

- system for myocardial gene delivery. *Circulation* **106**: 1756–1759.
19. Miyamoto, M, del Monte, F, Schmidt, U, DiSalvo, TS, Kang, ZB, Matsui, T *et al.* (2000). Adenoviral gene transfer of SERCA2a improves left ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci USA* **97**: 793–798.
20. del Monte, F, Williams, E, Lebeche, D, Schmidt, U, Rosenzweig, A, Gwathmey, JK *et al.* (2001). Improvement in survival and cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase in a rat model of heart failure. *Circulation* **104**: 1424–1429.
21. Dishart, KL, Denby, L, George, SJ, Nicklin, SA, Yendluri, S, Tuerk, MJ *et al.* (2003). Third-generation lentivirus vectors efficiently transduce and phenotypically modify vascular cells: implications for gene therapy. *J Mol Cell Cardiol* **35**: 739–748.

With a Little Help From My f(X)riends!: The Basis of Ad5-Mediated Transduction of the Liver Revealed

Pedro R Lowenstein¹

doi:10.1038/mt.2008.80

“Vorstellung schafft Wirklichkeit.”
—Richard Wagner

It has puzzled gene therapists that, despite many manipulations to the adenoviral capsid, it has been difficult to prevent adenoviral vectors from infecting the liver. Dissecting the molecular mechanisms of Ad5 infection has usually been performed using cultured cells. *In vitro* Ad5 infects cells through binding of the fiber to the Cocksackie and adenovirus receptor (CAR), followed by binding of an RGD domain within the penton base protein to integrins on the cell surface. These receptors having been identified, a proof-of-principle experiment was carried out to determine whether deletion of these binding sites on the adenovirus fiber and penton would abolish native viral tropism. Liver transduction *in vivo* is not eliminated by these mutations, which nevertheless block infection *in vitro*.¹ However, similar genetic mutations reduced transduction of muscle and brain.^{2–5} In the brain, eliminating binding

of Ad5 to CAR and integrins completely abolished transduction but, surprisingly, had no effect on the ability of adenovirus to induce inflammation.⁶ Recent studies have demonstrated that alternative adenoviral serotypes exhibit reduced liver tropism,⁷ which may be clinically advantageous. However, because the majority of vector development thus far has been performed using the subgroup C Ad2 and Ad5, a better understanding of the mechanisms that underlie the intrinsic hepatocyte tropism of these vectors is of great importance. Taken together, these data suggested the existence of other receptors for Ad5. A putative heparan sulfate proteoglycan binding site on the fiber shaft was identified, although the effect of mutation of this site may be due to changes to fiber structure or altering post-binding and post-internalization steps of adenovirus transduction.^{8–10} In summary, genetic mutations introduced into the adenoviral capsid have failed to abolish liver transduction.

Numerous groups have attempted to simultaneously de-target and re-target Ad5 vectors through the use of bispecific antibodies or peptide insertions in the fiber knob. Ad5 re-targeting is based on the idea of blocking the sites on both fiber and penton base that bind to CAR and integrins, through the use of antibodies or other molecules used as adapters to redirect viral infectivity. The addition of small molecules to the adapters could achieve re-targeting

to specific target cells. Re-targeting of adenoviruses via such adapters does indeed block Ad5 from infecting many cells, especially *in vitro*. Nevertheless, whatever the re-targeting approach, and whatever the efficiency of all these methods to achieve predictable re-targeting *in vitro*, all of these strategies failed to stop Ad5 from infecting the liver upon direct injection into the bloodstream.^{4,5}

Approaching from a different perspective, several groups determined that liver transduction by Ad5 could be abolished by pretreating animals with blood proteins from the coagulation cascade. Treatment of mice with warfarin, which depletes factors VII (FVII), IX (FIX), and X (FX), as well as protein C, completely eliminated Ad5 transduction of the liver.^{11–14} Thus, could Ad5 be entering liver cells using coagulation proteins as a bridge? Two recent articles published in *Cell*¹⁵ and the *Proceedings of the National Academy of Sciences USA*¹⁶ have solved the enigma of how Ad5 enters the liver.

In a series of very elegant experiments, Waddington *et al.*¹⁵ and Kalyuzhniy *et al.*¹⁶ demonstrated that FX binds to the hexon protein in Ad5. The groups followed slightly different routes to arrive at similar conclusions. Barker and collaborators¹⁵ first determined that FX binds in a Ca²⁺-dependent manner to Ad5 through the γ -carboxyglutamic acid domain. Using surface plasmon resonance they made the surprising determination that, instead of binding to fiber protein, FX actually binds to the hexon protein. Electron cryomicroscopy and three-dimensional reconstruction of Ad5 bound to FX confirmed that FX binds at the cup formed at the center of each hexon trimer (Figure 1). The stoichiometric analysis of 205 molecules of FX binding per virus particle is consistent with just one FX molecule binding to each hexon trimer. These results were further confirmed by replacement of the hypervariable region (HVR) of Ad5 with that from Ad48; such a virus did not bind FX and lacked FX-mediated enhanced binding and transduction. Transduction of liver was also shown to be mediated through a bridge formed by FX binding through its γ -carboxyglutamic acid domain to Ad5 hexon and through its serine protease domain to heparan sulfate proteoglycans. Furthermore, because CAR and integrin binding are dispensable for

¹Board of Governors' Gene Therapeutics Research Institute, Cedars-Sinai Medical Center, and Departments of Pharmacology and Medicine, University of California, Los Angeles, Los Angeles, California, USA

Correspondence: Pedro R Lowenstein, Research Pavilion, Davis Building, Room 5093, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA.

E-mail: lowensteinp@cshs.org

liver transduction, the experiments suggest that FX binding to hexon is necessary and sufficient for Ad5 binding to and transduction of the liver. The crucial experiment here would be to demonstrate that vectors unable to bind CAR or integrin, and unable to bind FX, fail to bind to and transduce the liver. Finally, Ad35- and Ad26-derived vectors, which bind to FX weakly or not at all, failed to transduce liver.

Independently, Shayakhmetov's group¹⁶ performed a series of similar experiments that led to similar conclusions. Using surface plasmon resonance they determined an affinity of FX for Ad5 1 log higher than had Waddington *et al.*¹⁵ Given the high affinity found for FX binding to Ad5, Kalyuzhnyi *et al.*¹⁶ isolated the FX binding protein from Ad5 by affinity chromatography. Following trypsin digestion and mass spectrometry, numerous peptides corresponding to Ad5 hexon were identified, suggesting that FX indeed binds to the hexon protein. This was confirmed by studying the characteristics of FX binding to hexon trimer, which were indistinguishable from the affinity of FX binding to whole Ad5 particles. Furthermore, a virus that contains a 71-amino acid insertion into the exposed hexon loop of HVR5 abolished binding to FX, strongly suggesting that FX binds to adenovirus through hexon protein. Electron cryomicroscopy and three-dimensional reconstruction further confirmed the binding of FX to hexon protein, especially to the central depression of each hexon trimer. Based on sequence comparisons across the HVR from various adenovirus serotypes, Kalyuzhnyi *et al.*¹⁶ postulated that HVR3 or 7 is the most likely direct binding site to hexon, with only one binding site per trimer. Thus, there is agreement with the data of Waddington *et al.*¹⁵ showing that one FX molecule binds per each hexon trimer. Functional analysis *in vitro* indicated that FX increases infectivity of serotypes able to bind FX but does not change infectivity of those lacking such binding. Nevertheless, absence of binding to FX did not influence the capacity to propagate such vectors on 293 cells. *In vivo* transduction followed the same pattern, with Ad5 trapping and expression in the liver being dependent on available FX, whereas those from a vector unable to bind FX were independent of circulating FX levels. Shayakhmetov and collaborators¹⁶ also suggest that the presence

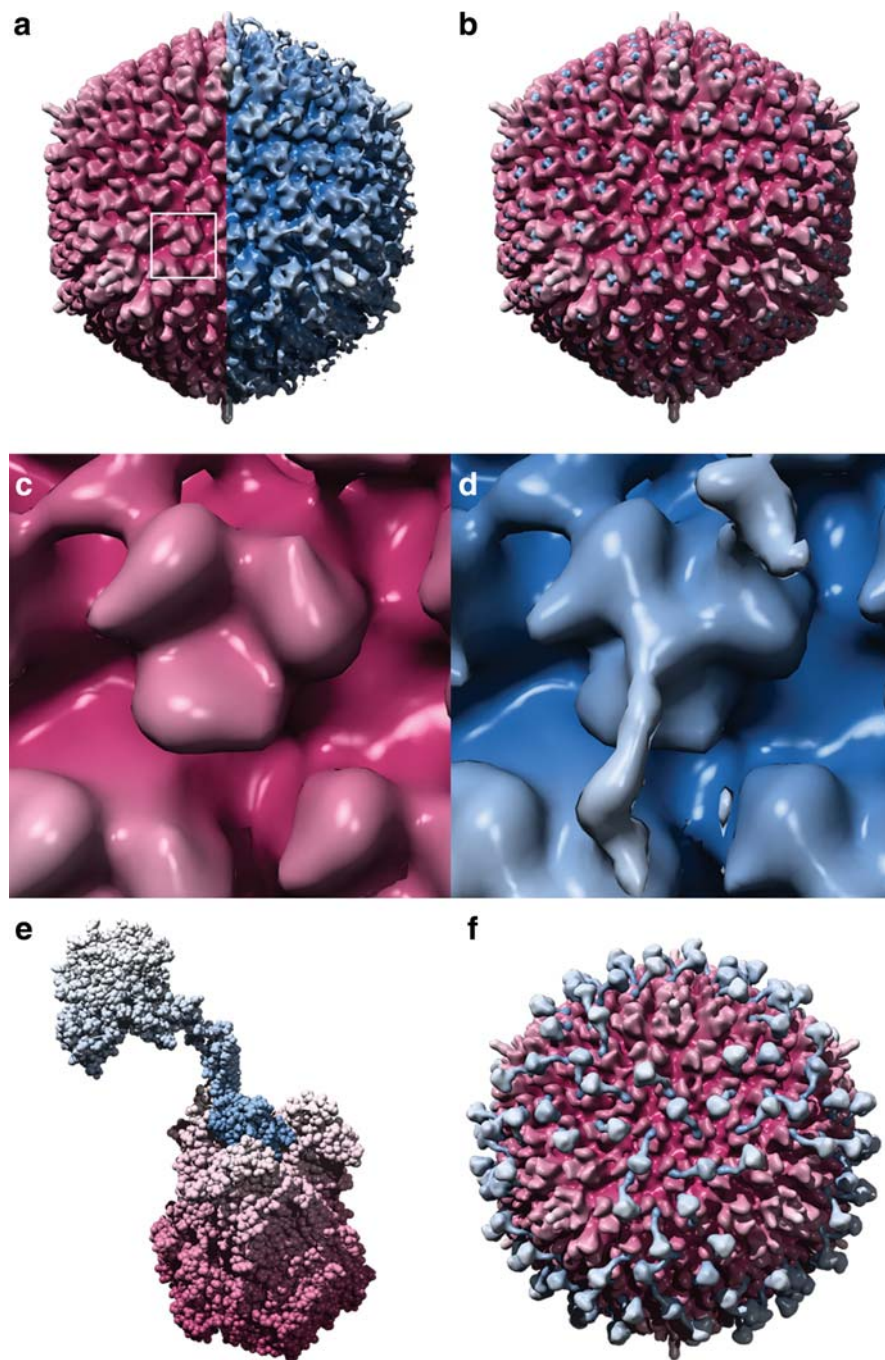


Figure 1 Electron cryomicroscopy of adenovirus serotype 5 (Ad5) and Ad5 complexed to Factor X (FX). **(a)** Three-dimensional reconstructions of unlabeled Ad5 (left, pink) and FX-labeled Ad5 (right, blue). **(b)** Overlaying the two reconstructions highlights the point of contact between FX and hexon. **(c and d)** Close-up views of a single hexon. **(d)** Symmetry in the Ad5 capsid led to steric collision between bound FX molecules, resulting in low occupancy and noisy density in the three-dimensional reconstruction. **(e and f)** Fitting of crystallographic coordinates for hexon^{17,18} and a molecular model of FX, combined with modeling experiments and SPR data, demonstrated that FX binds to hexon in one of three symmetry-related binding sites and allowed the synthesis of a high-resolution model of the interaction.

of binding to FX may influence the intracellular fate of internalized adenoviruses, and potentially endosomal escape and transduction.

In summary, AD5, unexpectedly, uses a heretofore unknown complex multiprotein bridge to enter the liver. The main receptors—CAR and integrins—most certainly

play an important role, but the binding of FX to Ad5 seems to be primary, because in its absence transduction is simply abolished, whereas elimination or masking of binding of Ad5 to CAR and integrins reduces but does not abolish liver transduction. These new data explain the resilience of Ad5 infection of the liver. What remains to be established is exactly how many proteins (e.g., low-density lipoprotein receptor-related protein, heparan sulfate proteoglycans) are involved in liver transduction of Ad5 mediated by FX, and whether these mechanisms are necessary for the transduction of different types of cells in the liver, or whether binding is specific to hepatocytes. These data are of strong clinical relevance and will help in devising new vectors that either completely avoid or are specifically targeted to the liver.

Finally, although the articles cited above concentrate on the role of plasma proteins on the transduction of liver cells, they are likely to have uncovered a novel means of virus infection of target cells, through their binding to plasma, extracellular, or other tissue proteins. In the brain, for example, vectors unable to bind to both CAR and integrins fail to transduce any brain cells yet cause a local inflammation that is indistinguishable from that caused by control vectors.⁶ Thus, it is likely that such bridge-mediated mechanisms of infection of target cells may be cell type-specific and extend to other proteins, viruses, and cell types.

REFERENCES

1. Alemany, R and Curiel, DT (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther* **8**: 1347–1353.
2. Wickham, TJ (2003). Ligand-directed targeting of genes to the site of disease. *Nat Med* **9**: 135–139.
3. Roelvink, PW, Mi Lee, G, Einfield, DA, Kovesdi, I and Wickham, TJ (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* **286**: 1568–1571.
4. Glasgow, JN, Everts, M and Curiel, DT (2006). Transductional targeting of adenovirus vectors for gene therapy. *Cancer Gene Ther* **13**: 830–844.
5. Waehler, R, Russell, SJ and Curiel, DT (2007). Engineering targeted viral vectors for gene therapy. *Nat Rev Genet* **8**: 573–587.
6. Thomas, CE, Edwards, P, Wickham, TJ, Castro, MG and Lowenstein, PR (2002). Adenovirus binding to the coxsackievirus and adenovirus receptor or integrins is not required to elicit brain inflammation but is necessary to transduce specific neural cell types. *J Virol* **76**: 3452–3460.
7. Stone, D, Liu, Y, Li, ZY, Tuve, S, Strauss, R and Lieber, A (2007). Comparison of adenoviruses from species B, C, E, and F after intravenous delivery. *Mol Ther* **15**: 2146–2153.
8. Kritz, AB, Nicol, CG, Dishart, KL, Nelson, R, Holbeck, S, Von Seggern, DJ *et al.* (2007). Adenovirus 5 fibers mutated at the putative HSPG-binding site show restricted retargeting with targeting peptides in the HI loop. *Mol Ther* **15**: 741–749.
9. Bayo-Puxan, N, Cascallo, M, Gros, A, Huch, M, Fillat, C and Alemany, R (2006). Role of the putative heparan sulfate glycosaminoglycan-binding site of the adenovirus type 5 fiber shaft on liver detargeting and knob-mediated retargeting. *J Gen Virol* **87**: 2487–2495.
10. Di Paolo, NC, Kalyuzhnyi, O and Shayakhmetov, DM (2007). Fiber shaft-chimeric adenovirus vectors lacking the KTK motif efficiently infect liver cells *in vivo*. *J Virol* **81**: 12249–12259.
11. Parker, AL, McVey, JH, Doctor, JH, Lopez-Franco, O, Waddington, SN, Havenga, MJ *et al.* (2007). Influence of coagulation factor zymogens on the infectivity of adenoviruses pseudotyped with fibers from subgroup D. *J Virol* **81**: 3627–3631.
12. Parker, AL, Waddington, SN, Nicol, CG, Shayakhmetov, DM, Buckley, SM, Denby, L *et al.* (2006). Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood* **108**: 2554–2561.
13. Waddington, SN, Parker, AL, Havenga, M, Nicklin, SA, Buckley, SM, McVey, JH *et al.* (2007). Targeting of adenovirus serotype 5 (Ad5) and 5/47 pseudotyped vectors *in vivo*: fundamental involvement of coagulation factors and redundancy of CAR binding by Ad5. *J Virol* **81**: 9568–9571.
14. Shayakhmetov, DM, Gaggar, A, Ni, S, Li, ZY and Lieber, A (2005). Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol* **79**: 7478–7491.
15. Waddington, SN, McVey, JH, Bhella, D, Parker, AL, Barker, K, Atoda, H *et al.* (2008). Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell* **132**: 397–409.
16. Kalyuzhnyi, O, Di Paolo, NC, Silvestry, M, Hoffner, SE, Barry, MA, Stewart, PL *et al.* (2008). Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. *Proc Natl Acad Sci USA* **105**: 5483–5488.
17. Rux, J and Burnett, R (2000). Type-specific epitope locations revealed by X-ray crystallographic study of adenovirus type 5 hexon. *Mol Ther* **1**, 18–30.
18. Venkateswarlu, D, Perera, L, Darden, T and Pedersen, L (2002). Structure and dynamics of zymogen human blood coagulation factor X. *Biophys J* **82**, 1190–1206.

Translation of Targeted Oncolytic Virotherapeutics from the Lab into the Clinic, and Back Again: A High-Value Iterative Loop

Ta-Chiang Liu¹, Tae-Ho Hwang^{1,2}, John C Bell^{1,3} and David H Kirn^{1,4}

doi:10.1038/mt.2008.70

As our knowledge and understanding of cancer biology have exploded over the past decade, some have proposed that this is a golden age of targeted cancer therapeutics. These novel therapeutics target specific molecules and pathways in cancers. Most notably, numerous monoclonal antibodies and small-molecule tyrosine kinase inhibitors have been developed and approved for a variety of cancers. Although these agents generate billions of dollars in sales, their impact on the overall survival of patients with metastatic solid tumors is in general minimal (although rare exceptions exist).¹ In addition, a major driving force behind the development of these more “elegant” and targeted therapies—the promise that toxic chemotherapy could

be avoided—has been undercut. In this Commentary we analyze the difference in development strategies and processes between molecular therapeutics and oncolytic virotherapeutics, and discuss how the field can benefit from a unique “iterative loop” that is feasible with the virotherapeutics class.

Standard drug development typically follows a relatively slow, sequential, and unidirectional process from target identification to lead identification, lead optimization, preclinical pharmacology and toxicology, and Investigational New Drug Application (IND) filing, followed by phase I, II, and III trials, and eventually, in a small fraction of cases, to approval. The entire process can take 10 or more years before a drug is approved for a cancer market. Product approval of bevacizumab (Avastin, Genentech) for example, took approximately 10 years from identification of the target vascular endothelial growth factor molecule. Estimated costs to approval of a new chemical entity vary, but most estimates are more than \$500 million. It is not at all surprising that once a molecule has been designated as an IND

¹Jennerex Biotherapeutics, San Francisco, California, USA; ²Pusan National University, Pusan, South Korea; ³Ottawa Health Research Institute, Ottawa, Ontario, Canada; ⁴Department of Clinical Pharmacology, University of Oxford Medical School, Oxford, UK

Correspondence: David H Kirn, Jennerex Biotherapeutics, One Market Street, Spear Tower, Suite 2260, San Francisco, California, USA. E-mail: dkirn@jennerex.com