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Abstract:	Recent studies have generated interest into the function of human adenovirus serotype 5 (HAd-V5) hexon: factor X (FX)-binding and subsequent hepatocyte transduction and interaction with the immune system. Here, we retargeted adenovirus serotype 5 vectors, ablated for FX interaction, by replacing amino acids in hexon HVR7 with RGD-4C or inserting the peptide into the fiber HI loop. These genetic modifications in the capsid were compatible with virus assembly, and could efficiently retarget transduction of the vector via the αvβ3/5 integrin-mediated pathway, but did not alter immune recognition by pre-existing human neutralising anti-HAdV-5 antibodies or by natural antibodies in mouse serum. Thus, FX-binding ablated HAdV-5 can be retargeted but remain sensitive to immune-mediated attack. These findings further refine HAd-V5 based vectors for human gene therapy and inform future vector development.

1 ***Retargeting FX binding-ablated HAdV-5 to vascular cells by inclusion of the RGD-4C***
2 ***peptide in hexon hypervariable region 7 and the HI loop***
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5 Running title: *Retargeting FX-ablated HAdV-5 by RGD-4C peptide insertions*
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43 **Abstract**

44

45 Recent studies have generated interest into the function of human adenovirus serotype 5

46 (HAd-V5) hexon: factor X (FX)-binding and subsequent hepatocyte transduction and

47 interaction with the immune system. Here, we retargeted adenovirus serotype 5 vectors,

48 ablated for FX interaction, by replacing amino acids in hexon HVR7 with RGD-4C or

49 inserting the peptide into the fiber HI loop. These genetic modifications in the capsid were

50 compatible with virus assembly, and could efficiently retarget transduction of the vector via

51 the $\alpha v\beta 3/5$ integrin-mediated pathway, but did not alter immune recognition by pre-existing

52 human neutralising anti-HAdV-5 antibodies or by natural antibodies in mouse serum. Thus,

53 FX-binding ablated HAdV-5 can be retargeted but remain sensitive to immune-mediated

54 attack. These findings further refine HAd-V5 based vectors for human gene therapy and

55 inform future vector development.

56

57　Adenoviral (Ad)-based vectors, particularly those based on human adenovirus serotype 5

58　(HAdV-5) are widely used clinically and experimentally. *In vitro,* and in local *in vivo*

59　applications, HAdV-5 transduces cells via the human coxsackie and adenovirus receptor

60　(hCAR) (Bergelson et al., 1997, Tomko et al., 1997). Our research has focussed on the use of

61　adenoviral vectors as a tool for *ex vivo* manipulation of coronary artery bypass material to

62　overexpress anti-proliferative genes (e.g. TIMP-3, p53) in coronary artery vascular smooth

63　muscle cells (VSMCs), to prevent their migration, proliferation and formation of a neointimal

64　lesion and ultimately graft reocclusion and failure following grafting (George et al., 2011). A

65　significant limitation in this strategy is that VSMCs express a very low level of CAR and are

66　thus refractory to transduction (Parker et al., 2013), necessitating high input titers of HAdV-5

67　to achieve therapeutic levels of transgene expression. For systemic *in vivo* applications,

68　HAdV-5 efficiently and selectively transduces hepatocytes (Huard et al., 1995) in a process

69　mediated though the engagement of the blood coagulation factor X (FX) with the

70　hypervariable regions (HVR) of the HAdV-5 hexon protein (Hofherr et al., 2008, Kalyuzhniy

71　et al., 2008, Waddington et al., 2008). Through selective modification of the HVRs, we

72　previously generated a vector (HAdV-5T*) devoid of FX interactions and consequently

73　hepatocyte transduction by introducing point mutations in key FX-interacting amino acids of

74　the hexon protein HVR, namely T270P and E271G (HVR5) and I421G, T423N, E454S,

75　L426Y and E451Q (all in HVR7) (Alba et al., 2009). Conversely, it has been reported that

76　FX may actually offer a protective role in gene delivery, by shielding HAdV-5 from immune

77　mediated attack by natural IgM and the classical complement system (Xu et al., 2013) that

78　interact with HVRs, neutralising the virus (Ma et al., 2015). The specific amino acids

79　responsible for immune recognition remain unknown, and the impact in humans remains

80　unconfirmed. Incorporation of FX binding HVRs from HAdV-5 into HAdV-26 (non-FX

81　binding Ad serotype) instilled liver transduction to this vector (Ma et al., 2015), reiterating

82　the importance of FX in determining viral hepatic transduction. Therefore, retargeting of FX-

83　ablated virus remains an area of interest for improving safety and efficacy of gene therapy

84　vectors.

85

86　In this study, we evaluate optimal locations compatible for inserting targeting peptides within

87　the HAdV-5T* vector. Previous studies identified the fiber HI loop as viable for peptide

88　incorporation for retargeting *in vitro* and *in vivo* (Krasnykh et al., 1998, Dmitriev et al., 1998,

89　Reynolds et al., 1999). Furthermore, the incorporation of RGD into HAdV-5 HVR5 was

90　shown to result in increased transduction in non-permissive VSMC using a non-modified

91   HAdV-5 vector with high background hepatocyte transduction (Vigne et al., 1999). Here,

92   three locations were selected for peptide incorporation: fiber HI loop (after amino acid

93   543G), and hexon HVRs 5 and 7 (Figs 1a - 1d). We selected the RGD-4C (CDCRGDCFC)

94   peptide to test retargeting because it efficiently binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expressed on

95   many cell types, including endothelial cells (Zitzmann et al., 2002). This peptide has

96   previously been widely used for adenoviral retargeting studies (Pasqualini et al., 1997,

97   Dmitriev et al., 1998). The RGD-4C peptide was inserted into the HVRs with (designated

98   "R" for replacement) or without (designated "I" for insertion) replacement of amino acids

99   upstream of amino acid 272C (in HVR5) or 432K (in HVR7) whilst replacements involved

100   removal of amino acids 272A–280L or 427T–435Q with simultaneous insertion of the

101   peptide (Figs. 1c and 1d).

102

103   RGD-4C was cloned in modified shuttle plasmids containing the T* modified sequence (Fig.

104   1b) (Alba et al., 2009) or fiber HI loop (Fig. 1d) (Alba et al., 2010). Vectors were linearised

105   and electroporated into BJ5183 bacteria cells with digested pAd5CMVlacZ for homologous

106   recombination. Adenoviral production was performed in HEK293 cells as described

107   previously (Alba et al., 2009). Peptide insertion in HAdV-5T*HVR7I proved incompatible

108   with virus assembly, suggesting limitations for peptide insertion within this locale. Virus

109   generation could be achieved for HAdV-5T*HVR5I, however titer assessment indicated very

110   poor virus particle: plaque forming unit (vp: PFU) ratios (Fig. 1e), again suggesting simple

111   insertion strategies within the HVRs appear to limit viral fitness, and this virus was therefore

112   excluded from subsequent analysis. All other viruses were successfully propagated, verified

113   by sequencing and quality control demonstrated consistent, high quality virus batches, as

114   assessed by silver staining, BCA assay, nanoparticle tracking analysis (NanoSight LM10,

115   Malvern) and plaque forming unit (pfu) assays (Figs. 1e and 1f), thus demonstrating amino

116   acid removal from HVR5 and HVR7 are non-essential for virus assembly.

117

118   Recombinant viruses were evaluated for cell binding and transduction in α_v integrin-positive

119   SKOV3 cells and A549 cells (Guo et al., 2009, Cannistra et al., 1995). Cells were transduced

120   with HAdV-5, HAdV-5T* and peptide-modified adenoviral vectors at 50 pfu/cell, and

121   transduction was assessed 48 hours post-transduction after a 3-hour exposure to each Ad by

122   measuring β-galactosidase activity using the Tropix Galacton Plus and Tropix accelerator II

123   kit (Applied Biosystems). Replacement of amino acids in HVR5 with RGD-4C or its

124   insertion in the HI loop failed to increase cell transduction compared to HAdV-5 or HAdV-

4

125   5T*. However, replacing amino acids in HVR7 (HAdV-5T*HVR7R) with RGD4C in the

126   HAdV-5T* background exhibited >10-fold increase in transduction compared to the parental

127   HAdV-5T* vector (Figure 2a and 2b), validating HVR7 as a candidate site for targeting

128   peptide insertion. Strategies to improve vascular tropism for effective gene delivery in

129   coronary artery bypass graft purposes need to efficiently target VSMC , therefore we tested

130   viral transduction in low passage (passage 2-5) human saphenous vein (HSV) primary

131   VSMC, isolated as previously described (Southgate and Newby, 1990). Using the RGD

132   targeted vectors (500 pfu/cell), we observed a robust 6-fold increase in transduction in HSV

133   VSMC transduced with HAdV-5T*HVR7R or HAdV-5T*HI loop compared to HAdV-5 or

134   HAdV-5T*, but not HAdV-5T*HVR5R (Fig. 2c). This contrasts with previous studies

135   demonstrating insertion of RGD in HVR5 in an otherwise wild type HAdV-5 capsid, which

136   did increase VSMC transduction (Vigne et al., 1999). This discrepancy could relate to the

137   different RGD peptide sequence used in each study, our peptide contained 4 cysteine

138   (CDCRGDCFC) in contrast to only two in the previous study (DCRGDCF) (Vigne et al.,

139   1999). Additionally, these differences may relate to conformation alterations introduced by

140   the specific combination of the FX-binding ablating mutations engineered in HVR5

141   combined with the RGD peptide incorporation.

142

143   Surface binding analysis was performed by incubating the recombinant vectors with the cells

144   at 4°C for 1 hour. DNA was then isolated using QIAamp DNA Mini Kit (QIAGEN) and cell-

145   membrane associated viral genomes were quantified by quantitative PCR as described

146   previously (Alba et al., 2009). No change in binding was observed for HAdV-5T*HVR5R in

147   any cell type, compared to HAdV-5T* (Figs. 2d, 2e, 2f), confirming that insertion of peptides

148   within HVR5 confers little re-targeting benefit, whilst significant increases in cell association

149   were observed for HAdV-5T*HVR7R (Figs. 2d and 2f). Increased binding to A549 cells was

150   observed following peptide insertion in the fiber HI loop, although this did not correlate with

151   increased transgene expression (Figs. 2b and 2e). In HSV VSMC, no increase in binding was

152   observed for HAdV-5T*HI loop, however transduction was increased (Fig. 2f). This

153   inconsistency could be due to differences in the ability of the virus to internalise and traffic

154   through different cellular compartments following uptake. The discrepancy is observed

155   across all of the cells type tested (SKOV3, A549 and HSV VSMC) indicating that the

156   insertion of RGD-4C in the fiber HI loop may influence the surrounding capsid structure and

157   hence effect trafficking to the nucleus. Further studies are required to fully delineate this

158   finding.

5

159

160　FX binding HAdV-5 has been shown to prevent IgM and complement-mediated

161　neutralization of the virus *in vivo* (Xu et al., 2013), with inhibitory serum components

162　binding sites within the HAdV-5 HVRs (1-3 and 5-7) (Ma et al., 2015). We tested if this

163　response was altered by the insertion of the RGD-4C peptide within these regions by

164　investigating the sensitivity of the vectors to murine serum *in vitro* using a snake venom-

165　derived factor X binding protein (X-bp) which binds to the Gla domain of FX preventing its

166　interaction with Ad hexon (Waddington et al., 2008, Atoda et al., 1998). Recombinant Ad

167　vectors (2×10^{10} vp/mL) were incubated with RPMI-1640 media, 90% C57BL/6 mouse serum

168　or 90% C57BL/6 mouse serum pre-incubated with 40 µg/mL X-bp, for 30 min at 37°C. Ad

169　vector suspensions were diluted 200-fold in serum-free media and 100 µL added to SKOV3

170　cells for 2 hours at 37°C before being replaced with RPMI-1640 media with 2% FCS.

171　Transgene expression was quantified 16 hours post-transduction as relative light units (RLU)

172　normalized to total protein. HAdV-5 mediated transduction significantly increased in the

173　presence of serum, and reduced following pre-incubation of serum with X-bp (to bind and

174　neutralize FX) (Waddington et al., 2008, Mizuno et al., 2001). HAdV-5T* demonstrated

175　reduced transduction in the presence of murine serum compared to media alone (Fig. 3a).

176　Peptide insertion failed to prevent virus neutralization and reduction in transduction,

177　indicating that these sites are not critical to natural antibody mediated binding and

178　neutralisation (Fig. 3a). To evaluate what effect, if any, peptide insertion might have on

179　evasion of pre-existing human anti-HAdV-5 immunity, we performed neutralisation assays

180　on HepG2 cells transduction following incubation with 1,000 vp/cell of Ad vectors in the

181　presence of 1 IU/mL of FX and in 2.5% serum isolated from 103 cardiovascular patients

182　(Parker et al., 2009). Reporter gene expression was quantified 48 hours post-transduction, and

183　the changes in transduction relative to vector in the absence of serum was assessed (Fig. 3b).

184　Incorporation of RGD-4C peptide into hexon or fiber had no discernible effect on evasion of

185　pre-existing immunity, with 39.6% evasion observed at the 90% neutralisation level for both

186　HAdV-5T*HVR7R and HAdV-5T*HI loop compared with 35.9% for the parental HAdV-

187　5T* (Fig. 3b).

188

189　This study successfully retargeted a FX-ablated HAdV-5 vector to human smooth muscle

190　cells and demonstrates that incorporation of the RGD-4C targeting peptide does not affect

191　neutralisation by natural antibodies in murine sera or recognition by pre-existing anti-HAdV-

192　5 immunity in the general population. Whilst ablation of FX interactions increases

6

193   neutralisation of Ad via IgM and complement, this has only been demonstrated in murine

194   models to date (Xu et al., 2013). It remains unclear whether this is replicated in humans and

195   this will be important to determine in the future. For intravascular delivery applications in

196   humans, optimised retargeting strategies, including those based upon FX-binding ablated Ad

197   vectors described herein, will be of key importance. Further studies will be required to

198   evaluate these vectors in *ex vivo* human vein culture model (Soyombo et al., 1990) and

199   suitable *in vivo* animal models to determine confirm the ability of these modified viruses to

200   target to vasculature using clinically relevant model systems.

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295

296

FIGURE LEGENDS

298

299 **Figure 1. RGD-4C peptide placement in HAdV-5T* and quality control analysis of**

300 **purified viruses.**

301 (a) Hexon trimer protein, arrows indicate RGD-4C insertion sites in HVR5 (orange) or

302 HVR7R (blue). (b) HAdV-5 hexon amino acid sequence; HVR5 and HVR7 are highlighted in

303 grey; T* point mutations are marked with asterisk (*). The amino acids removed for

304 generation of RGD-4C replacement vectors are designated by dashed line and RGD-4C

305 insertion point is indicated by black arrow. (c) Fiber protein, arrows indicate RGD-4C

306 insertion sites in HI loop (purple). (d) HAdV-5 fiber amino acid sequence; arrow indicated

307 insertion site of RGD-4C. (e) Details of vector production, indicating preparation in HEK293

308 cells and infectious titres measured by end-point dilution infection in HEK293 cells, physical

309 titers quantified by microBCA and nanoparticle tracking analysis (Nanosight) and the

310 respective VP: pfu ratios for each virus. (f) Analysis of viral proteins by SDS-PAGE and

311 silver staining.

312

313 **Figure 2. Assessment of HAdV-5T* retargeting by cell surface binding and viral**

314 **transduction in three cell types.**

315 Cells were transduced with HAdV-5, HAdV-5T* and peptide-modified adenoviral vectors

316 (HAdV-5T*HVR5R, HAdV-5T*HVR7R and HAdV-5T*HI loop) at a concentration of 50

317 pfu/cell for SKOV3 and A549 cells and 500 pfu/cell for human saphenous vein vascular

318 smooth muscle cells (HSV VSMC). (a - c) Transduction was assessed 48 hours post-

319 transduction after a 3-hour exposure to each HAdV by measuring β-galactosidase activity. (d

320 - f) Surface binding analysis was performed by incubating the HAdV with the cells at 4°C for

321 1 hour and cell-bound adenoviral vectors were quantified by measuring adenoviral genomes

322 by quantitative PCR as described in (Alba et al., 2009). Each experiment was performed in

323 technical triplicate and repeated 3 independent times. Mean ± Standard Deviation One-way

324 ANOVA with Bonferroni post-hoc analysis performed using Graphpad Prism v.5; ***

325 $p < 0.001$.

326

327 **Figure 3. Evaluation of the effect of peptide insertion on evasion of neutralising anti-**

328 **HAdV-5 immunity.**

329 (a) HAdV-5T* and its derivatives ($2x10^{10}$ vp/mL) were incubated with RPMI-1640 media,

330 90% C57BL/6 mouse serum or 90% C57BL/6 mouse serum preincubated with 40 µg/mL X-

331 bp, for 30 min at 37°C. Virus suspensions were diluted 200-fold in serum-free media and 100

332 μL added to SKOV3 cells for 2 h at 37°C before being replaced with RPMI-1640 media with

333 2% FCS. Transgene expression was quantified 16 h post-transduction as relative light units

334 (RLU) normalized to total protein. Transduction expressed as a percentage of control

335 (HAdV-5 transduction with serum free media alone); each experiment was performed in

336 technical quadruplicate and repeated two independent times. Mean ± SEM * $p<0.05$. (b)

337 Effect of neutralizing sera on HepG2 cells transduction following incubation with 1,000

338 vp/cell of HAdV vectors in the presence of 1 IU/mL of FX and 2.5% sera from patients

339 previously screened for anti-HAdV-5 Nabs (Parker et al., 2009) and stained for β-Gal

340 expression 48 h post-transduction. Experiment was performed four times, data presented are

341 mean ± SEM.

Figure 1

a

HVR7

HVR5

b

HVR5I – *CDCRGDCFC*

**

QFFSTTEAAAGNGDNLTPKVVYSEDVDIETPDTHISYMPTIKEGNSRELMGQQSMPNRPNYIAFR

HVR5R - *CDCRGDCFC*

DNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSY

HVR7I - *CDCRGDCFC*

* ** *   *

DPVRIIENHGTEDELPNYCFPLGGVINTETLTKVKPKTGQENGWEKDATEFSDKNERVGNNFAME

HVR7R - *CDCRGDCFC*

c

HI LOOP

d

HILOOP Insertion - *CDCRGDCFC*

KSHGKTAKSNIVSQVYLNGDKTKPVTLTITLNGTQETGWTQLQVHTLCHFHGTGLATTTLM

e

Adenovirus	Infectious titre (pfu/mL)	microBCA		NanoSight	
		Physical titre (VP/mL)	Ratio (VP/pfu)	Physical titre (VP/mL)	Ratio (VP/pfu)
HAdV-5T*	1.11×10^{11}	4.00×10^{12}	36.60	3.12×10^{12}	14.18
HAdV-5T*HVR5I	7.00×10^{8}	1.03×10^{12}	1471.42	1.11×10^{12}	1585.71
HAdV-5T*HVR5R	1.24×10^{11}	1.78×10^{12}	14.30	2.10×10^{12}	16.93
HAdV-5T*HVR7R	3.25×10^{10}	1.89×10^{12}	58.17	2.24×10^{12}	68.92
HAdV-5T*HILoop	1.76×10^{11}	2.60×10^{12}	14.77	2.79×10^{12}	15.85

f

HAdV-5T*
HAdV-5T*HVR5R
HAdV-5T*HVR7R
HAdV-5T*HILoop

kD
160 —
105 —
75 —
50 —
35 —
30 —
25 —
15 —

— II (Hexon)
— III (Penton)
— IIIa/IV (Fiber)
— V
— VI
— VII

Figure 2

Figure 2

Figure 3　　　　　　　　　　　　　　　　　Click here to download Figure Figure 3 JGV revised.pdf ⬇

A

B