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Cellular transduction mechanisms of adeno-associated viral vectors

Garrett Edward Berry[1,2,3] and Aravind Asokan[1,2,4]

Recombinant adeno-associated viral vectors (rAAV) are regarded as promising vehicles for therapeutic gene delivery. Continued development and new strategies are essential to improve the potency of AAV vectors and reduce the effective dose needed for clinical efficacy. In this regard, many studies have focused on understanding the cellular transduction mechanisms of rAAV, often with the goal of exploiting this knowledge to increase gene transfer efficiency. Here, we provide an overview of our evolving understanding of rAAV cellular trafficking pathways through the host cell, beginning with cellular entry and ending with transcription of the vector genome. Strategies to exploit this information for improving rAAV transduction are discussed.

Addresses
[1] Gene Therapy Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[2] Department of Genetics, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[3] Curriculum in Genetics and Molecular Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[4] Department of Biochemistry & Biophysics, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Corresponding author: Asokan, Aravind (aravind@med.unc.edu)

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Introduction

Gene therapy broadly describes strategies in which genetic material is introduced into a target cell in an effort to treat or cure disease. Of the approaches that are currently being explored, recombinant adeno-associated viral vectors (rAAV) have emerged as one of the most promising candidates. Adeno-associated virus (AAV) is a member of the *parvoviridae* family that was initially discovered as a contaminant in simian adenovirus preparations [1]. A small, icosahedral non-enveloped virus ∼25 nm in diameter that contains a single-stranded DNA genome [2], AAV is distinct from other members of the *parvoviridae* family due to its inability to replicate without the assistance of a helper virus, such as Adenovirus (Ad), Herpes simplex virus (HSV), human papilloma virus (HPV), or vaccinia virus [3]. Additionally, AAV can confer long term gene expression, has a range of serotypes that collectively have a range of tissue tropism, and can package any transgenes flanked by AAV inverted terminal repeats (ITRs) and with the total genome size not exceeding ∼5 kb [4]. All of these properties make AAV an excellent candidate for therapeutic gene delivery.

It is well known that eukaryotic mechanisms to sort and degrade internalized cargo are exploited by several viruses and pathogens for infecting and replicating within host cells. At the same time, several cellular factors act to restrict viral infection within the host. A similar dichotomy is apparent in case of rAAV, with host factors demonstrating the ability to aid or limit transduction efficiency. For instance, one study estimates that following cellular uptake, only ∼30% of AAV particles will successfully reach the nucleus [5]. Here, we review the cellular transduction mechanisms of rAAV vectors by breaking down the different steps leading to transgene expression. We also discuss strategies to overcome host restriction factors that act as barriers that limit the potential of rAAV-mediated gene therapy in the clinic.

Cellular uptake of rAAV

The first step in AAV infection requires binding to cell surface glycan receptors [6]. This key step mediates cell surface attachment of virions and triggers subsequent cellular internalization and trafficking leading to transduction. Cellular uptake of AAV particles into endocytic vesicles is thought to be mediated by integrins and/or different transmembrane receptors. However, our understanding of AAV receptor usage continues to evolve. Although it is currently unclear how different AAV serotypes exploit specific receptors for cellular uptake, it is well known that mammalian cells are known to internalize extracellular material by numerous endocytic pathways, several of which have been implicated in uptake of rAAV (Figure 1A). The first studies to investigate AAV uptake suggested that internalization of rAAV occurred via clathrin-mediated endocytosis. In these studies, transduction of AAV2 was inhibited by expression of a dominant-negative mutant of dynamin, a protein necessary for successful clathrin-mediated endocytosis [7,8]. Additionally, internalized AAV2 colocalized with transferrin, a protein known to be internalized by this mechanism. It is also worth noting that transcytosis of rAAVs has been

shown to occur in polarized cells in a serotype-dependent manner, and it has been suggested that this phenomenon is dependent upon caveolin [9].

A more recent study suggests that uptake of AAV2 is dependent on the clathrin-independent carriers and GPI-enriched endocytic compartment (CLIC/GEEC) endocytic pathway [10•]. This study demonstrated that AAV2 uptake was inhibited by dominant negative versions of Arf1, Cdc42, and GRAF1, three important effectors of the of the CLIC/GEEC pathway. In addition, AAV2 colocalized with cholera toxin B and GPI-anchored GFP, two markers of CLIC vesicles, after internalization. In addition, this study identified EIPA as an inhibitor of CLIC/GEEC endocytosis. However, it is worth noting that EIPA is classically known as an inhibitor of macropinocytosis. Consistently, other studies have suggested a role for macropinocytosis in rAAV uptake. One such study demonstrated that inhibition of Rac1 activation, a key effector of macropinocytosis, inhibits AAV internalization [11]. Another study used multiple small molecule inhibitors of macropinocytosis, including EIPA, to demonstrate that inhibition of macropinocytosis decreased transduction in some cell types, while demonstrating enhanced transduction in others [12•].

Although diverse endocytic mechanisms have been implicated in AAV cell entry, it is evident that certain uptake pathways lead to successful transduction, while other pathways in the same cells lead to a 'dead end' for AAV [10•,11]. Further, it is likely that such mechanisms are altered in a cell-type dependent manner [12•].

Post-entry trafficking of rAAV

After rAAV enters the cell, it must traffic toward the nucleus in order to successfully deliver its genetic cargo. Immediately after uptake, AAV is presumably trafficked to Rab5+ early endosomal compartment, which is a feature conserved amongst many parvoviruses [13]. From here, rAAV traffics through a number of different compartments. Studies have demonstrated that rAAV2 traffics through both Rab7+ late endosomes and Rab11+ recycling endosomes [14] (Figure 1B). As is the case with AAV cell entry, intracellular trafficking pathways likely differ in a cell line-dependent and serotype-dependent fashion. For instance, while rAAV9 was localized to Rab5+, Rab7+, and Rab11+ vesicles in neurons in cell culture, this serotype was shown to only traffic effectively along axons in Rab7+ endosomes [15].

Vesicle-entrapped rAAV particles have been shown by numerous studies to traffic to the Golgi apparatus [16–18]. One of the steps known to be required for efficient trafficking is endosome acidification, as the vacuolar H+-ATPase inhibitor bafilomycin A1 effectively blocks transduction [8,19]. Additionally, studies utilizing the small molecules brefeldin A and golgicide A, known to disrupt the Golgi apparatus, have shown that trafficking of rAAV through the Golgi apparatus is also required [19,20]. Recently, we demonstrated that inhibition of endoplasmic reticulum-associated degradation (ERAD) by eeyarestatin I (EerI) in HeLa cells reroutes rAAV to enlarged Lamp1+ lysosomes. This approach increased transduction, indicating that trafficking of rAAV through LAMP1+ vesicles may be an important step in infection [21]. It is important to note that, as of now, no studies have observed rAAV localization within the endoplasmic reticulum (ER) or implicated a role for rAAV trafficking through the ER prior to nuclear entry.

At the molecular level, a recent study showed that siRNA-mediated knockdown of syntaxin 5 (STX5), as well as disruption of STX5 by the small molecule Retro2.1, reduced rAAV transduction, suggesting that retrograde transport of rAAV to the *trans*-Golgi network (TGN) mediated by syntaxin 5 is important for transduction [20]. Another recent study utilized a screen based on haploid cells and identified KIAA0319L as a cellular factor required for infection of cells by multiple AAV serotypes, which they termed AAV receptor (AAVR) [22••]. CRISPR-based technology was utilized to knockout AAVR both *in vitro* and *in vivo* to demonstrate that AAV infection is severely inhibited in the absence of AAVR, and can be rescued by subsequent complementation by expression of ectopic AAVR. Interestingly, AAVR largely localizes to the Golgi, and contains a signal in the C-terminus that results in dynamic recycling of AAVR from the cellular surface to the TGN. While the precise mechanisms underlying AAVR-mediated AAV transduction remain to be understood, the data demonstrates that AAVR is an essential cellular factor that mediates AAV transport through the endomembrane system to the TGN for successful infection.

After trafficking of rAAV through the endomembrane system, rAAV escapes the endosome into the cytosol (Figure 1C). Endosomal escape is dependent on a phospholipase A2 (PLA2) domain located in the VP1 unique region of AAV [23,24]. Multiple studies have shown that mutation or deletion of the PLA2 domain prevents endosomal escape and subsequent transduction [25]. Thus, an absolutely essential outcome of these trafficking steps is the triggering of conformational changes in the capsid [26] leading to exposure of the N-terminal domains of VP1 and VP2, which are buried inside the capsid prior to infection [27]. Exposure of these domains for successful transduction is required, as it has been demonstrated that microinjection of both complete virions, as well as VP3-only virions, directly into the cytosol do not properly transduce the cell [28]. It has been suggested that the AAV capsid has protease activity that is pH-dependent, which could possibly be triggered by the acidification of the endosome [29]. However, it has yet to be determined if the self-cleavage events mediated by this activity also play a role in

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