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# Retargeted and detargeted adenovirus for gene delivery to the muscle

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ABSTRACT

We previously selected muscle binding peptides 12.51 and 12.52 from "context-specific" phage display libraries for introduction into adenovirus (Ad) vectors. In this work, these peptides were inserted into the hypervariable region (HVR) 5 loop of the Ad5 hexon protein to display 720 peptides per virions. HVR-12.51 and 12.52 increased transduction of C2C12 cells up to 20-fold when compared to unmodified Ad5. 12.51 increased *in vivo* muscle transduction 2 to 7-fold over unmodified Ad after intramuscular injection in mice and hamsters. 12.52 did not increase muscle transduction. Notably, insertion of 12.51 into the hexon reduced liver transduction 80-fold when compared to unmodified Ad5 after intravenous injection. Increased muscle transduction in mice translated into increased immune responses after gene-based vaccination. These data suggest there are merits to retargeting and detargeting benefits to modifying the hexons of Ads with peptide ligands.

## 1. Introduction

Adenoviruses are robust vectors for gene delivery and gene-based immunization (reviewed in (Barry et al., 2012; Lasaro and Ertl, 2009)). The archetype adenovirus used for the vast majority of these application has been human species C adenovirus serotype 5 (HAdV-C5 or Ad5). *In vitro,* Ad5 binds and enters cells through the combined interactions of its fiber and penton base proteins with cell surface receptors. The trimeric fiber binds the coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997). Cells that lack CAR are relatively resistant to infection unless they also express $\alpha_v$ integrins that can be bound by an RGD motif on the penton base (Huang et al., 1996; Wickham et al., 1993).

*In vivo,* these interactions are still utilized, but their importance varies by injection route. If injected directly into a solid tissue or tumor, CAR and integrin interactions dominate. If injected intravenously (IV), these interactions become secondary due to the effects of Ad5 binding to vitamin-K-dependent blood clotting factors (Baker et al., 2007; Kalyuzhniy et al., 2008; Waddington et al., 2008). Blood factor X (FX) binds with subnanomolar affinity to the hexons of Ad5 (Kalyuzhniy et al., 2008; Vigant et al., 2008; Waddington et al., 2008) and, consequently, enables Ad5 to efficiently transduce liver hepatocytes after IV injection. In the absence of FX, liver transduction is drastically reduced. It was originally thought that FX binding cross-linked the virus to heparin sulfate proteoglycans on hepatocytes to enhance transduction (Baker et al., 2007). Other data suggest that FX protects the Ad5 virion from being bound by natural antibodies and complement that will target the virus for destruction by liver Kupffer cells and other macrophages (Qiu et al., 2015; Xu et al., 2013).

Adenoviral vectors are somewhat unique in their ability to carry very large cDNA sequences of up to 36 kilobase pairs (kbp) when compared to other vectors like adeno-associated virus (AAV) vectors with only 4.5 kb of DNA sequence. This payload capacity justified early exploration of Ad vectors for muscle gene therapy when delivering very large transgenes like the 14 kbp dystrophin cDNA (Bouri et al., 1999; Clemens et al., 1996; Feero et al., 1997; Gilchrist et al., 2002; Jiang et al., 2001; Kochanek et al., 1996; Liu et al., 2001; Mitani et al., 1995). IV administration in newborn mice can mediate muscle gene delivery, but this ability is lost in adult mice (Ascadi et al., 1994; Ragot, 1993). The decreased transfection with age is due in part to the very large size of Ad virions (*i.e.* 100 nm) as well as the loss of CAR receptor on muscle cells with aging (Cao et al., 2001; Sewry and Partridge, 1996). The intramuscular (IM) route is by far the most popular route for gene-based vaccines when using Ad5 and other serotypes (Lasaro and Ertl, 2009) despite the fact that CAR is absent on skeletal muscle cells.

Therefore, Ad5 and other Ad serotype transduction of muscle can be

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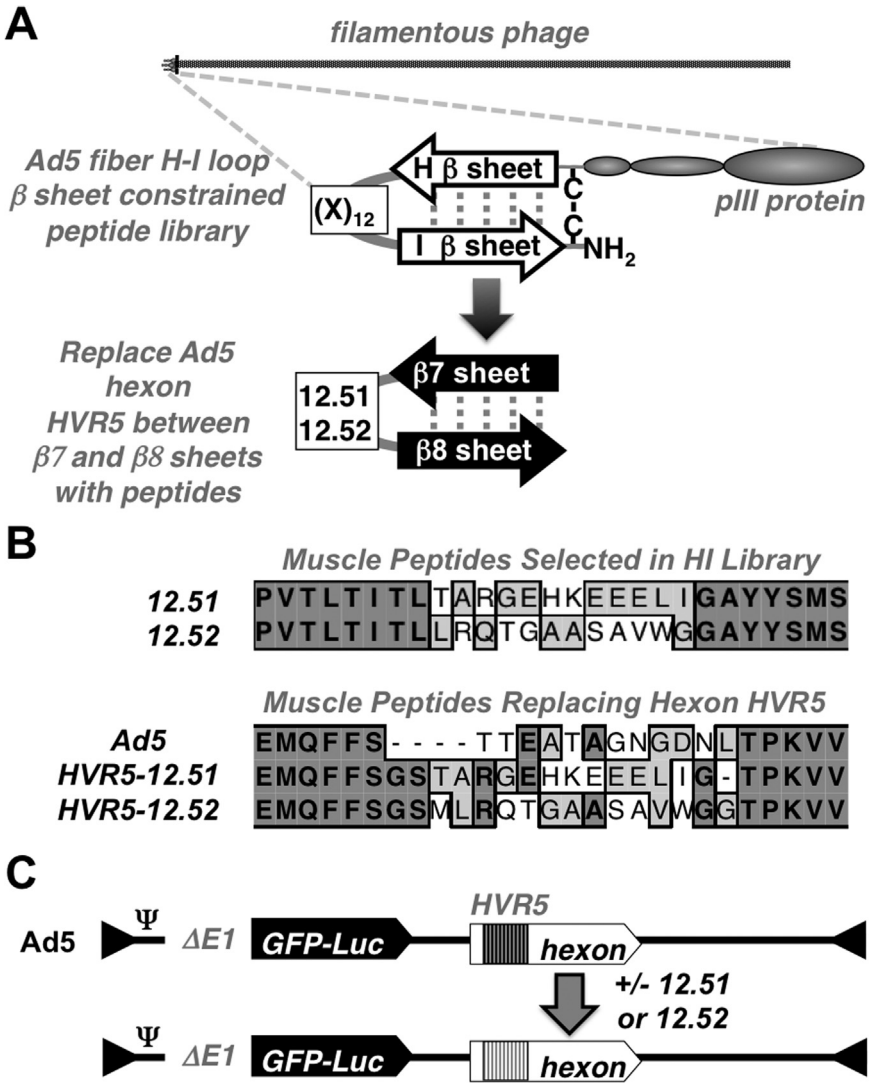


**Fig. 1.** Translation of context-specific peptides from phage to adenovirus. A) Diagram of a phage display library containing the Ad5 fiber HI β sheets that structurally constrain a random 12-mer peptide library. Shown below is a depiction of the structurally similar site between the β7 and β8 sheets in the Ad5 HVR5 hexon. B) Primary amino acid alignments of 12.51 and 12.52 in the HI library and their location when inserted into HVR5 of hexon. C) Representation of Ad5 GFP-Luc expressing viruses modified with the peptides.

adequate for gene therapy or gene-based vaccination. However, the absence of the virus' primary receptor in the muscle reduces the efficacy of the virus and requires more vector to be delivered to achieve desired effects. Given this, groups have worked to improve Ad vector transduction by introducing promiscuous or cell-specific ligands into viral proteins (reviewed in (Campos and Barry, 2007; Parrott et al., 2003a)). Proof of principle began by modifying the Ad fiber with non-targeting ligands (Michael et al., 1995) or promiscuous ligands like polylysine (Bouri et al., 1999; Wickham et al., 1997) or RGD motifs on fiber or in hexon (Bilbao et al., 2003; Martin et al., 2003; Vigne et al., 1999). These would be considered "gain of function" of viruses, since the ligands are not cell specific. In other applications, putative cell-specific ligands have been introduced to render Ads moderately to completely cell specific (Reynolds et al., 2000).

In many cases, there are no ligands available to target certain cells. To supply these for Ad and other vectors, we originally used peptide-presenting phage libraries to select cell binding peptides (Barry et al., 1996) and reviewed in Barry et al. (2002). Another more recent solution is to generate peptide libraries in Ad itself (Wu et al., 2010; Yamamoto et al., 2014). While these Ad library approaches have great merit in directly producing usable vectors, peptide library sizes in Ads are orders of magnitude smaller than peptide libraries generated in bacterial phage libraries and this may potentially limit the repertoire of available targeting ligands that can be screened (reviewed in (Barry et al., 2002)).

Although phage libraries have more diverse peptide libraries, two problems can occur when inserting phage-discovered ligands into viral capsid proteins: 1) inserting the ligand into the Ad protein can disable the protein function or 2) the ligand may fail to target when translated into the heterologous structure of the virus. These “context” problems are fundamental, since an ideal candidate peptide ligand may be identified, but cannot be applied because either the ligand or vector is disabled when combined.

To circumvent this "context" problem for muscle targeting, we engineered “context-specific” phage libraries by introducing the H and I β sheets of the Ad5 fiber on to the pIII protein of filamentous bacteriophage (Ghosh and Barry, 2005). A 12 amino acid (12-mer) random peptide library was inserted in place of the HI loop between the sheets which were constrained by disulfide bonds. This library was selected against mouse C2C12 myoblasts with pre-clearing against non-target cells (Ghosh and Barry, 2005). We selected peptides 12.51 and 12.52. The more prevalent peptide 12.51 was shown to bind better to muscle cells than benchmark integrin-binding RGD peptide. When 12.51 was translated back into the knob domain of Ad5, the peptide increased *in vitro* transduction 14-fold on C2C12 myoblasts and 2-fold on differentiated C2C12 myotubes.

In this work, we explored if inserting these muscle-selected peptides between two other β sheets that also constrains a hypervariable loop on the virus, could modulate tropism. Peptides 12.51 and 12.52 were introduced into the hypervariable region (HVR) 5 loop constrained by the