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Epidermal growth factor receptor is a co-receptor for adeno-associated virus serotype 6

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Abstract

A critical step in gene therapy is the efficient transfer of genes in a cell-type and tissue specific manner. To better understand the mechanism of AAV6 transduction we used CGA combined with pathway visualization software to identify a positive correlation between AAV6 transduction and EGFR expression. Subsequent experiments suggest EGFR is necessary for vector internalization and likely functions as a co-receptor for this virus.

Text

Adeno-associated virus vectors offer unique advantages over other vector systems in gene therapy applications. These replication-deficient parvovirus vectors have repeatedly demonstrated the ability to deliver DNA to specific tissues and offer long-term transgene expression in a variety of systems1,2. While many studies have looked at the tissue specific expression elicited by each of the AAV serotypes3, a true understanding of how AAV transduces these tissues is still unclear. Of the large AAV family, only a few receptors or co-receptors have been identified for any of the parvoviruses4. The ability to better target transduction to select tissues based on the receptors that each serotype uses for entry is essential to enable users to pick a serotype based on the receptor expression in specific tissue or to exploit their altered receptor expression under disease conditions.

AAV6 has been reported to effectively transduce muscle5, lung6, brain7 and multiple types of tumors, including gliomas7 and lung adenocarcinomas8, and to elicit lower serum neutralizing antibodies when compared with AAV29. Identifying the receptor and any intracellular signaling pathway used by AAV6 to transduce these tissues would enable further development of this vector for gene therapy applications and may shed light on other similar AAV serotypes, including AAV1, AAV(VR-195) and AAV(VR-355)10.

Previously, we have demonstrated the utility of a bioinformatics-based approach, called comparative gene analysis (CGA), to identify PDGFR as a receptor for AAV511. Additional

bioinformatics-based software packages were added to further prioritize potential AAV cell surface receptors. In this manuscript, we have used this refined approach to identify a positive correlation between EGFR expression (GC16212) and cells permissive to AAV6 (PCC value of 0.421, p=0.003, Supplementary Figure 1). Although EGFR was not returned with the highest rank order PCC value from COMPARE analysis, the extensive clustering of +PCC genes connected to the EGFR signaling pathway supported exploring the involvement of EGFR or its downstream signaling pathways in AAV6 transduction.

To test our *in silico* hypothesis, we measured the influence of EGFR expression on AAV6 transduction. Initially, 32D cells that lack EGFR expression were engineered to stably express EGFR (32D-EGFR) and transduced with multiple AAV serotypes (Figs. 1A, 1B). Wild type 32D cells were not permissive to any of the serotypes tested. In the presence of EGFR, AAV6 was able to efficiently transduce 54.1+/−0.3% of the 32D-EGFR cells. Like AAV6, AAV1 was able to transduce the 32D-EGFR cells, but to a lesser extent suggesting additional molecules may be necessary for optimal transduction activity with this vector. Lack of transduction by AAV2 or AAV5 in the presence or absence of EGFR suggests specificity for AAV6 like viruses. We next used EGFR specific siRNA to knockdown EGFR expression and evaluated the impact on AAV transduction in two cell lines HEK293T cells and HN13 cells. (Fig. 1C). In HEK293T and HN13 cells, EGFR expression was knocked down by 37% and 58%, respectively, using EGFR specific siRNA and, in accordance, AAV6 transduction was decreased by 40% and 70%, respectively.

To better understand the role of EGFR in AAV6 transduction, AAV6 vector transduction was measured +/− EGFR inhibitors AG1478 or IRESSA. AAV6 transduction of HEK293T cells was inhibited by 50% in the presence either inhibitor (Fig. 1D). Under the same conditions, AAV2 transduction was unchanged. Further analysis suggested that EGFR is involved in vector entry as AAV internalization was decreased by 6 fold in the presence of IRESSA (Fig. 1E). These results suggest functional signaling through EGFR is required for AAV6 transduction and vector internalization.

Although the above data suggests a direct interaction between EGFR and AAV6, EGFR could be functioning as a part of a complex or AAV6 could be using the same trafficking pathway as EGFR. To measure direct EGFR:AAV6 interaction, soluble recombinant human-EGFR Fc fusion protein (rhEGFR-Fc) or soluble FGFR (rhFGFR-Fc) was pre-bound to protein-A sepharose beads and incubated with AAV2, AAV5, or AAV6. Of the three serotypes used, AAV6 binding increased approximately 7 fold in the presence of rhEGFR-Fc (Fig. 1F). No significant increase in EGFR specific binding was noted with AAV2 or AAV5. Furthermore, AAV6 did not bind to rhFGFR-Fc coated beads suggesting a specific AAV6:EGFR interaction.

Prior studies have suggested an association between EGFR and intracellular signaling pathways that regulate AAV2 transgene expression12,13. Mah et. al. showed that by inhibiting EGFR with tyrphostin 23, AAV2 transduction was increased whereas using AG1478 resulted in no change AAV2 transduction. This is in agreement with our study where we noted no significant change in AAV2 transduction in the presence of AG1478. Further research will be required to determine which of the multiple EGFR downstream

signaling pathways is regulating AAV6 transduction compared to AAV2 transduction. It is possible that AAV6 has evolved the ability to manipulate intracellular signaling and enhance its infectivity through mechanisms that are distinct from AAV2.

Increased expression of EGFR correlates with aggressive head and neck squamous cell carcinomas (HNSCC) tumor growth and resistance to treatment15. We next assessed the utility of AAV6 to transduce and ablate specific HNSCC presenting with elevated EGFR expression using gene-directed enzyme prodrug therapy (GDEPT). Two HNSCC cell lines, HN12 and HEp-2, were selected to represent polarities of EGFR expression. HN12 cells express a higher level of membrane localized EGFR, compared with HEp-2 cells which express a lower, more diffuse pattern of EGFR expression14. In preliminary *in vitro* studies, HN12 showed an EGFR-dependent AAV6 transduction, whereas and HEp-2 cells were significantly less permissive to AAV6 and transduction was not altered in the presence of AG1478 (Supplementary Figure 2). To evaluate AAV6:EGFR dependence *in vivo*, xenograft tumor models of these two cell lines were developed and intratumorally injected with AAV6-CMV-luciferse. Upon receiving luciferin, the HN12 tumors that received AAV6-CMV-luciferase displayed a significantly elevated (15-fold) average radiance of 1.01E4+/−0.31E4 photons/s/cm^2/sr, after subtraction of average background radiance compared with the Hep-2 tumors (0.69E3+/−0.48E3 photons/s/cm^2/sr) (Fig. 2A). This difference in transduction activity was also confirmed by quantification of vector genomes isolated from the tumors (Supplementary Figure 3). The ability of AAV6 to efficiently transduce EGFR expressing tumors *in vivo* presented an opportunity to target and deliver cytotoxic transgenes to HN12 tumors presenting elevated, membrane-localized EGFR.

To test if the specificity and tropism of AAV6 for the EGFR expressing HN12 cell was sufficient to ablate tumor growth without damaging the surrounding EGFR expressing muscle, HN12 xenograft tumor were injected with AAV6 vectors encoding HSVtk followed seven days later by treatment with ganciclovir. At the culmination of the study (day 20), a 65% reduction in tumor growth was observed between tumors transduced with AAV6-CMV-HSVtk and ganciclovir treatment and tumors that received only ganciclovir treatment (Fig. 2B). Although not explored in this study, alternative methods of vector delivery such as convection may enhance AAV6 distribution and thus more wide spread tumor killing than the simple intratumoral injection tested in this study.

This study presents the first evidence that EGFR is a co-receptor for AAV6. The methodology detailed here can be exploited to identify the receptors for other AAV serotypes or other virus families. While our studies have identified EGFR as a co-receptor for AAV6, expression of other cell surface molecules, such as ICAM1, also positively correlated with AAV6 transduction and need further study. Additionally, the comparison of genes that correlate with multiple serotypes of AAV may be useful for identifying genes involved in common pathways between serotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Dong JY, Fan PD, Frizzell RA. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. Hum Gene Ther. 1996; 7:2101–2112. [PubMed: 8934224]
2. Lebherz C, et al. Long-term inducible gene expression in the eye via adeno-associated virus gene transfer in nonhuman primates. Hum Gene Ther. 2005; 16:178–186. [PubMed: 15761258]
3. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. Mol Ther. 2008; 16:1073–1080. [PubMed: 18414476]
4. Harbison CE, Chiorini JA, Parrish CR. The parvovirus capsid odyssey: from the cell surface to the nucleus. Trends Microbiol. 2008; 16:208–214. [PubMed: 18406140]
5. Blankinship MJ, et al. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. Mol Ther. 2004; 10:671–678. [PubMed: 15451451]
6. Halbert CL, Lam SL, Miller AD. High-efficiency promoter-dependent transduction by adeno-associated virus type 6 vectors in mouse lung. Hum Gene Ther. 2007; 18:344–354. [PubMed: 17430088]
7. Huszthy PC, et al. Widespread dispersion of adeno-associated virus serotype 1 and adeno-associated virus serotype 6 vectors in the rat central nervous system and in human glioblastoma multiforme xenografts. Hum Gene Ther. 2005; 16:381–392. [PubMed: 15812233]
8. Gilbert JL, et al. Comparative evaluation of viral, nonviral and physical methods of gene delivery to normal and transformed lung epithelial cells. Anticancer Drugs. 2008; 19:783–788. [PubMed: 18690089]
9. Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J Virol. 2000; 74:1524–1532. [PubMed: 10627564]
10. Schmidt M, et al. Identification and characterization of novel adeno-associated virus isolates in ATCC virus stocks. J Virol. 2006; 80:5082–5085. [PubMed: 16641301]
11. Di Pasquale G, et al. Identification of PDGFR as a receptor for AAV-5 transduction. Nat Med. 2003; 9:1306–1312. [PubMed: 14502277]
12. Mah C, et al. Adeno-associated virus type 2-mediated gene transfer: role of epidermal growth factor receptor protein tyrosine kinase in transgene expression. J Virol. 1998; 72:9835–9843. [PubMed: 9811719]
13. Zhong L, et al. A dual role of EGFR protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. Mol Ther. 2007; 15:1323–1330. [PubMed: 17440440]
14. Magne N, et al. Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 ("Iressa"). Br J Cancer. 2002; 86:1518–1523. [PubMed: 11986789]
15. Thariat J, et al. Combination of radiotherapy with EGFR antagonists for head and neck carcinoma. Int J Clin Oncol. 2007; 12:99–110. [PubMed: 17443277]

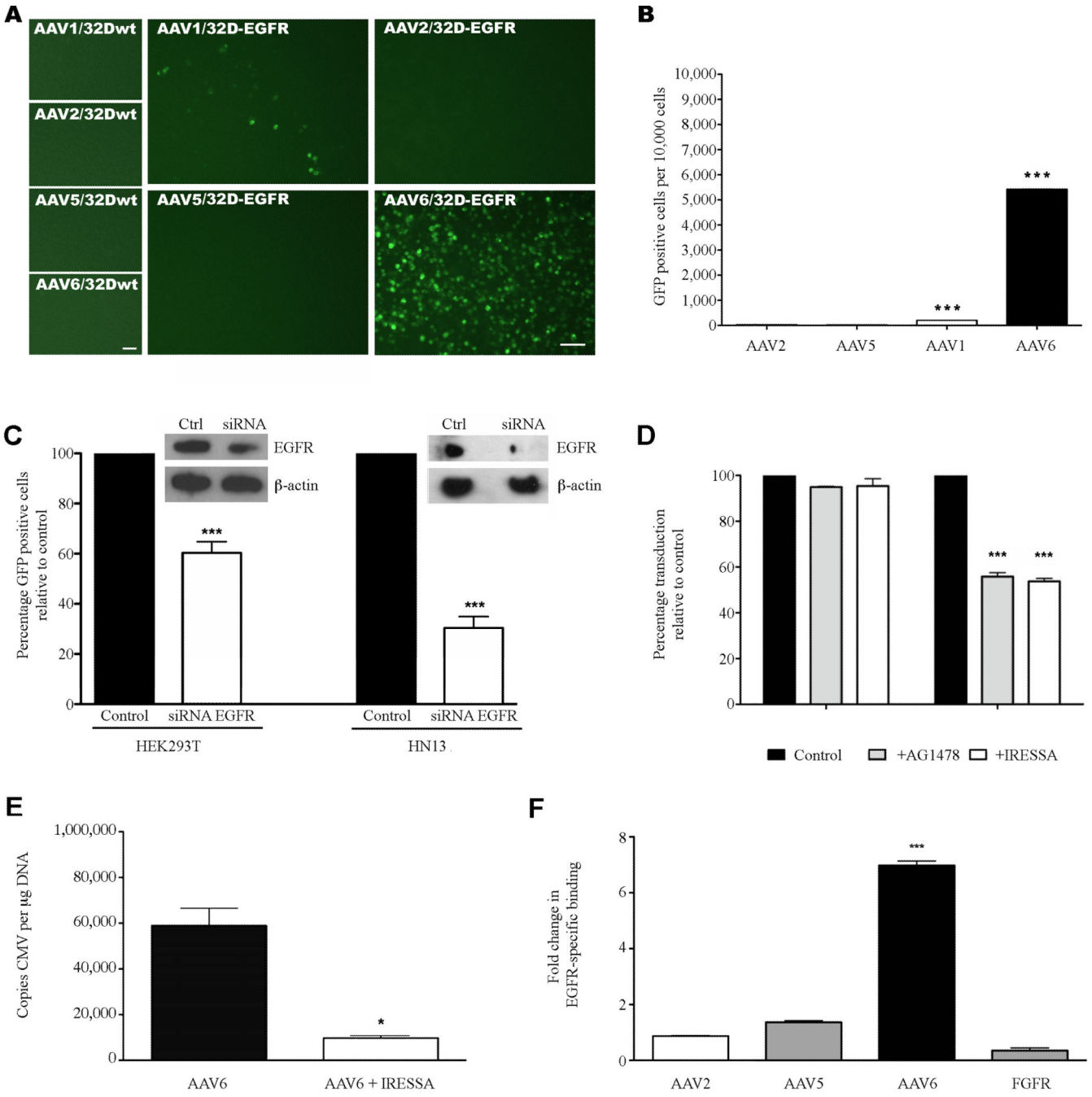

Figure 1.

A) A stable EGFR 32D clone (32D-EGFR) was used to evaluate the specific impact of EGFR expression on AAV transduction. Wildtype 32D (32Dwt) and 32D-EGFR cells were transduced with AAV1, AAV2, AAV5 or AAV6. Scale bar 50µm **B)** FACS analysis of 32D-EGFR cells 96 hours after being transduced with AAV2, AAV5, AAV1 or AAV6-CMV-eGFP. ***p<0.0001, n=3 **C)** HEK293T and HN13 cells were transfected with siRNA against EGFR showed a 37% and 58% knockdown in EGFR expression, respectively, and a similar 40% and 70% reduction in AAV6 transduction, respectively (***p<0.0001, n=3). **D)**

HEK293T cells preincubated with EGFR specific inhibitors, AG1478 or IRESSA blocked AAV6-mediated transduction by 50%. AAV2 transduction was not significantly influenced by EGFR inhibition. ***$p<0.0001$, n=3 **E)** 32D-EGFR cells + IRESSA inhibited internalization of AAV6 by 6-fold. *$p<0.01$, n=3 **F)** Protein A sepharose beads were incubated with AAV2, AAV5, or AAV6 alone or pre-coated with rhEGFR-Fc or rhFGFR-Fc. A 7-fold increase in AAV6 immunoprecipitation in the presence of rhEGFR-Fc suggests a serotype and receptor specific interaction between AAV6 and EGFR. ***$p<0.0001$, n=3

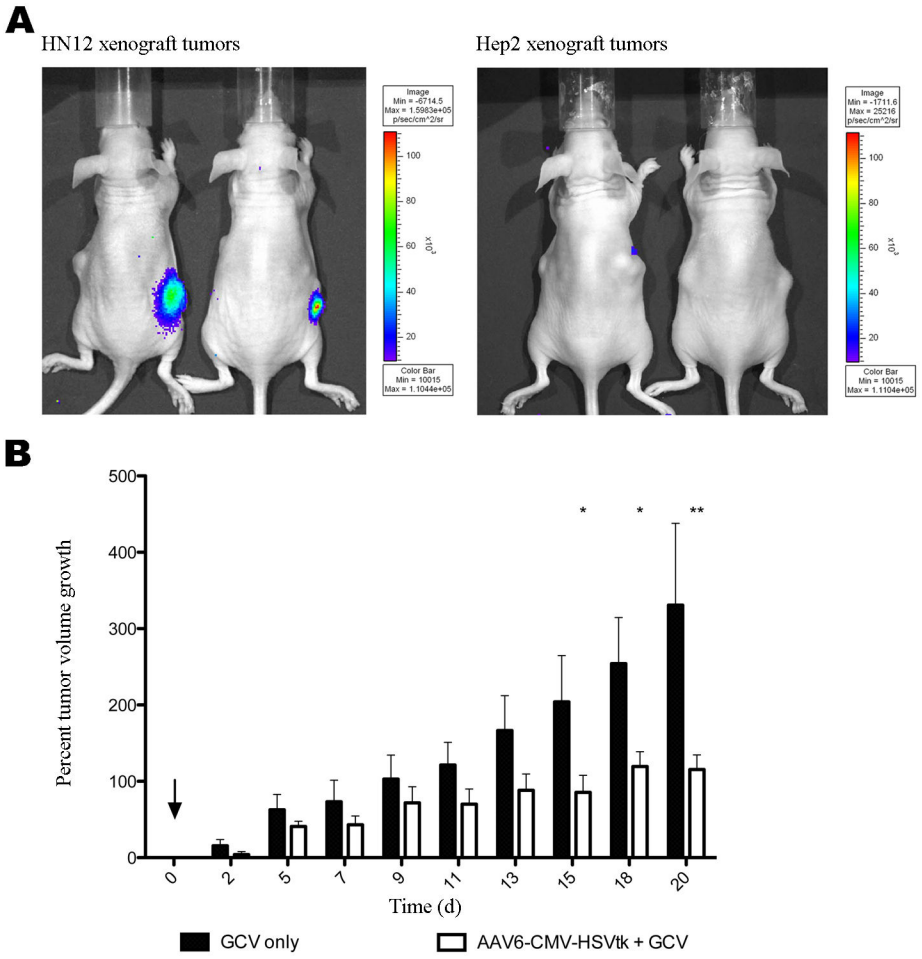

Figure 2.

A) Head and neck tumor cell lines, HN12 and HEp-2, were injected subcutaneously into the right and left flank of female nude mice. After tumors were established, AAV6-CMV-luciferase was introduced by direct intratumoral injection to the right flank tumors with the vehicle control injected into the left flank tumors. Ten days post AAV administration, *in vivo* luciferase activity was measured by bioluminescence following IP injection of luciferin (representative image, n=5). **B)** The HN12 xenograft tumors received intratumoral injections of AAV6-CMV-HSVtk. One week post AAV6 transduction, mice were started on daily ganciclovir (GC) injections. Arrow indicates day ganciclovir treatment was started. At culmination of study, tumors that received AAV6-CMV-HSVtk + GC showed a 65% reduction in tumor growth compared to tumors that received GC alone. *p<0.05 **p<0.001 n=9