity of TZM-bl cells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The cells were incubated with various concentrations of these compounds for 3 days. Measurement of intracellular ATP levels showed that DEAD dextran decrease metabolic activity of TZM-bl cells at the concentration 25 - 50 µg/ml (Fig. 21b), whereas treatment the cells with EF-C nanofibrils, polybrene and protamine sulphate has no toxic effect for the cells (Fig. 21b).

Figure 21. Comparison of infection enhancing properties of EF-C fibrils and others enhancers. (a) Indicated compounds were serially diluted and subsequently incubated with HIV particles. 20 µl of these mixtures were used to infect 180 µl TZM-bl cells. Infection rates were determined 2 days later by quantifying β-galactosidase activities in cellular lysates. Concentrations shown are those during virion treatment. (b) Indicated compounds were incubated with TZM-bl for 3 days and cellular ATP concentrations were measured by the Cell Viability Assay. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
In addition, the effect of EF-C, polybrene and protamine sulphate on transduction rates of KG-1 cells was tested by retroviral vector carrying the GaLV GP (Fig. 22). For this experiment, concentrations of polybrene and protamine sulphate were reduced to 8 and 16 µg/ml as recommended by literature (Davis et al., 2002; Cornetta et al., 1989). EF-C nanofibrils increased the percentage of GFP expressing cells from 1.5 % in the control to almost 56.2 % after treatment with 25 µg/ml EF-C, whereas only 2.6 % and 6.6 % cells were transduced after addition of 8 µg/ml of protamine sulphate or polybrene respectively (Fig. 22). In sum, compared to polybrene, protamine sulphate and DEAE dextran, EF-C nanofibrils are more effective and less toxic.

3.9 Immobilized EF-C nanofibrils capture virus and increase transduction.

Currently, the most effective method to boost retroviral gene transfer is based on the use of the fibronectin derivative RetroNectin (Williams et al., 1991; Hanenberg et al., 1996; Moritz et al., 1996; Hanenberg et al., 1997). RetroNectin needs to first be pre-coated onto plates. The heparin domain of RetroNectin binds the viral particles this allows to remove the utilize virus medium. Finally the target cells attach to the cell-binding domain of RetroNectin. The co-localization of virions and cells in close proximity increased cellular uptake of the virus. I analysed whether EF-C nanofibrils can be applied in an analogue

![Figure 22. Comparison of transduction efficiency of EF-C fibrils, polybrene (PB) and protamine sulphate (PS). Indicated concentrations of enhancers were incubated with GaLV pseudotyped retroviral particles and subsequently used to infect 2 x 10^5 KG-1 cells. Transduction rates were determined 5 days later by FACS. Concentrations shown are those during cells treatment.](image)
manner. For this, plastic slides were coated with Rho-EF-C and incubated with YFP-tagged retroviral particles. Confocal microscopy analyses showed that the fibrils were immobilised on the surface and efficiently captured virions (Fig. 23a). The binding of lentiviral particles to the dishes was demonstrated in a p24 ELISA (Fig. 23b). For that, various concentrations of EF-C were coated on routinely used microtiter plates (Costar, #3596) over night at 4°C. The next day, peptide was removed and plates were incubated with HIV-1 for 1 or 4 hrs. Then, the wells were washed once in PBS and bound virus was determined by p24 ELISA. The results showed that immobilised fibrils capture virions and this occurred in a time- and dose-dependent manner (Fig. 23b).

![Figure 23. Coated EF-C fibrils can capture viruses.](image)

To test if immobilized EF-C fibrils enhance viral infection, four microtiter plates obtained from different providers (A: Thermo Scientific NuncTM Nunc F96 MicroWell™ Plates Polystyrene Clear, #167008; B: Thermo Scientific NuncTM Nunc F96 MicroWell™ Plates Polystyrene Clear, #442404; C: CORNING (96 well) EIA/RIA Flat Bottom, #3590; D: CORNING (96 well) Multiple Well Cluster Plate TC Treated, #3596) were incubated with freshly diluted EF-C. The next day, the solutions were removed and HIV-1 added. After 4 hours of incubation, the virus inoculum was removed and TZM-bl reporter cells were plated. In some control samples, the virus inoculum has not been removed and cells were directly added to the virus (ctrl). Beta-galactosidase assays performed 2 days later revealed that infection rates of cells added to EF-C coated plates were strongly increased (Fig. 24a).
Maximum enhancement was observed for all 4 dishes between 1-10 µg EF-C per well (Fig. 24a). Importantly, infection rates observed in control samples were even lower than those observed in wells that were coated with nanofibrils and where the whole inoculum has been removed (Fig. 24a). Similar experiment was performed with GaLV pseudotyped retroviral vector and KG-1 cells. Analyse of flow cytometric data showed that EF-C nanofibrils coated onto plates increased the transduction rates of suspension cells by more than 5-fold (Fig. 24b). Thus, EF-C can be coated onto the surfaces of cell culture dishes allowing to increases viral infection and transduction rates.

To compare the ability of immobilized RN and immobilized EF-C nanofibrils to promote retrovirus transduction, both compounds were tested in a site by site approach. Microtiter plate from BD Bioscience (#351147) that was recommended by TAKARA BIO INC was coated with RetroNectin. Routinely used microtiter plate from Costar (#3596) was coated with EF-C. Both plates were then incubated with similar amounts of a RV-GaLV for 4 hours. RN coated microplate was washed as recommended by the manufacturer and EF-C fibrils coated microplate was washed with 100 µl PBS. Thereafter, KG-1 cells were plated and the transduction rates were measured by FACS analysis. The results showed that both

![Figure 24. Immobilized EF-C nanofibrils capture virus. (a) EF-C fibrils that have been coated on different microtiter plates facilitate viral infection. See text for detail information. (b) Immobilized EF-C fibrils boost lentiviral vector transduction into KG-1 cells. Freshly diluted EF-C was added to Costar 96 well plates and was incubated over night at 4°C. Peptide was then removed and 50 µl of a lentiviral vector containing the GaLV GP was added to each well. 4 hrs later, the inoculum was removed and wells were either washed once in PBS or left untreated. Thereafter, 1 x 10\(^6\) KG-1 cells were added. After 5 days, transduction rates were determined using FACS analysis. ctrl, control in which the inoculum was not removed. (Reprinted with friendly permission from Yolamanova, Meier et al., 2013, Nature Nanotechnology, 8(2):130-136. Copyright © by Macmillan Publishers Limited)
compounds dose-dependently increased the rate of GFP expressing cells to a maximum of ~11% (Fig. 25a). This value was reached in the presence of 1.2 μg of RetroNectin or 10 μg of EF-C. Interestingly, transduction rates in the absence of RetroNectin in BD Bioscience microtiter plates were lower than those in the absence of EF-C in tissue culture treated plates from Costar (Fig. 25a), suggesting that cell growth in non-tissue BD Bioscience plates might be compromised.

To compare the effects of soluble RetroNectin and EF-C, HIV-1 virus stocks containing 1 ng p24 antigen were incubated with 0, 0.8, 4, 20 μg/ml enhancer and used to infect TZM-bl cells. EF-C nanofibrils strongly enhanced infection whereas treatment of virus stocks with RetroNectin had no effect (Fig. 25b). Additionally, the activities of EF-C fibrils and RetroNectin were compared under conditions in which the target cells were incubated first with compounds. TZM-bl cells were incubated with 0, 0.8, 4 or 20 μg/ml enhancer and subsequently infected with HIV-1. Also under these conditions, EF-C nanofibrils potently favoured infection, whereas levels of infection in cells treated with RetroNectin were comparable to those not treated by any enhancer (Fig. 25b). In sum these results demonstrate that in contrast to RetroNectin that is active only if bound on surfaces, EF-C potently enhanced viral infection when added to virions directly, or when used for treatment target cells before infection.
Finally, it was examined whether EF-C fibrils and RetroNectin can be used in combination. The plate from BD Bioscience was coated with 6 µg of RetroNectin and incubated overnight at 4 °C. The microplate was washed as recommended by the manufacturer and retroviral particles pseudotyped with GaLV Env were inoculated. After 1 hour virus stock was removed and 10^6 KG-1 cells were plated in each well. Further RV-GaLV stocks were treated with EF-C nanofibrils or PBS and after 5 min incubation these mixture were added to the KG-1 cells. The results showed that fibrils-treatment increases the strong enhancement of lentiviral transduction of KG-1 cells by RetroNectin treatment even further by up to 5-fold (Fig. 26). Hence, both methodologies can be combined to achieve maximal gene transfer efficiencies.
4. DISCUSSION

4.1 Application of EF-C nanofibrils as enhancer of transduction efficiency.

Retroviral gene transfer is the method of choice for the stable introduction of genetic material into cells and offers many prospects for basic research and for the treatment of genetic disorders, malignancies and infectious diseases. The low titer of infectious virions in the stocks is one of the major limitations of retroviral gene transfer. To produce high-titered virus stocks various approaches like the development of new viral vectors systems, the optimization of transfection protocols and ultracentrifugation were established (Kotani et al., 1994; Reiser, 2000; Bajaj et al., 2001; Gatlin et al., 2001; Zhang et al., 2001). Furthermore, work with retrovirus vectors is often hampered by low transduction rates, particularly into primary cells. Transduction efficiency is determined by rate of virus attachment to the target cell. Viral adsorption can be divided into three steps: (i) passive diffusion of virions to the proximity of the cell, (ii) nonspecific binding of the virus to the cell surface and (iii) binding of the viral glycoproteins to a specific cell surface receptor that triggers the subsequent fusion (Davis et al., 2002). Passive diffusion can hamper by electrostatic repulsion forces from the negatively charged phospholipid bilayers of retrovirus and cell. Frequently used strategies to overcome this obstacle are low speed centrifugation of virions together with their target cells and/or treatment with cationic compounds, such as the hexadimethrine bromide (polybrene), the diethylaminoethyl (DEAE)-dextran, protamine sulfate, poly-L-lysine, or cationic liposomes (Hodgson and Solaaiman, 1996; Vogt, PK, 1967; Cornetta et al., 1989; Hennemann et al., 2000). These compounds are thought to reduce the repulsion forces between the cell and the virus and mediate the binding of retroviral particle to the cell surface resulting in a higher efficiency of transduction. The big disadvantage of some cationic polymers, like polybrene and DEAE-dextran, is their cytotoxicity. Therefore, these compounds are used at only low concentrations (4–20 μg/ml) (Toyoshima and Vogt, 1969; Manning et al., 1971; Cornetta and Anderson, 1989). Furthermore, application of polybrene has a negative impact on cell proliferation in primary cells, such as keratinocytes or human mesenchymal stem cells, even when it is used at low concentrations (Seitz et al., 1998; Lin et al., 2010). Currently, the most common method to increase the efficiency of retroviral transduction is coating of the cell cultures dishes with RetroNectin, a fibronectin derivative which has been discovered almost 20 years ago (Hanenberg et al., 1996; Moritz et al., 1996; Hanenberg et al., 1997). When RetroNectin is coated onto cell culture dishes it induces the colocalization
of virus particles and target cells in close proximity resulting in increased transduction efficiencies. However, utilization of RetroNectin is relatively time consuming and expensive. Thus, improved convenient, flexible and effective transduction enhancers are still needed. Novel classes of transduction enhancers are peptide-derived amyloidogenic fibrils. It has been found that fibrils derived from human semen strongly enhance HIV-1 infection (Münch et al., 2007) and allow to improve viral gene transfer (Wurm et al., 2010; Wurm et al., 2011). These fibrils, termed semen-derived enhancer of virus infection (SEVI), bind virions and increase their adsorption to cells and subsequent fusion. However, SEVI also has some drawbacks for a use as transduction enhancer. Firstly, the amyloidogenic peptides that form SEVI are relatively large (38 amino acids) and thus expensive to produce. Secondly, it takes hours to generate the active fibrils (Münch et al., 2007). Finally, very large aggregates that are less effective in boosting retroviral transduction may form after long-term storage (Münch J, personal communication).

Newly, Fenard et al. identified the new viral entry enhancer, Vectofusin-1. This histidine-rich cationic amphipathic peptide efficiently enhance gene transfer of lentiviral vectors into human CD34+ cells by promoting the adhesion and the fusion between viral and cellular membranes, without inducing toxicity (Fenard et al., 2013). Vectofusin-1 shows similar efficiencies as RetroNectin with the advantage that it can be easily synthetized and purified.

In this study a small HIV-1 gp120-derived peptide named EF-C that instantaneously formed nanofibrils which greatly increased HIV-1 infection was investigated. This peptide effectively boosted the efficiency of lenti- and retroviral gene delivery into various cell types. EF-C nanofibrils increased the efficacy without affecting the specificity of retro- or lentiviral gene delivery suggesting that the fibrils might also boost recently developed lentiviral vectors that are based on specific single chain antibodies recognizing their cognate cell surface antigens (Anliker et al., 2010). This is noteworthy, because many gene therapy approaches aim to target specific cell types to avoid possible side effect.

Moreover, EF-C allowed to cross-link viral particles and to precipitate them by low speed centrifugation. The precipitated virions remained fully infectious and could be dissolved in the medium and volume of choice prior to transduction. Thus, EF-C represents a convenient and effective tool to concentrate virions, and to remove toxic compounds that may affect the functionality of the target cells. EF-C facilitated higher efficiency of viral transduction compared to polybrene, protamine sulphate, DEAE dextran and SEVI. EF-C was tested as non-cytotoxic for several cell lines and primary cells. Additionally, to
exclude negative effect of EF-C on hematopoietic stem cell differentiation capacity, our colleagues from Institute for Clinical Transfusion Medicine and Immunogenetics (DRK Blood Service Baden-Württemberg, Ulm, Germany) examined the CD34+ cells in Colony Forming Cell Assays. The results showed that EF-C did not affect differentiation capacity of human stem cells (Yolamanova, Meier et al., 2013). Thus, in contrast to the former enhancers, it can be used for gene transfer into sensitive primary human cells, such as macrophages or hematopoietic stem cells. Lack of cytotoxicity in these cell types has also been reported for amyloid SEVI fibrils (Wurm et al., 2011). When compared to RetroNectin EF-C fibrils can be used to promote viral gene transfer in a more flexible and simple manner, because it is/was added to the virions or to the target cell culture prior to transduction. Usage of EF-C nanofibrils instead of RetroNectin reduces the workflow from 100 minutes (not including the overnight incubation after coating and the second spin-infection step) to 10 minutes. Notably the highest transduction efficiencies were observed when RetroNectin and EF-C nanofibrils were combined. Thus, treatment with EF-C has significant advantages over other methods that are currently employed to increase lentiviral and retroviral transduction efficiencies, EF-C fibrils have no effect on cell viability of the transduced cells and differentiation capacity of stem cells and might be suitable tool for gene therapy.

In mice studies, which were carried out in the Department of Experimental Dermatology and Allergic Diseases (Ulm University, Ulm, Germany), the effects of EF-C nanofibrils and RetroNectin on viral gene delivery were compared. For this, lineage depleted bone marrow cells were transduced ex vivo (i) in the absence of enhancers; (ii) using a commonly used multi-step RetroNectin protocol including a spin transduction (Zhou et al., 2001); (iii) or a one-step approach using EF-C-treated virus. The results showed that RetroNectin and EF-C fibrils increased transduction rates from 3.1 % in the control to 23.9 % and 18.9 %, respectively. Transplantation of these cells into recipient mice showed no obvious side effects, and engraftment was determined by blood cell analysis 4 and 12 weeks later. The results showed a significantly higher proportion of transduced cells in the RetroNectin (19.7 % and 12.3 %) and EF-C (13.5 % and 6.1 %) groups compared to the control mice (0.1 %) (Yolamanova, Meier et al., 2013). Notably, brief EF-C treatment resulted in almost similar transduction and engraftment rates as the multi-step RetroNectin protocol, which involves an additional spin-transduction step and thus exposure of the cells to twice as much virus. Taken together, EF-C represents a convenient and effective tool to boost lentiviral and retroviral gene delivery, to concentrate virions, and to remove toxic
compounds that may affect the functionality of the target cells, and is suitable for ex vivo gene therapy applications.

A possible drawback of EF-C for clinical application is that it attached to and was taken up by the cells and would thus be transferred to the patients. However, in the mice studies we did not observe any side effects after transplantation of EF-C treated cells over 12 weeks of follow-up. The lack of toxicity in cell culture suggests that the amyloid fibrils may just be degraded without causing undesired effects. The possible induction of antibodies to EF-C cannot be excluded but would not represent a major caveat because most clinical gene transfer studies aim for a single treatment. However, follow-up studies on the fate of nanofibrils in the cells or tissues seem highly necessary.

4.2. Analysis of the mechanism of fibril-mediated enhancement of viral infection.

Beside the application of amyloidogenic fibrils as efficient transduction enhancer, the mechanism of fibril-mediated enhancement of viral infection is of great interest. It is highly likely that EF-C fibrils promote virion attachment by serving as a bridge between the virions and the cells allowing them to overcome the repulsion between the negatively charged membranes as it has been proposed for cationic compounds like polybrene and protamine sulphate (Davis et al., 2002). Indeed, it has been previously established that the cationic properties of SEVI and other semen-derived amyloidogenic peptides are necessary for efficiently promoting virus infection (Münch, et al, 2007; Roan et al., 2009; Arnold et al., 2012). Roan et al. showed that a negatively charged SEVI variant, in which lysines and arginines were replaced by alanines, was capable to form amyloid fibrils, but does not efficiently enhance HIV-1 infection (Roan et al., 2009). Moreover, addition of polyanionic compounds like heparin to fibrils or to the targets cells abrogate the enhancing activity of SEVI and other semen-derived fibrils as heparin shields the charged surface of the fibrils (Roan et al., 2009; Arnold et al., 2012). Recently, Zhang et al. identified another 13-residue peptide in the HIV-1 gp120 glycoprotein which also form amyloid fibrils and augment HIV-1 infection. They termed this peptide P13 and extend them with three lysine amino acids at its C-terminus to increase its cationic charge (Zhang et al., 2013). The modified peptide was designated as P16 and exhibited a pI of 10.48 and a net charge of +3. It has been also observed that mutation form of P16, which contained alanine residues instead of tryptophan, formed fibrils but much less enhanced infection. Furthermore, anionic polymer completely blocked the activity of peptide to boost virus infection. These
4. DISCUSSION

interesting observations also show that electrostatic interaction is driving in fibril mediated HIV infectivity enhancement.

Indeed the EF-C sequence contains positively charged basic residues and has a net charge of +2 and an isoelectric point of 9.9. Zeta potential measurements revealed that EF-C nanofibrils display a positively charged surface (+17.7±1.7 mV). Furthermore, our colleagues from Institute of polymer science (University of Ulm, Germany) constructed a structural model of the peptide nanofibrils. The resulting fibril model suggests that the lysine side chain forms a hydrophilic surface with a high density of cationic charges at physiological pH (Yolamanova, Meyer et al., 2013). Besides, it is conceivable, that the magnitude of the enhancing effect will depend on the intrinsic capability of both virions and cells to interact. The results derived from pseudotyped experiment with lentivirus based virion showed that EF-C fibrils had only a moderate effect on transduction rates of VSV-G carrying particles (Fig. 17a). The similar results were observed in the same experiments with SEVI (Münch J, personal communication). I hypothesised that amount of glycopolymers on the viral envelope can play substantial role in the mechanism of enhancing activity of amyloid fibrils. HIV-1 virions carry usually only 8 to 10 Env trimers per virus particle (Zhu et al., 2003), whereas about 400 envelope glycoprotein incorporated in the VSV-G particle (Brown et al., 2010). The high density of glycopolymers on the surface of VSV-G particle probably shields the negatively charge of phospholipid bilayer of virus and makes the amyloid fibrils less effective for enhance infection. It would be interesting to control in additional experiments whether the degree of virus infection enhancement indeed correlates with amount of envelope glycopolymers. For this purpose pseudopolymers with low, median and high amounts of VSV-G glycoprotein should be generated and used for comparative analyses with amyloidogenic peptides.

Noteworthy, cationic properties do not seem to be an absolute requirement for the enhancement of HIV infection by amyloid fibrils. Another interesting aspect in the mechanism of enhancement activity is the self-assemble of peptide into fibrils. It has previously been shown that amyloid fibrils in semen promote HIV-1 infection, whereas the monomeric peptide has no effect (Münch et al., 2007; Roan et al., 2011; Arnold et al., 2012). Albeit the EF-C peptide possesses a positive net charge, H2O or PBS dissolved EF-C had no effect on infection. However dilution of DMSO-dissolved peptide in H2O or PBS potently enhanced infection and transduction rates, this solution became turbid and formation of fibrils could be observed by Thioflavin T binding and Congo red staining. Existence of amyloid fibrils was further confirmed by atomic force microscopy, X-Ray
powder diffraction and circular dichroism spectroscopy (Yolamanova, Meyer et al., 2013). Like SEVI, EF-C adopts a classic cross β amyloid structure in which β-sheets run perpendicular to the axis of the fibrils. This indicates that in addition to cationic properties of EF-C, fibril formation is a prerequisite for EF-C enhancement activity. Moreover, EF-C is usually even more effective than SEVI in boosting retro- and lentiviral gene transfer. The reason for this is most likely that EF-C consists of small amyloid nanofibrils, whereas SEVI forms large amyloid fibrils that may be too immotile to make efficient contact with the target cells. Interestingly, negatively charged SEVI was capable to slightly enhance virus infection (Roan et al., 2009). This can be explained by formation of a fibril network, which captures the virus via hydrophobic interaction. Fibrils-virus complexes, which due to their large size rapidly sediment onto the target cells, increase the rate of infection. Since the cross β-sheet structure is common to all amyloid proteins but the ability to enhance viral infection possess not all amyloid fibers even with positively charge surface. For example, bacterial amyloid curly protein which also formed positively charged fibrils (net charge of +5, pI of 9.9) do not enhance viral infection (Hartmann et al., 2012). Hence, enhancement of virus infection rates is an intrinsic property of amyloid structure.

An interesting question is whether it is just coincidence that gp120 fragments form fibrilar virion attachment factors or whether HIV-1 generates its own enhancer to facilitate viral spread. It has been estimated that the sum of soluble, cell- and virion-associated gp120 in HIV-infected individuals reaches up to 5 µg per ml blood (Cummins et al., 2010). Even if all gp120 molecules are degraded to EF-C fragments, this would only result in concentrations of approximately 50 ng/ml of the fibrils, which is far below those required to enhance HIV infection in vitro. As mentioned above fibril formation is a prerequisite for EF-C mediated transduction enhancement. For fibril formation EF-C has to be dissolved first in organic solvent, conditions that are not found in vivo. Altogether, it seems unlikely that gp120 fragments play a significant role for HIV-1 replication in vivo. Other amyloids, however, may well play a relevant role in virus spread and the pathogenesis of AIDS because amyloid deposits are fairly common (Green et al., 2005) and β-amyloid fibrils associated with Alzheimer’s or other diseases also promote HIV-1 infection (Wojtowicz et al., 2002).

In sum, EF-C is an effective, simple, and inexpensive tool to boost viral gene delivery into various cell types. Moreover, this peptide has great potential as enhancer of viral transduction because it can be easily modified with various functional groups that raise its natural ability to self-assemble into nanofibrils and increase their stability in vivo models.
Therefore application of EF-C and its derivatives will significantly facilitate the utilization of retro- and lentiviral vectors in basic research and potentially in the treatment of human diseases.
5. SUMMARY

Low transduction efficiencies are a common problem in retro- and lentiviral gene transfer. Here, I have presented a new approach to boost retroviral gene transfer using self-assembled nanofibrils formed by the 12-amino acid peptide termed EF-C (Enhancement Fragment C). This peptide was derived from the HIV-1 external envelope glycoprotein gp120. The fibrils form instantaneously upon dilution of peptide in aqueous media and adopt a typical amyloid structure that was observed due to Thioflavin T and Congo Red staining and atomic force microscopy. Measurement of $\zeta$-potential charge of aqueous solution of EF-C revealed that the nanofibrils have positively charged surface, supposed that their enhance infection by neutralizing the repulsion between the negatively charged viral and cellular membranes. My data obtained using confocal fluorescence microscopy shown that fibrils bind virions and increase their adsorption to cells and subsequent infection. Addition of EF-C to virus stocks or target cells boosted gene delivery into multiple cell types, including primary T cells, macrophages and CD34+ stem cells, and was substantially more effective than other reagents commonly used to increase transduction. Moreover, like RetroNection, the most commonly used reagent to increase retroviral transduction, EF-C potently boosts transduction also when immobilized on cell culture dishes. This effect was confirmed with retro- and lentiviral particles carrying various viral envelope proteins frequently used for gene transfer and therapy. The enhancing effect was independent of the viral glycoprotein but still required the respective cellular receptor(s). Furthermore, the fibrils allow a fast and convenient concentration of virions without the need of ultracentrifugation. The result presented in this thesis demonstrate that EF-C nanofibrils provide a convenient, flexible and effective means to increase retro- and lentiviral gene transfer in basic research and clinical applications.
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