Dose-dependent Toxicity of Humanized Renilla reniformis GFP (hrGFP) Limits Its Utility as a Reporter Gene in Mouse Muscle

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Methods: Original Article

Gene therapy has historically focused on delivering protein-coding genes to target cells or tissues using a variety of vectors. In recent years, the field has expanded to include gene-silencing strategies involving delivery of noncoding inhibitory RNAs, such as short hairpin RNAs or microRNAs (miRNAs). Often called RNA interference (RNAi) triggers, these small inhibitory RNAs are difficult or impossible to visualize in living cells or tissues. To circumvent this detection problem and ensure efficient delivery in preclinical studies, vectors can be engineered to coexpress a fluorescent reporter gene to serve as a marker of transduction. In this study, we set out to optimize adeno-associated viral (AAV) vectors capable of delivering engineered miRNAs and green fluorescent protein (GFP) reporter genes to skeletal muscle. Although the more broadly utilized enhanced GFP (eGFP) gene derived from the jellyfish, Aequorea victoria was a conventional choice, we were concerned about some previous studies suggesting this protein was myotoxic. We thus opted to test vectors carrying the humanized Renilla reniformis-derived GFP (hrGFP) gene, which has not seen as extensive usage as eGFP but was purported to be a safer and less cytotoxic alternative. Employing AAV6 vector dosages typically used in preclinical gene transfer studies (3×10^10 –1 × 10^11 particles), we found that hrGFP caused dose-dependent myopathy when delivered to wild-type (wt) mouse muscle, whereas identical titers of AAV6 carrying eGFP were relatively benign. Dose de-escalation at or below 8 × 10^9 AAV particles effectively reduced or eliminated hrGFP-associated myotoxicity, but also had dampening effects on green fluorescence and miRNA-mediated gene silencing in whole muscles. We conclude that hrGFP is impractical for use as a transduction marker in preclinical, AAV-based RNA interference therapy studies where adult mouse muscle is the target organ. Moreover, our data support that eGFP is superior to hrGFP as a reporter gene in mouse muscle. These results may impact the design of future preclinical gene therapy studies targeting muscles and non-muscle tissues alike.

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Subject Category: Methods section

Introduction

We are developing RNA interference-based gene therapies as prospective treatments for neuromuscular diseases with dominant phenotypes.1–4 Our strategy typically involves delivering engineered microRNA (miRNA) expression cassettes (miRNA shuttles) to mouse muscle using myotropic adenoviral (AAV) vectors, such as AAV6.1–5 The resultant miRNA products are difficult to visualize in processed tissue, and impossible to detect in living animals. Thus, to circumvent this detection problem and permit indirect monitoring of vector transduction and miRNA expression in live animals, we also include separate green fluorescent protein (GFP) reporter genes in our vectors.2,3,6

GFP, originally discovered in the jellyfish, Aequorea victoria, has become one of the most important tools in modern biology.7–9 Indeed, the 2008 Nobel Prize in Chemistry was awarded to Shimomura, Chalfie, and Tsien for their discovery and development of GFP as a biological reporter gene.10–12 Over the years, numerous variants of the wild-type GFP (wtGFP) protein were created to improve stability and brightness, and optimize expression in mammalian cells. The most commonly used variant was enhanced GFP (eGFP), which was codon-optimized for mammalian cell expression (humanized), and engineered with a serine-65 to threonine mutation that made it 35 times brighter than the wtGFP protein.8

The utility of eGFP as a biological reporter spurred the development of alternative fluorescent proteins from other organisms, including the sea pansy, Renilla reniformis.13,14 A humanized form of Renilla reniformis GFP (hrGFP), introduced to market several years ago, has been used as a fluorescent marker in several animal studies, including those involving vector-mediated gene transfer.2,14–21 Because some reports suggested Aequorea-derived eGFP could be toxic in striated muscle, and Renilla hrGFP was billed as a potentially safer alternative for which no obvious toxicity was previously noted, we used hrGFP as a reporter in our first generation AAV6 miRNA shuttle vectors.2,13,15,22–26 In our original proof-of-concept study using this vector system, we used constitutively active promoters (U6 and cytomegalovirus (CMV))
to co-deliver therapeutic or control miRNAs and hrGFP to muscles of newborn mice. We found no overt evidence of vector toxicity in diseased or wt mouse muscles 4 months after injection of 1-day-old mice. Although this initial work focused on prevention of muscular dystrophy in neonatal animals, we were also interested in reversing pre-existing pathologies in adult animals. To do this, we first tested our delivery strategy using an AAV6 vector carrying only the hrGFP expression cassette (CMV.hrGFP). Upon delivery to adult animals, we were surprised to find that hrGFP caused severe dose- and time-dependent toxicity in wt adult mouse muscles, whereas identical doses of CMV.eGFP vectors were benign by comparison. Lowering the vector load reduced or prevented hrGFP-associated myopathy, but subtoxic levels of hrGFP vectors coexpressing therapeutic inhibitory RNAs were incapable of effectively silencing a disease gene target. Our results have important implications for future preclinical muscle gene delivery studies using GFP reporter genes.

Results

The initial intent of this work was to optimize AAV6 delivery to adult mouse muscle in our laboratory, with the ultimate goal of expressing therapeutic inhibitory RNAs. We began by injecting 1 × 10¹¹ AAV6 particles (“high dose”) carrying a CMV.hrGFP reporter cassette into tibialis anterior (TA) muscles of 6 weeks old wt C57BL/6 mice (Figure 1a). We observed robust expression by 1 week as indicated by hrGFP epifluorescence in whole muscles (Figure 1a). Upon closer histological examination, we were surprised to find massive inflammatory lesions 2 weeks after injection, indicating vector toxicity (Figure 1b). We ruled out endotoxin contamination as the source of this toxicity, as endotoxin levels were low (<0.85 endotoxin units (EU)/ml, Table 1). We therefore hypothesized that the hrGFP protein was the source of the observed muscle lesions. To test this, we compared histological sections of TA muscles injected with identical titers of AAV6.CMV.eGFP and AAV6.CMV.hrGFP vectors, 1, 2, and 4 weeks after vector delivery. At 1 week, comparable levels of green fluorescence were present in whole muscles and neither vector showed any histological indications of toxicity (Figure 1a–c). However, 2 and 4 weeks after injection, muscles expressing eGFP had markedly reduced or nearly absent inflammation compared with hrGFP-injected counterparts, despite significantly higher fluorescence in eGFP-treated muscles at both timepoints (Figure 1a). In contrast to the massive lesions associated with AAV6.CMV.hrGFP injection, muscles receiving identical titers of AAV6.CMV.

Figure 1 Humanized Renilla reniformis green fluorescent protein (hrGFP) is toxic to adult mouse muscle. (a) Transduction of tibialis anterior (TA) muscles with AAV6 vectors carrying GFP reporters. Top, schematic of AAV proviruses containing enhanced GFP (eGFP) and hrGFP. Bottom, low power photographs under fluorescent excitation showing typical transduction of 1 × 10¹¹ DNase-resistant particle (DRP) eGFP and hrGFP AAV6 vectors, 1, 2, and 4 weeks after injection with quantification of fluorescence units using Bioquant software. Fluorescence intensity was significantly higher in eGFP mice, 2 and 4 weeks following injection (t-test, P < 0.001; N = 4 legs per virus). (b) Low-power photomicrographs of hematoxylin and eosin stained mouse TA muscles injected with 1 × 10¹¹ DRP of indicated vectors, 2 weeks prior. Muscles expressing AAV6.CMV.hrGFP developed widespread inflammatory lesions, whereas those containing comparable eGFP vectors did not. (c) Time course shows regeneration (as indicated by myofibers containing central nuclei) in mouse muscles injected with 1 × 10¹¹ DRP AAV6.CMV.hrGFP by 4 weeks. In contrast, muscles expressing identical doses of AAV6.CMV.eGFP were relatively normal at 4 weeks, by comparison, although focal inflammation, occasional central nuclei, and histological indications of myonecrosis were evident in some areas (top right panel). (d) hrGFP persisted in some regenerated myofibers 4 weeks post-injection. White arrows indicate examples of GFP-negative, centrally nucleated myofibers; yellow arrows indicate examples of GFP-positive, centrally nucleated myofibers; asterisk centers a cluster of GFP-positive myofibers with nuclei at the normal peripheral location. AAV, adeno-associated virus; CMV, cytomegalovirus; ITR, inverted terminal repeat; PA, polyadenylation signal.
eGFP showed only occasional focal inflammatory infiltrates and some evidence of muscle regeneration (as indicated by presence of myofibers with centrally located nuclei). In comparison, by 4 weeks hrGFP-injected muscles were almost completely regenerated, as evidenced by myofibers with centrally located nuclei throughout the injected muscle. Upon closer histological examination of transduced areas in 4-week cryosections, we found hrGFP-positive myofibers with and without central nuclei (Figure 1d; yellow and white arrows, respectively), and presumably undamaged hrGFP-positive myofibers containing only peripheral myonuclei (Figure 1d; asterisk indicates a cluster). This suggested that some hrGFP expression was tolerable, and we therefore hypothesized that hrGFP-associated toxicity was dose-dependent. To test this, we determined the effect of decreasing AAV6.CMV.hrGFP vector dose on muscle toxicity. Muscles injected with the lowest vector dosage (3 × 10⁹ particles) were histologically normal at 2 and 4 weeks post-injection into C57BL/6 tibialis anterior muscles. 3 × 10⁹ DNAse-resistant particle (DRP) was well tolerated at both time points. Focal lesions were evident by 4 weeks in muscles that received 8 × 10⁹ DRP, and by 2 weeks in those injected with 3 × 10¹⁰ DRP of AAV6.CMV.hrGFP. Muscle regeneration was evident in the 3 × 10¹⁰ group by 4 weeks, as indicated by widespread central nuclei in myofiber clusters. (b) Despite abundant degeneration and regeneration, lesions in muscles injected with 3 × 10¹⁰ DRP AAV6.CMV.hrGFP were less pronounced (indicated by arrow) than in the high-dose group (3 × 10¹¹; Figure 1), despite hrGFP being present throughout the muscle (right panel). AAV, adeno-associated virus; CMV, cytomegalovirus.

**Table 1** AAV6.CMV.eGFP and AAV6.CMV.hrGFP vectors had baseline levels of endotoxin

| Sample                | Concentration (EU/ml) | Concentration x dilution | Mean (EU/ml) ± SD |
|-----------------------|-----------------------|--------------------------|------------------|
| AAV6.CMV.eGFP         | 0.050                 | 0.50                     | 0.85 ± 0.43      |
| 10                    | 0.048                 | 0.48                     |                  |
| 50                    | 0.026                 | 1.32                     |                  |
| 10                    | 0.022                 | 1.10                     |                  |
| 50                    | 0.048                 | 0.48                     |                  |
| AAV6.CMV.hrGFP        | 0.045                 | 0.45                     | 0.51 ± 0.05      |
| 10                    | 0.011                 | 0.55                     |                  |
| 50                    | 0.011                 | 0.55                     |                  |
| 10                    | 0.325                 | 3.25                     |                  |
| AAV6.CMV.eGFP, 5 EU spike control | 0.040                 | 4.04                     | 3.66 ± 0.52      |
| 10                    | 0.063                 | 3.17                     |                  |
| 50                    | 0.083                 | 4.17                     |                  |
| 10                    | 0.345                 | 3.45                     |                  |
| AAV6.CMV.hrGFP, 5 EU spike control | 0.345                 | 3.45                     | 3.81 ± 0.43      |
| 10                    | 0.081                 | 4.04                     |                  |
| 50                    | 0.092                 | 4.31                     |                  |

AAV, adeno-associated virus; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; EU, endotoxin units; hrGFP, humanized Renilla reniformis GFP.
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Figure 3 Subtoxic doses of AAV6.hrGFP vector are impractical for in vivo gene-silencing studies. (a) Dose-dependence of humanized Renilla reniformis green fluorescent protein (hrGFP) expression is evident in whole tibialis anterior muscles injected 2 weeks prior with the indicated particles of AAV6 vectors carrying CMV.hrGFP and a separate U6.miFRG1 cassette. (b) Relative FRG1 expression determined by real-time PCR in FRG1−high animals receiving the indicated vector doses, 2 weeks prior. AAV, adeno-associated virus; CMV, cytomegalovirus; WT, wild-type.

Discussion

Fluorescent reporter genes have numerous uses in biology, including serving as important tools for visualizing vector transduction in gene transfer experiments.2,3,6,15,17,27 The choice of which fluorescent reporter is indicated for an experiment may depend upon a number of factors, including the wavelength of fluorescent light desired, brightness, and photostability of the fluorophore.28 Potential toxicity is another issue, as some fluorescent proteins have proven toxic to various cells and tissues.22,23,25,26,28–30 In this study, we were concerned about some published reports suggesting the prominently used Aequorea eGFP gene could be myopathic, and we therefore developed an AAV6 vector utilizing the hrGFP, based on the hypothesis that it was potentially less deleterious to adult mouse muscles.13,14,22,23,25,26,29 Contrary to expectations, we found that hrGFP caused dose-dependent muscle toxicity. The most severely injured muscles regenerated normally by 4 weeks, but this regeneration did not result in complete clearance of hrGFP-positive myofibers. Indeed, damaged/regenerated muscles still showed widespread and persistent hrGFP expression, although gross hrGFP levels seemed to plateau between 2 and 4 weeks (Figure 1a). In comparison, eGFP expression increased with time (Figure 1a). Within individual myofibers, we found both GFP-positive and GFP-negative fibers containing central nuclei, as well as GFP-positive myofibers containing only peripheral nuclei. These data suggested that some myofibers tolerated hrGFP expression, whereas others were negatively impacted and underwent degeneration and subsequent regeneration. We do not know which transduced myonuclei contributed hrGFP expression to centrally nucleated myofibers. We hypothesize that hrGFP was sourced from transduced myonuclei located at the periphery of mature myofibers that had undergone segmental repair (and thus harbored some central nuclei). It is also possible that our AAV6.CMV.hrGFP vectors transduced satellite cells, which then contributed GFP-expressing myonuclei upon repair of damaged myofibers. However, we note that it is currently uncertain if AAV6 vectors are capable of transducing muscle satellite cells in vivo. Regardless of their source, the fact that hrGFP expression persisted following the initial acute injury and subsequent regeneration is consistent with the observation that muscle cells can tolerate some level of hrGFP expression. Indeed, lower doses of hrGFP vectors were non-toxic by 4 weeks but also failed to show robust gross hrGFP fluorescence (Figures 2 and 3). Although our histological analyses here represent snapshots in time, and we cannot track individual myofiber degeneration/regeneration cycles in vivo, we hypothesize that differences in AAV.CMV.hrGFP transduction accounted for the differential turnover of individual myofibers within an individual muscle. Specifically, since hrGFP elicits dose-dependent myopathic effects (Figure 2), we propose that regenerated myofibers were more highly transduced and expressed hrGFP above a toxic threshold, whereas histologically normal hrGFP-positive myofibers received less vector. Following regeneration (by 4 weeks), we found no obvious histological indications that hrGFP-positive regenerated muscles were undergoing another round of degeneration, thereby suggesting that hrGFP-associated damage was acute and transient in adult animals (Figure 1a,c,d). Indeed, hrGFP intensity in whole muscles plateaued between 2 and 4 weeks (Figure 1a). In a previous study, we used hrGFP as a reporter gene to track AAV6 vectors carrying therapeutic miRNAs targeting the FRG1 gene in FRG1−high transgenic mice.4 In this work, we found sustained hrGFP expression, significant FRG1 gene silencing, and associated improvements in FRG1-associated...
myopathic phenotypes, 4 months after intramuscular delivery to newborn FRG1-high mice. Moreover, we detected no evidence of vector toxicity (indicated by the presence of inflammatory lesions or abundant centrally nucleated myofibers), in 4 months old wt animals injected with hrGFP-expressing vectors as neonates. Thus, hrGFP was a useful reporter in that study. Nevertheless, the FRG1 study differed from the current one in the ages of the mice used, although not the strain background, as FRG1 mice are inbred on the C57BL/6 background. Since immunity in newborn mice may not be as well developed as in adults of the same strain, it is possible that the toxic effects we observed in the current study are at least partially attributable to an immune response against high doses of the hrGFP protein in adult C57BL/6 mice. Alternatively, since we did not examine our newborn-injected animals at intermediate timepoints, if any acute damage did occur without significant loss of vector, we would likely not have detected it 4 months later. Regardless, we do not currently understand the mechanisms by which hrGFP elicits myopathic effects, beyond the dose dependency we reported here. Since the original intent of this study was to optimize vector transduction for miRNA delivery in vivo, exploring the mechanisms of hrGFP toxicity is beyond our current focus.

Compared with hrGFP, high-dose eGFP was largely well tolerated, although we did find some focal inflammation and evidence of muscle damage (Figure 3). Nevertheless, we never observed the massive inflammatory lesions with eGFP that were evident in hrGFP-injected muscles using high AAV6 dosages. Thus, we conclude that our data generally support that eGFP is a safe and practical choice for muscle gene delivery, although an argument could be made that the minor inflammation we observed 4 weeks after high-dose injection was consistent with some previous work showing that eGFP can injure striated muscles and/or stimulate inflammatory immune responses when delivered by certain AAV serotypes.

Based on our data here, it is difficult to draw any concrete conclusions about potential mechanisms underlying eGFP-associated toxicity, if it indeed arises at all. Nevertheless, these mechanistic questions are beyond the scope of this study.

In conclusion, we found that the AAV6.CMV.hrGFP vectors caused dose-dependent myopathy in adult mouse muscles, whereas similar levels of AAV6.CMV.eGFP were well tolerated by comparison. Although both systems can be used as reporter genes in muscle, the requirement to express hrGFP at comparatively lower levels limits its utility for studies in which high amounts of vector transduction are indicated or when expression levels are difficult to control. Thus, because eGFP is safer for use across a broader expression range than hrGFP, the former is a superior choice for muscle gene delivery studies using AAV6 vectors.

Materials and methods

AAV production. The AAV.CMV.hrGFP and AAV.CMV.eGFP vectors were cloned as previously described. Both vectors contained a CMV promoter and SV40 polyadenylation signal flanked by two AAV2 inverted terminal repeats. AAV6 vectors were generated by the Viral Vector Core Facility at The Research Institute at Nationwide Children’s Hospital (TRINCH). Titers were determined by quantitative PCR and reported as DNAse-resistant particles. Endotoxin was measured at two different dilutions for each vector using a kinetic chromogenic LAL assay (Cape Cod Associates, East Falmouth, MA) following manufacturer’s instructions. Data were reported in EU/ml, and normalized to a reference control containing 5 EU/ml of endotoxin, where 5 EU/kg is considered the minimum pyrogenic threshold in preclinical animal studies.

Mouse injections. All animal studies were approved by Institutional Animal Care and Use Committee at TRINCH. Six to eight weeks old C57BL/6 females received a 50 μl intramuscular injection of indicated doses of AAV.CMV.hrGFP or AAV.CMV.eGFP into the TA muscle. In vivo transduction was determined using a fluorescent dissecting microscope (Leica M165FC; W. Nuhsbaum, McHenry, IL), and fluorescence intensity in whole muscle was measured using the Bioquant image analysis software (Bioquant Image Analysis, Nashville, TN).

Histological analysis. TA muscles were dissected from injected mice at 1, 2, and 4 weeks post-injection for histological analysis (n = 4 muscles per group at each timepoint for each dose). Muscles were frozen in OCT using liquid nitrogen-cooled isopentane, and 10 μm cryosections were hematoxylin and eosin stained using previously described methods.

Real-time PCR. Indicated doses of AAV6.hrGFP.miFRG1 vectors, or contralateral saline controls, were injected into the TA muscles of adult FRG1-high mice using previously described methods. Two weeks after injection, muscles were harvested, photographed using identical conditions under a fluorescent dissecting microscope (M165FC; Leica), and cryosectioned at 50 μm for RNA collection (TRI Reagent; Molecular Research Center, Cincinnati, OH). Following random-primed reverse transcription, human FRG1 levels were measured using Taqman assay (Life Technologies, Grand Island, NY) as previously described. Data were normalized to saline-injected animals that received 8 × 10^6 particles in the contralateral leg.

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