Exploiting high-throughput screens to optimize Adeno-Associated Viral Vectors for gene transfer into primary human keratinocytes

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Der Ursprung der Wissenschaft liegt im Wissen, dass wir nichts wissen.

Fernando Pessoa (*13.06.1888 - 30.11.1935*)

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Zusammenfassung

Die Heilung chronischer Wunden, wie z.B. diabetischer Ulzera oder großflächiger Verbrennungswunden, stellt ein nicht unerhebliches medizinisches Problem dar. Der Heilungsprozess kann sehr langwierig und schmerzvoll sein und schränkt dadurch die Lebensqualität der Patienten massiv ein. Mit den traditionellen Vorgehensweisen und Maßnahmen zur Behandlung akuter Erkrankungen allein kann auf Grund der Vielzahl negativer Einflussmöglichkeiten kein optimales Ergebnis erzielt werden. Daher ist die Einführung neuer, innovativer, therapeutischer Strategien von Nöten, wie zum Beispiel die Verwendung von primären humanen Keratinozyten für die Herstellung autologer Hauttransplantate. Gentherapeutische Vektorsysteme könnten das Anwachsen von Hauttransplantaten durch z.B. gezielte, aber transiente Bereitstellung von Wachstumsfaktoren mittels Gentransfer verbessern. Rekombinante adeno-assoziierte Virus Vektoren (rAAV) wären hierfür ein potentiell geeignetes System. Sie sind wenig immunogen und stabil, lassen sich mit hohen Titern herstellen und sind als nicht-integrierende Vektoren in proliferierenden Zellen nur transient vorhanden. Allerdings scheint die Haut ein schlechtes Zielorgan für AAV Vektoren des Serotypes 2, sowie pseudotypisierte AAV Vektoren mit Kapsiden anderer AAV Serotypen zu sein, da sich primäre humane Keratinozyten nur unzureichend von AAV transduzieren lassen. Ein Grund hierfür wurde im Rahmen dieser Arbeit gefunden. Es konnte gezeigt werden, dass primäre humane Keratinozyten den AAV2-Primärrezeptor Heparansulfat-Proteoglykan (HSPG) nur unzureichend oder gar nicht exprimieren.

Kürzlich wurde demonstriert, dass die genetische Modifizierung des AAV-Kapsids durch Insertion rezeptorspezifischer Liganden („AAV targeting“) die Transduktion von Zellen unabhängig vom Vorhandensein der natürlichen AAV-Rezeptoren ermöglicht. Die „AAV-targeting“-Technologie bietet einen möglichen Lösungsansatz um spezifische rAAV2-Targeting-Vektoren für primäre humane Keratinozyten zu generieren.

Im Rahmen dieser Arbeit wurden neue, vielversprechende rAAV Vektoren für die Modifikation primärer humaner Keratinozyten generiert. Mit Hilfe einer „AAV

peptide display“ Bibliothek wurden drei rAAV Peptidinsertionsmutanten (Kera1, Kera2 und Kera3), die sich in der inserierten Sequenz unterscheiden, selektioniert. Die AAV2-Bibliothek besteht aus Mutanten, die 7-mer Peptide mit zufälliger Sequenz im Kapsid in der Position 587 präsentieren. Um „targeting“-Vektoren mit einem veränderten Tropismus zu generieren, wurde die AAV-Bibliothek optimiert, indem Mutanten die an HSPG binden können vor der Selektion durch Heparinaffinitätschromatographie abgereichert wurden. Eine weitere Optimierung des Selektionsschemas wurde durch die Verwendung von verschiedenen Keratinozyten-Spendern in jeder Selektionsrunde erzielt. Das erhöhte die Wahrscheinlichkeit Mutanten mit Spezifität für einen allgemeingültigen Rezeptor für primäre humane Keratinozyten zu selektionieren. Die auf diese Weise selektionierten Mutanten Kera1 (RGDTATL), Kera2 (PRGDLAP) und Kera3 (RGDQQSL) weisen eine außergewöhnliche Änderung des Tropismus auf. Sie transduzieren primäre humane Keratinozyten mit einer hohen Effizienz und Spezifität, was selbst in Mischkultur-Experimenten mit Nicht-Ziel-Zellen zu einer präferentiellen Transduktion von Keratinozyten führte. In dieser Arbeit wurde zudem erstmalig die neue bioinformatische Methode der komparativen Genanalyse (CGA) zur Identifizierung des Ziel-Rezeptors eines rAAV-targeting Vektors angewandt. In Kooperation mit Giovanni Di Pasquale (NCI/NIH, Bethesda, USA) wurde zu diesem Zweck ein Zellscreening auf der NIH Zelllliniensammlung durchgeführt. Für die Mutante Kera2 konnte mit Hilfe dieses Verfahren eine hohe Affinität zu dem Integrin-Rezeptor beta8 festgestellt werden. Die Integrin beta8 Untereinheit bildet mit der Integrin alpha V Untereinheit ein Heterodimer. Das Intergin $\alpha_V\beta_8$ wird tatsächlich auf der Oberfläche von primären Keratinozyten expressioniert. Durch Experimente mit blockierenden α_V- oder $\alpha_V\beta_8$-Antikörpern konnte nachgewiesen werden, dass das Integrin $\alpha_V\beta_8$ als Rezeptor für Kera2 fungiert.

Außerdem war es möglich differenzierte Keratinozyten einer 3D Kultur nach topischer Anwendung der „targeting“-Vektoren Kera1, Kera2 und Kera3 zu transduzieren. Zusammenfassend lässt sich sagen, dass die drei in dieser Arbeit entwickelten und charakterisierten „targeting“-Vektoren Kera1, Kera2 und Kera3 Schlüsselfunktionen für die klinische Anwendung erfüllen.

Abstract

Chronic non-healing wounds such as diabetic ulcers or burns represent a devastating health problem with significant clinical, physical and social implications. The healing can be frustrating and painful for patients. The difficult healing process requires advanced therapeutic strategies such as the use of primary human keratinocytes (HK) as autologous transplants, which may be considered for clinical use. To improve engraftment or to introduce therapeutic genes into primary HK, efficient and safe vectors are required. One of the most promising vector systems today is based on the adeno-associated virus (AAV), a member of the parvovirus family. Recombinant AAV (rAAV) vectors possess a number of attractive properties including low immunogenicity, high stability and the potential to integrate site-specifically without known side-effects. Unfortunately, cell entry into primary HK of rAAV2 is barely detectable and consequentially, HK are poor targets of rAAV2-mediated transductions. As demonstrated in this thesis, primary HK do not express AAV2´s primary receptor heparan sulphate proteoglycan (HSPG), the presence of which, however, is required for binding to AAV2´s internalization receptors. Cell surface targeting allows re-directing the viral vector tropism towards a novel receptor mediating thereby transduction of cells in absence of AAV's natural receptors. These AAV capsid mutants have displayed improved transduction efficiency in wild-type-AAV non-permissive cells and have provided the opportunity of rAAV-mediated, cell-type-specific gene transfer.

As documented in this study, new rAAV vectors were developed as promising tools for modifying primary HK. Using an AAV peptide display library that displayed 7mer peptides of random sequence at capsid position 587; three AAV peptide insertion mutants differing in sequence of inserted ligand (Kera1, Kera2 and Kera3) were selected and subsequently analyzed. To select rAAV targeting vectors with a re-directed tropism, the library was optimized by depleting mutants capable of binding to HSPG prior to selection by heparin affinity chromatography. Furthermore, the selection was performed on primary HK obtained from different donors to target a common receptor and the selection pressure was continuously increased by decreasing the vector genomes per cell ratio to select for the fittest variant. The thereby developed rAAV targeting vectors Kera1 (RGDTATL), Kera2

(PRGDLAP) and Kera3 (RGDQQSL) showed a remarkable change in tropism, transducing primary HK with high efficiency and specificity even in mixed cultures of target and non-target cells. In this study, a novel microarray based bioinformatic approach (comparative gene analysis (CGA)), was used for the identification of the receptor that targeted the mutant that showed the most striking change in tropism, Kera2. Briefly, in cooperation with Giovanni Di Pasquale (NCI/NIH, Bethesda, USA), a screening of the NIH cell line panel was performed, pointing towards the involvement of beta8 integrin subunit for cell transduction by Kera2. Beta8 is unique as it is solely described as heterodimer with alpha V and the integrin $\alpha_V\beta_8$ could be detected on cell surface of primary human keratinocytes. By blocking experiments with blocking α_V- or $\alpha_V\beta_8$-antibodies experimental evidence was provided that the integrin $\alpha_V\beta_8$ serves as receptor for Kera2. Finally, this study has shown that the targeting vectors Kera1, Kera2 and Kera3 transduced airlifted differentiated keratinocytes in organotypic 3D cultures. In summary, the three rAAV targeting vectors Kera1, Kera2 and Kera3, selected from an optimized library and using a novel selection strategy, are excellent candidates for successful application in clinical use.

1 Introduction

1.1 Adeno Associated Virus (AAV)

Adeno-Associated Viruses (AAVs) belong to the genera of *Dependovirus* and the subfamily *Parvovirinae* that infects vertebrates. *Parvovirinae* together with the insect-infecting *Densovirinae* form the family of *Parvoviridae.* These include viruses with a linear, single-stranded DNA genome of about 4.7 kb and a non-enveloped icosahedric capsid of 18-30 nm in diameter [1].

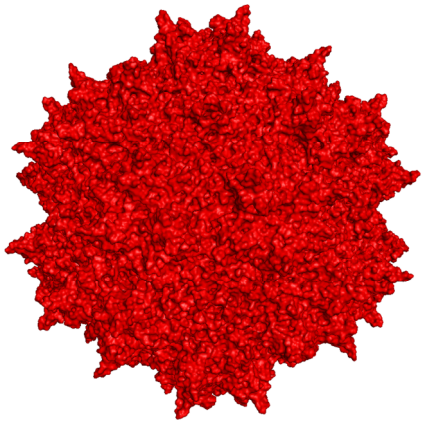


Figure 1: Atomic structure of AAV serotype 2. Figure was kindly provided by J. Boucas.

AAV were first described in 1965 as DNA-containing particles in preparations of a simian adenovirus [2]. Later, AAV was defined as a unique virus family. For replication and initiation of a productive infection cycle, AAV is, as the name implicates, dependent on co-infection by a helper virus. Known helper viruses are Adenoviruses, Herpes Simplex Viruses, human Cytomegaloviruses (CMV) and Papillomaviruses [3], [4]. In absence of co-infection with a helper virus, AAV establishes a latent infection where the viral DNA is either maintained as episomes or integrated into the host genome. Integration occurs in 70% of cases, site-specific into the human chromosome 19 at position 13.4-qter (AAVS1) [5], [6], [7]. After super-infection with a helper virus, the provirus enters the lytic cycle, leading to viral gene expression, rescue and replication of the AAV genome with subsequent production of viral progeny (see 1.1.2), [8]. Thus far, 12 different serotypes (AAV1-12) and over 100 variants of AAV have been isolated from

adenoviral isolates and tissue samples [4]. They differ in the amino acid (aa) composition of their capsids, but show similar capsid morphology, genome length and genome organization. AAV serotype 2 (AAV2), the best-characterized serotype, is frequently applied in human gene therapy [9].

1.1.1 Viral genome and AAV proteins

The single stranded DNA genome of AAV2 contains four functional units, the open reading frame (ORF) for the Rep proteins *(rep)*, the *cap* ORF (ORF1), the alternative *cap* ORF (ORF2) and the inverted terminal repeats (ITR) flanking these ORFs (Figure 2). The alternative *cap* ORF was just recently discovered and encodes a 23 kDa protein, which was named assembly-activating protein (AAP), required for initiation of capsid formation [34].

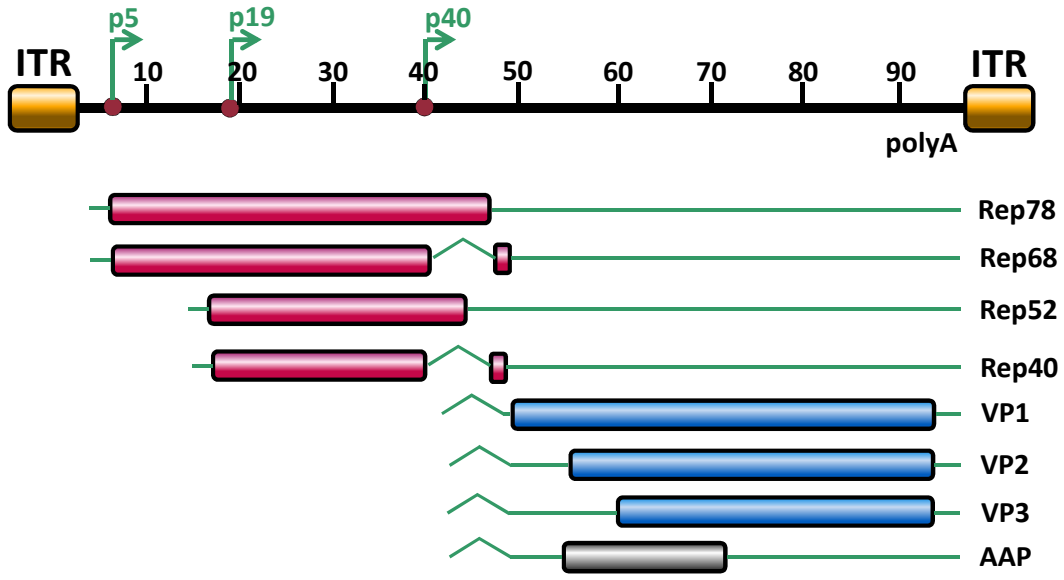


Figure 2: Genome organization of AAV2
The AAV2 genome is flanked by the ITRs, spans 4680 nt divided into 100 map units. Shown are the three promoters p5, p19 and p40 at map position 5, 19 and 40 and the polyadenylation signal (polyA) at position 96. The open reading frames are indicated by rectangles, translated regions in red, blue or grey, untranslated regions by thin solid lines, while introns are marked as nicks. The p5 promoter controls expression of the large Rep proteins (Rep78, Rep68), while the p19 promoter is responsible for expression of the small Rep proteins (Rep52, Rep40). Rep68 and Rep40 are splice variants of Rep78 and Rep52, respectively. The expression of capsid proteins VP1, VP2, VP3 and AAP is controlled by the p40 promoter. Figure was kindly provided by N. Huttner [10] and modified according to F. Sonntag [11].

The genome contains three promoters (p5, p19 and p40) and a single polyadenylation signal (poly A). The 5´-ORF *rep* encodes four *Rep* proteins. These are multifunctional, non-structural proteins that are termed by their molecular

weights (Rep78, Rep68, Rep52, and Rep40). Transcription of the larger Rep proteins (Rep78, Rep68) is controlled by the p5 promotor, while the smaller ones (Rep52, Rep40) are transcribed by the p19 promotor [12]. Rep68 is a splice variant of Rep78 and Rep40 is a splice variant of Rep52. The larger Rep proteins are necessary for site-specific integration into AAV2, thus they possess site- and strand-specific endonuclease activity. In addition, they are required for transcription of the viral ORFs, control of viral replication (see below) and packaging of the viral genome. Specifically, Rep78 and 68 possess DNA binding, ATPase, DNA helicase, and endonuclease activities [13], [14], [15], [16], [17], while the Rep proteins are involved in accumulation and packaging of the single-stranded DNA genome into the preformed capsid [18], [19]. Furthermore, all Rep proteins contain in the common C-terminal part a nuclear localization signal (NLS) [15], [20]. The smaller Rep proteins seem to be involved in accumulation and packaging of single-stranded DNA into the preformed capsid [18], [19].

The 3´-located ORFs encode the capsid proteins, VP1, VP2, VP3 and AAP, the latter of which is required for viral capsid assembly. The VP proteins are expressed from ORF1, while ORF2 encodes for AAP [11]. Expression of the AAV2 capsid proteins is controlled by the p40 promoter. The three VP proteins assemble the viral capsid in a 1:1:10 ratio [21]. The capsid proteins VP1 and VP2 share identical sequences at the C-terminus but differ in their N-terminal sequences. The translation of VP1 is regulated by alternative splicing of the p40 -transcripts [22].

Translation of VP2 is initiated from an alternative start codon (ACG) [23]. All three VP proteins use the same stop codon. The molecular weights of the capsid proteins are 90 kDa (VP1), 72 kDa (VP2) and 60 kDa (VP3). All capsid proteins are required for the formation of infectious particles, while intact non-infectious capsids assemble in absence of VP1 or in absence of VP1 and VP2, when AAP is present to mediate nuclear transport of VP3 [11]. VP2 seems to be dispensable for the formation of infectious particles, at least in an *in vitro* application [24], [25]. The capsid formation takes place in the cell nucleus [26], [27].

The 5´- and the 3´-end of the viral genome are formed by the ITRs consisting of 145 nt. Due to their palindromic sequence, a hairpin structure is formed by the first 125 bp [28], [29]. The ITRs serve as signal sequence – recognized by the viral Rep proteins - for packaging of the viral genomes into the capsid. In addition, the ITRs serve as origin of replication (ori). For this function, a Rep binding site (RBS),

a specific cleavage site for Rep proteins (terminal resolution site, TRS) and a certain distance between the former two sites are required [14], [30], [31]. The ITRs play a key role in the site-specific integration into AAVS1, as well as in the subsequent rescue of viral DNA from the integrated state in the presence of helper viruses [32], [33], [34], [35].

1.1.2 AAV infectious biology

A successful infection of cells by AAV is a multistep process including attachment, uptake, intracellular trafficking, nuclear translocation and replication of the virus (Figure 3). Many steps of the AAV-cell interaction are still unknown. As single virus tracing studies have revealed, AAV2 contacts the cell membrane several times before entering the cell. On average, AAV contacts the cell 4.4 times [36]. For AAV2, the widely expressed cell surface receptor heparan sulfate proteoglycan (HSPG) has been identified as primary receptor [37]. This contact is mediated by binding motives present on the AAV capsid, that are formed by residues R484, R487, K532, R585 and R588 in the common VP3 region [38]. Binding to HSPG is believed to induce a conformational change in the capsid, which is required for internalization into the cell [39]. For efficient internalization, co-receptors are required. So far, five co-receptors have been described for AAV2. Human fibroblast growth factor receptor 1 (FGFR-1), hepatocyte growth factor receptor (HGFR) and laminin receptor seem to support virus-cell interaction, facilitating the HSPG-induced structural rearrangement of the capsid [40], [41], [42]. The integrins $\alpha_V\beta_5$ and $\alpha_5\beta_1$ are thought to mediate endocytosis of AAV2 [39], [43]. In addition, integrin binding subsequently leads to the activation of the small GTPase Rac1 and phosphatidylinositol-3 kinase (PI3K), resulting in cytoskeletal rearrangements that promote clathrin-dependent internalization of AAV2 as well as trafficking of AAV2 from the cell periphery towards the nucleus [43], [44], [45], [46].

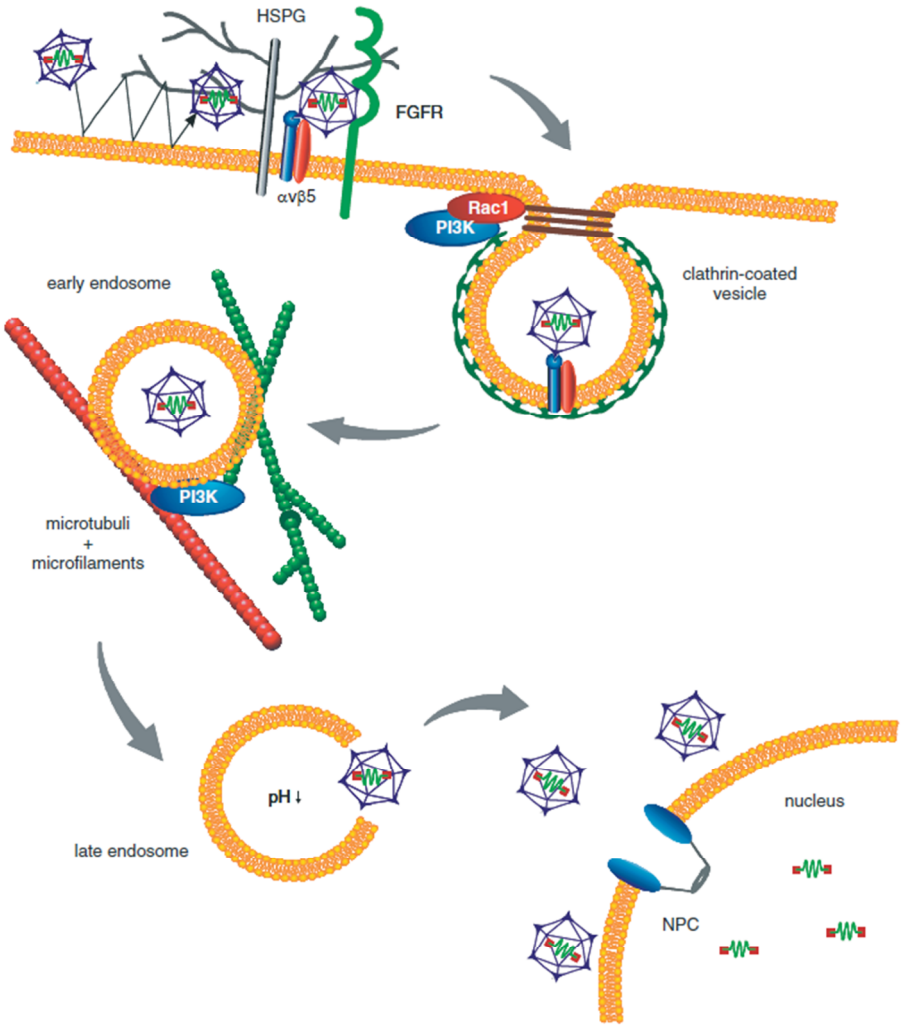


Figure 3: Infectious pathway of AAV2 in HeLa cells
Following multiple contacts with the cell, AAV binds to HSPG on the cell membrane. The attachment is likely enhanced by co-receptors such as FGFR1 and/or HGFR. Subsequent binding to integrins lead to endocytosis via clathrin-coated pits. Integrin binding activates the small GTP binding protein Rac1, which stimulates the PI3K pathway. The resulting rearrangement of the cytoskeleton allows for trafficking of AAV2-containing endosomes. Acidification of the endosome may lead to conformational changes in the AAV2 capsid and its release. Once inside the nucleus the AAV genome is replicated (lytic phase; requires the presence of helper virus), stays episomally or is integrated into the host genome (latent phase) [47]. NPC: nuclear pore complex. Picture was kindly provided by H. Büning © 2008

Once internalized, AAV is trafficked mainly inside endosomes [36], [44], [45], [48], [49], [50]. The transport of the endosomal vesicle takes place via motor proteins along microtubules and microfilaments [43], [44], [45]. AAV particles remain in the endosomal compartment until late stages. When and how AAV escapes from the endosome is still subject of debate and may be cell type specific [45], [50]. Acidification inside the endosomes appears to be essential for priming AAV for nuclear entry. This assumption is based on the observation that microinjection of AAV2 particles directly into the cytoplasm (instead of natural infection) did not

result in gene expression [51]. The same effect can be reached by the addition of inhibitors of acidification like bafilomycin A1 or ammonium chloride [45]. The acidification of endosomes during maturation may lead to a conformational change of the viral capsid, leading to exposure of a phospholipase A2 (PLA_2) homology domain, present within the N-terminus of VP1 [52], [53]. The PLA_2 domain is conserved among parvoviruses [54] and AAV2 requires this domain for endosomal escape through lipolytic pore formation [53], [55]. When AAV2 is released from the endosome the capsids are target for ubiquitination, which is a general signal for proteasomal degradation [56]. Several groups have shown that the addition of proteasome inhibitors results in an enhancement of transgene expression at least in some cell lines [44], [57] [58], [59], [60]. Though, the mechanism remains unclear, studies suggested that, conceptually proteasome inhibitors block capsid degradation, facilitate vector uncoating and lead to an increased perinuclear accumulation or translocation into the nucleus [57], [56].

It is still unknown how the virus enters the nucleus and where viral uncoating occurs. Viral particles start to accumulate in the perinuclear area between 15 and 30 min post infection (p.i.) [45], [36]. The majority of these virions still have intact viral capsids containing viral genomes [25]. Several studies have reported of intact AAV particles in the nucleus. But there are controversial reports concerning the mechanism and efficiency of capsid import as well as their role in viral infection [25], [43], [45], [50], [61]. Lux *et al.* showed that when using a low number of virions for infection, viral genomes, but no intact capsids, are found within the nucleus, whereas intact full and empty capsids were still evident in the perinuclear area [25]. This study suggested that viral genomes rather than intact capsids are transported into the nucleus. In contrast, Sonntag and colleagues blocked AAV infection completely by injection of capsid specific antibodies into the nucleus. These results suggest that viral genomes are transferred into the nucleus by intact viral capsids and that the uncoating event takes place there [55]. Moreover, whether AAV and/or AAV genomes enter the nucleus through the nuclear pore complex (NPC) or in a NPC-independent way is still discussed [62].

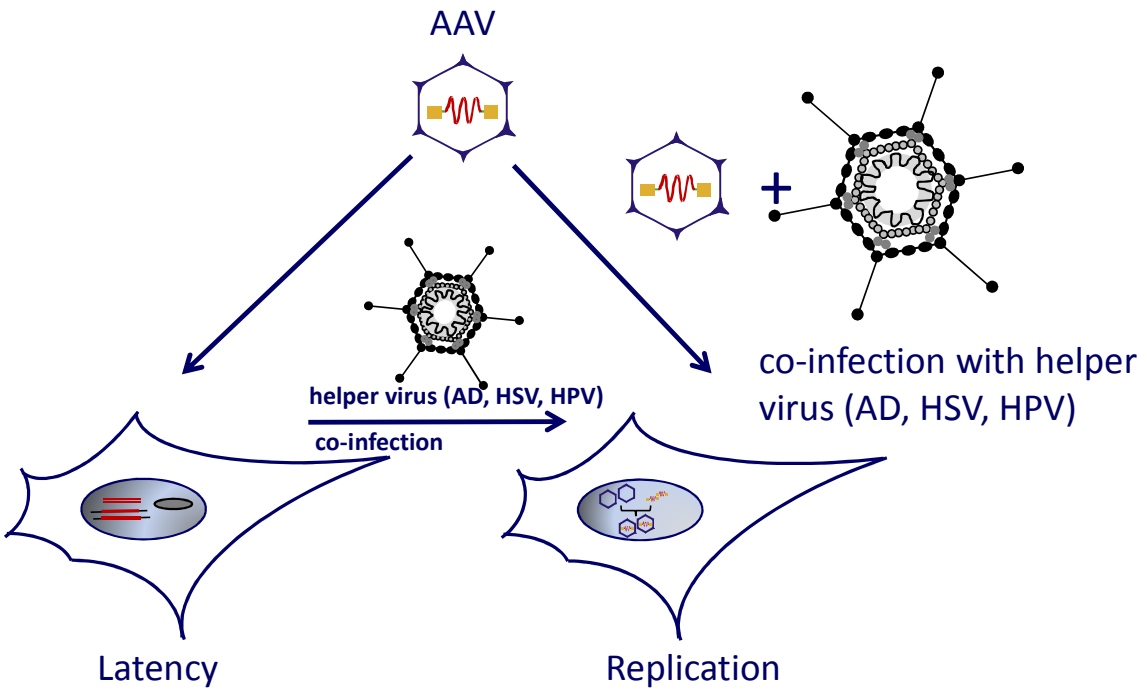


Figure 4: Schematic representation of latent and lytic life cycle of AAV2
Infection of cells with AAV, in the absence of a helper virus results in the establishment of a latent infection that is characterized by the persistence of viral DNA - frequently integrated within the host genome - and by absence of viral gene expression. In the presence of a helper virus, wild type AAV2 enters a productive cycle leading to the replication of viral DNA, expression of viral genes and packaging of viral DNA into pre-assembled capsids. AD = adenovirus, HSV herpes simplex virus, HPV = human papillomavirus

Inside the nucleus, the presence or absence of a helper virus determines whether AAV enters a lytic or latent life cycle. In the absence of helper viral functions second-strand synthesis of the single-stranded virus genome and the basal expression of the Rep proteins are activated [63], [64]. First, second-strand synthesis of the single-stranded virus genome and a basal expression of the Rep proteins are activated [63]. In presence of the large Rep proteins (Rep78, Rep68) and intact ITRs, integration occurs, although not exclusively, at the so-called AAVS1 site on the human chromosome 19 (19q13.3-qter) [65], [66]. The AAVS1 locus resides a Rep binding element (RBS) and a terminal resolution site (TRS) equivalent to the AAV genome [67], [68], [69]. Usually, proviral sequences are integrated as viral concatemers in a head-to-tail conformation [67]. Helper viral superinfection can rescue the integrated provirus initiating a lytic, productive life cycle (Figure 4), [8]. Alternatively, AAV genomes can form episomes, which at least in non-dividing cells, also results in a latent life cycle.

In the presence of a helper virus, AAV can undergo a productive infection. During viral replication, the 3'-OH end of the ITR serve as the primer for second-strand

synthesis [3]. The large Rep proteins unwind the ITR by their helicase activity, leading to exposure of the TRS, which is nicked by the Rep endonuclease enabling complete synthesis of the second-strand by switching templates [13], [63]. The single-stranded DNA is then converted into a parental duplex replicative form where production of viral progeny can proceed.

1.1.3 Adenovirus-free AAV production and recombinant AAV vectors (rAAV)

The structural properties of the AAV capsid allow for the production of recombinant viral particles that package a DNA genome of approximately 5 kb [70]. For the generation of rAAV vectors, all ORFs are deleted leaving only the ITR sequences of the parental virus. The ITRs are the solely required *cis* elements necessary for the production of viral particles (replication and packaging). The deleted ORF sequences are replaced by an exogenous DNA sequence (transgene expression cassette).

A successful approach to produce rAAV vectors at high titers for laboratory scale uses triple transfection of AAV vector, AAV helper and adenoviral helper plasmids (Figure 5A). The vector plasmid contains the transgene flanked by the ITRs. The AAV-specific ORF required in *trans*, *rep* and *cap/AAP*, are cloned onto the helper plasmid, which lacks the ITR sequences [34], [71]. These plasmids are co-transfected with the third plasmid carrying the essential adenoviral genes VA, E2A and E4, necessary for AAV replication (Figure 5B) [71], [72], [73]. For viral particle production, HEK293 cells, which are transgenic for the adenoviral genes E1a and E1b (also required for AAV progeny production), are commonly used. After transcription and translation of *rep* and *cap/AAP* proteins and replication of the vector genome, the vector genome is shuttled into preformed AAV capsids. Finally, vector particles are harvested and purified by density gradient centrifugation (CsCl or Iodixonal) and/or column chromatography [74], [75].

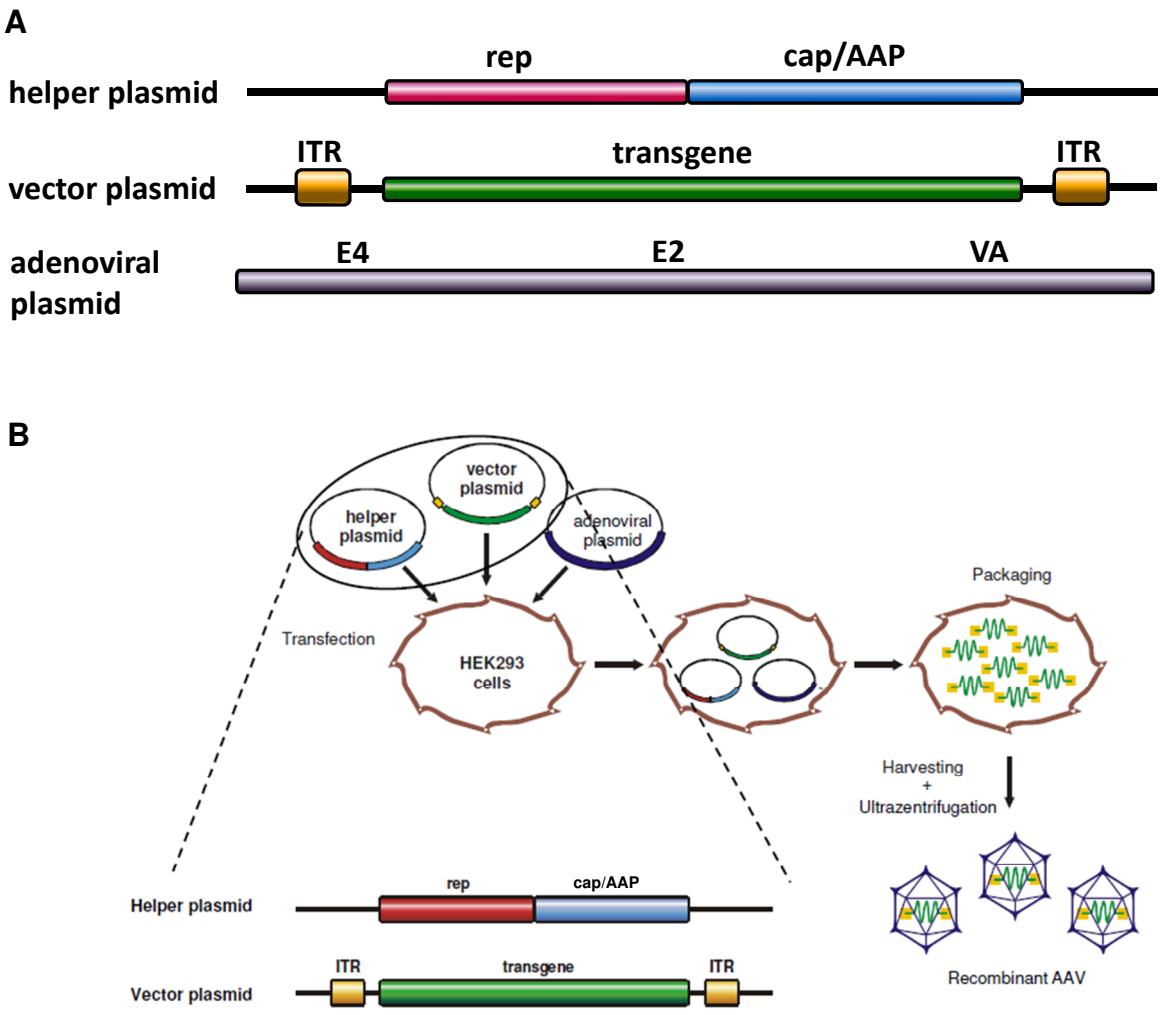


Figure 5: Packaging of recombinant AAV (rAAV) vectors
*(**A**) Plasmid constructs used for packaging of rAAV vectors. The vector plasmid is devoid of all viral genes, only the ITRs are left, which flank the transgene expression cassette ("transgene") and serve as packaging signal. The helper plasmid encodes for the non-structural, multifunctional Rep proteins (rep) and proteins required for capsid production (cap/AAP). These proteins are necessary for replication of the vector genome, production and assembly of the capsid and the subsequent packaging of the vector genome into preformed capsids. The adenoviral plasmid carries the essential adenoviral genes for rAAV production (VA, E2A and E4).*
*(**B**) Packaging of rAAV vectors. AAV vector plasmids, AAV helper plasmids, and adenoviral helper plasmids are transfected into HEK293 cells. After replication and assembly of viral vector particles, cells are lysed and vector particles are harvested and purified by e.g. iodixanol gradient centrifugation [47]. Figure A was kindly provided by N. Huttner and Figure B by H. Büning.*

1.2 AAV in Gene Therapy

Gene therapy is based on the idea of introducing genetic material into an organism in order to cure or improve the status of a disease [76]. A key factor for the success of gene therapy is the development of gene delivery systems that combine efficiency and safety. Currently, viral as well as non-viral vectors have been developed for this purpose. Whereas the viral systems include adeno-, retro-, vaccinia-, pox-, herpes simplex- and adeno-associated-viral vectors, the non-viral vector strategy uses naked DNA within lipoplexe or polyplexe [77], [78]. However, each vector has its own advantages and disadvantages. The simplest way of gene delivery is injecting naked DNA encoding the transgene expression cassette. But this strategy lacks efficiency [79]. Viral vector systems are very efficient at transferring DNA into host cells but are in general more immunogenic, more sophisticated to produce and are limited in the size of foreign DNA that can be delivered. AAV has many features that make it attractive for use as a gene therapy vector. Briefly, rAAV vectors are based on a non-pathogenic virus [80], [81] and transduce dividing as well as post-mitotic or quiescent cells [82], [83]. Furthermore, they show a broad tissue tropism infecting diverse organs such as brain, liver, muscle, lung, retina and heart [84], [85], [86], [87], [88]. Moreover, in non-dividing cells or tissues AAV mediates long-term expression without the need for integration. Examples of such tissues are muscle or liver where e.g. in a muscle-directed trial transgene expression was sustained for at least four years in a canine hemophilia B model [85]. Another important aspect that – as already mentioned – AAV in contrast to lenti- or retroviral vectors stays as episomes [89], [90], [91], reducing thereby the risk for insertional mutagenesis. Moreover, if integration is required, expression of Rep proteins can be exploited to direct AAV towards integration at AAVS1 [5], [10]. The immunological reactions to AAV are low comparing to adenovirus [92], [93]. As such, AAV have only a minimal inflammatory potential. Nevertheless, in a clinical trial of liver-directed gene transfer, re-direction of memory T cells caused failure of long-term gene expression [94]. Recently, our group demonstrated that primary human liver cells, like Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC) are capable of sensing AAV. The AAV capsid represents pathogen-associated molecular patterns (PAMPs) that are detected by the pattern recognition receptors (PPR) Toll-like

receptor-2 (TLR-2) [95] known to activate innate immune response. Minimizing this recognition will be a key to improving rAAV-mediated gene transfer and reducing side effects in clinical trials due to immune responses against rAAV [95].

Disadvantages of the AAV vector system include the small genome size limiting the coding capacity for transgenes including ITRs to approximately 5 kb [96] and the broad tissue tropism interfering with a cell-specific in *in vivo* gene transfer. To date, AAV vectors have been applied in over 80 clinical trials (Table 1).

Table 1: Examples of clinical trials using AAV gene transfer [97]

Disease	**Transgene product**	**Serotype**	**Route administration**	**Clinical trial**	**Clinical Trials. gov identifier**	**Refs**
AAV clinical trials for inherited disease						
α1antitrypsin deficiency	α1 antitrypsin	AAV2	Intramuscular	Phase I/II	NCT00377416	[98], [99]
		AAV1			NCT00430768	
Batten's disease	CLN2	AAV2	Direct intracranial administration	Phase I/II	NCT00151216	[100]
		AAVrh10			NCT01161576	
Canavan's disease	Aspartoacylase	AAV2	Direct intracranial administration	Phase I	NA	[101]
Cystic fibrosis	CFTR	AAV2	Direct instillation to maxillary sinus, bronchoscopy to right lower lobe, aerosol to whole lung	Phase I/II	NCT00004533	[102], [103], [104], [105]
Haemophilia B	FactorIX	AAV2	Intramuscular	Phase I/II	NCT00076557	[106], [107]
			Hepatic		NCT00515710	
		AAV8	Intravenous	Phase I/II	NCT00979238	
Muscular dystrophy: Duchenne	Microdystrophin	AAV1-AAV2 hybrid	Intramuscular	Phase I	NCT00428935	[108]
AAV clinical trials for acquired diseases						
Severe heart failure	SERCA2a	AAV1	Antegrade epicardial coronary artery infusion	Phase I/II	NCT00454818	[109]
		AAV6			NCT00534703	
Parkinson'sdisease	AADC	AAV2	Intracranial	Phase I/II	NCT00229736	[110], [111]
	GAD				NCT00643890, NCT00195143, NCT01301573	[112], [113]
	Neutrophin				NCT00252850, NCT00985517, NCT00400634	[114]

AADC, aromatic-L-amino-acid decarboxylase; AAV, adeno-associated virus; CFTR, cystic fibrosis transmembrane regulator; CLN2, also known as tripeptidyl peptidase 1 (TPP1); GAD, glutamic acid decarboxylase; SERCA2a, sarcoplasmic reticulum calcium ATPase 2a

Early published data dealt with the monogenic diseases cystic fibrosis and hemophilia B in gene therapy trials. Administration of the cystic fibrosis transmembrane conductance regulator (CFTR) as a transgene on the nasal sinus and bronchial epithelium resulted in an improvement of pulmonary function and partial correction of hyperinflammatory responses and electrophysiological defects [104], [105], [103]. AAV was approved for safe usage in these clinical settings as well as in the treatment of hemophilia B by intramuscular, intrahepatic or intravenous vector administration [115], [107], [106], [116]. Evidences for transduction were found in all patients of the muscle-directed study as well as the intravenous study and long-term expression of the therapeutic gene, coagulation factor IX (FIX), could be detected albeit at low levels.

Further success was achieved by Bainbridge *et al.*, Cideciyan *et al.* and Hauswirth *et al.*. They used AAV2-based *RPE65* gene replacement therapy to treat patients, afflicted with *RPE65* Leber congenital amaurosis. All three groups observed an increase in visual sensitivity [32], [117], [118].

In November 2012, the first AAV based gene therapy drug (Glybera®) was approved by regulatory authorities in Europe. This drug was developed by uniQure (former Amsterdam Medical Therapeutics) for treating patients suffering from lipoprotein lipase deficiency (LPLD). In 2004, Rip and colleagues reported on the rAAV1-lipoprotein lipase $(LPL)^{S447X}$ vector, which aims to introduce episomal copies of a functional LPL gene variant into muscle tissue of patients with LPLD [119], [120], [121]. After several interventional clinical studies, conducted in the Netherlands and in Canada, the therapy was judged to be successful, based on tolerance, safety and efficiency, and Glybera® was authorized for patients suffering from LPLD.

Despite these successes, AAV´s broad host range remains a challenge as higher vector doses have to be applied and only those transgenes that do not harm the patient when expresses off-target are applied. In case of cancer therapy with suicide genes e.g., unspecific transduction of neighboring tissue would cause severe damage [122]. The specificity is not only important because of safety aspects but also helpful in reducing the number of particles required to be delivered [122], [123].

1.2.1 Improvements of naturally occurring AAVs

Increasing the efficiency of vectors is possible by modifying the viral tropism through capsid engineering, improving thereby gene delivery properties. There are different methods for modification of the viral capsid (Figure 6).

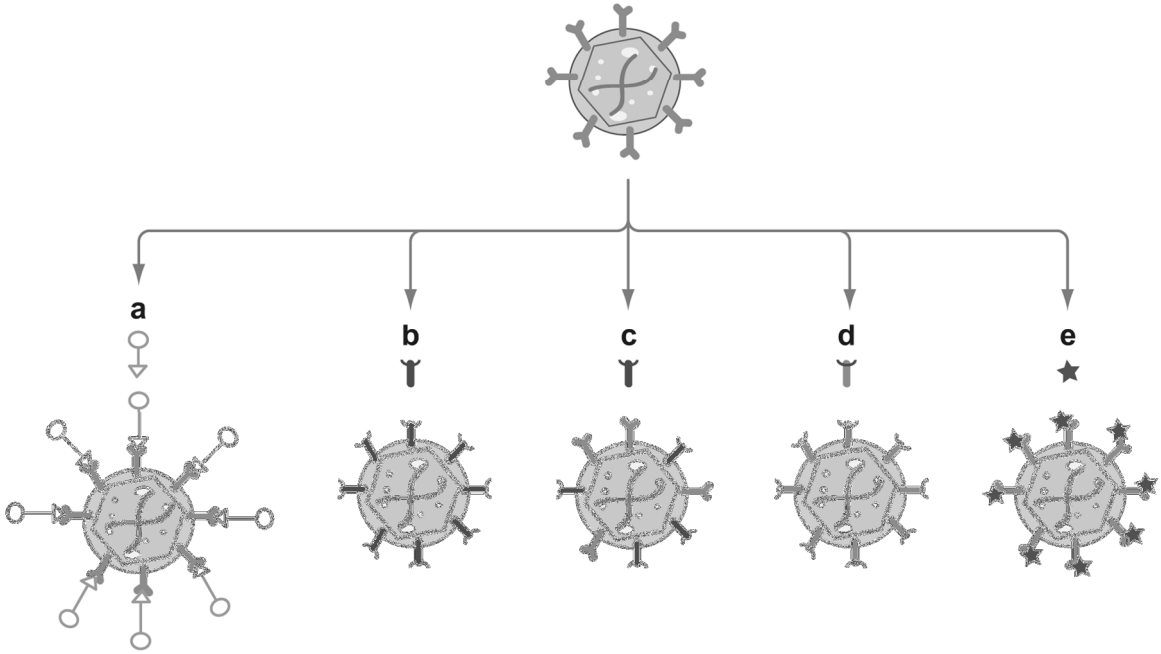


Figure 6: Overview for modifications of viral capsids
Rational design methods include (a) the use of a bispecific adaptor, (b) pseudotyping with an alternate serotype capsid (c, d) the generation of mosaic or chimeric particles, and (e) genetic engineering of the capsid sequence by peptide insertion or point mutations. Adapted by permission of Annual Reviews, Inc: Annual review of biomedical engineering [124] © 1999.

1.2.1.1 Mosaic rAAV vectors

A possible method to change the feature and to expand the tropism of rAAV vectors is the combination of capsid proteins from different serotypes resulting in viral capsids that accumulates the attributes of the respective serotypes [47], [125]. For example, an AAV1/AAV2 mosaic vector achieved gene expression levels similar to those of AAV1 in muscle and AAV2 in liver and could be purified by Heparin affinity chromatography like wild-type AAV2 [126]. However, since these vectors are produced by transfection of plasmids encoding the capsid proteins of the different serotypes, such viral preparations consist of virions with non-uniform capsid compositions, which in turn make standardization of this technology difficult [47], [125].

1.2.1.2 Chimeric rAAV vectors

Chimeric rAAV vectors contain capsid proteins that have been modified by domain or aa swapping between different serotypes [125]. Bowels and colleagues generated isolated virions, co-transfected by a non-functional, HSPG-deficient AAV2 capsid mutant and an AAV3 capsid sequence in AAV replication supporting cells. This allowed for the rescue of chimeric functional viruses from these cells, which showed HSPG binding ability (the parental AAV variant was deficient in HSPG binding) and transduced the target cells [127].

1.2.1.3 Pseudotyped rAAV vectors

Pseudotyping is the process of producing viral particles that incorporate foreign viral proteins. A pseudotyped AAV vector containing the ITRs of serotype X encapsulated with the proteins of serotype Y and will be designated as AAVX/Y. For example, a vector plasmid carrying a transgene flanked by AAV2 ITRs is co-transfected with an AAV helper plasmid coding simultaneously for Rep proteins derived from AAV2 and for capsid proteins and AAP from the serotype of choice [47]. Initial studies testing these vectors for gene delivery demonstrated far superior transduction efficiency for retina with AAV4 and AAV5 in comparison to AAV2 [128], [129], [130]. This method leads to broadening the viral tropism and may circumvent pre-existing immunity to one serotype by using a different capsid [131]

1.2.2 Generation of rAAV targeting vectors with increased transduction efficiencies

The possibility to engineer viral particles displaying selective binding domains that enable stringent interaction with target cell specific receptors (vector targeting) is desirable. Vector targeting allows the transduction of cell types that are refractory to infection with natural occurring AAVs [47]. Two main strategies have been used to achieve an altered tropism of AAV in the past; non-genetic (indirect) targeting and genetic (direct) targeting (Figure 7).

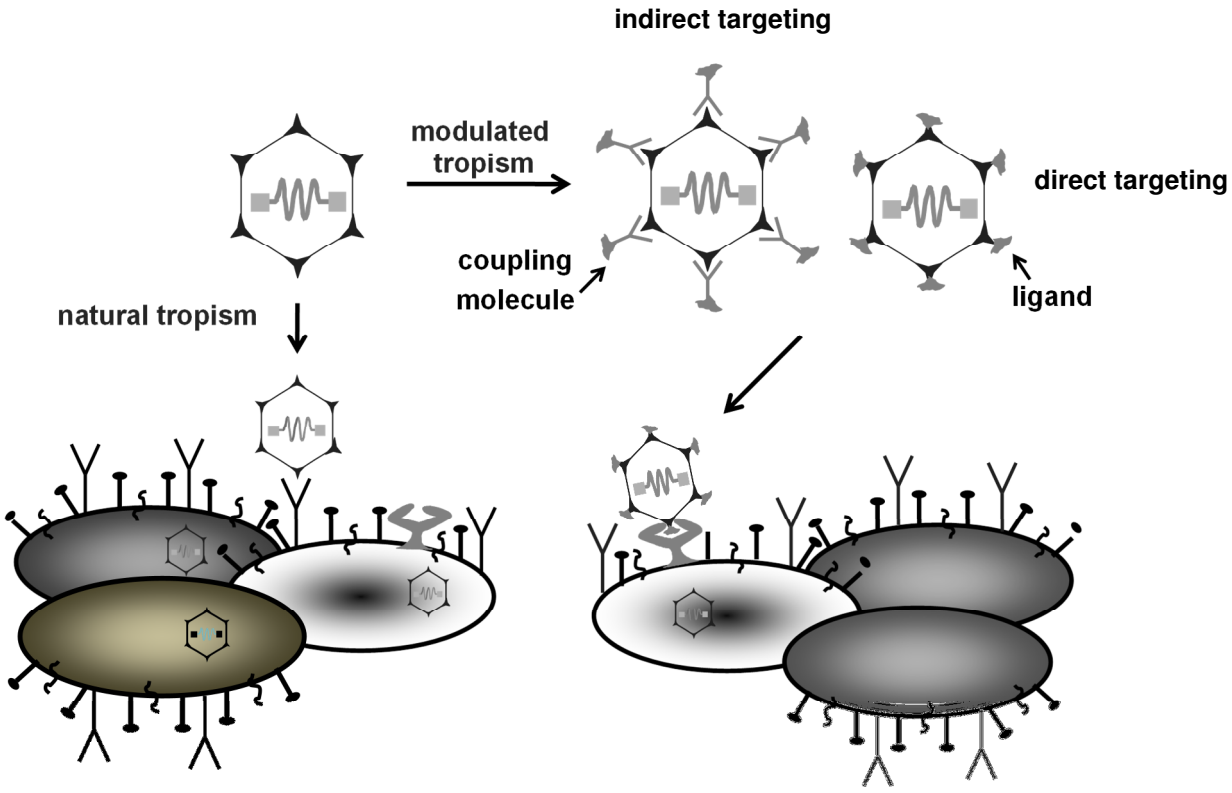


Figure 7: Principle of cell surface targeting using the example of AAV2
By natural tropism AAV2 binds to the cell surface molecules HSPG for cell attachment and integrins $\alpha_V\beta_5$ or $\alpha_5\beta_1$ for internalization. These receptors are very common and hence AAV2 shows a broad tropism, which may result in off-target transduction. It is possible to redirect the natural tropism of AAV to a more specific receptor. Furthermore, certain cell types do not express AAV receptors and therefore it would be beneficial to expand tropism to a receptor present on these cells. This constraint can be circumvented by modulating the tropism using adaptor molecules (indirect targeting) or by the insertion of peptide ligands (direct targeting) into the capsid. Figure was kindly provided by H. Büning.

1.2.2.1 Non-genetic vector targeting using adaptors

The non-genetic (indirect) targeting approach uses an adaptor molecule, which acts as a bridge between the viral capsid surface and a specific cell surface molecule (Figure 7), [47]. This technique is applicable even with limited knowledge of the viral structure [132]. This method allows for high flexibility as different adaptors can readily be coupled to the same vector and do not induce changes in capsid structure that may negatively effects vector gene transfer efficiency and packaging efficiency. Most adaptors can achieve the two main goals of targeted delivery: ablating native tropism and conferring novel tropism towards the desired target [132].

Barlett *et al.* used a bi-specific F(ab´Y)^2 antibody that was subsequently linked to the capsid of AAV2. The capsid-antibody linked rAAV vectors were retargeted successfully to $\alpha_{IIb}\beta_3$-expressing cell lines. Results showed an increased transduction by up to 70-fold in receptor-positive cell lines [122]. Another approach used avidin-linked epidermal growth factor (EGF) or fibroblast growth factor (FGF) fusion proteins conjugated to biotinylated AAV capsids to transduce human ovarian cancer and megakaryocytic cell lines [133]. Despite the promising and successful studies of diverse adaptor systems *in vitro*, their usability in an *in vivo* setting remains to be demonstrated. Obstacles in this regard are maybe the stability of the vector-adaptor complex, in particular when host factors compete with adaptor binding [132].

1.2.2.2 Genetic vector targeting

By using the genetic vector targeting approach, cell specific targeting of the vector is mediated by genetically incorporating ligands into viral capsid proteins by simultaneously shielding the natural binding receptor (Figure 7), [134].

A first attempt to use this strategy was reported by Yang *et al*. [135] who fused a single-chain antibody to the N-terminus of VP2 to target CD34+ cells. Although the study showed the incorporation of the targeting ligand, vector titer was extremely low. Several groups were able to show the incorporation of small peptides to the N-terminus of VP1 or peptides within VP1 and simultaneously to the N'-terminus of VP2, which resulted in functional virions with an expanded tropism of AAV [24], [136], [137]. More recent approaches demonstrated that the N-terminus of VP2 also accepts large insertions. Lux and colleagues genetically incorporated enhanced green fluorescent protein (GFP) into AAV capsid by replacement of wild-type VP2 by GFP-VP2 fusion proteins to visualize viral trafficking [25]. Furthermore, Münch and colleagues used the N-terminus of VP2 for insertion of Designed Ankyrin Repeat Protein (DARPin) into an AAV2 vector with ablated HSPG binding. The DARPin insertion confers the AAV vector with a high cell type specificity of vector genome delivery thereby enabling the safe delivery of suicide genes following systematic application into tumor bearing mice [138].

The first successful modification of AAV´s capsid by direct targeting was achieved by Girod *et al.*. They demonstrated that the insertion of peptides into the common regions of all three AAV capsid proteins (aa position 587) retargeted AAV2´s

natural tropism to mouse melanoma cells (B16F10). Later, the results of Girod *et al.* were confirmed by Grifman *et al* [139], who inserted the tumor-targeting NGRAHA sequence at the same position, 587, leading to up to 20-fold increased transduction efficiencies on several tumor cell lines expressing CD13 (a receptor expressed in angiogenic vasculature and in many tumor cell lines). Further, Shi and Bartlett demonstrated that the aa position 588 is also suitable for peptide insertion. They introduced a 4c-RGD peptide, CDCRGDCFC, which is known to bind with high affinities to the integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$, into the AAV capsid resulting in vectors that transduce cells HSPG independent, but through the above mentioned integrin [140]. Later Boucas *et al.* identified also aa position 453, located at the highest peaks on AAV2´s capsid, as possible site for peptide insertion [141].

To generate targeting vectors with a novel and restricted tropism, natural receptor binding elimination is necessary [47]. Notably in this context, insertions at the positions 587 interfere with the binding of two (R585 and R588) of the five positively charged aa of the AAV2 HSPG-binding motif [38], [142], explaining the ablation of HSPG binding of some re-targeted vectors [123], [143], [139], [144], [145]. In some cases, binding was only partially affected or even restored, when ligands were inserted at amino acid position 587 [139], [144], [146], [147]. This loss or maintenance of HSPG binding exemplified a dependence on the nature of the inserted ligand sequence as follows: insertion of bulky or negatively charged peptides resulted in AAV2 capsid mutants unable to bind to HSPG due to sterical or charge interference, while insertion of positively charged peptides can lead to an HSPG-binding phenotype by reconstituting a binding motif with one of the original arginines (R585 or R588) or independently of them [148].

1.3 AAV peptide display

Although rational design has generated viral vectors with novel gene delivery properties, the successful application of rational approaches often requires detailed mechanistic knowledge of AAV's infection process and on suitable receptor-binding peptides (ligands) capable of mediating efficient and cell-type specific vector entry [124]. As an answer to these challenges, the AAV display technology has been developed. This technology based on a high-throughput

screening technique consisting of a library of AAV capsid mutants carrying insertion of peptides with random sequences. Briefly, the AAV display library is used to infect desired target cells. The pool of *de novo* produced AAV variants is harvested from the cells and is used for further rounds of selection until an enrichment of viral particles, possessing the ability to successfully transduce the target cells has taken place. Thus, several AAV peptide libraries have been developed. Two very promising libraries are based on AAV2, consisting of mutants carrying 7-mer peptides with a random sequence at aa position 587 [112] or 588 [113] (Figure 8).

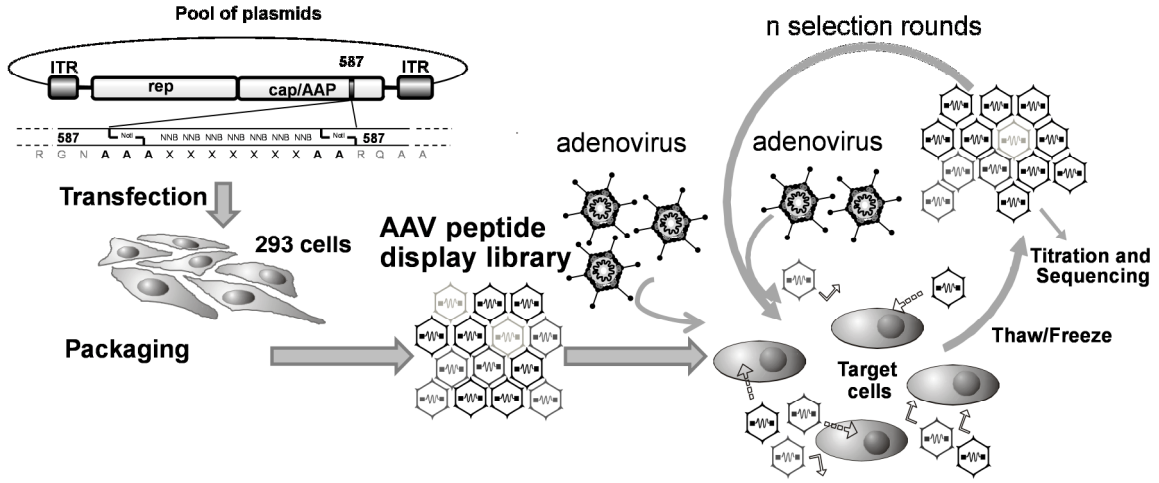


Figure 8: AAV peptide display
Schematic representation of the construction of the library of AAV2 capsid modified particles and selection protocol for the isolation of retargeted mutants is depicted. A pool of oligonucleotides with random sequence is cloned into an AAV2 genome encoding plasmid at the site corresponding to aa position 587 of the viral capsid proteins. Following a standard AAV production protocol, a library of approximately $4x10^6$ different capsid modified AAV2 clones can be generated. For the selection of retargeted mutants, target cells are co-infected with the pool of AAV2 mutants and with adenovirus. The viral progeny collected 48 h p.i. is used for the next infection round.

Perabo and colleagues performed five selection rounds with an AAV peptide display library on megakaryocytic cells (MO7e) and B-cell derived chronic lymphocytic leukemia cells (Mec1) [144], which both are non-permissive for wild type AAV2. In two separate selections, they were able to isolate RGD-containing peptides (RGDAVGV and RGDTPTS) from the selection on MO7e cells. In transduction experiments performed with rAAV vectors displaying the selected peptides on the capsid surface, an up to 100-fold increased efficiency in M-07e cells was observed [144]. The rAAV vectors displaying the selected peptides on

the capsid surface were successful in transducing the target cells. Totally different peptide motives were selected on Mec1 cells (GENQARS and RSNAVVP).
A similar approach was applied by Müller *et al.*. Their library contained a 7-mer peptide of random sequence inserted into the AAV2 capsid at amino acid position 588. They selected peptides able to mediate the transduction of human coronary artery endothelial cells [149]. Most of the selected peptides fitted into the consensus sequence NSVRDL$^{G/S}$ and NSVSSX$^{S/A}$ displaying remarkably higher transduction levels than AAV2 with unmodified capsid on the target cells.
Recently, Varadi *et al.* successfully generated an AAV9 peptide library with a randomized insertion of heptapeptides in aa position 589. They were able to show up to 40-fold improved transduction efficiencies on coronary artery endothelial cells in vitro by using AAV9 library selected mutants in comparison to wild-type AAV9 vectors [150].
The above-mentioned and several other studies concerning the AAV peptide display library technology [148], [151], [152], [153] demonstrate the successful identification of capsid mutants with increased transduction efficiencies on the concerning target cells. These mutants own the characteristics of receptor-specific cell entry and successful intracellular processing, which both are essential for an efficient AAV targeting vector.

1.4 Skin

The skin is the largest organ of the body. In a 70 kg individual the skin weights over 5 kg covering a surface of 2 m^2. Human skin consists of a stratified epidermis and an underlying dermis of connective tissue, which is organized into basal (stratum basale), spinous (stratum spinosum), granular (stratum granulosum) and cornified layers (stratum corneum), each layer consisting of keratinocytes of a specific morphology and state of differentiation (Figure 9), [154], [155], [156], [157], [158].

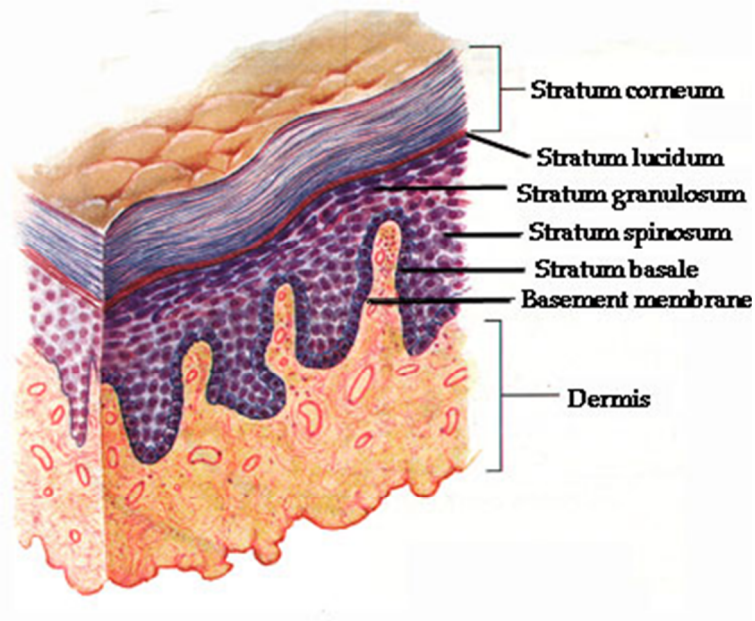


Figure 9: Structure of the human skin (www.physioweb.org)

The keratinocytes account for more than 80% of the cells of the epidermis. They function as a barrier and contribute to skin repair and regeneration [159]. Important structural proteins of the vertebrate epidermis are keratins constituting up to 85% of differentiated keratinocytes [160]. 20 different keratins are described for the human skin [161], [162], [163]. Typical keratins expressed in the mitotically active cells of the basal layer are keratins K5 and K14, which are considered to be biochemical markers of the epidermis [164]. K5 and K14 form intermediate filaments that assemble into strong networks, and anchor the epidermis to underlying layers of the skin. The network of keratin intermediate filaments provides strength and resiliency to the skin and provides protection from being damaged by friction and other everyday physical stresses [164], [165], [166]. Other important keratins are K1 and K10, which are the most abundant proteins in the upper epidermis where they polymerize to form intermediate filaments. In addition to their well-established function in providing epidermal stability, K1/K10 intermediate filaments are supposed to be important for terminal epidermal differentiation and barrier formation [167]. Point mutations of keratin genes can lead to severe diseases, many of which manifest as blistering skin diseases [166]. The most prominent of these inherited skin fragility disorders is epidermolysis bullosa simplex (EBS), of which the various variants are caused by a spectrum of point mutations of K5 or K14 [168], [169], [170].

Also, present in the basal layer of the epidermis are integrins, which are essential for cell-cell and cell-matrix interactions. The major types in the epidermis are $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins [171], [172], [173]. Integrins are heterodimeric transmembrane receptors consisting of an α and a β subunit that links the extracellular matrix (ECM) to the cytoskeleton, and "integrates" the extra-cellular environment with the cell interior [174].

A characteristic feature of the epidermis is the formation of the cornified cell envelope [175], which is crucial for barrier function of the epidermis [176]. The cornified cell envelope is a highly insoluble structure and contains a complex mixture of specific proteins such as involucrin, loricrin, small proline-rich proteins, XP-5 family members, cystatin A, elafin, S100 family members, and lipids that are covalently cross-linked by transglutaminases [177], [178], [179], [180], [181].

Other important cells found in the epidermis are Langerhans cells, melanocytes and Merkel cells. Langerhans cells provide immunological protection, while melanocytes absorb UV light, and the Merkel cells are sensors for mechanical events at the skin´s surface and within the epidermal compartment.

1.4.1 Organotypic skin co cultures

The epidermis is a surface epithelium with its upper cell sheet exposed to the outer environment. *In vivo,* formation and maintenance of the mature epidermis consists of four layers that rely on a continuous process of keratinocyte proliferation and terminal differentiation (see 1.4). The epidermal organization and tissue homeostasis are regulated by mesenchymal influences [182] and the proliferation of basal cell attachment to the basement membrane. Culturing primary human keratinocytes (HK) in 2D completely alternates the system since in conventional 2D culture, cells grow either as mono-layers on solid, impermeable surfaces or as uniform suspensions. The cells are nourished from above and lack the basement membrane, depriving the cells of mesenchymal support. To generate more natural growth conditions for primary HK, *in vitro c*ulturing skin explants were explored. While this allowed the keratinocytes to migrate from the explants, the differentiation program was only rudimentary, and the cells eventually became senescent or detached. Pioneering work was done by Rheinwald and Green 1975, demonstrating that single cell suspensions of keratinocytes could be grown on feeder layers of irradiated fibroblasts [183].

Although, keratinocytes could be further propagated in such cultures, neither cell polarization or structural organization, nor the expression of the differentiation markers filaggrin, keratin K1/10 and loricrin were improved [184], [185], [186], [187]. Henceforward, multiple versions were developed to mimic the *in vivo* situation of normal skin using a current method of cultivating the air-exposed keratinocytes on various substrates that serve as dermal equivalents (organotypic skin 3D culture, Figure 10). The dermal equivalents were composed of porous membranes, which were either coated with a cell-free extracellular matrix or with fibroblasts at the lower side of the filter [188], [189]. Similar to an *in vivo* situation, organotypic skin co-cultures have been generated with air-exposed primary cultured keratinocytes grown on top of a type I collagen gels containing fibroblast, which mimic an appropriate substratum for the development of the polarized and stratified epithelium (Figure 10), [190], [191], [192]. Here, the fibroblasts nourish the keratinocytes by diffusion from the medium which is restricted to the base of the collagen gel. The keratinocytes growing in the organotypic culture can develop into a stratified epidermis-like epithelium, consisting of several nucleated cells as well as fully keratinized layers, closely resembling the epidermal strata. In this system, function of diffusible factors mediating epithelial-mesenchymal interactions has been demonstrated by Smola *et al*. [193] and Maas-Szabowski *et al*. [194].

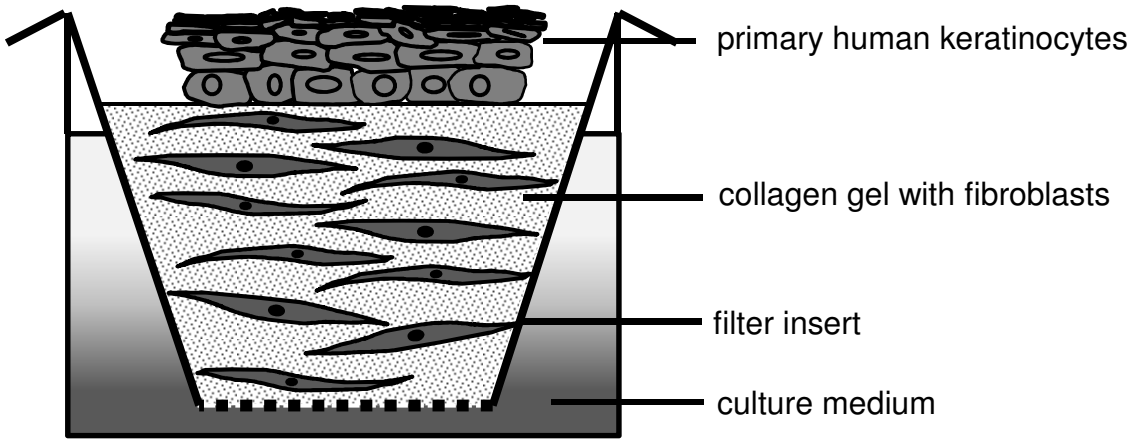


Figure 10: Schematic drawing of an organotypic skin co-culture

1.4.2 Wounds and wound healing therapies

Once the protective barrier of the skin is damaged, the physiologic process of wound healing is immediately set in motion. Generally, the human adult wound healing process can be divided into 3 distinct phases: the inflammatory phase, the proliferative phase, and the remodeling phase [195], [196]. Upon injury, a series of biochemical events takes place. Minutes post-injury, platelets (thrombocytes) aggregate at the injury site to form a fibrin clot. During the inflammatory phase, ichor (including chemokines and cytokines) is released to attract cells that phagocytose debris, bacteria, and damaged tissue, in addition to releasing signaling molecules that initiate the proliferative phase of wound healing. During blood clotting (2–7 days after injury), fibrin and fibronectin cross-link together to form a plug that traps proteins and particles, preventing further blood loss [197]. In the third phase, wound remodeling, fibroblasts are transformed into myofibroblasts that mediate wound contraction and collagen is deposited in abundance. The keratinocytes close the wound surface with a neoepidermis. This third phase of wound healing shows a transition from granulation tissue to scar tissue, a continued spreading of collagen and constant remodeling of the scar that lasts for months [195], [196]. The important molecules responsible for mediating wound healing are proteins known as growth factors (EGF family, EGF, TGF-α, TGF-β, PDGF, VEGF etc.) [198]. They interact as mediators and receptors and play an essential role in linking each step of wound healing [198]. These growth factors are synthesized and secreted by many types of cells, involved in tissue repair, like platelets, inflammatory cells, fibroblasts, epithelial cells and vascular epithelial cells [199], [200].

The wound repairing process fails in conditions of large cutaneous burns and chronic wounds. For example, a decreased production and/or secretion of growth factors in addition to local inflammation impairs wound healing in the case of diabetic ulcers [201]. Furthermore a loss of growth factors leads to macromolecular leakage of fibrogen, α-macroglobin, and albumin, which could cause venous stasis ulcers or diabetic ulcers [200], [202], [203].

On the cellular level, the process of wound healing can be supported and promoted by gene delivery. The skin is easily accessible for both *in vivo* and *ex vivo* gene transfer and for monitoring of the treatment site. The epidermis is a self-

renewing tissue containing stem cells in the basal layer, which proliferate throughout the whole life span, replace themselves upon division and give rise to keratinocytes committed to terminal differentiation. Some promising strategies have been reported for the treatment of severe skin diseases by genetic manipulations. Freiberg and colleagues developed a retroviral expression vector for human steroid sulfatase arylsulfatase (STS) to provide corrective gene delivery to human keratinocytes for patients suffering from X-linked ichthyosis (XLI). Afterwards, they utilized these corrected cells for the regeneration of fully functional normal human epidermis *in vivo* on immunodeficient mice [204].

Another group, Mavilio and colleagues published a study on the treatment of patients suffering from epidermolysis bullosa (EBS) through *ex vivo* retroviral vector transduction of autologous epidermal stem cells with a normal copy of the defective gene, followed by reconstitution of the patient's skin with epithelial sheets that are grown from these genetically corrected cells [205]. A disadvantage of retroviral gene transfer is related to the potential mutagenicity of retroviral vectors due to their random integration into the host's genome, which may cause insertional mutagenesis if it disrupts a tumor suppressor gene or activates an oncogene [76].

An alternative vector thought to offer some additional advantages might be the rAAV vector. However, the use of AAV vectors for the treatment of inherited skin diseases or in wound healing has been hindered by the lack of suitable AAV variants that allow efficient transgene delivery [206], [207], [208]. So far, AAV2 as well as vectors pseudotyped with capsids of alternate serotypes such as AAV5, -7 or 8 had to be used at multiplicities of infection (MOI) of > 100,000, which is not feasible in a clinical setting [208], [209]. A possible strategy to overcome this limitation is to re-direct the viral tropism towards a novel receptor by genetic modification of the viral capsid (targeting) (Figure 8).

1.5 Objective

Skin-directed gene transfer is believed to be a promising strategy to treat a multiplicity of skin diseases [210], [211], [212] including cancer, burns or chronic non-healing wounds [213], [214] or inherited diseases such as epidermolysis bullosa, ichthyosis and xerderma pigmentosum [215], [216], [217], [218].

Therefore, the generation of vectors providing high transduction efficiency and sustaining gene expression for a period of time by applying low vector doses and a general lack of toxicity would be an important tool for gene therapy. A very promising vector system is based on AAV2 [219]. However, cell entry of rAAV2 occurs inefficient [209] and as a consequence very high numbers of vector particles have to be applied, which is not feasible in a clinical setting [208].

In this context, the main objective was the selection and characterization of rAAV targeting vectors with improved gene transfer efficiencies for primary HK. In the absence of knowledge of an appropriate receptor to target, a high-throughput selection screen of AAV capsid mutants on primary HK with an AAV peptide display library had to be performed. Previous results of our group proved that selection with the AAV2 display library, depleted for HSPG-binding ligands, resulted in neutral charged, highly efficient and cell-type-specific rAAV2 targeting vectors transducing target cells via an HSPG independent and clathrin-dependent mechanism [144], [220]. Therefore, pre-selection, the library had to be depleted for HSPG-binding ligands by heparin chromatography. Furthermore, it should be tested if the randomly inserted peptide ligand mediates the cell entry of the appropriate rAAV2 targeting vector since the specificity of ligand defines the tropism of the targeting vectors. The selected rAAV2 targeting vectors should be highly specific in order to restrict gene transfer into primary HK as they are frequently co-cultured with feeder cells. To elucidate receptor candidates of the selected rAAV targeting vectors, the comparative gene analysis (CGA) [221] was to be applied in cooperation with the NIH (USA). In summary, this work´s main objective is the development of new tools for tissue engineering and a strategy to map the targeting receptor.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, solutions and enzymes

Product	Company
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Invitrogen, Karlsruhe, Germany
Anti-Anti, Antibiotic-Antimitotic Solution	Life Technologies GmbH**,** Darmstadt, Germany
Aqua bidest. (Ampuwa)	Fresenius Kabi, Homburg, Germany
Bovine Serum Albumin	AppliChem, Darmstadt, Germany
Calcium Chloride	Sigma-Aldrich, Taufkirchen, Germany
Chlorpromazine	Sigma-Aldrich, Taufkirchen, Germany
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe, Germany
Collagen G	Biochrom AG, Berlin, Germany
Dispase II	Sigma-Aldrich, Taufkirchen, Germany
EDTA	Roth, Karlsruhe, Germany
Eosin Y solution, alcoholic	Sigma-Aldrich, Taufkirchen, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium Bromide	Roth, Karlsruhe, Germany
37% Formaldehyde-solution	Merck, Darmstadt, Germany
Genistein	Sigma-Aldrich, Taufkirchen, Germany
Glycerol	Roth, Karlsruhe, Germany
Hematoxylin solution, Meyer`s	Sigma-Aldrich, Taufkirchen, Germany
Heparin	ROTEXMEDICA GmbH, Trittau, Germany
Hepes	Roth, Karlsruhe, Germany

Instamed 9.55 g/l PBS Dulbecco	Biochrom AG, Berlin, Germany
Iodixanol	Sigma-Aldrich, Taufkirchen, Germany
IS Mounting Medium DAPI	Dianova GmbH, Hamburg, Germany
Isopropanol	Roth, Karlsruhe, Germany
Magnesium Chloride	Roth, Karlsruhe, Germany
MassRuler DNA Ladder Mix	MBI Fermentas, St. Leon-Rot, Germany
Peptone/Tryptone	Roth, Karlsruhe, Germany
Phusion™ DNA Polymerase	Finnzymes, Keilaranta, Finland
Proteinase K	Sigma-Aldrich, Taufkirchen, Germany
Rat-tail Collagen, high concentration	Cellsystems, Troisdorf, Germany
Saccharose	Merck, Darmstadt, Germany
Sodium Hydroxide	Roth, Karlsruhe, Germany
Sodium Phosphate	Roth, Karlsruhe, Germany
T4 DNA Ligase	MBI Fermentas, St. Leon-Rot, Germany
TRIS Hydrochloride	Roth, Karlsruhe, Germany
Tissue-Tek®	Sakura Finetek, Zoeterwoude, Netherlands
Yeast Extract	Roth, Karlsruhe, Germany

All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany) or Carl Roth GmbH & Co. (Karlsruhe, Germany).

2.1.2 Standard kits

Product	Company
BigDye® Terminator v 3.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, USA
DNeasy® Blood & Tissue Kit	Qiagen, Hilden, Germany
EndoFree® Plasmid Kits	Qiagen, Hilden, Germany
AAV2 Titration ELISA	Progen, Heidelberg, Germany
Gel extraction Kit	Qiagen, Hilden, Germany
LightCycler® 480 SYBERGreen Master	Roche, Mannheim, Germany
LightCycler ® Fast Start DNA Master SYBER Green I	Roche, Mannheim, Germany
PCR Purification Kit	Qiagen, Hilden, Germany

2.1.3 Plasmids

pGFP self-complementary:

AAV vector plasmid that encodes the GFP gene is controlled by the human CMV promoter. The transgene cassette is flanked by the AAV2 ITRs. The plasmid contains an Ampicillin-resistance gene (beta-lactamase). A deletion in one of the terminal resolution sites interferes with strand displacement resulting in a self-complementary genome conformation, which is packaged into the viral capsid [58].

pRC:

pRC is an AAV based helper plasmid containing the AAV2 Rep and Cap ORFs but lacks the viral ITRs. pRC contains an Ampicillin-resistance gene [143].

pRC "Kotin":

The AAV based helper plasmid contains the AAV2 Rep and Cap ORFs but lacks the viral ITRs. The plasmid contains a SnaBI and a BsiWI cloning site within the CAP ORF. pRC "Kotin" possesses an Ampicillin-resistance gene and was kindly provided by Anne Girod.

pRC "Kotin"-Kera1-587, pRC "Kotin"-Kera2-587, pRC "Kotin"-Kera3-587:
The AAV based helper plasmid contains the AAV2 Rep and Cap ORFs but lacks the viral ITRs. This plasmid carries an insertion at the position that corresponds to aa 587. Three different plasmids were cloned during this work: pRC-Kera1 with the insertion (-AARGDTATLAA-), pRC-Kera2 with (-AAPRGDLAPAA-) and pRC-Kera3 with (-AARGDQQSLAA-). The plasmids possess an Ampicillin-resistance gene.

pRGD-4C-587:
AAV based helper plasmid containing the AAV2 Rep and Cap ORFs but lacks the viral ITRs, the RGD4C peptide -ACDCRGDCFCA- is inserted at a site that corresponds to aa 587. The plasmid contains an Ampicillin-resistance gene [141].

pXX6-80:
Adenoviral helper plasmid encoding for VA, E2A and E4 and Ampicillin resistance; pXX6 was kindly provided by J. Samulski (University of North Carolina, Chapel Hill, USA). The plasmid contains an Ampicillin-resistance gene [73].

2.1.4 Enzymes

Benzonase	Merck, Darmstadt, Germany
Restriction enzyme	MBI Fermentas, St. Leon-Rot, Germany; New England Biolabs, Frankfurt am Main, Germany

2.1.5 Primers

All primers were synthesized by Invitrogen (Karlsruhe, Germany).

Sequencing primer

wt_4066_rev	5' – ATG TCC GTC CGT GTG TGG – 3'

Primers for qPCR

GFP_fw	5' – GCTACCCCGACCACATGAAG – 3'
GFP_rev	5' – GCTCATGCCGAGAGTGATCC – 3'

Plat_fw	5’ – ACCTAGACTGGATTCGTG – 3’
Plat_rev	5’ – AGAGGCTAGTGTGCAT – 3’

Primers for amplification of selected clones:

BsiWI_fw	5’ – TAC CAG CTC CCG TAC GTC CTC GGC – 3’
NewSnaBI_rev	5’ – CGC CAT GCT ACT TAT CTA CG – 3’

2.1.6 Antibodies

2.1.6.1 Direct labeled antibodies

Anti-Feeder	monoclonal, APC conjugated mouse anti-human	Miltenyi, Bergisch Gladbach, Germany

2.1.6.2 Primary antibodies

Anti-$\alpha_v\beta_5$ (MAB1961)	monoclonal, mouse anti-human	Millipore, Schwalbach/Ts., Germany
Anti-$\alpha_5\beta_1$ (MAB1999)	monoclonal, mouse anti-human	Millipore, Schwalbach/Ts., Germany
Anti-α-V (MAB1953)	monoclonal, mouse anti-human	Millipore, Schwalbach/Ts., Germany
Anti- $\alpha_V\beta_8$, 37E5	monoclonal, IgG2a, mouse anti-human	kindly provided by S. Nishimura, UCSF, USA
Anti-human heparan sulfate delta	monoclonal, IgG2b, mouse anti-human	USBiological, Massachusetts, USA
Anti-Collagen Type IV	monoclonal, IgG2b, mouse anti-human	Progen, Heidelberg, Germany

2.1.6.3 Secondary antibodies

IgG (ab7002-500)	monoclonal, PE-conjugated goat anti-mouse	abcam, Cambridge, UK
IgG (1030-09s)	polyclonal, PE-conjugated goat anti-mouse	SouthernBiotech, Eching, Germany

2.1.7 Peptides

H-Gly-Arg-Gly-Glu-Ser-OH	Bachem Distribution Service, Weil am Rhein, Germany
H-Gly-Arg-Gly-Asp-Ser-OH	Bachem Distribution Service, Weil am Rhein, Germany

2.1.8 Bacteria strain

E.*coli*DH5α:

F-, *lac*1-, *rec*A1, *end*A1, *hsd*R17, _(*lac*ZYA-*arg*F), U169,F80d*lac*Z_M15, *sup*E44, *thi*-1, *gyr*A96, *rel*A1; [222]

2.1.9 Eukaryotic cells

For culturing and media conditions, please refer to 2.2.

2.1.9.1 Immortalized cell lines

A375

Human malignant melanoma cells; American Type Culture Collection (ATCC) number: CRL1619™; [223]

BLM

Human melanoma cells; were kindly provided by C. Mauch (Department of Dermatology and Venereology, Cologne).

DU145

Human prostate cancer cells; ATCC number: HTB-81; [224]

HEK293

Human embryonic kidney cells, transformed with Ad5 DNA and containing the adenoviral genes *E1a* and *E1b*; ATCC number: CRL-1573; [225]

HeLa

Human epithelial cervix adenocarcinoma cells; ATCC number: CCL-2™; [226]

HepG2

Human hepatocellular carcinoma cells; ATCC number: HB-8065; [227]

NIH3T3

Mouse embryonic fibroblast cells; ATCC number: CRL-1658TM; [228]

SW480

Human colon adenocarcinoma cells, were kindly provided by S. Nishimura, San Francisco; [229]

SW480-$\alpha_V\beta_8$

Human colon adenocarcinoma cells expressing $\alpha_V\beta 8$ integrin, were kindly provided by S. Nishimura, San Francisco; [229]

2.1.9.2 Primary human keratinocytes

Primary human keratinocytes isolated from human foreskin (see 2.2.4.6).

2.1.9.3 Primary murine keratinocytes

Primary murine keratinocytes were kindly provided by the group of Carien Niessen (Department of Dermatology, CECAD and CMMC Cologne, Germany).

2.1.10 Culture Media and Supplements

Product	Company
Accutase	Invitrogen, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Taufkirchen, Germany
Chelex100 Resin	Biorad, Munich, Germany
CnT Basal Medium 1	CELLnTEC Advanced Cell Systems AG, Bern, Switzerland
DMEM Medium + GlutaMAXTM-I	Invitrogen, Karlsruhe, Germany
Epidermal Growth Factor (EGF)	Sigma-Aldrich, Taufkirchen, Germany
FCS	Invitrogen, Karlsruhe, Germany
HBSS-Hank´s Balanced Salt Solution	Sigma-Aldrich, Taufkirchen, Germany
MEM Non-Essential Amino Acid	Life Technologies GmbH, Darmstadt, Germany
PBS	Invitrogen, Karlsruhe, Germany
Penicillin/Streptomycin	Invitrogen, Karlsruhe, Germany
Puromycin-Dihydrochlorid	Roth, Karlsruhe, Germany
RPMI-1640 medium + GlutaMAX^{TM}-I	Invitrogen, Karlsruhe, Germany
Sodium L-ascorbate	Sigma-Aldrich, Taufkirchen, Germany
Sodium Pyruvate	Invitrogen, Karlsruhe, Germany
TGF-α (Transforming Growth Factor-α)	Sigma-Aldrich, Taufkirchen, Germany
Trypsin/EDTA	Invitrogen, Karlsruhe, Germany

Media compositions for cell types are listed below:

A375, HeLa, HEK293, NIH3T3 and SW480 cells:

- DMEM Medium + GlutaMAXTM-I
- 10% FCS
- 100 U/ml penicillin and 100 µg/ml streptomycin

DU-145 cells

- DMEM Medium + GlutaMAX^{TM}-I
- 10% FCS
- 100 U/ml penicillin and 100 µg/ml streptomycin

HepG2 cells:

- DMEM Medium + GlutaMAX^{TM}-I
- 10% FCS
- 100 U/ml penicillin and 100 µg/ml streptomycin
- 2 mM L-Glutamine
- 1 mM Sodium Pyruvate
- 1x MEM Non-Essential Amino Acid

BLM cells:

- RPMI-1640 medium + GlutaMAX^{TM}-I
- 10% FCS
- 100 U/ml penicillin, 100 µg/ml streptomycin

SW480-$\alpha_V\beta_8$

- DMEM Medium + GlutaMAXTM-I
- 10% FCS
- 100 U/ml penicillin and 100 µg/ml streptomycin
- 4 µg/ml Puromycin-Dihydrochlorid

Primary human keratinocytes:

- CnT Basal Medium 1
- 100 U/ml penicillin, 100 µg/ml streptomycin

Organotypic human skin co-cultures:

- DMEM Medium + GlutaMAX™-I
- 10% FCS
- 100 U/ml penicillin, 100 µ/ml streptomycin
- 10 µg/ml TGF-α
- 10 µg/ml Epidermal Growth Factor (EGF)
- 50 µg/ml Sodium L-ascorbate

2.1.11 Laboratory equipment and disposables

Product	Company
Balance Adventurer Pro	Ohaus, NJ, USA
Beckman Coulter Rotor Type E70Ti	Beckman Coulter GmbH, Krefeld, Germany
Beckman Coulter Optima™ L-80 XP Ultracentrifuge	Beckman Coulter GmbH, Krefeld, Germany
BiodocAnalyze live Ultracentrifuge tubes	Kendro/Thermo Fisher Scientific, Germany
Captairbioflow	Cologne, Germany
Cell Culture Plastic Ware	TPP AG, Trasadingen, Switzerland
Centrifuge Z 216 MK	Hermle, Wehingen, Germany
Centrifuge Z 233 M-2	Hermle, Wehingen, Germany
Centrifuge Z 383 K	Hermle, Wehingen, Germany
Centrifuge 5415 D	Eppendorf, Hamburg, Germany
Centrifuge Avanti J-E	Beckmann Coulter, Krefeld, Germany
Cell scrapers	Corning Incorporated, New York, USA
Cell strainer	BD Falcon™ BD Biosciences,

	Erembodegem, Belgium
CO_2 Incubator MCO-20AIC	Sanyo, Munich, Germany
Companion 6 well plates for cell culture filter application	BD Falcon™, BD Biosciences, Erembodegem, Belgium
Cover slips 10 mm	Roth, Karlsruhe, Germany
Cryostat LEICA CM1850	Leica, Nussloch, Germany
FACS Calibur	Becton Dickinson, Heidelberg, Germany
FACS tubes	Becton Dickinson, Heidelberg, Germany
Filter tips	Sarstedt, Nümbrecht, Germany
General laboratory ware	VWR, Darmstadt, Germany
Glass rings, 20+/-0.25 mm x wall thickness 1.8 mm,10 mm size	Custom product by Brennstein Laborbedarf, Markt Schwaben, Germany
Heater/Magnetic stirrer	Heidolph MR 3001 Heidolph Instruments, Schwabach, Germany
Hera -80ºC freezer	Heraeus/Thermo Fisher Scientific, Germany
HiTrap Heparin Affinity Columns (1 ml)	Amersham /GE Healthcare, Freiburg, Germany
HiTrap ProteinA HP Columns (1 ml)	Amersham /GE Healthcare, Freiburg, Germany
Incubator Shaker	Multitron Standard Infors HAT, Bottmingen-Basel, Switzerland
Laminar Air Flow	BioWizard Golden Line Kojair, Vilppula, Finland
Laminar Air Flow	BioWizard Xtra Kojair, Vilppula, Finland
LightCycler 480 II	Roche, Mannheim, Germany
Light Cycler plates and foils	Roche, Mannheim, Germany
LightCycler Capillaries	Roche, Mannheim, Germany
LightCycler carousel centrifuge	Roche, Mannheim, Germany

Membrane application for 6 well plates, 3 µm, $8x10^5$ pores/cm^2	BD Falcon™, BD Biosciences, Erembodegem, Belgium
Microcentrifuge	Roth, Karlsruhe, Germany
Microscope Olympus CKX41	Olympus, Hamburg, Germany
Microscope Olympus IX81	Olympus, Hamburg, Germany
Microscope slides Superfrost®Plus	Thermo Fisher Scientific Inc., Braunschweig, Germany
Microtome Blades	Leica, Nussloch, Germany
NanoDropTM 1000	Thermo Fisher Scientific Inc., Braunschweig, Germany
Parafilm	Pechinery Plastic Packaging, Chicago, USA
pH Meter Seven Easy	Mettler-Toledo, Schwerzenbach, Switzerland
Pipettes	Eppendorf, Hamburg, Germany
Power Supply	Renner, Dannstadt, Germany
Pump P-1	Amersham/GE Healthcare, Freiburg, Germany
Reaction tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Reaction tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht, Germany; Becton Dickinson, Heidelberg, Germany
Rotary Microtome Leica RM2255	Leica, Nussloch, Germany
Scalpels Feather Safety	Razor Co. Ltd., Japan
Syringes and cannulas	B. Braun Melsungen, Melsungen, Germany
Thermocycler, T3000	Biometra, Göttingen, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Tissue Processor Leica ASP 300S	Leica, Nussloch, Germany
Vortex Genie 2	Scientific Industries, NY, USA
Waterbath Medingen W6	Medingen, Freital, Germany

2.1.12 Data treating Software

Clone Manager, Picasa, Roche LightCycler480 SW1.5, Roche LightCycler3.5 Microsoft Excel, Microsoft Word, RelQuant, WinMDI, FACS DIVA, specific software for the respective instruments.

2.2 Methods

2.2.1 Bacteria culture

2.2.1.1 Cultivation of bacteria

Bacteria were grown in LB medium at 37°C under vigorous shaking overnight. For generating single clones, bacteria were plated on plates containing LB agar and 100 µg/ml Ampicillin.

LB medium: 10 g tryptone
5 g yeast
5 g NaCl
15 g agar (for plates)
add 1 l distilled H_2O

2.2.1.2 Preparation of chemically competent bacteria

All solutions were autoclaved before use. Equipment and solutions were pre-cooled. 3 ml LB medium without antibiotics were inoculated with DH5alpha E*. coli* and incubated overnight at 37°C in a shaker at 220 rpm. The overnight culture was added to 400 ml LB medium and incubated at 25-30°C until the absorbance at 600 nm was approximately 0.5. The culture was chilled on ice for 10 min. For a gentle handling of the bacteria, all the following steps were done on ice. The bacteria suspension was centrifuged for 7 min at 1600 rcf at 4°C. After removal of the supernatant the pellet was resuspended in 10 ml ice-cold $CaCl_2$-solution and the bacteria suspension, was further centrifuged for 5 min at 1100 rcf and 4°C. Then, the pellet was gently resuspended in 20 ml ice-cold $CaCl_2$-solution and the cell suspension was chilled on ice again for 30 min. After an additional centrifugation step for 5 min at 1100 rcf, aliquots of 100 µl were produced, and the cell suspension was shock frozen in liquid nitrogen. Aliquots were stored at -80°C.

$CaCl_2$-solution: 60 mM $CaCl_2$ x $2H_2O$
10 mM PIPES, pH 7
10% Glycerin

2.2.1.3 Transformation of bacteria

Transformation of bacteria was done by using the heat shock method. 50 µl competent bacteria were thawed on ice for 15 min. DNA (approx. 50-100 ng) was added to the bacteria and mixed very gently. After 30 min incubation on ice, bacteria were exposed to a heat shock of 42°C for 1 min followed by a two-min-incubation. 400 µl LB medium without antibiotics was added. Bacteria were placed in a shaker (37°C at 250 rpm, 30 min). The suspension was plated on a LB agar plate containing antibiotics. Plates were incubated overnight by 37°C.

2.2.2 Working with nucleic acid

2.2.2.1 Plasmid amplification and extraction

To isolate plasmid DNA from bacteria, anion exchange columns were used. The preparation was done in “Mini”, “Maxi” and “Mega” measuring units according to the standard protocols and EndoFree system kits 50, 500 and 2500 of Qiagen.

2.2.2.2 DNA quantification

DNA concentration was measured at a wavelength of 260 nm (DNA) and 280 nm (protein impurities) by making use of NanoDropTM 1000. Purity of the nucleic acid preparation is defined by the ratio Abs 260 nm /Abs 280 nm. DNA of high purity has a ratio of 1.8, lower values point to contaminations with proteins and aromatic substances, whereas higher ratios indicate possible contaminations with RNA.

2.2.2.3 Restriction Digest of DNA

Digestion with restriction enzymes was performed according to the manufacturer’s instructions in a final volume of 20 µl containing 1 µg of DNA, 1-10 units of restriction enzyme per 1 µg DNA and 1x buffer.

2.2.2.4 Gel Electrophoresis

Analytic or preparative agarose gel electrophoreses were performed in 1xTAE buffer. Depending on the fragment size, the concentration of the agarose gel varied between 0.8 and 1.2%. The agarose was solved in 1xTAE buffer and mixed with the DNA intercalating substance ethidium bromide (0.1 µg per 1 ml gel volume) and poured onto a gel casting tray. To analyze the DNA fragments, from a

restriction digest approximately 200 ng DNA was mixed with 1x loading dye to reach an end volume of 10 µl. DNA ladders were used as reference.

TAE Buffer (50x): 242 g Tris base (2 mol/L)
57.1 ml Glacial acetic acid (1 mol/L)
18.6 g EDTA pH 8.0 (0.05 mol/L)
add 1 l H_2O

Extraction of DNA fragments or PCR products from agarose gels was performed using the Qiagen Gel Extraction Kit according to the manufacturer's instructions.

2.2.2.5 DNA extraction from eukaryotic cells

DNA was extracted from eukaryotic cells using the QIAGEN DNeasy Blood & Tissue Kit according to the protocol for "Purification of Total DNA from Animal Blood or Cell". Column-bound DNA was eluted in 200 µl 10 mM Tris/HCl pH 8.5.

2.2.2.6 Polymerase chain reaction

After the fifth round of selection (see 2.2.2.9), DNA isolated from viral progeny was amplified by PCR using the primer BsiWI_fw and New SnaBI_rev. The 1.2 kb fragment containing the insertion at aa position 587 (nt 5311 to nt 6532 of CAP ORF) were subsequently cloned into pRC-Kotin. The PCR reaction conditions are described below.

Pipetting scheme for PCR reaction mix:

5 µl template DNA
10 µl 5x Phusion reaction buffer
0.5 µl PhusionTM DNA Polymerase
2 µl dNTPs (10 mM)
2 µl BsiWI_fw (10 µM)
2 µl New SnaBI_rev (10 µM)
ad 50 µl H_2O

PCR cycling program:

PCR step	Time	Temperature	Number of cycles
Denaturation	30 sec	98°C	1
Denaturation	10 sec	98°C	35
Annealing	30 sec	56°C	35
Elongation	40 sec	72°C	35
Final elongation	10 min	72°C	1
Final hold	∞	4°C	

2.2.2.7 Quantitative real-time PCR (qPCR)

QPCR was used to determine the vector copy number following transduction experiments (relative quantification of target versus reference gene) or the genomic titer of rAAV vector stocks or wtAAV (absolute quantification). Measurements were done at the Light Cycler System LightCycler® 480 II or Capillary LightCycler (Roche) by making use of the LightCycler® 480 SYBR Green Master for LightCycler® 480 II or, LightCycler® FastStart DNA Master SYBR Green I for Capillary LightCycler kits. For absolute quantification, a standard was generated containing $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ plasmid molecules per µl. For normalization the RelQuant software for Capillary LightCycler or the LightCycler® 480 Software 1.5 for LightCycler® 480II was used for relative quantification.

Pipetting scheme:

2 µl template DNA
1 µl Primer fw (20 µM)
1 µl Primer rev (20 µM)
4 µl Mix (including FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, SYBRGreen I dye and $MgCl_2$) ad 20 µl H_2O

qPCR cycling program:

Program	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per°C)
Denaturation	1	None	95	None	00:05:00	4.4	
Amplification	40	Quantification	95	None	00:00:15	4.4	
			60	None	00:00:10	2.2	
			72	Single	00:00:15	4.4	
Melting	1	Melting Curve	95	None	00:00:01	4.4	
			68	None	00:00:15	2.2	
			95	Continuous			5
Cooling	1		40	None	00:00:30	2.2	

Genomic titers of the AAV peptide display library and of viral progeny were determined using the wild-type AAV (wtAAV) qPCR cycling program.

wtAAV qPCR cycling program:

Program	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per°C)
Denaturation	1	None	95	None	00:15:00	4.4	
Amplification	40	Quantification	95	None	00:00:10	4.4	
			60	None	00:00:03	2.2	
			72	Single	00:00:35	4.4	
Melting	1	Melting Curve	95	None	00:00:01	4.4	
			68	None	00:00:10	2.2	
			95	Continuous			5
Cooling	1		40	None	00:00:30	2.2	

2.2.2.8 Sequencing

Sequencing of single DNA clones was carried out in an ABI 3730 Sequencer at the Cologne Center for Genomics, University of Cologne, Germany. For the

sequencing reaction, the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) was used.

Sequencing reaction mix: 200 ng template DNA
0.5 µl 10x Buffer
0.5 µl Primer 4066 (10 pmol/µl)
1 µl BigDye v3.1
ad 5 µl H_2O

PCR cycling program:

PCR step	Time	Temperature	Number of cycles
Denaturation	2 min	94°C	1
Denaturation	20 sec	94°C	25
Annealing	30 sec	50°C	25
Elongation	4 min	60°C	25
Final elongation	4 min	60°C	1
Final hold	∞	4°C	

2.2.2.9 Molecular cloning

2.2.2.9.1 Cloning of CAP fragment

pRC “Kotin” plasmid, used as helper plasmid backbone, was digested with SnaBI and BsiWI enzymes, purified and dephosphorylated. For ligation, 80 ng of vector backbone was mixed with 5-fold excess of the purified PCR product, which had been digested with SnaBI and BsiWI (2.2.2.6). The reaction mixture was incubated at 16°C overnight and transformed into chemically competent bacteria (2.2.2.8). Sequencing (Qiagen Sequencing Services, Hilden, Germany) of bacterial clones was performed using Primer 4066 after picking single colonies of the plated cultures.

2.2.3 Capsid ELISA

The capsid titers of rAAV vector preparations were determined by ELISA, using the AAV 2 Titration ELISA kit (Progen, Heidelberg) according to the manual.

2.2.4 Eukaryotic cell culture

2.2.4.1 Cultivation of cells

Cells were cultured at 37°C in humid atmosphere containing 5% CO_2. For culture media, please refer to chapter 2.1.10.

2.2.4.2 Counting

10 µl of the cell suspension was transferred into a “Neubauer” chamber. Four squares were counted and an average was calculated. The number of cells (n) in one square equals n x 10^4 per ml.

2.2.4.3 Seeding and culturing

Cells were transferred into a new culture dish in a suitable dilution of pre-warmed, fresh medium. Agitation of the culture plates and flasks was used to ensure homogenous distribution of the cells. For culturing primary HK and primary murine keratinocytes, the culture plates were pre-coated with 1:100 diluted collagen G in PBS (either 1 h at 37°C or 24 h at 4°C).

2.2.4.4 Seeding of primary human keratinocytes as mixed culture with mouse embryonic fibroblast cells (NIH3T3)

Primary HK and NIH3T3 cells were seeded in a ratio of 1:1. Briefly, NIH3T3 cells were seeded in DMEM Medium + GlutaMAXTM-I (10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin) in a collagen pre-coated 24-well-plate and shifted to 37 ° for 5 h. Following this step, the medium was carefully aspirated and the same number of primary HK in CnT Basal Medium 1 (100 U/ml penicillin, 100 µg/ml streptomycin) was added to NIH3T3 cells.

2.2.4.5 Freezing and thawing of cells

Cells were trypsinized and pelleted before resuspending them in 1 ml freezing solution containing 90% FCS and 10% DMSO. Immediately, the suspension was put on ice and then stored in liquid nitrogen. For thawing, the freezing vial was taken out of the liquid nitrogen tank and transported on ice. The suspension was thawed in a water bath at 37°C until only some rests of ice were left. Then, the cells were transferred into a 15 ml plastic tube containing the pre-warmed medium

before pelleting the cells at 1000 x rpm for 5 min at room temperature. After resuspension in fresh medium, the cells were plated in culture dishes.

2.2.4.6 Isolation of primary human keratinocytes (monolayer)

The cells were isolated from human foreskin, gained from the children's medical surgery of Dr. med. Hikmet Ulus, Cologne (Z-Project, SFB 829 Ethikvotum 12-163). Human foreskin samples were stored in serum free keratinocytes medium (CnT Basal Medium 1) at 4°C (not longer than 10 days). Skin was intensely cleaned using PBS and treated with Antibiotic-Antimitotic Solution (1:50 dilution in PBS) for 20 min. The foreskin was separated into dermis and connective tissue by using scalpel and forceps. Subsequently, the dermis was milled into small pieces of 1x1 cm^2, transferred into Dispase II solution (10 mg/ml in DMEM Medium + GlutaMAXTM-I pure) and incubated for 24 h at 4°C. Using two forceps, the epidermis was detached from the dermis. To lyse the epidermis into single cells, pieces of epidermis were incubated with 5 ml Trypsin/EDTA for 5 min at 37°C under constant stirring. The reaction was stopped by addition of 5 ml DMEM Medium containing 10% FCS. The suspension was filtered through a 70 µm cell strainer and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 5 ml CnT Basal Medium1, and for further application either $5x10^5$ cells were seeded on a 10 cm culture dish or the cells were frozen and stored in liquid nitrogen.

2.2.4.7 Preparation of organotypic human skin co-cultures

A collagen type I gel with integrated fibroblasts was prepared by mixing the 4 mg/ml rat tail collagen solution with 10x Hank`s balanced salt solution. After neutralization with NaOH, FCS containing mouse dermal fibroblasts in suspension ($1.5\text{-}2x10^5$ cells/ml) was added. All solutions added to the collagen were chilled and the mixture was kept on ice during manipulation. 2.5 ml of this mixture was poured onto filter inserts placed in BioCoat six-well plates. After 1 h incubation at 37°C, glass rings (20 mm diameter) were gently pressed onto the gels to press out excess liquid and to provide a defined area of epithelial growth. After 1 h incubation at 37°C, the liquid was removed and the gels were allowed to equilibrate, submerged overnight in humid atmosphere.

Collagen gel mixture:	
rat tail collagen (4 mg/ml)	80%
Hank`s solution (10x)	10%
FCS	10%
NaOH (5 M)	several drops; until the gel changes color from yellow to light pink

Primary human keratinocytes in 1 ml medium were seeded on to the gels ($1x10^6$ cells/insert). After another 24 h, the glass rings were removed and the cultures were lifted to the air-liquid interface by removing all medium from the surface and incubate the cultures in only 10 ml medium in each well. From this point onward the cultures were only nourished through the collagen gel (Figure 10), [230]. Medium was changed 3x a week.

2.2.5 Vector production and purification

2.2.5.1 AAV library and vector packaging

AAV particles were produced in HEK293 cells by the adenovirus-free production method using pXX6-80 to supplement the adenoviral helper functions [73]. Briefly, $7.5x10^6$ HEK293 cells were seeded in 15 cm^2 cell culture plates. 24 h later (at an approximate confluence of 80%), the medium was exchanged and 2 h later, co-transfection of the three packaging plasmids was performed by the calcium phosphate method with a total of 37.5 µg plasmid DNA per 15 cm^2 cell culture dish.

For rAAV2 vector and rAAV peptide insertion variants:

7.5 µg AAV helper plasmid
(pRC/ pRC-Kera1/ pRC-Kera2/ pRC-Kera3)
7.5 µg scGFP
22.5 µg pXX6-80

For each plate a solution of 1 ml $CaCl_2$ (250 mM) was mixed with the plasmid DNA, and 1 ml of the HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM NaP, pH 6.8) was dropped onto the solution that was subsequently incubated for 2 min and then pipetted onto the plate. After 24 h incubation at 37°C and 5% CO_2, medium was exchanged with DMEM containing 2% FCS to reduce proliferation rate. The

transfected cells were harvested by scraping (48 h post transfection) and pelleted by low-speed centrifugation. The pellet was resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.5)) and subjected to repeated freeze and thaw cycles. Cell lysate was treated with 50 U/ml Benzonase for 30 min at 37°C and cleared by centrifugation (30 min at 4°C and 3.220x g). The supernatant was transferred to a new tube and again centrifuged.

2.2.5.2 Iodixanol gradient purification

Discontinuous iodixanol gradient centrifugation was used to concentrate the vector preparation and to remove cellular debris. Vector suspension was filled into an ultracentrifugation tube. The different phases of the iodixanol gradient, beginning with 15%, were sub-layered by using a syringe connected to an Amersham Biosciences Pump P-1. 8, 6, 5 and 6 ml of the respective solutions were applied. The tube was filled up with PBS/$MgCl_2$ (1 mM)/KCl (2.5 mM), closed and centrifuged at 63,000 rpm at 4°C for 2 h (Beckman Coulter Ultracentrifuge). Subsequently, the 40% iodixanol phase, containing the vector particles, was harvested.

	15%	25%	40%	60%
10x PBS	5 ml	5 ml	5 ml	/
1M $MgCl_2$	50 µl	50 µl	50 µl	50 µl
2.5M KCl	50 µl	50 µl	50 µl	50 µl
5 M NaCl	10 ml	/	/	/
Optiprep	12.5 ml	20 ml	33.3 ml	50 ml
0.5 Phenolred	150 µl	75 µl	/	25 µl
H_2O	ad 50 ml	ad 50 ml	ad 50 ml	ad 50 ml

2.2.5.3 Vector titration

For extraction of the vector genome from the viral particles, the Qiagen DNeasy Blood & Tissue Kit was used according to the protocol for Isolation of “Purification of total DNA from Animal Blood or Cell“. The genomic titer was then determined by qPCR as described in 2.2.2.7.

2.2.5.4 Coupling of pheno- and geno-type of mutants

The cells were seeded 24 h prior to infection in a 15 cm culture dish. First, the AAV library (GOI 1000) was used to infect the cells in 12.5 ml medium. 2 h p.i. the medium was removed; cells were washed with PBS and infected with 10 µl adenovirus in 25 ml medium. After 2 h, the medium was removed, cells were washed with PBS and fresh medium was added. 48 h later the cells were harvested by scraping, followed by a low speed centrifugation. The supernatant was removed and stored at -80°C. The pellet was lysed by the thaw/freeze method and treated with Benzonase. The amount of progeny was determinate by qPCR (see 2.2.2.7).

2.2.5.5 Transducing titer of viral vectors encoding for GFP

48 h after transfection of primary HK with serial dilution of purified AAV vector preparations, cells were harvested and washed with PBS. The total number of cells per well was determined by counting. The number of GFP-expressing cells was measured by flow cytometry. Based on the amount of cells per well, the amount of transgene expressing cells from each dilution and the respective dilution factor, the transducing titer was determined using 10% positive cells (10% equals MOI = 0.1; each transgene expressing cell is a result of a single vector transduction) as reference.

2.2.5.6 Heparin affinity chromatography

To separate the AAV capsid mutants according to their HSPG binding ability, affinity chromatography using HiTrap Heparin Affinity Columns (1 ml) from Amersham Pharmacia Biotec was performed. First, the column was equilibrated with PBS/$MgCl_2$ (1 mM)/ KCl (2.5 mM) (abbrev. PBS M/K), while the library solution was diluted 1:10 in the same buffer and applied to the column. After a washing step with 20 ml PBS M/K, vector was eluted with PBS M/K plus 1 M NaCl in 500 µl steps.

2.2.5.7 AAV peptide display on primary HK

The AAV peptide display technology was previously developed in our lab [144]. Here, $5x10^5$ primary HK per well were seeded in a 6-well-plate. 3 wells were transduced with $1x10^3$ g.p. of the HSPG-non-binder library. 2 h p.i. at 37°C the

library was removed, cells were washed twice with PBS and fresh keratinocyte medium was added. Subsequently, the cells were superinfected with 1 µl 1:100 diluted wild-type adenovirus type 5 (wtAd5). Two hours post super-infection, wtAd5 containing medium was removed and the cells were washed again with PBS. After leaving the cells in keratinocyte medium for 48 h at 37°C, cells were harvested by using a cell scraper, pelleted and resuspended in 200 µl lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8)). To disrupt the cell membrane and collect the viral progeny, three cycles of freezing and thawing were performed. Adenovirus was inactivated by heat (56°C for 30 min). The viral progeny was used for further selection rounds. To raise the selection pressure, the initial amount of viral particles given to the cells were reduced from 1000 g.p. per cell in the first two selection rounds, 100 g.p. in third-, 10 in the fourth- and finally 1 genomic particle per cell in the last selection round. After each selection round, the genomic titer was determined by qPCR using the wtAAV protocol (see 2.2.2.7).

2.2.6 Cell transduction by rAAV vectors

2.2.6.1 Quantification of vector entry efficiency

24 h prior to transduction, cells were seeded sub-confluent in collagen pre-coated 24-well-plates. Cells in one well were counted to determine the cell number. Then cells were incubated with 500 g.p./cell in 500 µl of medium. To allow vector binding, 30 min incubation on ice was performed before cells were shifted to 37°C and 5% CO_2. One hour later, supernatant was removed and cells were washed twice with PBS. To ensure removal of membrane-bound vector particles and to detach the cells, cells were harvested by trypsin treatment [46], [231]. After pelleting of cells at 500x g for 5 min, they were washed twice with 1x PBS. Total DNA was isolated as described before (see 2.2.5.3). Relative quantification of vector genomes (GFP) and reference gene (Plat) was performed by qPCR.

2.2.6.2 Drug treatment

Chlorpromazine (16 µg/ml final concentration) or Genistein (175 µg/ml final concentration) was used to inhibit clathrin- or caveolin-mediated endocytosis. All drugs were added to cells 30 min prior to transduction and remained present until transduction was stopped by washing and trypsin treatment.

2.2.6.3 Cell transduction assays

24 h prior to transduction, cells were seeded sub-confluent in collagen pre-coated 24-well-plates. The cells of one well were counted and incubated with vectors at indicated vector per cell ratios as described before (see 2.2.6.1). To stop the treatment with Chlorpromazine and Genistein after 2 h, cells were washed twice with 1x PBS and harvested by trypsin treatment. Cells were re-seeded in fresh medium for 48 h at 37°C and 5% CO_2. Percentage of transduced cells was determined by flow cytometry using a BD FACS Calibur system. A minimum of 10000 cells were measured in the FITC channel and the background fluorescence was set to 1%.

2.2.6.4 Heparin competition assay

24 h prior to transduction, cells were seeded sub-confluently in collagen pre-coated 24-well-plates. After determination of the cell number per well, vectors were incubated with 470 U/ml of soluble Heparin in 500 µl medium for 5 min and subsequently applied to the cells. 48 h post transduction cells were harvested and the percentage of GFP-expressing cells was determined by flow cytometry (see 2.2.6.3).

2.2.6.5 Peptide and α_V blocking-antibody competition assay

24 h prior to transduction, cells were seeded sub-confluently in collagen pre-coated 24-well-plates. After determining the cell number per well, 300 µM of peptides (competing and non-competing) or 2 µg/ml α_V blocking-antibody were combined with 200 µl fresh medium and incubated with the cells for 15 min at 37°C [141]. Then for the peptide competition assay $5x10^3$ g.p./cell of rAAV2 and $7.5x10^2$ g.p./cell of the rAAV peptide insertion variants and for the α_V blocking-antibody competition assay $6x10^2$ g.p./cell of rAAV2 and targeting vectors were incubated for 4 h at 37°C. Cells were washed twice with 1x PBS and re-seeded [46], [231]. 48 h post transduction percentage of GFP-expressing cells was defined by flow cytometry (see 2.2.6.3).

2.2.6.6 $\alpha_V\beta_8$ antibody competition assay

24 h prior to transduction, cells were seeded sub-confluently in 24-well-plates. Cells were washed with ice cold PBS, followed by incubation with 200 µg/ml of

$\alpha_V\beta_8$ antibody for 30 min. $7.5x10^2$ g.p per cell of rAAV2 and rAAV variants, respectively, were incubated 60 min on ice. Subsequently the medium was removed. Cells were washed with PBS/10% FCS and supplied with fresh, pre-warmed medium. 48 h post transduction GFP-expressing cells were determined by flow cytometry (see 2.2.6.3).

2.2.6.7 Transduction of mixed cultures

24 h prior to transduction, primary HK and NIH3T3 cells were seeded sub-confluent in collagen pre-coated 24-well-plates (see 2.2.4.6). Cells were incubated with $5x10^3$ g.p. per cell of vectors (number of particles were calculated according to the number of HK cells). 48 h p.t. cells were stained with the anti-feeder antibody to discriminate between primary HK and NIH3T3 cells, and analyzed by flow cytometry for percentage of GFP expressing cells (see 2.2.6.3).

2.2.6.8 Transduction of organotypic human skin co-cultures

A sterile glass ring was placed by forceps onto the keratinocyte top layer and filled with 200 µl DMEM with 10% FCS (see 2.1.9) containing $1.5*10^{10}$ g.p.of rAAV2 or selected variants (see 2.2.6.7), respectively. The culture was allowed to incubate for 2 h at 37°C and 5% CO_2. Vector containing medium was removed by pipetting. Culture was incubated for further 72 h at 37°C and 5% CO_2. For fixation, the filter including the fibroblast gel and the stratified keratinocytes were detached by scalpels. The samples were fixed in 2% PFA in 3.5% Sucrose/ PBS for 30 min at room temperature, then embedded in Tissue-Tek® and stored at -80°C. For preparing cryosections Cryostat LEICA CM1850 was used.

2.2.7 Immunohistochemistry

2.2.7.1 Immunofluorescence staining of cryosections of organotypic human skin co-cultures

Cryo-sections were mounted by IS Mounting Medium DAPI. This mounting medium is fortified with DAPI which is a counter-stain for DNA and is used for nuclear staining. Since the vectors used for transduction carried GFP as a transgene, GFP-expressing cells in cryo-sections, were analyzed by fluorescence microscopy.

3 Results

Considering the options to cure chronic wounds, inherited skin diseases or skin cancer, there is a need for new therapeutic strategies. Gene therapy has the potential to play an important role in wound healing. The purpose of gene therapy in this setting might be either to promote wound healing by gene delivery of growth factors or to improve engraftment of autologous skin transplants by overexpression of anti-inflammatory or pro-angiogenic factors through genetic engineering of keratinocytes [232]. A key factor in the success of these approaches is the development of gene delivery systems that are capable of efficient and safe gene transfer [233]. Viruses are natural vehicles for gene delivery [234]. Thus, they are a promising basis for the development of gene delivery tools in gene therapy. Here, the focus was on Adeno-associated viral vectors, which showed so far an excellent safety profile and an increasing number of reports on clinical benefit [47].

3.1 Characterization of cell surface receptors of primary human keratinocytes

Gene delivery vectors are required to be efficient in cell transduction [208], but previous studies revealed that primary human keratinocytes (HK) are resistant to transduction by AAV vectors [206], [207], [208]. In line, transduction of primary HK, isolated and cultivated in our laboratory were incubated with, $5x10^3$ vector particles of rAAV2 per cell but did not exceed 5% (Figure 11).

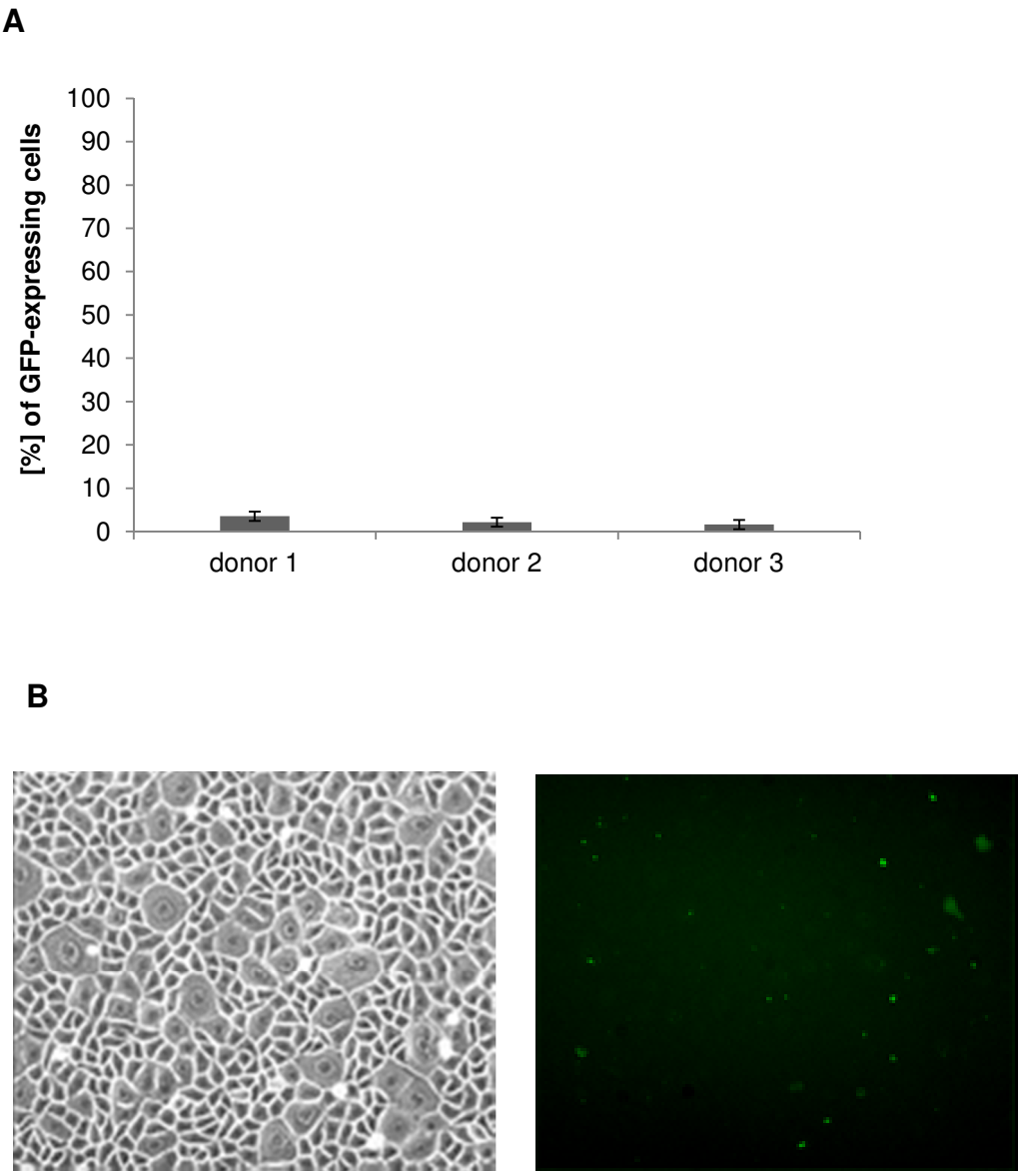


Figure 11: Transduction efficiencies of rAAV2 with wild-type capsid on primary HK of different donors.
***A**: Cells were incubated with rAAV2 (5x10^3 g.p./cell) encoding for GFP in a self-complementary genome conformation. Percentage of transduced cells was determined by flow cytometry 48h p.t. Values represent the mean of three independent experiments, and error bars show SD.*

***B:** Primary HKs were seeded sub-confluently on collagen pre-coated cover slips and incubated with 5x10^4 g.p./cell of rAAV. Cells were analyzed 24 h p.t. by immune fluorescence (x40 magnification). The microscopic images were kindly provided by the group of Fernando Laguzzi Larcher, Ciemat, Madrid.*

Aiming to gain insight on the cause of the refractoriness towards AAV2, a characterization of cell surface receptors on primary HK was performed. Specifically, the presence of heparan sulfate proteoglycan (HSPG), [37] was determined and AAV2s' internalization receptors $\alpha_V\beta_5$ and $\alpha_5\beta_1$ integrin [39], [235], was tested by flow through cytometry using the corresponding antibodies. As a

control, the human cervix carcinoma cell line, HeLa, was used because it is highly permissive for rAAV2 (Figure 12).

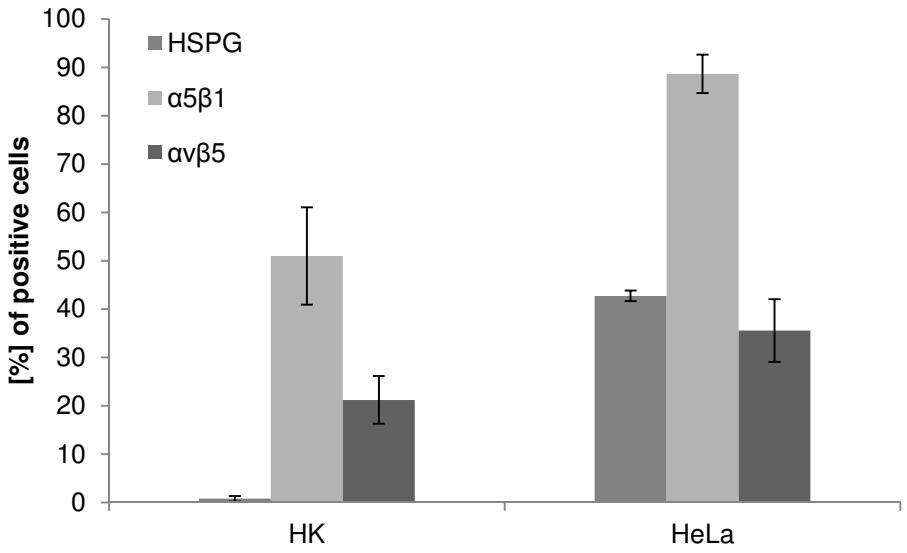


Figure 12: Characterization of cell surface receptors on primary HK and HeLa cells
The primary antibodies against HSPG and the integrins ($\alpha_V\beta_5$, $\alpha_5\beta_1$) were separately incubated with the cells, followed by a PE-labeled IgG secondary antibody. Cells were measured by flow cytometry. The experiment was performed three times independently, error bars show SD.

In contrast to HeLa cells HSPG is barely detectable on primary HK (Figure 12), while the integrins are expressed on HeLa and the target cells. According to the current model of AAV2 infection, binding of the capsid to HSPG induces a conformational change required for binding to $\alpha_V\beta_5$ or $\alpha_5\beta_1$ integrins and ultimately for inducing virus/vector internalization [39]. The lack of HSPG expression on primary HK likely hinders efficient rAAV2 transduction. A possible solution to overcome the lack of receptor expression is cell surface targeting, i.e. directing the viral vectors towards a novel receptor. Recently in our group, a high-throughput method, AAV peptide display, was developed to identify suitable ligands [144]. In this thesis, this technology was used to develop AAV variants with improved transduction efficiency compared to rAAV2 on primary HK.

3.1.1 Selection of rAAV targeting vectors from a library enriched for non-HSPG binding mutants

To select AAV capsids variants with high transduction efficiency on primary HK, the AAV peptide display library was used. The AAV peptide display library was packaged in HEK293 cells followed by coupling of geno- and phenotype (PCT/EP2008/004366), [144]. The latter is required to ensure that the genome encodes the peptide that is displayed on the capsid. The AAV2 display library was then depleted for mutants displaying HSPG-binding peptides by affinity chromatography (see 2.2.5.6). The genomic titer of the HSPG-non-binder library (NB) was $5.8x10^{10}$ g.p/ml as determined by qPCR (2.2.2.7). Next, primary HK, isolated from foreskin of different healthy donors were subjected to five rounds of AAV peptide display selection.

Briefly, in the first selection round, primary HK were incubated with $1x10^{3}$ genomic particles (g.p.) per cell of the library for two hours. The viral particles, which failed to enter the cells, were removed by exchanging the medium followed by superinfection with adenovirus to provide helper virus function. Viral progeny was harvested from the cells 48 h p.i. and the genomic titer was determined by qPCR. The viral progeny was used for next selection round. To raise the selection pressure, the amount of viral particles given to the cells were subsequently reduced from initially 1000 g.p./cell to 1 g.p./cell (Figure 13). Isolated from progeny of the final selection round, viral genomes were sequenced in order to determine the sequence of the peptide inserts. Specifically, viral genomes were amplified by PCR using primers that flank the insertion site and cloned into pRC Kotin (see 2.2.2.9) for bacterial transformation. Single colonies were picked and sequenced (see 2.2.2.8).

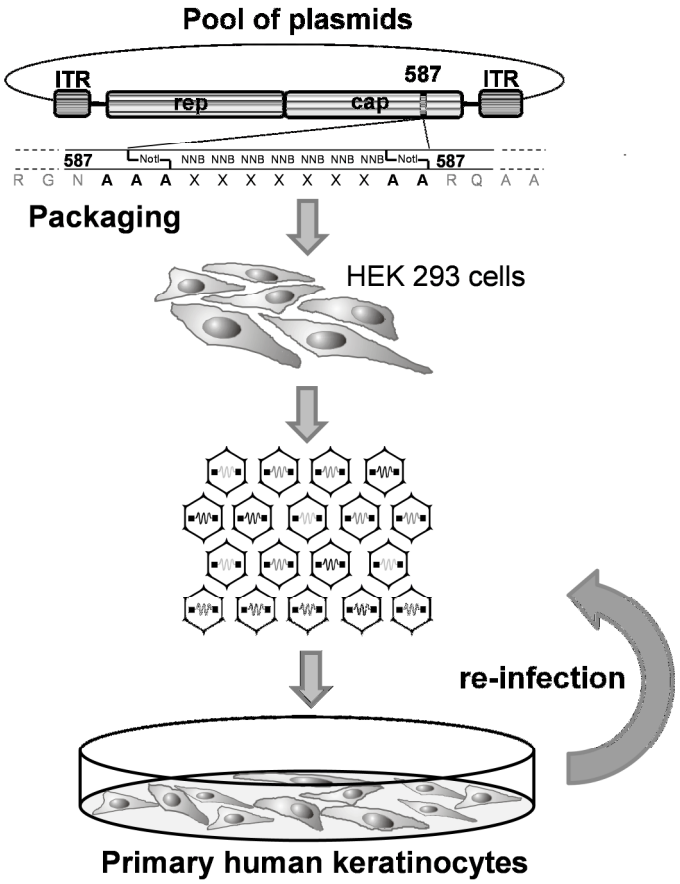


Figure 13: Schematic representation of AAV peptide display selection on primary HK

A total of 35 single viral clones were analyzed (Table 2). With the exceptions of two motifs, PRGDLRP and RGDQHSL, all sequences show an overall neutral net charge. 89% of the sequences resemble classical integrin binding ligands with RGD tripartite motif or contained a RSD motif, described to be functionally equivalent to RGD [236], [237].

Table 2: Sequences identified after the fifth selection round
Sequences are given in one letter code. Bold letters represent charged aa.

frequency	sequence	net charge
17	**R**G**D**TATL	neutral
2	P**R**G**D**LAP	neutral
4	**R**G**D**QQSL	neutral
3	**R**S**D**LASL	neutral
1	P**R**G**E**LAP	neutral
2	G**R**G**D**LAP	neutral
1	**R**G**D**TASL	neutral
4	P**R**G**D**L**R**P	positive
1	**R**G**D**Q**H**SL	positive

In four out of nine sequences, the RGD/RSD binding ligand is followed by either an LA or LR. Out of the nine selected targeting variants, three motifs with a neutral charge were chosen for further analysis. The AAV clones with the sequences RGDTATL (Kera1) and RGDQQSL (Kera3) were chosen because of the prevalence of their occurrence. In addition, the peptide sequence PRGDLAP (Kera2) was included. Kera2 was of interest because it contains the prominent LA motif adjacent to the potential integrin-binding motif flanked and a proline residue at position 1 and 7 of the insert. The three mutants and the parental serotype rAAV2 were packaged as recombinant vectors encoded for enhanced green fluorescent protein (GFP) in a self-complementary vector genome conformation (see 2.2.5.1). Genomic titers were determined by qPCR (see 2.2.2.7) and capsid titers were measured by A20 ELISA (see 2.2.3). The values were used to calculate the total-to-full particle ratio to judge whether the peptide insertion impacts on efficiency with which the viral vector genome is packaged into the pre-formed capsids (“packaging efficiency”). Although the ratios for the targeting vectors were found to be increased in comparison to rAAV2, the ratios were still in the range defined as “wild-type” phenotype [38].

Table 3: Characterization of selected rAAV peptide insertion variants
Genomic titers were determined by qPCR. Capsid titers were determined by A20 ELISA. The packaging efficiency is specified by calculated the capsid-to-genome ratio.

vector	sequence in 587	net charge	genomic titer [/µl]	capsid titer [/µl]	capsid/genome ratio
Kera1	RGDTATL	neutral	$2.40x10^8$	$2.40x10^9$	8.25
Kera2	PRGDLAP	neutral	$2.56x10^8$	$2.12x10^9$	8.28
Kera3	RGDQQSL	neutral	$1.34x10^8$	$1.39x10^9$	10.37
rAAV2	----------	----------	$6.41x10^8$	$9.19x10^8$	1.42

3.2 Characterization of rAAV peptide insertion variants regarding cell entry and transduction efficiency on primary HK

The cell entry efficiency of the selected targeting variants was studied on the target cells in comparison with rAAV2. Therefore, primary HK, isolated from healthy donors, was incubated with equal numbers/cells of rAAV2, Kera1, Kera2 and Kera3. Cells were harvested by trypsin treatment 90 min p.t. and total DNA was isolated (see 2.2.6.1), [220]. To determine intracellular vector genomes, qPCR was performed for vector DNA (GFP) and the single-copy gene plasminogen activator (PLAT). Melting peak analysis was accomplished to proof specificity of PCR products. The target gene (GFP) was normalized to the reference gene (PLAT) and values obtained for rAAV2 were set to 1 (see 2.2.2.7). Kera2 was the most efficient variant with a 2500-fold increase in cell entry efficiency in comparison to rAAV2, followed by Kera3 (1700-fold increase) and Kera1 (1600-fold increase), (Figure 14).

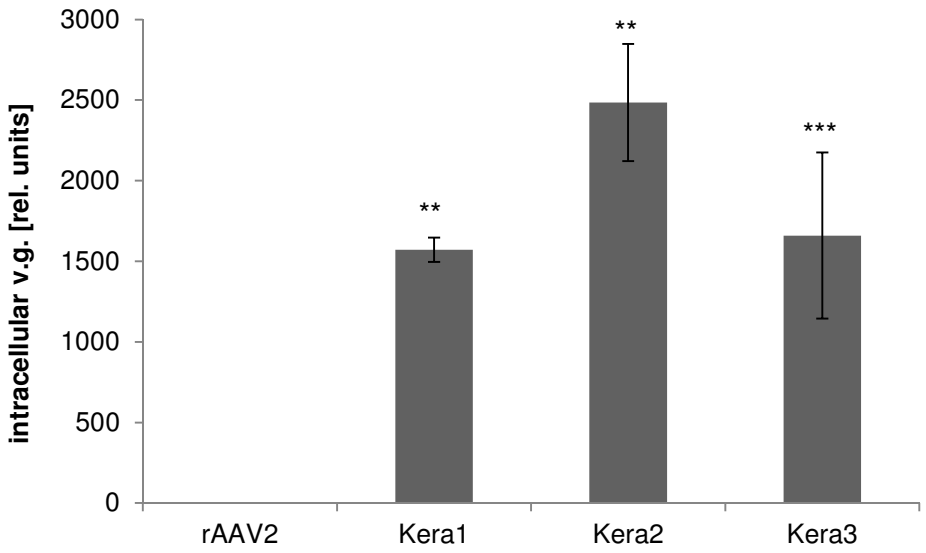


Figure 14: Cell entry efficiencies of indicated vectors
*Primary HK were transduced with the rAAV peptide insertion variants and rAAV2, respectively. 90 min p.t. total DNA was isolated from the cells and the number of intracellular vector genomes were determined by qPCR. Normalization of target (GFP) to reference gene (PLAT) was done and the normalized target-reference ratio for rAAV2 was set to 1. Values represent the mean of three independent experiments; error bars show SD. To define statistical significance between entry efficiencies of rAAV2 and the selected peptide capsid variants, Student's t-test was performed. **p<0.01; *** p<0.001, n=3*

The transduction efficiency of the rAAV peptide insertion variants was determined by microscopy and by FACS analysis (see 2.2.6.3). For imaging, primary HK were seeded on collagen pre-coated cover slips and transduced with $5x10^4$/cell. 24h p.t. cells were fixed and analyzed by fluorescent microscopy for GFP expression with a magnification of 40x. These experiments were kindly performed by the group of Fernando Laguzzi Larcher, Ciemat, Madrid.

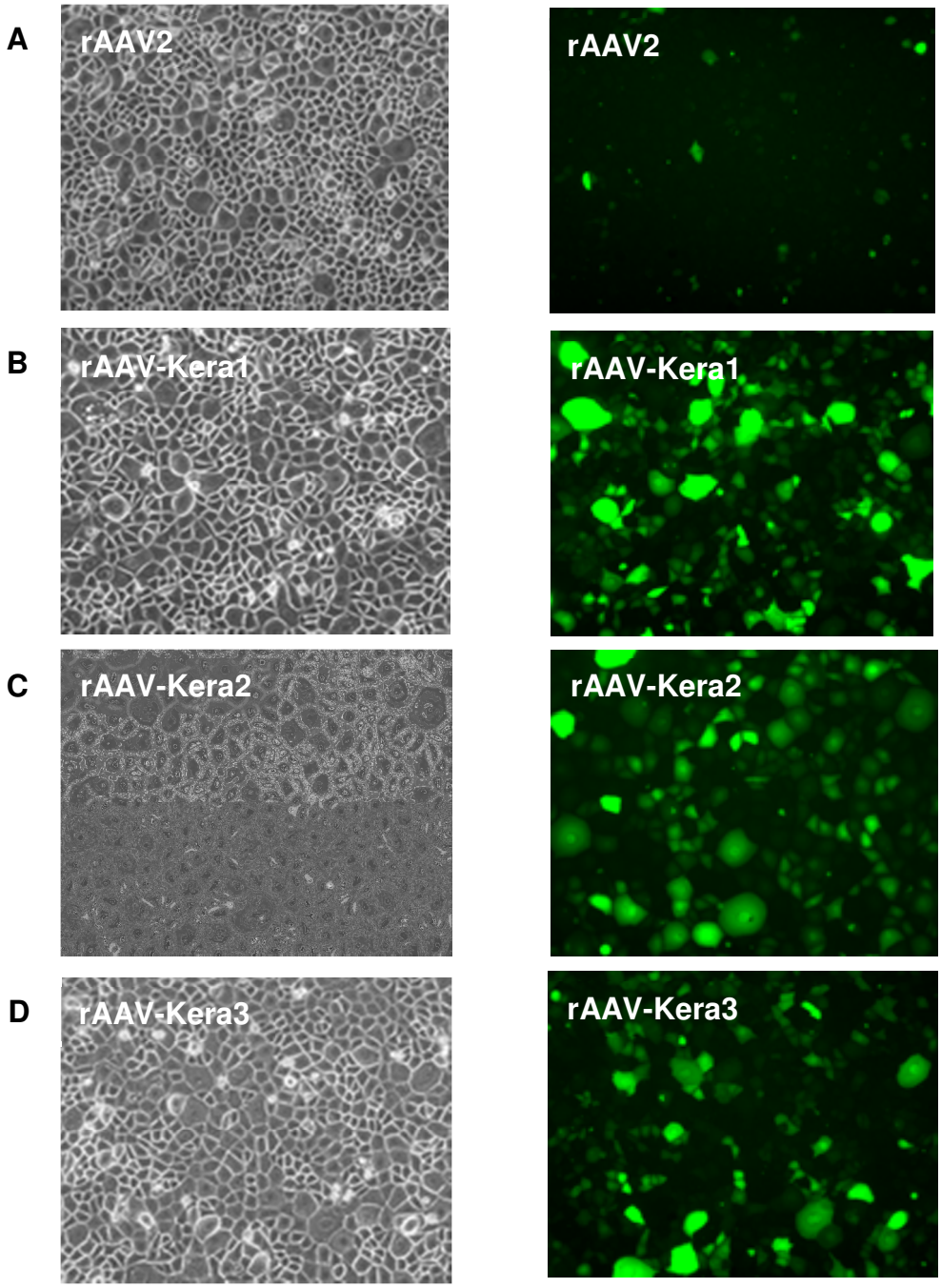


Figure 15: Microscopic images of primary HK transduced with rAAV2, Kera1, Kera2 and Kera3 24 h p.t. primary HK were fixed and analyzed by fluorescence microscope with 40x magnification. The pictures were kindly provided by the group of Fernando Laguzzi Larcher, Ciemat, Madrid.

As indicated in (Figure 15), transduction efficiencies of the selected targeting variants Kera1, Kera2 and Kera3 significantly exceeded those of rAAV2. This result was supported by flow cytometric measurements. Briefly, equal numbers of g.p./cell of the rAAV peptide insertion variants and rAAV2, respectively, were incubated with primary HK. Determination of the percentage of transgene expressing cells was performed 48 h p.i. (Figure 16).

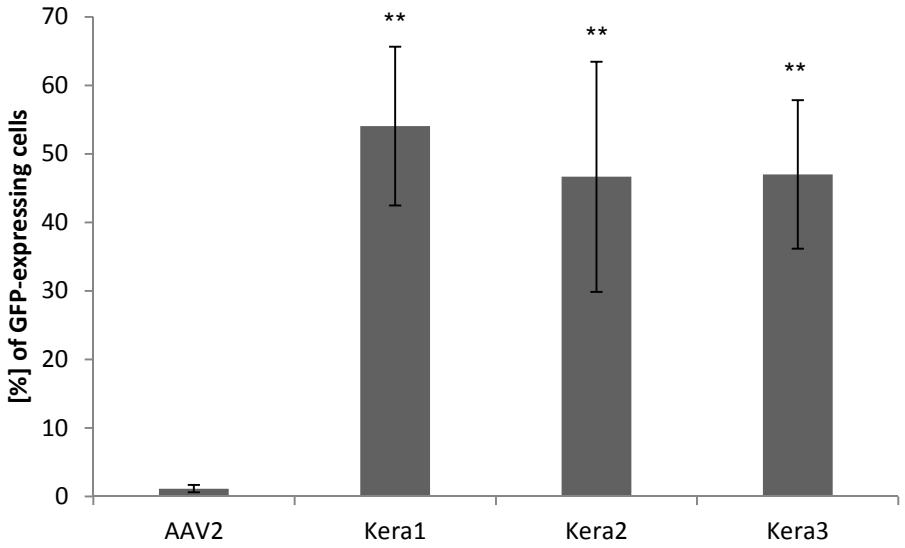


Figure 16: FACS analysis of rAAV2 and rAAV peptide insertion variants on primary HK.
*Primary HK were transduced with 5x10^3 g.p./cell of rAAV2, Kera1, Kera2 and Kera3 and incubated at 37°C in a humidified CO_2 incubator. 48 h p.t. the number of GFP-expressing cells was determined by flow cytometry. Values represent the mean of three independent experiment; error bars show SD. To define statistical significance between transduction rAAV2 and the selected peptide capsid variants, Student's t-test was performed. ** p<0.01, n=3*

Kera1 showed the highest transduction efficiency (54% +/- 11.6%) followed by Kera2 (47% +/- 16.8%) and Kera3 (47% +/- 10.8%), while the transduction efficiency of rAAV2 is only 1.2% +/-0.5%. Thus, Kera1, Kera2 and Kera3 showed a significant enhanced entry efficiency and transduction efficiency (Figure 14, Figure 15 and Figure 16).

3.2.1 Infectivity of rAAV2 and rAAV2 selected peptide insertion variants on primary human keratinocytes

To compare the different vectors with regard to infectivity independent of the volume of the preparation [38], transducing titers on the target cells and capsid titers were used to determine the infectivity for all four vector preparations. Infectivity is determined as ratio of transducing-to-capsid titer (Table 4).

Table 4: Transducing titer and infectivity of rAAV2 and rAAV peptide insertion variants determined on primary HK
The infectivity was calculated as the ratio of capsid (Table 3) to transducing titer. According to Kern and colleagues the wild-type phenotype on HeLa cells corresponds to a ratio of ≤10^4; reduced infectivity corresponds to ratios >10^4 to <10^6; and low infectivity corresponds to ratios of ≥10^6.

	transducing titer	infectivity
vector	**[infectious particles/ml]**	**no. of capsid/no. of infectious particles**
Kera1	$4.70x10^7$	$5.11x10^3$
Kera2	$3.16x10^7$	$8.10x10^3$
Kera3	$3.10x10^7$	$1.24x10^3$
rAAV2	$1.32x10^6$	$4.90x10^5$

As depicted in Table 4, Kera1, Kera2 and Kera3 showed comparable transducing titers on target cells, which are up to 37x higher than the value obtained for rAAV2. The highest infectivity was determined for Kera1 and Kera2, followed by Kera3.
In summary, the selected rAAV peptide insertion variants display a much higher infectivity on the target cells than rAAV2.

3.3 Transduction efficiencies of rAAV2 and rAAV2 peptide insertion variants in presence or absence of Heparin

As mentioned previously (see 1.1.2), HSPG serves as primary receptor for AAV2. Heparin resembles the heparan sulphate residues on HSPG and is therefore used as its soluble analogue. Heparin binds to the capsid of AAV2, impairing the binding to HSPG on the cell surface in a competitive way [37]. Although, it was shown that primary HK did not express HSPG (see 3.1), it was determined whether the presence of Heparin impacts on cell transduction of rAAV peptide insertion variants Kera1, Kera2 and Kera3. Therefore they were incubated with Heparin and

then added to primary HK. As depicted in Figure 17 Heparin did not interfere with cell transduction of Kera1, Kera2 and Kera3 on primary HK.

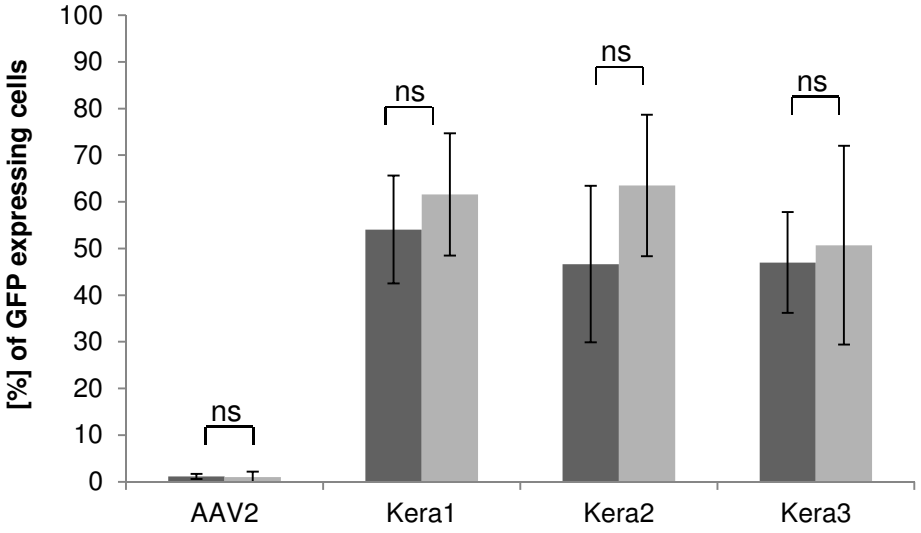


Figure 17: Heparin competition assay on primary HK
Transduction rate of peptide insertion variants and rAAV2 were assessed by flow cytometry after pre-incubation of viral preparation without (dark grey) or with (light grey) 470 u/ml soluble Heparin. Values represent the mean of three independent experiments; error bars show SD. To define statistical significance between cells treated with and without Heparin, Student's t-test was performed. ns = non-significant.

3.4 Peptide competition of selected rAAV2 peptide insertion variants on human primary keratinocytes

In order to assay whether the peptides displayed at position 587 mediates cell transduction of the corresponding AAV peptide insertion variant, a peptide competition assay on HK was performed. Specifically, primary HK were transduced with Kera1, Kera2, Kera3 and rAAV2 in presence or absence of 300 µM GRGDS or GRGES peptides, respectively. Additionally, the capsid variant rAAV-RGD4C587 displaying the CDCRGDCFC motif in position 587 and transducing cells through $\alpha_V\beta5$ and $\alpha_V\beta3$ integrins [141] was used as positive control. Notably, in order to increase the likelihood of measuring cell transduction by rAAV2 and RGD4C587 on HK, seven times higher particle per cell numbers compared to the selected rAAV peptide insertion variants were used.

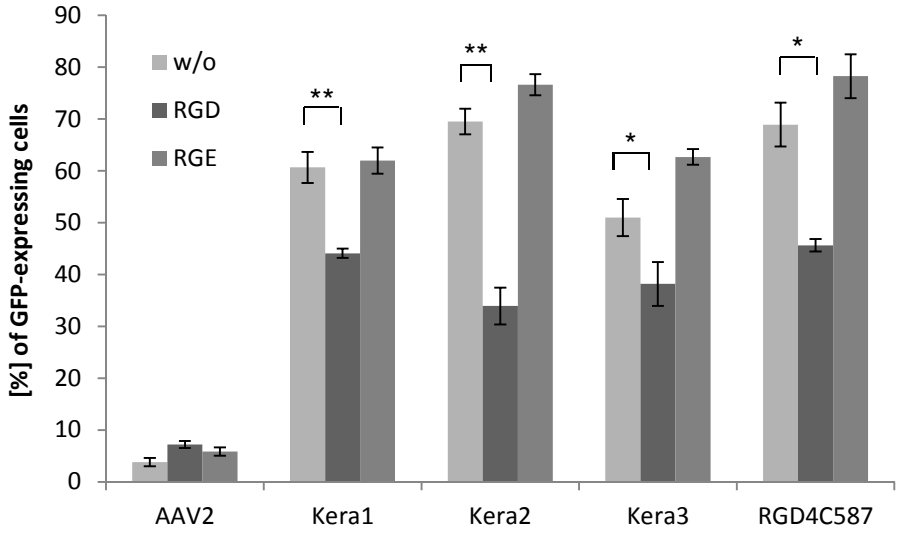


Figure 18: Peptide competition on primary HK
*Transduction efficiencies of the rAAV peptide insertion variants, rAAV2 and rAAV-RGD4C587 were determined after incubation of primary HK in the presence or absence of 300 µM of G**RGD**S (competing peptide) or G**RGE**S (non-competing peptide) peptide, respectively, by flow cytometry. Values represent the mean of a technical triplicate. Error bars show SD. To define statistical significance between cells treated with and without peptides, Student's t-test was performed. * p<0.05; ** p<0.01*

Due to the low transduction efficiency of rAAV2, a judgment was impossible. The transduction efficiency of rAAV-RGD4C587 was significantly reduced in presence of the RGD-containing peptide while transduction efficiency was not affected in presence of RGE-containing peptides (Figure 18). However, to gain transduction efficiency on primary HK with RGD4C587 comparable to Kera1, Kera2 and Kera3 it was necessary to apply a substantially higher number of g.p./cell although the cells expressing $\alpha_V\beta_5$ integrin.

The three selected rAAV peptide insertion variants showed a significant reduced efficiency in the presence of the GRGDS but not GRGES peptides. The most dramatic effect was observed for Kera2. In summary and in line with previous studies, the selected rAAV2 peptide insertion variants transduced the target cells peptide-dependent.

3.5 Selected rAAV2 peptide insertion variants enter via clathrin-mediated endocytosis

RGD motifs are frequently found as part of ligands that bind to an integrin [238]. Integrins are transmembrane proteins that can be endocytosed either through the clathrin- or the caveolin-pathways [239]. To determine the pathway involved in the uptake of the AAV capsid variants, inhibition studies using Genistein or Chlorpromazine (CPZ) were conducted. Genistein blocks caveolae-mediated internalization through inhibition of protein tyrosine kinases [240]. Primary HK were incubated with 175 µg/ml Genistein for 30 min at 37°C, followed by addition of $5x10^3$ g.p./cell of rAAV2 and the selected rAAV peptide insertion variants, respectively. As control, cells were incubated with the vectors in the absence of Genistein. Cell transductions were stopped 2 h p.i. by re-seeded the cells into a new, freshly collagen pre-coated culture plate. The GFP-expression of the cells was determined by flow cytometry 48 h p.i. (Figure 19).

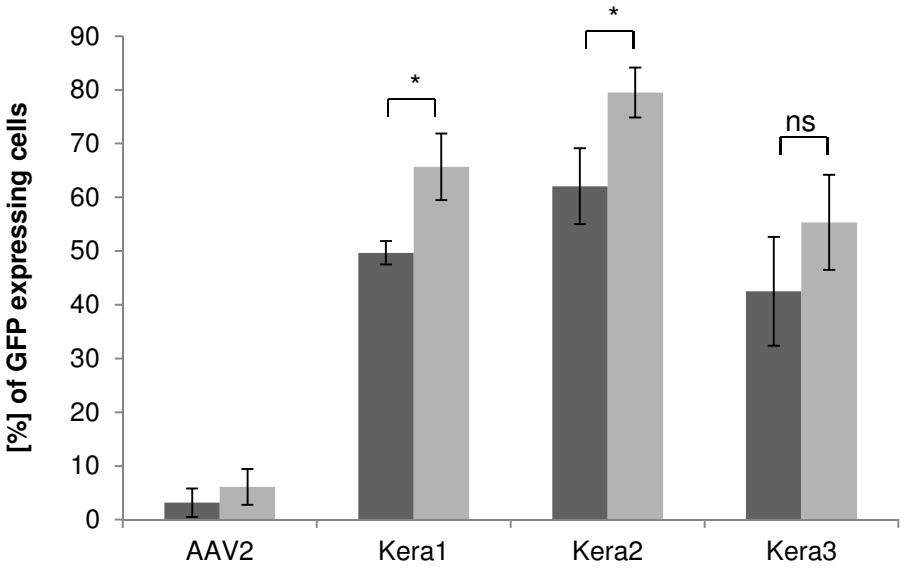


Figure 19: Cell transduction in presence and absence of Genistein
*Flow cytometric analysis of primary HK incubated with indicated vectors without (dark grey) or with (light grey) Genistein. Values represent the mean of three independent experiment; error bars show SD. To define statistical significance between cells treated with and without Genistein, Student's t-test was performed. ns = non-significant, * p<0.05*

No impairment in transduction efficiency was observed for the capsid insertion variants in the presence Genistein. Of note, the low transduction efficiency of rAAV2 on HK did not allow for a conclusive judgment (Figure 19). Next, the effect

of CPZ on cell transduction was determined. CPZ is known to inhibit clathrin-mediated endocytosis by leading to a miss-assembly of clathrin lattices [241]. Primary HK were incubated with a final concentration of 16 µg/ml CPZ for 30 min at 37°C, followed by addition of $5x10^3$ g.p./cell of rAAV2 and $3x10^3$ g.p./cell of Kera1, Kera2 or Kera3, respectively. As a control, cells were incubated with the indicated vector preparations in the absence of CPZ. To stop the infection process, cells were re-seeded 2 h p.i. on a pre-coated collagen plate. After 48 h at 37°C, the number of GFP-expressing cells was determined by flow cytometry.

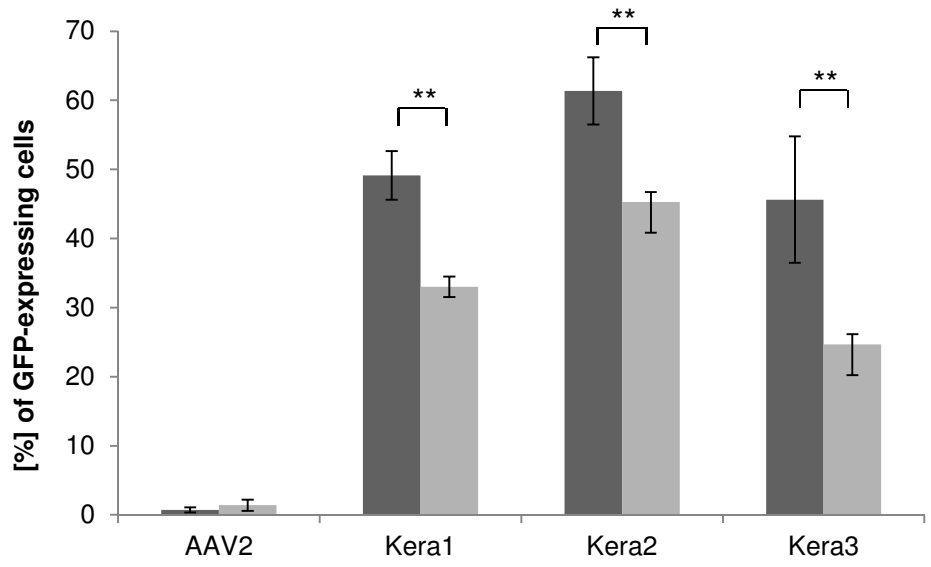


Figure 20: Cell transduction in presence and absence of CPZ
*Primary HK were incubated with indicated vectors without (dark grey) or with (light grey) CPZ as described. Number of transgene expressing cells was determined by flow cytometry. Values represent the mean of three independent experiments; error bars show SD. To define statistical significance between cells treated with and without CPZ Student's t-test was performed. ** p<0.01*

The presence of CPZ significantly reduced the transduction efficiency of Kera1, Kera2 and Kera3 (Figure 20). Thus, these data pointed towards a clathrin-, rather than caveolin-dependent transduction of primary HK by Kera1, Kera2 and Kera3.

3.6 rAAV2 peptide insertion variants show altered tropism

To characterize the specificity of the rAAV2 peptide insertion variants, cell lines representing potential non-target cell types were transduced. Specifically, the cell lines BLM and A375 were chosen as example of human melanoma cells. The human prostate cancer cell line DU-145 was selected as an example of human epithelial cells. The human hepatoma cell line HepG2 was chosen since AAV2 vectors tend to accumulate in liver tissue after systemically as well as local application [141]. NIH3T3 cells were chosen as an example for fibroblasts. Moreover, fibroblasts are frequently found in the skin and are often used as feeder layer for ex vivo keratinocyte cultures during tissue engineering. BML cells, HepG2 cells and NIH3T3 cells were transduced with increasing numbers of g.p./cell by the rAAV2 peptide insertion variants and rAAV2, respectively (Figure 21). To transduce A375 and DU-145 cells $5x10^3$ g.p./cell of the rAAV2 peptide insertion variants and rAAV2 were applied, respectively (Figure 22). 48 h p.t. the cells were analyzed by flow cytometry (see 2.2.6.1).

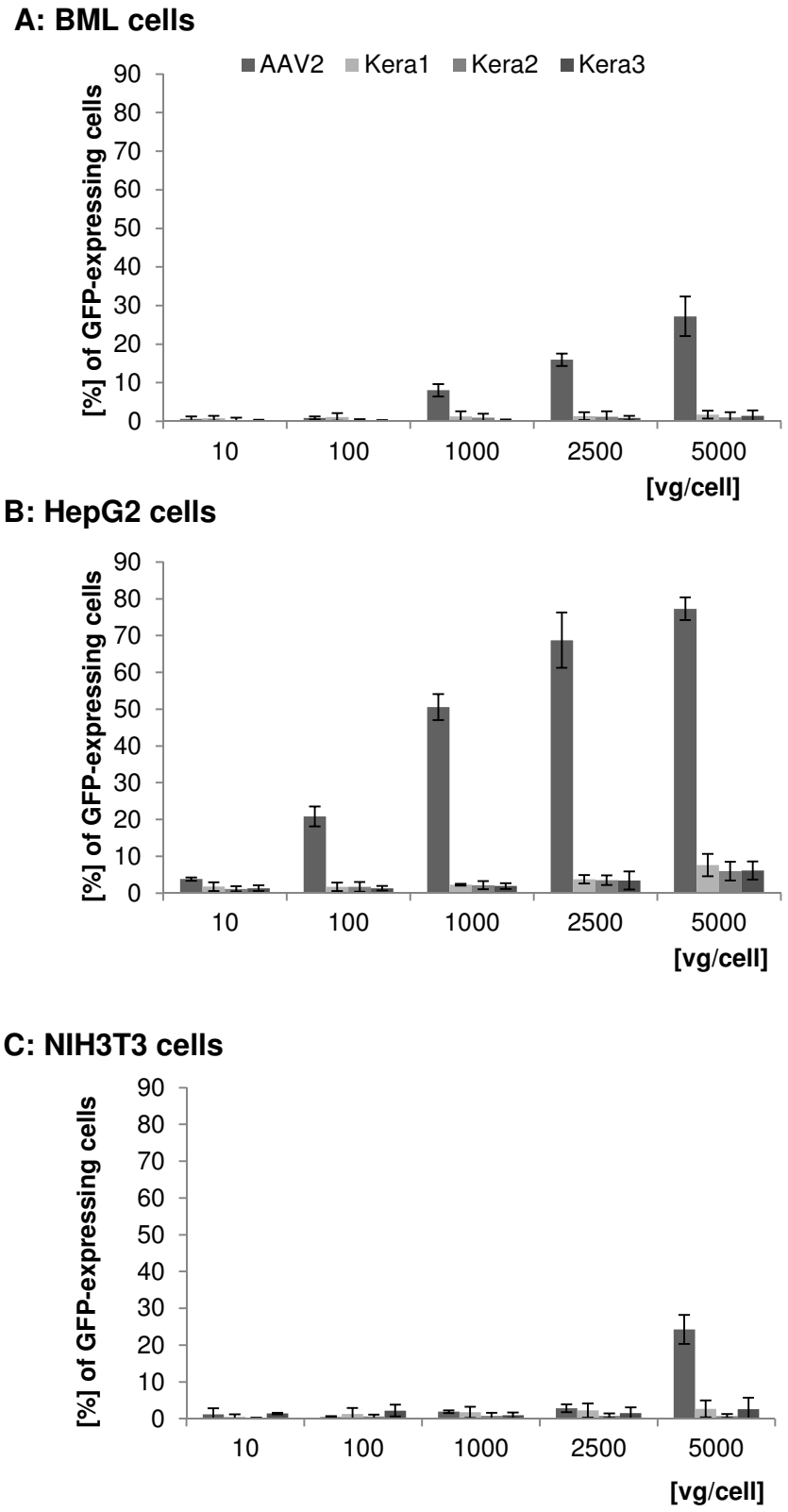


Figure 21: Transduction experiments of indicated vectors on non-target cells
*Different non-target cells (**A**: the human melanoma cell line BLM, **B**: the hepatoma cell line HepG2 and **C**: the mouse fibroblast cell line NIH3T3) were transduced with 10, 100, 1000, 2500 and 5000 g.p/cell and percentage of GFP-expressing cells was determined by flow cytometry 48 h p.t. All experiments were performed three times independently; error bars show SD.*

rAAV2 show the highest transduction efficiency for HepG2 cells with up to 77.3% +/- 3.1% of GFP-expressing cells, followed by BLM cells (27.2% +/- 5.1%). The lowest transduction efficiency for rAAV2 was measured in NIH3T3 cells (24.3% +/- 3.6%). In contrast, none of the three cell lines were transduced by Kera1, Kera2 or Kera3 above the background level even when applying $5x10^3$ g.p./cell (Figure 21).

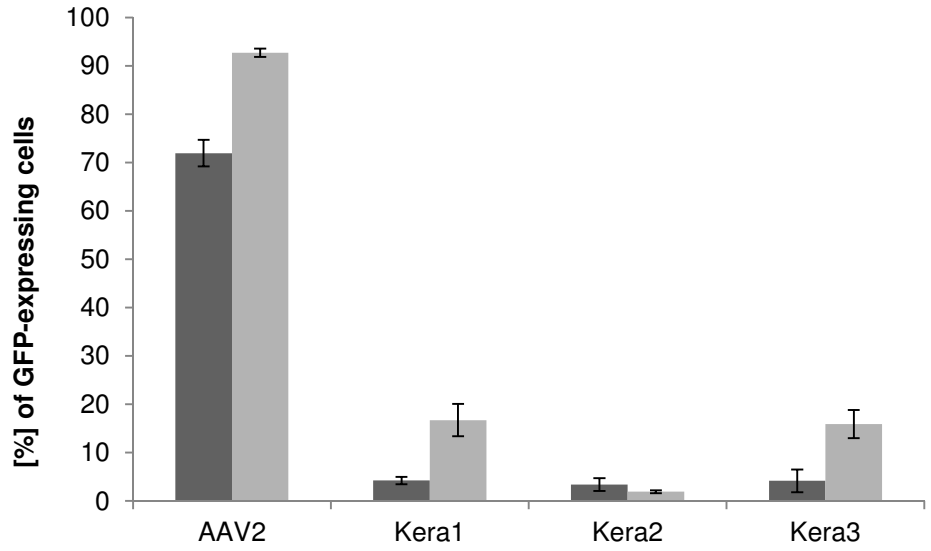


Figure 22: Transduction efficiencies of indicated vectors on DU-145 cells (dark grey) and A375 cells (light grey)
The number of GFP-expressing cells was determined by flow cytometry 48 h p.t.. The experiments were performed three times independently error bars show SD

While rAAV2 achieved transduction efficiencies higher than 90% on A375, transduction efficiencies of Kera1, Kera2 and Kera3 were below 16% (+/- 3.3%). The target vector, Kera2, in particular, did not transduce these cells. Also, DU-145 cells were efficiently transduced by rAAV2 (72.0% +/- 2.4%). Kera1, Kera2 and Kera3 again, showed transduction efficiencies below 5% (Figure 22).

3.6.1 Cell transduction of rAAV2 peptide insertion variants on feeder cultivated primary human keratinocytes

Primary HK are frequently cultured in the presence of fibroblasts, which function as feeder cells [183]. Therefore, primary HK and NIH3T3 cells were co-cultured and transduced with the rAAV2 peptide insertion variants with $5x10^3$ g.p./cell. Cells were harvested 48 h p.t. and stained with anti-Feeder antibody to discriminate between NIH3T3 feeder cells and primary HK, followed by flow cytometry

measurement (see 2.1.6.1). Kera1, Kera2, Kera3 show remarkable preference for the target cells (Figure 23). For Kera1 and Kera3 transduction efficiencies of 65% and, 69% for primary HK and of 20% and 11% for fibroblasts were measured, respectively. The most considerable results were obtained for Kera2, which transduced about 83% of primary HKs, while only 7.1% the NIH3T3 feeder cells were positive for GFP.

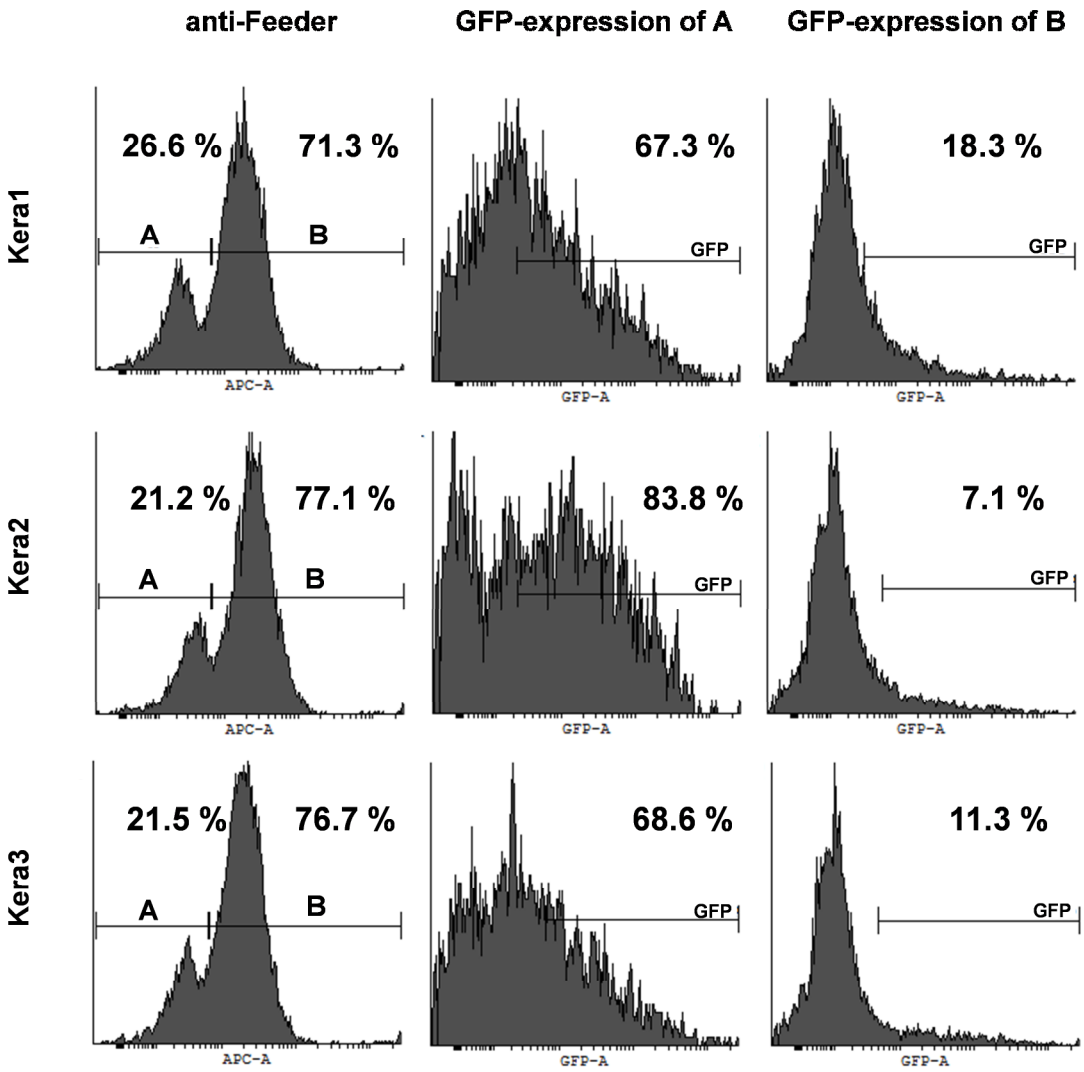


Figure 23: Target cell specificity of indicated vectors in mixed cultures
*Primary HK were co-cultured with NIH3T3 feeder cells in a 1:1 ratio and then transduced with the rAAV2 peptide insertion variants. Cells were stained with an anti-feeder antibody and analyzed by flow cytometry. The anti-Feeder antibody was used to discriminate between HK (**A**) and feeder cells (**B**). The primary HK were gated out from A to determine the GFP-expressing HK. To determine the transgene expressing NIH3T3 cells the cells from B were gated out. Here one representative experiment out of three is shown.*

Target-to-noise ratios are calculated as indicator of vector specificity. Briefly, transduction efficiency on target and non-target cells obtained with the same

g.p./cell ratio is determined and divided by each other. A value of 1 represents equal tropism for target and non-target cells. Here, primary HK were chosen as target, NIH3T3 cells as non-target cells. Cells were transduced with equal numbers of Kera1, Kera2 and Kera3, respectively, followed by flow cytometric analyses 48 h p.t. The best score was determined for Kera2, which transduced primary HK 15x better than the feeder cells followed by Kera3 (9x) and Kera1 (3x).

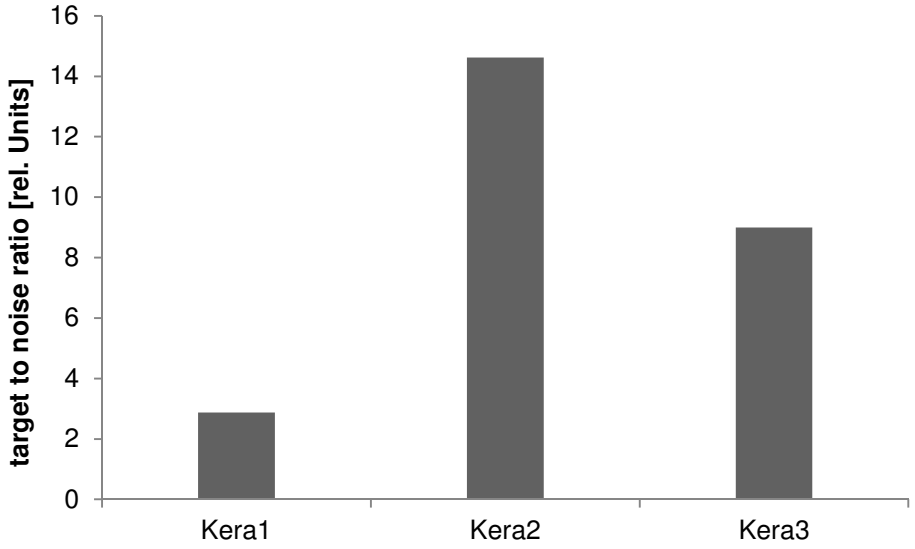
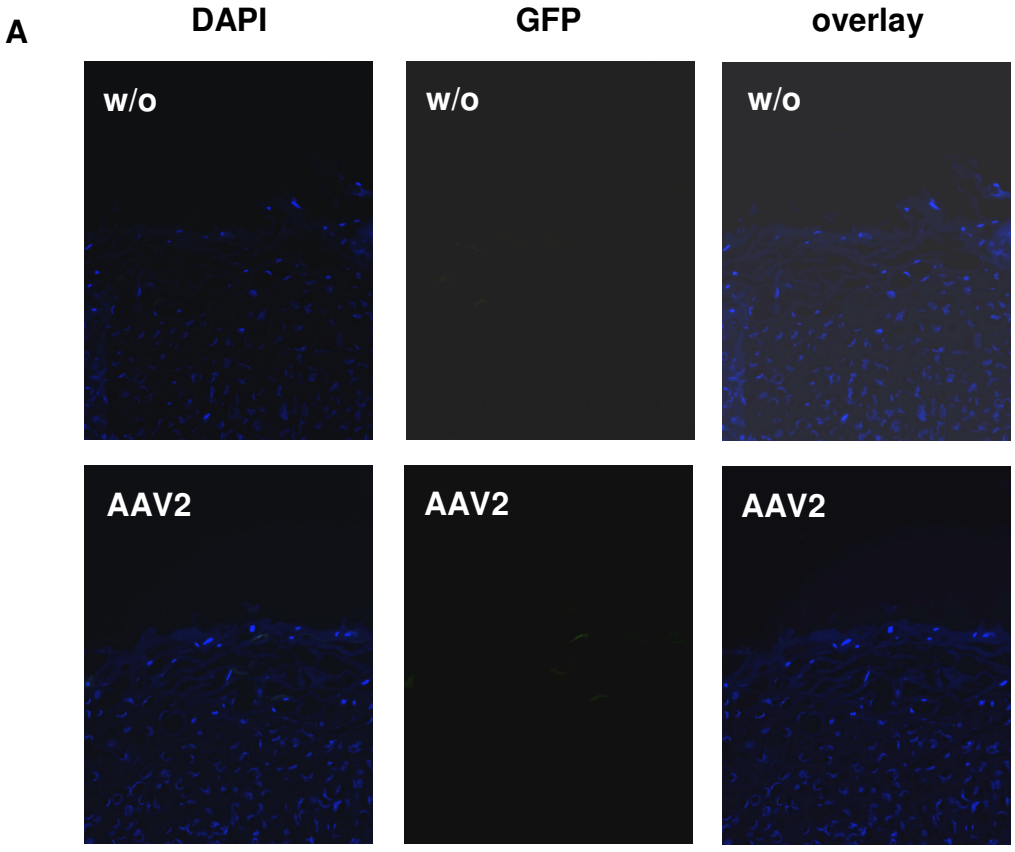


Figure 24: Target to-noise ratio of indicated vectors
NIH3T3 cells and primary HK were cultured as monolayer and incubated with the indicated vectors, respectively Here "target" stands for the transduction rate obtained on primary HK and "noise" stands for the transduction rate of NIH3T3 cells. The target-to-noise ratio was calculated by dividing the values obtained for primary HK by the values obtained for NIH3T3 cells.

3.7 Efficient and specific transduction of differentiated keratinocytes in human organotypic skin cultures

All previous experiments were performed on primary HK growing as monolayer in two dimensional (2D) cell cultures. 2D cultures do not reflect the situation in normal epidermis and therefore organotypic skin cultures were developed [242], [243], [244], [245], [246], [247], [248], [249]. In cooperation with the group of Carien Niessen (CECAD, Cologne, Germany), the transduction efficiencies of Kera1, Kera2 and Kera3, selected on 2D cultures, were investigated on human organotypic skin cultures in comparison with rAAV2.

As outlined in the introduction, organotypic skin cultures, keratinocytes grow air-air lifted on collagen matrix containing dermal fibroblasts (Figure 10). Here 16-day-old human organotypic skin co-cultures were used. A sterile glass ring was placed by forceps onto the air-exposed side of the 3D cultures and filled with PBS as control or $3.5x10^8$ g.p. of rAAV2 or AAV2 peptide insertion variants (see 2.2.6.7), respectively. 72 h post transduction the samples were fixed and processed for cryosections. The sections were embedded by mounting medium containing DAPI for nuclear staining and analyzed for GFP expression by microscopy (Figure 25).



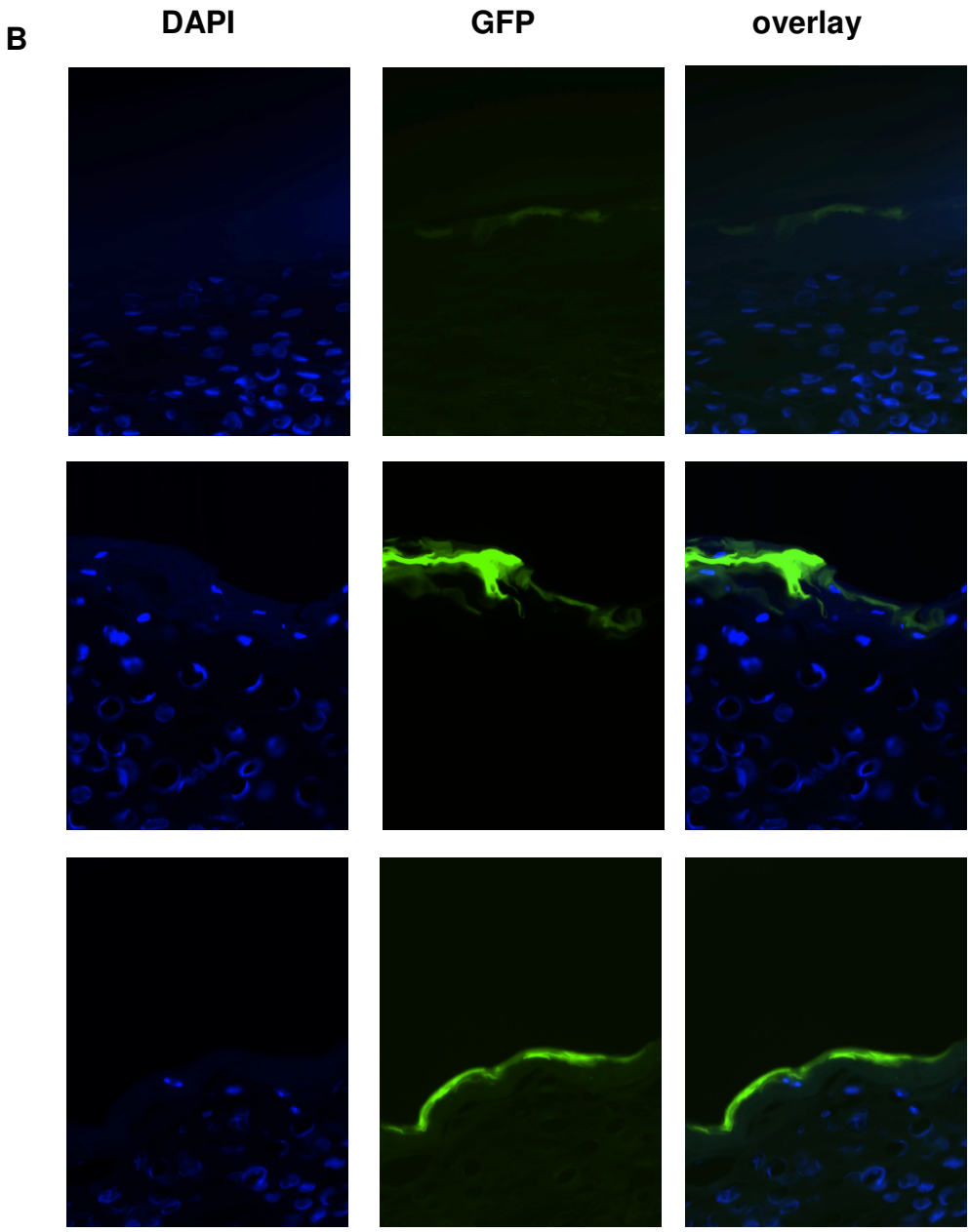


Figure 25: Histological examination of cryosections of human organotypic skin co-cultures
After 3D cultures were incubated with indicated vectors or PBS as control, 5 µm thick cryosections were mounted with mounting medium containing DAPI. The samples were analyzed by microscopy. ***A****: x20 magnification,* ***B****: x40 magnification, w/o = PBS treated control*

The PBS-treated and the rAAV2-treated samples appeared completely negative for GFP expression (Figure 25A). GFP-positive cells were detected in the most upper layer of these 3D cultures transduced with Kera1, Kera2 and Kera3. Only weak GFP signals were detected in the Kera1-treated culture, suggesting that Kera1 was less efficient in transducing differentiated keratinocytes. Strong positive GFP signals were seen in the most upper layers of Kera2- Kera3-treated cultures, Kera2 also showed strong staining in lower keratinocytes layers (Figure 25B). In

summary, these findings indicate that the AAV peptide insertion variants gained the ability to transduce differentiated keratinocytes.

3.7.1 Efficient transduction of primary murine keratinocytes

Mice have routinely been used as experimental models for skin biology and skin diseases [250], [251]; therefore, the transduction efficiencies of the rAAV2 peptide insertion variants on primary murine keratinocytes were investigated.

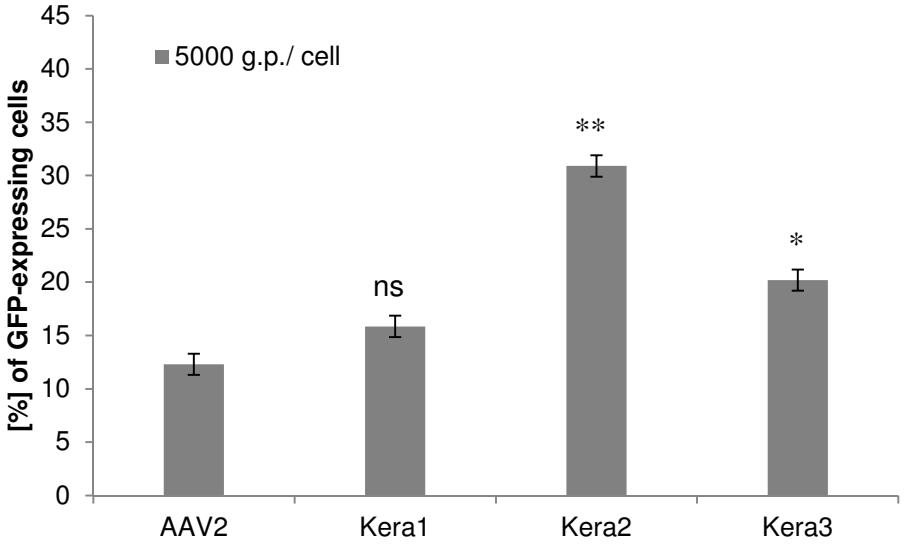


Figure 26: Flow cytometric measurements of primary murine keratinocytes incubated with indicated vector preparations
*Values represent the mean of a technical duplicate; error bars show SD. To define statistical significance between transduction rAAV2 and the rAAV peptide insertion variants Student's t-test was performed. ns =.non-significant, *p<0.05; ** p<0.01, n=3*

Primary murine keratinocytes (kindly provided by the group of Carien Niessen CECAD, Cologne, Germany) were transduced with $5x10^3$ g.p./cell of the rAAV2 targeting peptide insertion variants and rAAV2 respectively. 72 h p.t. GFP-expressing cells were analyzed by flow cytometry. As indicated in Figure 26, primary murine keratinocytes can be transduced by Kera2 and Kera3 with efficiencies of 30.9% +/- 1.4% and 20.2% +/- 2.5%, respectively. Kera1 reaches a transduction efficiency of 15.9% +/- 2.3%. Notably, in contrast to primary HK where transduction by rAAV2 was barely detectable, rAAV2 achieved a transduction efficacy of 12.3% +/- 0.8%.

3.8 Identification of candidate receptor for Kera2

Of the three variants, Kera2 showed the most prominent change in tropism. Based on these results, this mutant was used to establish, in collaboration with Giovanni Di Pasquale (NCI/NIH, Bethesda, USA), a method for the identification of receptors targeted by AAV peptide variants. The method has previously been applied by Di Pasquale and colleagues to identify PDGRF-α as receptor of AAV5. This method based on the NIH cell collection, which contains 60 well-annotated cell lines of different origin. Specifically, for each cell line of this collection, the gene expression profile is known and stored in a microarray database [201]. Di Pasquale transduced this panel with Kera2 and, for comparison, with rAAV2, respectively. In Figure 27 the pattern obtained for the two vectors is shown. Of note, for performing bioinformatics (COMPARE algorithm), only the relative but not the absolute transduction efficiencies of the different cell lines were of interest.

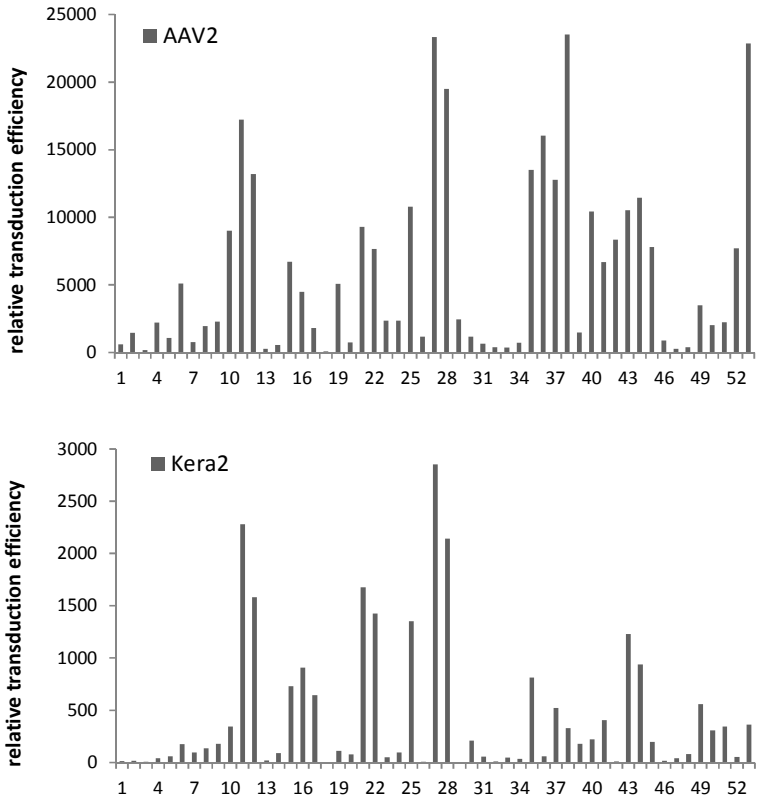


Figure 27: Transduction profiles of rAAV2 and Kera2 on NCI60 cell panel
***A**: The transduction efficiency of rAAV2 was determined by transducing 52 cell lines out of the NCI60 panel with 1µl of rAAV2 in triplicates in a 96 well plate in serial dilution. 48 h p.t. cells were harvested and GFP-expression was measured by flow cytometry. The same approach was done for Kera2 (**B**).Each bar on the graphs represents a different cell line of the panel. The order of the cell lines is the same in each graph. The experiment was kindly performed by Giovanni Di Pasquale (NCI/NIH, Bethesda, USA).*

The COMPARE algorithm determines the similarities of patterns between the given query and others within a database by creating a scalar index of similarity expressed quantitatively as the Pearson correlation coefficient [221]. The Pearson correlation explains the correlation between two variables reflecting the degree to which the variables are related. The range is from +1 to -1. A result of -1 means that there is a perfect negative correlation between the two values at all, while a result of +1 means that there is a perfect positive correlation between the two variables. A result of zero means that there is no linear relationship between the two variables [252], [253]. The scores received by COMPARE program are displayed as a rank-ordered list where the most highly correlated patterns from the databases are listed (Table 5).

Table 5: The output of the COMPARE analysis.
Shown are the first six cell membrane genes that were associated with the Kera2 transduction profile of the NCI60 cell panel. The frequency of occurrence and the range of scores as Pearson correlation coefficient are also listed.

gene	frequency	Pearson correlation coefficient
Integrin, beta8	11	0.72 to 0.5
Glypican 4	3	0.67 to 0.65
Enabled homolog (Drosophila)	3	0.63 to 0.53
Transmembrane and coiled-coil domain family 1	4	0.6 to 0.51
Prostaglandin-endoperoxide synthase 1	3	0.59 to 0.51
Kinesin family member 3A	2	0.56 to 0.52

The highest score (0.72) for Kera2 was observed for ITGB8, the β_8 integrin subunit. In contrast, no significant correlation for the β_8 integrin subunit was detectable for rAAV2. According to literature, the β_8 integrin is expressed by keratinocytes as $\alpha_V\beta_8$ integrin and is expressed on keratinocytes of the suprabasal layers [171], [254], [255].

3.8.1 $\alpha_V\beta_8$ integrin inhibition blocks Kera2 transduction

First, the expression of $\alpha_V\beta_8$ integrin on primary HK was confirmed by flow cytometry (Figure 28A). Subsequently, the expression of $\alpha_V\beta_8$ integrin on different non-target cells (BLM, HepG2 and NIH3T3 cells) was also examined by flow cytometry. As control SW480 cells that had been transfected with beta8 integrin to stably express $\alpha_V\beta_8$ integrin [229], [256], [257] were exploited. As depicted in Figure 28B, none of the non-target cells (NIH3T3, HepG2 and BLM) expressed the integrin $\alpha_V\beta_8$ in utmost contrast to the control cell line. This result is in line with the previously observed refractoriness of these cell lines for Kera2-mediated transductions (Figure 21).

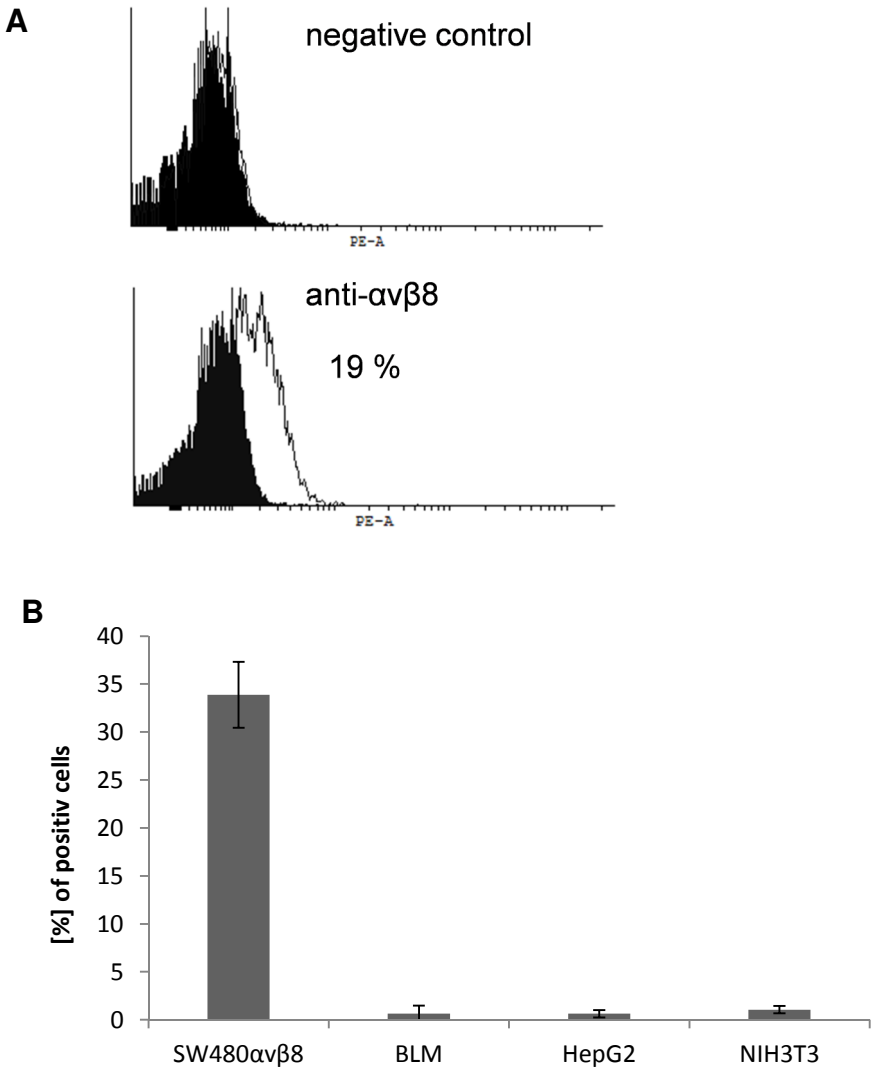


Figure 28: $\alpha_V\beta_8$ integrin expression on primary HK (A) and non-target cells (B)
Primary HK, NIH3T3 cells, HepG2 cells, BLM cells and as control SW480 $\alpha_V\beta_8$ cells were incubated with $\alpha_V\beta_8$-antibody followed by an IgG polyclonal goat anti-mouse secondary antibody. The cells were analyzed by flow cytometry. Values represent the mean of three independent experiment, error bars show SD

In line with a report by Jackson and colleagues, parental SW480 cells express the RGD-binding integrins $\alpha_V\beta_5$ and $\alpha_5\beta_1$, but not $\alpha_V\beta_8$ integrin, while SW480 $\alpha_V\beta_8$ cells express the same set of integrins as well as $\alpha_V\beta_8$ integrin [229]. Therefore, SW480 $\alpha_V\beta_8$ cells and parental SW480 cells are ideal model cell lines to prove whether $\alpha_V\beta_8$ integrin plays a crucial role for Kera2 in cell transduction, by antibody-blocking experiments.

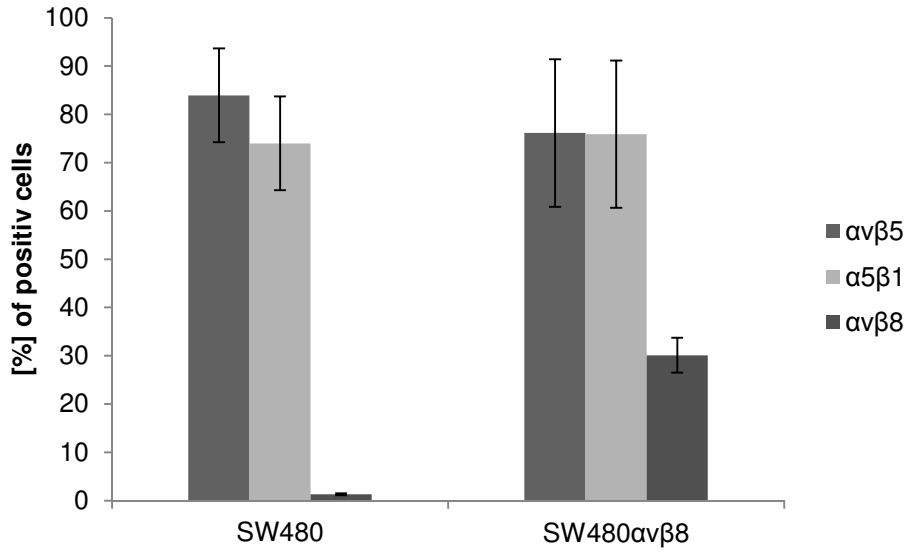


Figure 29: Characterization of RGD-binding integrins expressed on SW480 $\alpha_V\beta_8$ cells and parental SW480 cells

The cells were incubated with the indicated integrin antibody, respectively, followed by an IgG polyclonal goat anti-mouse secondary antibody. The cells were analyzed by flow cytometry. Values represent the mean of three independent experiments; error bars show SD.

Initially, SW480 $\alpha_V\beta_8$ cells and parental SW480 cells were incubated with a blocking antibody for the α_V integrin subunit, followed by incubation with Kera2 or rAAV2, respectively. As a control, cells were transduced in the absence of the blocking antibody. 4 h p.t. cells were treated with trypsin to remove membrane-bound vector particles. The number of transgene expressing cells was determined 48 h p.t. by flow cytometry.

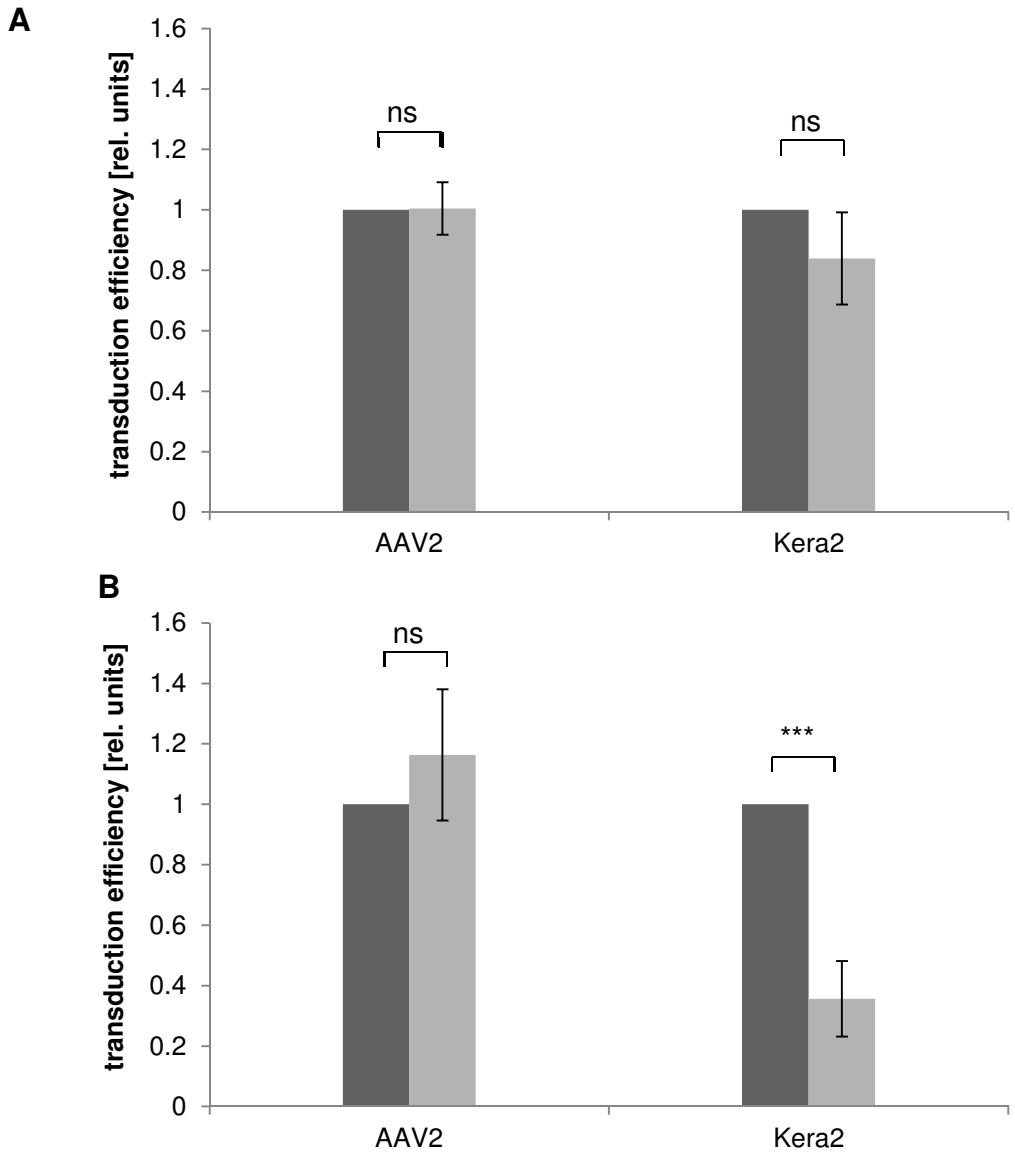


Figure 30: Blocking experiment using MAB specific for the α_V chain
*Flow cytometric analysis of cells incubated with indicated vectors in absence (dark grey) or presence (light grey) of α_V blocking antibody. **A:** SW480 cells. **B**: SW480 $\alpha_V\beta_8$ cells. Values obtained for cells incubated with vectors in absence of antibody were set to 1. Values represent the mean of three independent experiments; error bars show SD. To define statistical significance between cells treated with and without α_V blocking-antibody, Student's t-test was performed. ns = non-significant, ** p<0.001.*

The addition of the α_V-blocking antibody did not affect cell transduction by rAAV2 in neither SW480 nor SW480 $\alpha_V\beta_8$ cells. Conversely, transductions by Kera2 were significantly inhibited in SW480 $\alpha_V\beta_8$ cells by 64.3% (Figure 30B), which points towards a dependency on the α_V integrin subunit for transducing SW480 $\alpha_V\beta_8$ cells. Next, an experiment was performed using a blocking $\alpha_V\beta_8$ integrin antibody.

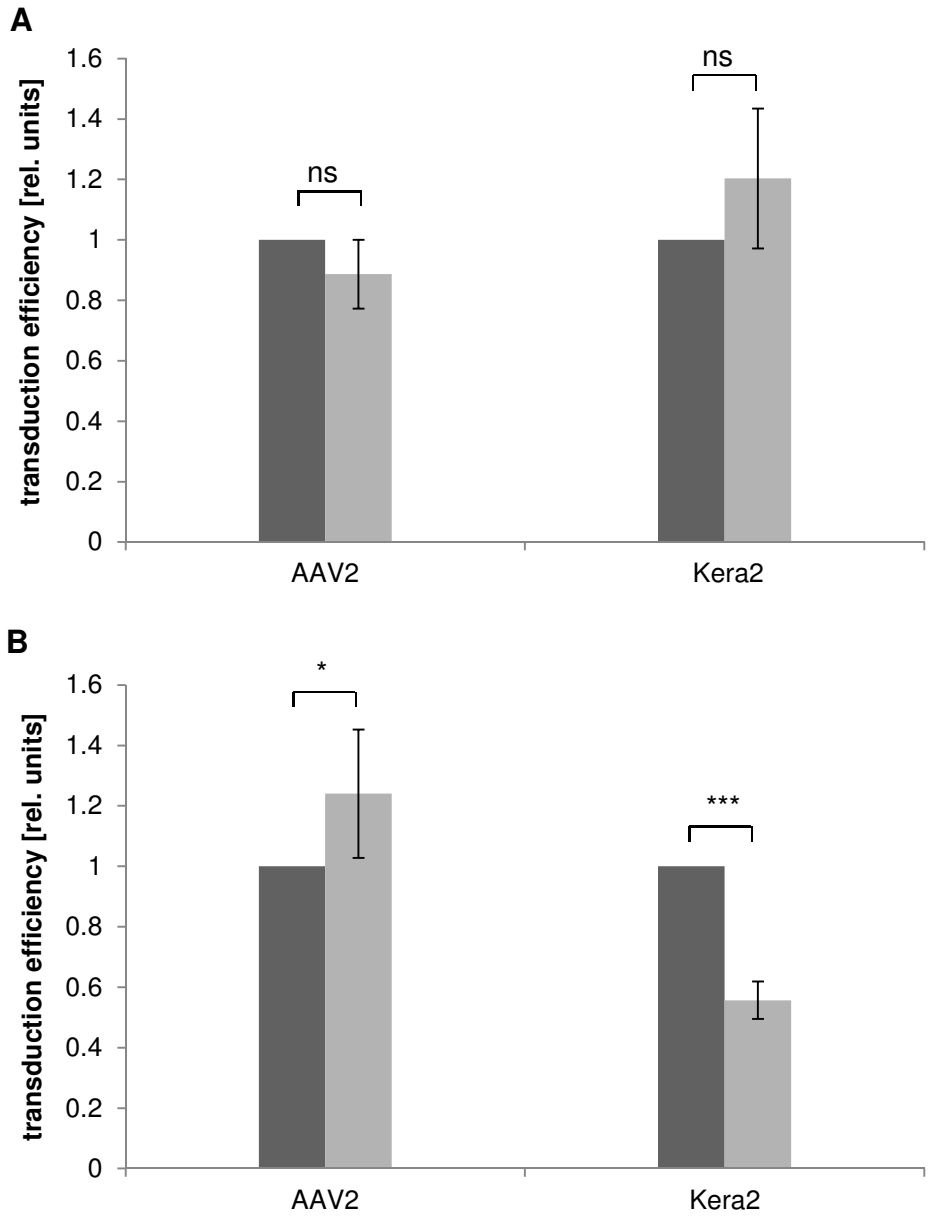


Figure 31: Blocking experiment using an $\alpha_V\beta_8$ integrin antibody
Transduction efficiencies of Kera2 and rAAV2 were determined after pre-incubation of the cells without (dark grey) or with (light grey) anti-$\alpha_V\beta_8$-antibody by flow cytometry. ***A:*** *SW480 cells.* ***B:*** *SW480 $\alpha_V\beta_8$ cells. Values for cell transduction in the absence of blocking antibody were set to 1 and represent the mean of three independent experiments; error bars show SD. To define statistical significance between cells treated with and without $\alpha_V\beta_8$ blocking-antibody, Student's t-test was performed. ns= non-significant, *** p<0.001.*

Briefly, SW480 $\alpha_V\beta_8$ cells and, as control, parental SW480 cells were incubated for 30 min on ice in presence or absence of 200 µg/ml of $\alpha_V\beta_8$-antibody. Thereafter, cells were incubated with Kera2 ($3x10^3$ g.p./cell) and rAAV2 ($7.5x10^2$ g.p./cell) for 1 hour followed by a washing step. Cells were analyzed 48 h p.t. by flow

cytometry. As indicated in Figure 31 $\alpha_V\beta_8$ antibody did not affect transduction of SW480 cell (A) and SW480 $\alpha_V\beta_8$ cells (B) by rAAV2. While the blocking antibody had no effect of Kera2 transduction on SW480 cells, it significantly inhibited transduction of SW480 $\alpha_V\beta_8$ by 44% (Figure 31B).

4 Discussion

Cutaneous gene therapy is becoming a promising strategy to treat inherited or acquired skin diseases. Skin, however, is a poor target for viral vector gene transfer [206], [207], [208]. Therefore, the objective of the research described in this thesis has been to develop vectors based on the adeno-associated virus serotype 2 (AAV2), as novel tools for genetic modification of primary human keratinocytes (HK). This chapter briefly recapitulates the results and discusses the potential of AAV vectors for gene delivery to the skin.

To develop novel AAV vectors that overcome the resistance of keratinocytes towards transduction with natural AAV serotypes, the AAV2 peptide display library was used. Sequencing of isolates obtained by this screening revealed that with exception of one variant, all variants selected displayed peptides that contained RGD/RSD motifs resembling thereby integrin-binding ligands. Three of them, Kera1, Kera2 and Kera3, were chosen for further analysis. All three variants, when produced as recombinant vectors, transduced human primary keratinocytes with significant improved efficiency compared with the parental serotype (Kera1 = 54.1% +/- 11.6%, Kera2 = 46.7% +/- 16.8%, Kera3 = 47.0% +/- 16.8% and rAAV2 = 1.2% +/- 0.5; (Figure 18). Furthermore, the three AAV variants transduced the target cells by the inserted peptide (Figure 18) through the clathrin entry pathway (Figure 20). Even differentiated keratinocytes in air-lifted organotypic 3D co-cultures were transduced following topical vector application (Figure 25). For the variant with the most prominent change in tropism, Kera2, the targeted receptor could be identified as integrin $\alpha_V\beta_8$ by comparative gene analysis (CGA), (Figure 27, Figure 30 and Figure 31).

4.1 Selection of AAV capsid variants

Chronic wounds caused by pressure, venous stasis or diabetes mellitus, as well as burns or inherited skin diseases still represent a major clinical problem worldwide with significant morbidity and no effective therapies available. Gene therapy is a promising strategy for treatment of both inherited and acquired disorders. As mentioned previously, the skin is an ideal candidate for gene transfer

not only due to its excellent accessibility but also due to the ease with which the keratinocytes are biopsied and expanded in culture. Frequently, engraftment as well as wound closure is enhanced if the transplant secrets anti-inflammatory cytokines and/or growth factors including angiogenic mediators [206], [258], [259], [260], [261]. For successful gene delivery, the selection of an appropriate vector is of paramount importance [262]. Over the years, a number of viral vector systems have been developed as tools for gene therapy [263], [264], [265]. Vectors based on adeno-associated viruses serotype 2 (AAV2) have been studied intensively. Deodato and colleagues [266] and Galeano and colleagues [267] developed a model for external gene delivery of vascular endothelial growth factor A by rAAV2 into wound bed. Wound healing in the rat showed significant acceleration and a well-structured granulation and vascularization [266]. However, the authors attributed the increased vascularization to the excellent tropism of the vector to the skeletal muscle layer underlying the skin in rodents. This thin muscle layer is not available in humans and generally caused wounds in rats to heal faster, indicating that this vector might not be as potent when used in humans [198], [266], [267]. This is in line with observations of Gagneoux and colleagues [206] and other groups [207], [208] who reported that primary human keratinocytes (HK) are not permissive to rAAV2. Also, findings of this thesis have shown that primary HK were insufficiently transduced by rAAV2 (Figure 11). Receptor analysis revealed that primary HK do not express HSPG (Figure 12). HSPG serves as primary receptor for AAV2 [37] and is required for binding of AAV2 to its internalization receptors $\alpha_V\beta_5$ or $\alpha_5\beta_1$ integrin [39], [235]. Thus, it is hypothesized that the lack of expression of AAV2´s primary receptor is a pre-entry-barrier towards AAV2-mediated gene transfer into HK. Furthermore, Braun-Falco and colleagues reported two post-entry barriers. They noted that rAAV2 mediated gene transfer into HK is influenced by ubiquitin/proteasome pathway and the epidermal growth factor receptor tyrosine kinase (EGF-R TK) [182]. This barrier could be attenuated by the addition of the proteasome inhibitor MG132, or the epidermal growth factor receptor tyrosine kinase inhibitor AG1478 [207]. If a target cell does not express receptors that are naturally used by AAV for cell infection, the AAV peptide display technology can offer an elegant solution to identify a ligand-receptor interaction for cell transduction [47]. Specifically, AAV peptide display leads to the identification of ligands enabling rAAV2 mutants displaying the respective ligand to enter target

cells. Moreover, AAV peptide display selections tackle the problem of post-entry barriers. This is because viral mutants are only selected when fulfilling the whole viral life cycle. To identify capsid variants displaying peptide insertions able to bind to a suited receptor for primary HK transduction, the AAV2 display library, initially described by Perabo and colleagues, [144] was used.

As mentioned before, parental rAAV2 vectors are internalized via HSPG. HSPG binding to rAAV2 leads to a strong vector-cell attachment [38], [142]. This confers rAAV2 vectors with a broad tropism, which is an undesired feature in cell targeting. Capsid mutants, engineered to bind to HSPG share the same features as rAAV2 vectors [220]. Therefore, an assumption is that re-direction of AAV´s tropism requires depletion of the HSPG binding ability. As previously described the AAV2 display library consist of mutants displaying 7-mer random peptides at amino acid position 587. Using this position for peptide insertion results in mutants receptor blinded for natural receptor as the two main residues of HSPG binding motif, R585 and R588, become separated [37], [142]. The hypothesis that depleting the AAV display library of HSPG binding ability resulted in ligands that confer rAAV2 vectors with the ability to enter target cells HSPG-independent and to select for mutants able to overcome post-entry barriers was validated. The here selected mutants (Kera1, Kera2 and Kera3) showed an impressively higher entry and transduction efficiency compared with rAAV2 (Figure 14 - Figure 16). As characterization of primary HK revealed the lack of AAV2´s primary receptor HSPG, a HSPG independent cell entry of Kera1, Kera2 and Kera3 is hypothesized.

4.2 Kera1, Kera2 and Kera3 transducing target cells peptide-dependent through the clathrin entry route

An unexpected finding of the AAV peptide display selection on primary HK using the NB library (library depleted for HSPG binding mutants) was the nearly exclusive selection of clones displaying a RGD/RSD containing peptide sequence (Table 2). Indeed, peptide competition experiments proved that Kera1, Kera2 and Kera3 transduced primary HK through the RGD-containing ligands (Figure 18). RGD motifs are classical integrin binding ligands [268] and reports have shown that RGD serves as cell attachment site for different viruses e.g. Foot and Mouth

Disease virus (FMDV), [269] and Coxsackie virus [270]. As noted, previous selections of our group resulted in ligands resembling integrin binding motifs and the packaged rAAV2 vectors displaying the selected peptide on the capsid efficiently transduce cells in a peptide dependent manner [144], [148]. Furthermore, the insertion of a known RGD-integrin binding ligand at position 587 of AAV2´s capsid resulted in targeting vectors transducing their respective target cells with high efficiencies [143]. In Table 7, integrins of the epidermis are depicted in relationship to their major ligands, expression in epidermis and RGD recognition sequence.

Table 6: Keratinocyte integrins [174], RGD recognition sequence [238]

Integrin	Major ligand	Expression	RGD recognition sequence
$\alpha_2\beta_1$	Collagen	Constitutive	-
$\alpha_3\beta_1$	Laminin	Constitutive	-
$\alpha_6\beta_4$	Laminin	Constitutive	-
$\alpha_5\beta_1$	Vitronectin	Weak	+
$\alpha_V\beta_5$	Fibronectin	Induces in culture, on wounding, under pathological conditions	+
$\alpha_V\beta_6$	Fibronectin; tenascin	As $\alpha_V\beta_5$	+
$\alpha_9\beta_1$	Tenascin	Upregulated during wound healing	-
$\alpha_V\beta_8$	Vitronectin	Suprabasal	+

Integrins appear to be important receptors for different viruses and also for rAAV2 based targeting vectors. One reason for this relationship might be specific intracellular conditions induced upon integrin binding of AAV2 for successful intracellular trafficking. Specifically, AAV2 binds to its integrin receptors, which induces cytoskeleton rearrangements and uptake into clathrin-coated pits [43]. AAV is transported with the endosome along the cytoskeleton towards the nuclear area [44], [56]. The N terminus of VP1 carries a phospholipase activity and facilitates escape of viral particles by breaking down the endosomal membrane [55] followed by nuclear delivery of the vector genomes, which is believed to be achieved by nuclear localization signals [271]. Clathrin-mediated endocytosis seems to be a successful entry route also for other viruses for example Kaposi's

sarcoma-associated herpesvirus (KSHV) [272], Human cytolomegalovirus (HCMV) [273] and the related autonomous parvovirus Canine parvovirus [274]. Of note, our group recently reported that cell transduction through clathrin-mediated endocytosis is associated with an efficient intracellular processing of rAAV targeting vectors [220]. Therefore, cell transduction on primary HK were performed in the presence of Chlorpromazine that inhibits assembly of clathrin lattices [220]. As control, Genistein was used, which inhibits caveolin-mediated uptake. While Genistein treatment had no effect on transduction, presence of Chlorpromazine significantly reduced the transduction rate of Kera1, Kera2 and Kera3 indicating a clathrin-dependent internalization pathway (Figure 20).

4.3 Kera2 possesses the highest receptor specificity

Cell type-specific gene delivery in a clinically setting is of utmost importance to avoid off-target transduction and to improve the safety and efficiency of gene therapy [275]. Therefore, the tropism of the three rAAV2 peptide insertion variants was characterized on different cell types representing off-target cells in cutaneous gene therapeutic approaches. Transduction experiments on 2 different melanoma cell lines (BLM and A375), the human hepatoma cell line HepG2 and the fibroblast cell line NIH3T3 revealed an altered tropism for Kera1, Kera2 and Kera3. Despite applying a high number of viral particles to the cells the transduction efficiency remained at background level, while rAAV2 transduced all cell types (Figure 21 and Figure 22). Potentially, this data suggest that Kera1, Kera2 and Kera3 are specific for a receptor, which is not as prevalent as the naturally occurring receptors for rAAV2. Similarly, high target cell specificity was demonstrated by transduction experiments with the rAAV peptide insertion variants on primary HK co-cultured with NIH3T3 feeder cells. Feeder cells are often used to support the growth of primary HK in the culture. The experiment revealed that the rAAV peptide insertion variants transduced primary HK with high efficiency in contrast to the feeder cells (Figure 23). This result further supported the hypothesis that receptors with a relative restricted expression pattern are targeted by Kera1, Kera2 and Kera3, respectively.

4.4 Kera1, Kera2 and Kera3 are capable of transducing differentiated keratinocytes in human organotypic skin co-cultures

Vectors developed in this thesis transduce differentiated keratinocytes in organotypic human skin co-cultures, a feature not described for AAV vectors before. Especially Kera2 and Kera3 showed impressive transduction efficiencies (Figure 25). The ability to transduce differentiated keratinocytes is an important prerequisite for potential clinical use across the skin barrier. This feature opens the door for research areas including regenerative medicine and basic life science research. Especially these vectors might be applicable for topic *in vivo* applications for transient overexpression of growth-factors to enhance wound healing [214], [276], [277] or for vaccination [278].

4.5 $\alpha_V\beta_8$ integrin serves as receptor for Kera2

Identification of cellular receptors engaged by the ligand displayed by AAV targeting vectors facilitates transition from “bench to bedside”. This task was difficult to accomplish, despite the obvious importance. This is due to the selection process, in which the library is screened for capsid variants with tropism for a certain cell type. In most of the cases, knowledge on potential suitable receptors or the receptor profile is lacking. Furthermore, each cell type possesses more than one receptor that in principle could mediate cell entry and processing intracellular signal cascades. This impedes selection of mutants with specificity for a beforehand chosen receptor. The NCI/NIH department (USA) has developed a microarray based bioinformatic approach, which has been successfully applied to identify viral receptors [221], [279]. In this thesis, this approach (named comparative gene analysis (CGA)) was used for the first time for the identification of a receptor engaged by a rAAV targeting vector. The on- and off-target transduction analysis, pointed towards a high target receptor specificity of Kera2, which was therefore chosen for exploiting the usability of this method for target receptor identification. As outlined in detail in the results, CGA pointed to ITGB8, the β_8 subunit of an integrin, as candidate (Figure 27). The β_8 cytoplasmic domain is 65 aa long sharing no apparent homology with the highly conserved cytoplasmic

domains of other β subunits or any other known protein [255]. β_8 is unique as it is solely described as heterodimer with α_V [280], [281]. Cambier and colleagues found that the $\alpha_V\beta_8$ integrin is expressed in airway epithelial cells *in vivo* and *in vitro* [256]. Later they were able to show that $\alpha_V\beta_8$ integrin is also expressed in perivascular cells process surrounding developing human cerebral blood vessels as well as in primary cultures of astrocytes or freshly dissociated immature neuroglial cells [282]. Further, Stepp reported that $\alpha_V\beta_8$ integrin is also found in suprabasal layer of the epidermis [254]. The integrin belongs to a group of integrins present in human skin (Table 6), [229], [238]. The only high-affinity ligand of this integrin is the latency associated peptide (LAP) of the transforming growth factor β (TGF-β) complex [257]. By comparing the ligand sequence of LAP (**RGDLA**TI) [229], a striking homology to the Kera2 peptide sequence (P**RGDLA**P) is noticeable. Further evidence that $\alpha_V\beta_8$ integrin plays a critical role in vector-cell binding is provided by the efficient transduction of cells expressing $\alpha_V\beta_8$ integrin with Kera2, while receptor-negative cells were refractory. Furthermore, receptor-blocking experiments revealed a significant reduction in cell transduction when $\alpha_V\beta_8$ integrin is blocked (Figure 30 and Figure 31).

4.6 Summary and outlook

The here-developed AAV-based vectors Kera1, Kera2 and Kera3 are potential tools for genetic manipulation of human skin. Specifically, these three rAAV2 peptide insertion variants were highly efficient in transducing primary human keratinocytes (HK). Of the three vectors, Kera2 demonstrated the most striking change in tropism, i.e. targeting of HK and detargeting from potential off-target cell types such as hepatocytes or fibroblasts. Furthermore, the here reported study is the first describing a strategy to identify candidate receptors engaged by capsid-modified rAAV vectors. Exploiting CGA revealed $\alpha_V\beta_8$ integrin as candidate receptor for Kera2, which was confirmed in subsequent experiments. Of particular interest for basic and translational research, the changed tropism conferred by the inserted peptide ligands enabled the three rAAV2 peptide insertion variants, Kera1, Kera2 and Kera3, to efficiently transduce differentiated keratinocytes in organotypic 3D cultures. Thus, the three selected rAAV2 peptide insertion variants (Kera1, Kera2 and Kera3) appear to be a potent tool in cutaneous gene therapy.

Another prospective candidate, identified by the AAV peptide display selection on primary HK, might be the AAV clone with the sequence RSDLASL. According to Hamidpour and colleagues, the motif RSD possesses the potential to act as a mimic of the RGD motif [236]. Assuming that RSD and RGD are indeed identical, this variant is likely similar efficient as Kera2 in binding $\alpha_V\beta_8$ integrin as its sequence (**RSDLA**SL) is strikingly similar to Kera2 (P**RGDLA**P) and the sequence of the former mentioned LAP peptide (**RGDLA**TI) of the TGF-β complex (known to bind $\alpha_V\beta_8$ integrin). Hence, this motive could be an alternative rAAV vector binding to the $\alpha_V\beta_8$ integrin. Further, the selection screen with the AAV2 display library performed during this thesis resulted in ligands including various motifs that are potentially able to bind to integrins (Table 2). It would be of interest to package and characterize the remaining mutants maybe resulting in targeting vectors binding to different integrin receptors facilitating cutaneous gene transfer.

For Kera2, as mentioned above, the $\alpha_V\beta_8$ integrin was identified as receptor. $\alpha_V\beta_8$ integrin is not only expressed in skin but also in different other tissues or organs like dendritic cells [283], airway epithelial cells *in vivo* and *in vitro* [256], astrocytes [284] as well as in epithelial cells of kidney [285], [286]. In principle, Kera2 may be able to mediate gene transfer in every tissue/cells expressing $\alpha_V\beta_8$ integrin. Thus, Kera2 potentially, could function as targeting vector not only for skin diseases. Further work must be done to prove this hypothesis by verifying expression of $\alpha_V\beta_8$ integrin on these tissues/cells, followed by transduction experiments to test efficiencies of Kera2 in these tissues.

Chapter 3.7.1 demonstrated that Kera1, Kera2 and Kera3 were able to transduce primary murine Keratinocytes (Figure 26). After rAAV2 showed relative high transduction efficiency, further research could proof the presence of HSPG (AAV2´s primary receptor) on primary murine keratinocytes. Nevertheless, Kera2 clearly outperformed rAAV2, which indicates higher transduction efficiency of murine keratinocytes. Therefore, of further interest, might be the characterization of RGD-binding integrins expressed on murine keratinocytes, particularly $\alpha_V\beta_8$ integrin.

Experiments with human organotypic skin cultures revealed that Kera1, Kera2 and Kera3 were able to transduce differentiated human keratinocytes (Figure 25). Another consideration may be transduction experiments on murine organotypic skin cultures since the three selected rAAV peptide insertion variants were able to

transduce primary murine keratinocytes in 2D culture. This experiment might open the door for *in vivo* applications on mouse models.

As mentioned before, the human epidermis is a self-renewing tissue and therefore any persistent genetic defect is present in the stem cells, with expression passed to daughter cells at each division [287]. Epidermal stem cells play a central role in homeostasis and wound repair [258]. They possess the ability to self-renew and are responsible for long-term maintenance of the tissue [259]. Thus, for prolonged gene expression in epidermis, integration into the genome of stem cells is required. Kera2 is probably not suitable for transduction of epidermal stem cells due to the lack of $\alpha_V\beta_8$ integrin receptor expression. Kera1 and Kera3 possessing a more unspecific tropism for primary HK seen in the mixed culture experiment (see 3.6.1) and in the transduction experiment of human organotypic skin cultures (see 3.7). It can thus not be excluded that one of them or both might successfully transduce epidermal stem cell for gene delivery. Further, it might be possible that one of the not yet characterized mutants selected during this thesis, when packaged as rAAV vector, would be able to transduce epidermal stem cells successfully due to target a different integrin receptor. A further possibility for successful gene delivery into epidermal stem cells would be to exploit the here optimized AAV2 display library on the stem cell for selection of new mutants.

Identification of genes that are responsible for genetic diseases opens the possibility for treatment of gene therapeutic approaches. Attempts to correct genetic defects using gene therapy include different forms of epidermolysis bullosa or lamellar ichthyosis [232]. Petek and colleagues demonstrated efficient targeting of KRT14 in normal and epidermolysis bullosa- (EB) affected human keratinocytes [189]. EB simplex is caused by point mutations in KRT14 gene [257]. They used an AAV gene targeting vector of serotype 6 with promoter trap design to disrupt the mutated allele resulting to a success rate of 50%. They observed that cells with disruption of transcription from the mutant allele dominate targeted cell populations after a short growth period in culture and they reported histologically normal skin grafts after transplantation to athymic mice [208]. Since Kera2 demonstrated significant higher entry efficiency into primary HK than the former mentioned rAAV6 mutant, Kera2 might be an alternative for correction of KRT14 genes in EB.

List of Abbreviations

aa	amino acid
AAP	assembly-activating protein
Ad	adenovirus
bp	base pair
BSA	bovine serum albumin
Cap	Open reading frame for capsid proteins
CPZ	Chlorpromazine
d	day
DAPI	4´,6-diamidino-2-phenylindol
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide trisphosphate
DNase	deoxyribonuclease
(ds)DNA	(double stranded) deoxyribonucleic acid
(ss)DNA	(single stranded) deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene-di-amine-tetra-acetic acid
(e)GFP	(enhanced) green fluorescent protein
EGF(R)	epidermal growth factor (receptor)
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF(R)	fibroblast growth factor receptor
FITC	fluorescein-5-isocyanate
g.p.	genomic particles
GOI	genomic particles per cell
h	hour
HBS	HEPES buffered solution
HCMV	human cytomegalo virus
HEPES	4-3-hydroxyethyl-1-piperazineethanesufonic acid
HK	human keratinocytes
HSPG	heparan-sulphate proteoclycan
HSV	Herpes Simplex Virus
IL	interleukin

ITR	inverted terminal repeat
kb	kilo bases
LB	Luria-Bertani
min	minute
NB	HSPG-non-binder
NDS	normal donkey serum
nt	nucleotide
ns	non-significant
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet derived growth factor receptor
PFA	paraformaldehyde
p.i.	post infection
PIPES	piperazine-N,N`-bis(2-ethanesulfonic acid)
Plat	plasminogen activator
qPCR	quantitative PCR
rAAV	recombinant adeno-associated viral vector
REP	REP protein
rep	open reading frame for REP proteins
rpm	rounds per minute
RT	room temperature
TAE	tris-acetate EDTA
TBS	tris-buffered saline
Tris	tris-(hydroxymethyl)-amino-methane
trs	terminal resolution site
(V)EGF(R)	(vascular) endothelial growth factor (receptor)
vg	vector genomes
VP	viral protein

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