

# Coagulation factor X mediates adenovirus type 5 liver gene transfer in non-human primates (*Microcebus murinus*)

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## Abstract

**FX-binding ablated adenovirus type 5 (Ad5) vectors have been genetically engineered to ablate the interaction with coagulation factor X (FX) resulting in substantially reduced hepatocyte transduction following intravenous administration in rodents. Here, we quantify viral genomes and gene transfer mediated by Ad5 and FX-binding ablated Ad5 vectors in non-human primates. Ad5 vectors accumulated in and mediated gene transfer predominantly to the liver while FX-binding ablated vectors primarily targeted the spleen but showed negligible liver gene transfer. Therefore, the Ad5-FX pathway mediating liver gene transfer in rodents is also the mechanism underlying Ad5 hepatocyte transduction in *Microcebus murinus*.**

**MESH Keywords** Adenoviridae; genetics; metabolism; Animals; Cell Membrane; metabolism; Cheirogaleidae; Factor X; genetics; metabolism; Gene Targeting; methods; Gene Therapy; Gene Transfer Techniques; Genetic Vectors; administration & dosage; genetics; metabolism; Genome, Viral; Heparan Sulfate Proteoglycans; metabolism; Hepatocytes; cytology; metabolism; Immunohistochemistry; Liver; cytology; metabolism; Protein Binding; Spleen; cytology; metabolism

Following intravenous (i.v.) administration adenovirus type 5 (Ad5) viral particles predominantly accumulate in and transduce the liver in rodent and non-human primate models(5, 15, 19). Although the coxsackie and adenovirus receptor (CAR) acts as a primary receptor for Ad5 *in vitro*, modifications of the Ad5 capsid ablating the interaction with CAR and/or its secondary receptors ( $\alpha\beta 3$  and  $\alpha\beta 5$  integrins) does not alter the biodistribution of Ad5 following i.v. delivery(6, 8, 11). Several studies have documented the role of blood coagulation factors in mediating liver gene transfer *in vivo* and the mechanism underlying Ad5 hepatocyte transduction(2, 14, 17, 20, 21). We demonstrated that coagulation factor X (FX) binds to the hexon protein of Ad5 at high affinity (~2 nM) and that this interaction mediates hepatocyte transduction following i.v. delivery in mice and rats(21). FX-binding ablated Ad5 vectors have been mutated in the hexon hyper-variable region (HVR)5 and/or HVR7 as described previously(2) and showed substantially reduced hepatocyte transduction following i.v. administration in rodent models(1, 2). Just recently, our group has highlighted the importance of heparan sulfate proteoglycans (HSPGs) as receptors for the Ad5-FX complex and the requirement of the integrin binding motif Arg-Gly-Asp (RGD) for efficient uptake, internalisation and correct trafficking when Ad5 vectors use this pathway(3).

However, many other interactions and anatomical features, including fenestrae size in liver sinusoids and interactions with key tropism-determining capsid proteins can differ between different species(7). Limited data obtained from necropsies in human clinical trials suggest that Ad5 may target the liver and spleen after intravenous administration(4), although definitive data has not been reported from previous or ongoing clinical trials. However, several studies performed in non-human primates have shown that Ad5 primarily transduces the liver and spleen following i.v. delivery(9, 10, 15, 18). Ablation of CAR- and integrin-binding motifs in the Ad5 capsid did not alter Ad5 biodistribution in cynomolgus monkeys, indicating that an alternate transduction pathway may be responsible for liver transduction in these animals(18). Importantly, none of the reported modifications incorporated in the Ad5 capsid, via ablation of the binding to natural receptors(18) or fiber substitution(10), have shown significantly reduced hepatic transduction or viral genome accumulation in non-human primates. In the present study, we document the transduction profiles and viral genome accumulation of Ad5 and FX-binding ablated Ad5 vectors in *Microcebus murinus* to identify whether the Ad5-FX pathway modulates Ad5 biodistribution in non-rodent species. To evaluate the biodistribution of these viruses 48h after i.v. delivery, anaesthetized animals were injected intravenously with  $1 \times 10^{11}$  vp/animal of Ad5 and FX-binding ablated Ad5-HVR5\*7\*E451Q vectors (approximately  $1 \times 10^{12}$  vp/Kg). Animals were sacrificed 48h later and tissues harvested for analysis. All animal experimentation was approved by the local Ethic Committee (authorization #CEEA-LR-1013) in accordance with the European Community Council directives of November 24, 1986. The dose used in this study ( $1 \times 10^{12}$  vp/Kg) has been proved to be safe in previous studies with Ad5 vectors and non-human primates(13). Doses above  $1 \times 10^{13}$  vp/Kg have resulted lethal in baboons(9) or highly toxic associated with transaminitis in rhesus monkeys(13). Viral genome accumulation was quantified using SyBR green real time PCR (7900HT Sequence Detection System, Applied Biosystems, UK) using 100ng of purified DNA and 0.2  $\mu$ M hexon primers as previously described(2). DNA was extracted from tissues using the QIAamp DNA

mini kit (QIAGEN, UK) following the manufacturer's instructions. As shown in Figure 1, Ad5 accumulated mainly in the liver although viral genomes were also detected in spleen, heart, lung and kidney. In addition, we also detected low levels of viral genomes in thymus, pancreas, intestines and lymph nodes. Importantly and in agreement with previous results(1), the FX-binding ablated Ad5CMVlacZ-HVR5\*7\*E451Q vector showed significantly reduced accumulation in the liver and were found primarily in the spleen (Fig. 1). In addition, negligible levels of Ad5CMVlacZ-HVR5\*7\*E451Q viral genomes were detected in most of the tissues analyzed (Fig. 1). Importantly, these results suggest that the Ad5-FX pathway may mediate viral genome accumulation not only in the liver but in the majority of the organs analyzed indicating the presence of HSPGs on the surface of targeted cells.

To test if viral genome accumulation correlated with gene transfer, beta-galactosidase protein quantification was carried out using the  $\beta$ -Gal enzyme-linked immunosorbent (ELISA) kit (Roche, UK) according to the manufacturer's instructions. In concordance with the biodistribution data, Ad5 vectors efficiently transduced the liver with lower levels of detection in the rest of the organs analyzed (Fig. 2). Of note, although high levels of viral genomes were found in several organs (spleen, heart, lung and kidney) including the liver, the levels of transduction were substantially lower for these organs, in agreement with results obtained previously (1). Contrary to Ad5CMVlacZ, FX-binding ablated Ad5 vectors (Ad5CMVlacZ-HVR5\*7\*E451Q) presented significantly lower levels of transduction in the liver and limited levels of transduction in the spleen as previously observed in rodent models at similar doses(1, 2). Interestingly, only basal levels of transduction were observed for Ad5CMVlacZ-HVR5\*7\*E451Q in the majority of tissues analyzed, with the exception of the spleen (Fig. 2). In order to confirm and visualize the  $\beta$ -gal expression of Ad5CMVlacZ, we performed histochemical analysis in livers and spleens of non-human primates 48 hours post-injection. Livers and spleens were embedded in O.C.T (Tissue-Tek), and frozen at  $-80^{\circ}\text{C}$  immediately post-necropsy. Six micrometers liver sections were analyzed by immunohistochemistry using the rabbit anti- $\beta$ -galactosidase polyclonal antibody (8.4  $\mu\text{g}/\text{ml}$ ; MP Biomedicals, UK) or matched rabbit IgG non-immune control (8.4  $\mu\text{g}/\text{ml}$ ; Invitrogen, Paisley, UK) and then detected using an Alexa-488 fluorescent anti-rabbit polyclonal antibody (Invitrogen, Paisley, UK). Frozen sections were blocked for 30 min in 10% normal goat serum (GS) (Vector laboratories, UK). Primary antibodies were incubated with sections for 16h at  $4^{\circ}\text{C}$ , followed by secondary antibody incubation (diluted 1:500 in PBS+Tween 0.05% with 2% GS) and incubated for 1h at room temperature. Slides were mounted with ProLong Gold+DAPI (Invitrogen, UK) and images were captured using an Olympus BX60 fluorescence microscope and analyzed using Cell<sup>TM</sup> software (Olympus, UK). As previously observed(1), animals injected with Ad5CMVlacZ exhibited high levels of hepatocyte transduction while very low levels of  $\beta$ -gal expression were detected in liver sections from animals injected with Ad5CMVlacZ-HVR5\*7\*E451Q vectors (Fig. 3). Negligible levels of  $\beta$ -gal were observed in spleen sections from both Ad5CMVlacZ and FX-binding ablated Ad5CMVlac-HVR5\*7\*E451Q (Fig. 3). In summary, transduction profiles and immunohistochemical studies correlated with the levels of viral genomes detected in the liver. Importantly, this work identifies the FX pathway as the main mechanism of gene transfer to hepatocytes following intravenous delivery of Ad5 particles in *Microcebus murinus*.

During the last decade, there have been many efforts to retarget Ad5 vectors from the liver to other tissues. Nevertheless, the ablation of CAR- and integrin-binding motifs did not reduce Ad5 mediated liver gene transfer following i.v. delivery(6, 8, 18). FX-binding ablated Ad5 vectors have shown substantially reduced hepatocyte transduction in mice and rats following intravenous delivery(1). However, this is the first time to our knowledge that Ad5 vectors have been detargeted from the liver in a non-human primate model. Here, we show FX-binding ablated Ad5 vectors with minimal modifications in the Ad5-hexon protein (2 amino acids in HVR5 and 5 amino acids in HVR7) accumulate in the spleen. However, the levels of transduction of FX-binding ablated Ad5 vectors observed in the spleen were lower compared to Ad5CMVlacZ at the dose administered. Moreover, it has been described a loss of white pulp following the intravenous administration of Ad5 vectors in non-human primates (rhesus monkeys) at substantially higher doses(15). Histochemical studies with *Microcebus murinus* spleen samples confirm normal histology of spleen sections compared with previous histochemical studies performed in this model (data not shown). The interaction of the Ad5 capsid with CAR receptors expressed on erythrocytes has been shown in humans and rats(12, 16). Removal of the CAR-binding motif in combination with FX-binding ablation may therefore improve the *in vivo* biodistribution of Ad5 vectors for detargeting/retargeting purposes. The varying size of fenestrated capillaries between different species (e.g. in humans  $107\pm 1.5$  nm) is also an important issue to take into account when Ad5 is administered intravenously(7, 22). The present study indicates that the fenestrated capillaries of non-human primates did not impair hepatocyte transduction by Ad5. In addition, the high levels of Ad5 viral genomes found in other organs (spleen, lung, kidney and heart) indicate the size of fenestrated capillaries present in *Microcebus murinus* did not restrict the extravasation of Ad5 particles into different organs following i.v. delivery. Furthermore, this work shows the relevance of the Ad5-FX pathway not only in the liver but in most of the organs analyzed and the abundance of HSPGs receptors on the surface of targeted cells(3). Therefore, these results have relevant implications for Ad5 liver targeting in humans and detargeting strategies based on FX-binding ablated Ad5 vectors.

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## References:

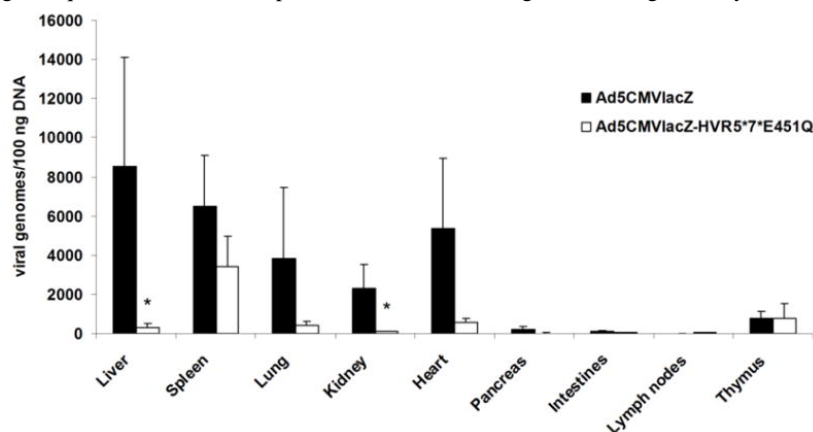
Bibliography

1. Alba R, Bradshaw AC, Coughlan L, Denby L, McDonald RA, Waddington SN, Buckley SM, Greig JA, Parker AL, Miller AM, Wang H, Lieber A, van Rooijen N, McVey JH, Nicklin SA, Baker AH. Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood*.
2. Alba R, Bradshaw AC, Parker AL, Bhella D, Waddington SN, Nicklin SA, van Rooijen N, Custers J, Goudsmit J, Barouch DH, McVey JH, Baker AH. 2009; Identification of coagulation factor (FX) binding sites on the adenovirus serotype 5 hexon: effect of mutagenesis on FX interactions and gene transfer. *Blood*. 114: 965 - 71
3. Bradshaw AC, Parker AL, Duffy MR, Coughlan L, van Rooijen N, Kahari VM, Nicklin SA, Baker AH. Requirements for Receptor Engagement during Infection by Adenovirus Complexed with Blood Coagulation Factor X. *PLoS Pathog*. 6:
4. Hamid O, Varterasian ML, Wadler S, Hecht JR, Benson A 3rd, Galanis E, Uprichard M, Omer C, Bycott P, Hackman RC, Shields AF. 2003; Phase II trial of intravenous CI-1042 in patients with metastatic colorectal cancer. *J Clin Oncol*. 21: 1498 - 504
5. Jaffe HA, Danel C, Longenecker G, Metzger M, Setoguchi Y, Rosenfeld MA, Gant TW, Thorgeirsson SS, Stratford-Perricaudet LD, Perricaudet M. 1992; Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. *Nature Genetics*. 1: 372 - 378
6. Koizumi N, Kawabata K, Sakurai F, Watanabe Y, Hayakawa T, Mizuguchi H. 2006; Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alphav integrin, and heparan sulfate binding reduce *in vivo* tissue transduction and toxicity. *Hum Gene Ther*. 17: 264 - 79
7. Lievens J, Snoeys J, Vekemans K, Van Linthout S, de Zanger R, Collen D, Wisse E, De Geest B. 2004; The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. *Gene Ther*. 11: 1523 - 31
8. Mizuguchi H, Koizumi N, Hosono T, Ishii-Watabe A, Uchida E, Utouchi N, Watanabe Y, Hayakawa T. 2002; Car- or  $\alpha$ V integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. *Gene therapy*. 9: 769 - 776
9. Morral N, O'Neal WK, Rice K, Leland MM, Piedra PA, Aguilar-Cordova E, Carey KD, Beaudet AL, Langston C. 2002; Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum Gene Ther*. 13: 143 - 54
10. Ni S, Bernt K, Gaggari A, Li ZY, Kiem HP, Lieber A. 2005; Evaluation of biodistribution and safety of adenovirus vectors containing group B fibers after intravenous injection into baboons. *Hum Gene Ther*. 16: 664 - 77
11. Nicklin S, Wu E, Nemerow G, Baker A. 2005; The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther*. 12: 384 - 393
12. Nicol C, Graham D, Miller W, White S, Smith T, Nicklin S, Stevenson S, Baker A. 2004; Effect of adenovirus serotype 5 fiber and penton modifications on *in vivo* tropism in rats. *Mol ther*. 10: 343 - 353
13. Nunes FA, Furth EE, Wilson JM, Raper SE. 1999; Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. *Hum Gene Ther*. 10: 2515 - 26
14. Parker AL, Waddington SN, Nicol CG, Shayakhmetov DM, Buckley SM, Denby L, Kembell-Cook G, Ni S, Lieber A, McVey JH, Nicklin SA, Baker AH. 2006; Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood*. 108: 2554 - 61
15. Schnell MA, Zhang Y, Tazelaar J, Gao GP, Yu QC, Qian R, Chen SJ, Varnavski AN, LeClair C, Raper SE, Wilson JM. 2001; Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol Ther*. 3: 708 - 22
16. Seiradake E, Henaff D, Wodrich H, Billet O, Perreau M, Hippert C, Mennechet F, Schoehn G, Lortat-Jacob H, Dreja H, Ibanes S, Kalatzis V, Wang JP, Finberg RW, Cusack S, Kremer EJ. 2009; The cell adhesion molecule "CAR" and sialic acid on human erythrocytes influence adenovirus *in vivo* biodistribution. *PLoS Pathog*. 5: e1000277 -
17. Shayakhmetov D, Gaggari A, Ni S, Li ZY, Lieber A. 2005; Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol*. 79: 7478 - 7491
18. Smith TA, Idamakanti N, Marshall-Neff J, Rollence ML, Wright P, Kaloss M, King L, Mech C, Dinges L, Iverson WO, Sherer AD, Markovits JE, Lyons RM, Kaleko M, Stevenson SC. 2003; Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum Gene Ther*. 14: 1595 - 604
19. Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, Barsoum J, Fawell SE. 2001; Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol ther*. 3: 28 - 35
20. Vigant F, Descamps D, Jullienne B, Esselin S, Connault E, Opolon P, Tordjmann T, Vigne E, Perricaudet M, Benihoud K. 2008; Substitution of hexon hypervariable region 5 of adenovirus serotype 5 abrogates blood factor binding and limits gene transfer to liver. *Molecular Therapy*. 1 - 7
21. Waddington SN, McVey JH, Bhella D, Parker AL, Barker K, Atoda H, Pink R, Buckley SM, Greig JA, Denby L, Custers J, Morita T, Francischetti IM, Monteiro RQ, Barouch DH, van Rooijen N, Napoli C, Havenga MJ, Nicklin SA, Baker AH. 2008; Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*. 132: 397 - 409
22. Wisse E, Jacobs F, Topal B, Frederik P, De Geest B. 2008; The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. *Gene Ther*. 15: 1193 - 9

**Figure 1**

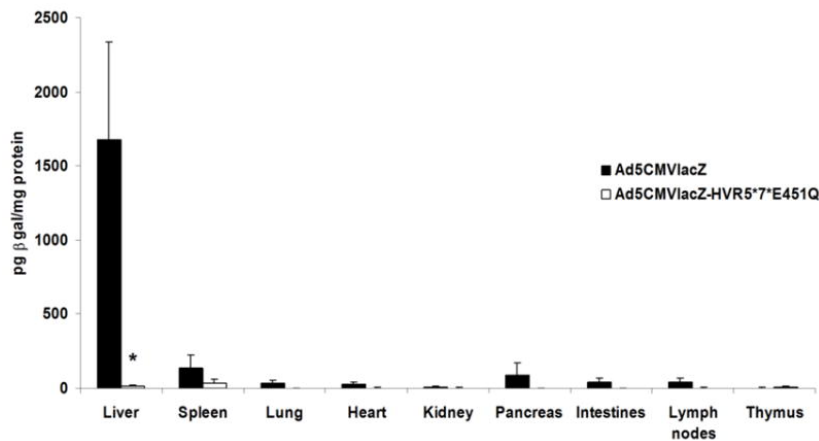
Biodistribution of Ad5CMVlacZ and FX-binding ablated Ad5CMVlacZ-HVR5\*7\*E451Q vectors at 48 hours

Viral genome accumulation was measured in different tissues after the administration of  $1 \times 10^{11}$  vp of control and FX-binding ablated Ad5 vectors in non-human primates (*Microcebus murinus*). Viral genomes were quantified from 100 ng of purified DNA and analyzed by SYBR green qPCR. Statistics were performed with natural log values using one way ANOVA (\* $<0.05$  Vs Ad5CMVlacZ, n=5).



**Figure 2**

Transduction profiles of Ad5CMVlacZ and FX-binding ablated Ad5CMVlacZ-HVR5\*7\*E451Q vectors at late time points (48h)  $\beta$ -galactosidase protein expressed from viral vectors was quantified using the  $\beta$ gal ELISA assay (Roche, UK) from non-human primate (*Microcebus murinus*) samples following the intravenous injection of  $1 \times 10^{11}$  vp/animal. Statistics were performed with natural log values using one way ANOVA ( $* < 0.05$  Vs Ad5CMVlacZ,  $n = 5$ ).



**Figure 3**

$\beta$ -galactosidase visualization by immunohistochemical studies  $\beta$ -gal expression in liver and spleen sections from non-human primates (*Microcebus murinus*) was analyzed by immunohistochemistry after the intravascular administration of  $1 \times 10^{11}$  vp of Ad5CMVlacZ and FX-binding ablated Ad5CMVlacZ-HVR5\*7\*E451Q at low magnification (40X).

