nNOS upregulation to improve DMD pathogenesis and knock-in efficiency for restoring full-length dystrophin

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Abstract

Duchenne Muscular Dystrophy (DMD) is the most common X-linked neuromuscular disease, affecting 1 in 3600 boys. Mutations that disrupt the open reading frame of the DMD gene cause the disease by eliminating dystrophin expression in skeletal and cardiac muscles. Without dystrophin, the dystrophin glycoprotein complex (DGC) at the sarcolemma is disrupted, leaving muscles vulnerable to damage during contraction. This increased fragility results in systemic muscle wasting, loss of ambulation, and significantly reduced life expectancy. Exacerbating the severity of DMD is the accompanying reduction in the muscle microvasculature and expression of neuronal nitric oxide synthase (nNOS). A curative gene therapy for DMD deletions is possible now due to the discovery of the genome editing CRISPR/Cas9 tool and homology-mediated enjoy joining (HMEJ) DNA repair pathway. We propose using these systems to correct the DMD patient deletion of exons 52-54 through the simultaneous upregulation of nNOS and knock-in of these deleted exons via Cas9 and HMEJ. This combinatorial system should improve DMD pathogenesis and permanently restore full-length dystrophin. In vitro and in vivo delivery of the gene editing components to a mouse model representative of the patient mutation will utilize AAV9s. Recovery of nNOS functionality should additionally increase muscular blood flow, improving the effectiveness of the AAV9 gene therapy in vivo. The results of this project will demonstrate the efficacy of a gene therapy utilizing a combined CRISPR/Cas9-mediated upregulation and exon knock-in to cure DMD.

Background

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is the most common X-linked recessive neuromuscular disease, affecting 1 in 3600 boys[5]. Individuals experience progressive wasting of skeletal and cardiac muscles throughout the body. DMD results from the loss of functional dystrophin protein within myotubes due to mutations in the DMD gene which disrupt the open reading frame (ORF). The majority of DMD patients (~70%) possess deletion mutations[3]. Dystrophin is localized to the sarcolemma at the myotube periphery, being a major component of the dystrophin glycoprotein complex (DGC) (Fig. 1)[9]. Numerous proteins make up the DGC, linking the extracellular matrix and sarcolemma to the actin cytoskeleton. The DGC confers the necessary structural integrity for myotubes to withstand damage during contraction-induced stress while co-localizing proteins of varied functions[15].

Without dystrophin, the DGC destabilizes, leaving myotubes vulnerable to damage, culminating in cell death. Hallmarks of DMD pathology are centralized nuclei (due to regenerating myotubes), fibrosis, inflammation, heterogenous myotube size, and elevated creatine kinase (CK)[10]. These features are reproduced in the mdx mouse model which contains a premature stop codon in exon 23 of dystrophin[20].

DMD leads to escalating muscle weakness and a complete loss of ambulation around age ten[1]. Cardiorespiratory failure commonly causes death in the early to mid teens[1]. Unfortunately, current medical interventions can only extend life expectancy to roughly 30 years at best; no cure exists.

Impaired microvasculature and sympatholysis in DMD

In DMD afflicted muscles the microvascular network is significantly reduced as well as muscular blood supply (Fig. 2)[18]. DMD patient biopsies also reveal abnormal thickening of the capillary basal lamina, increasing their distance from myotubes[4]. Strikingly, exercise-induced vasodilation is entirely ablated in DMD[21]. Normally, the increased metabolic demand of muscles during exercise triggers local vasodilation to attenuate basal vasocontriction; the process called sympatholysis[21]. Sympatholysis is absent in DMD patients and mdx mice as noted by reduced tissue oxygenation during exercise, decreased capillary perfusion, and increased susceptibility to post-activity fatigue, resulting in functional ischemia and exacerbation of the disease (Fig. 3)[13,21].

The role and impact of nNOS in healthy and dystrophic muscle

Neuronal nitric oxide synthase (nNOS) is part of the DGC, associating with dystrophin via the adapter protein synthrophin (Fig. 1)[9]. Nitric oxide (NO), a short lived, short distance messenger, is generated by nNOS[9]. Within muscle, nNOS has two primary spliced isoforms: nNOSμ and nNOSβ[11].

The majority of muscular nNOS is nNOSμ, functioning primarily in sympatholysis[7,21]. Muscle contraction activates nNOSμ, increasing the production of NO which diffuses into neighbouring capillaries[9]. Here, NO triggers a signal cascade that relaxes smooth muscle and induces vasodilation[9].

The less characterized nNOSβ isoform does not impact sympatholysis[11]. Instead, evidence suggests a more diverse range of functions including: inhibiting inflammation, promoting muscle hypertrophy, resistance to muscle fatigue, and protection from contraction stress[11].

nNOSμ is part of the DGC whereas nNOSβ associates with the Golgi complex[9,11]. DGC loss in DMD causes both isoforms to mislocalize to the cytosol[9,11]. Combined with substantial protein loss, nNOSμ activity and NO signalling are decreased by 80%[22]. The transcript level of nNOSμ is also reduced[6]. Studies using nNOS knockouts and transgenic expression in mdx mice confirm that all primary nNOS functions are impaired in DMD[11,22]. No expression data for nNOSβ in a DMD model exists, but

compensatory upregulation of nNOSβ may occur in response to nNOSμ loss since mdx knockouts for both nNOS isoforms yield a more severe DMD phenotype[11]. If nNOSβ is upregulated in healthy muscle, nNOSμ may carry out functions originally attributed to nNOSβ, but this has yet to be evaluated experimentally.

I therefore aim to correct DMD deletion mutations to restore full-length dystrophin as well as rescue nNOS expression to reduce DMD pathogenesis with the use of the genome editing technology clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9).

CRISPR/Cas9: a genome editing tool

The CRISPR/Cas9 system used for genome editing was discovered in archae and bacteria, functioning as an adaptive immune system to counter viruses[12]. Invading viral DNA is integrated into the host genome's CRISPR array, then transcribed and processed into mature crRNA[12]. When crRNA, tracrRNA, and the endonuclease Cas9 are present, the crRNA targets Cas9 to a homologous DNA sequence if a downstream protospacer adjacent motif (PAM) sequence is present (Fig. 4A)[12]. This PAM varies between Cas9s of different species. Successful crRNA guidance and binding to the complementary DNA allows Cas9 to introduce a double strand break (DSB), cleaving the DNA[12]. The crRNA and tracrRNA can be combined into a single guide RNA (sgRNA), simplifying design for Cas9 targeting[12].

Transcriptional upregulation with catalytically active Cas9

A catalytically dead Cas9 (dCas9) fused to transcriptional activators has been commonly used to upregulate genes by localizing these transcription factors to promoters and enhancers[17]. Cas9 DNA cleavage requires sgRNA of 20 bases or longer[17]. When shortened to 12-16 bases, these dead sgRNAs (dsgRNAs) allow Cas9 to retain efficient DNA targeting but prevent cutting[17]. Therefore, Cas9 fused to a transcriptional activator can upregulate genes while retaining the potential to cut DNA (Fig. 4B).

Efficient DNA knock-in using Cas9

Multi-exon deletions affect approximately 70% of DMD patients[3]. A knock-in of the deleted cDNA can correct these DMD mutations at their genetic source, permanently restoring full-length dystrophin. Cas9 enables exogenous DNA integration when a knock-in construct is present during repair of the DSB[16]. However, methods using common DNA repair pathways suffer from poor efficiency, particularly in post-mitotic cells, such as skeletal and cardiac myotubes[23].

Homology-mediate end joining (HMEJ) is a more promising DNA repair system for knock-ins. Its mechanism is summarized in Figure 5. Yao et al. used CRISPR/Cas9- mediated HMEJ to knock-in a construct in vivo in the neurons of mouse brains[23]. Knock-in efficiency was very high at 58% in neurons that received the construct and Cas9[23]. Since neurons and muscle cells are post-mitotic they may share similar DNA repair pathways, suggesting similar efficiencies using HMEJ may be possible in muscle.

Adeno-associated viruses (AAVs) in delivery of Cas9 and knock-in components

AAV vectors will be used to effectively deliver all required Cas9 components and knock-in constructs to muscle in vitro and in vivo. These non-enveloped viruses can package ~4.7 kb of single stranded linear DNA[8]. Within a cell, the second strand is synthesized, allowing transcription and translation of the incorporated genes via host machinery[8]. AAVs are applicable to DMD gene therapy because of their ability to infect non-dividing cells such as myotubes[14]. The AAV9 serotype will be utilized in my project as it efficiently targets skeletal and cardiac muscle[24].

nNOS restoration to improve DMD pathology and systemic gene editing

DMD in mdx mice is exacerbated when both nNOS isoforms are knocked-out while transgenic nNOS expression alleviates several major DMD pathological traits (Fig. 6)[11,22]. These beneficial effects still occur despite nNOS being mislocalized, suggesting that a sufficiently large increase in nNOS levels can partially overcome impaired NO signalling due to mislocalization. Therefore, targeting nNOS for upregulation by CRISPR/Cas9 can improve DMD progression.

DMD gene therapies suffer from poor editing using systemic AAV delivery despite significant editing in vitro or with in vivo intramuscular injections. Lower blood supply to dystrophic muscle may partially explain these observations as the muscle microvasculature and sympatholysis in mdx mice is impaired[18]. nNOS restoration may normalize functional sympatholysis, increasing blood supply to contracting muscle. Thus, CRISPR/Cas9 nNOS upregulation could improve the effectiveness of DMD gene therapies by enhancing blood flow, and thereby AAV delivery, to muscle during exercise despite their impaired vasculature.

Project Objectives

My thesis is centered around a DMD patient deletion of exons 52-54 (Δ52-54) which disrupts the ORF of dystrophin (Fig. 7). These exons are commonly lost in DMD and our lab has already generated a Δ52-54 DMD mouse model as well as the appropriate sgRNA for knock-in of exon 52-54 cDNA[2]. My objectives to improve the phenotype of the DMD Δ52-54 mutations are as follows:

1) Restore full-length dystrophin in vitro by Cas9 mediated HMEJ knock-in in cultured Δ52-54 DMD mouse myotubes
2) Upregulate nNOS in vitro using dsgRNA and Cas9 in cultured Δ52-54 DMD mouse myotubes
3) Systemic in vivo delivery of AAV9 to simultaneously upregulate nNOS and perform HMEJ knock-in of exons 52-54 in Δ52-54 DMD mice

1) Restore full-length dystrophin in vitro by Cas9 mediated HMEJ knock-in in cultured Δ52-54 mouse myotubes

Knocking-in the deleted exons will allow correction of DMD patient deletions. Previous work in our lab identified the 52-54 deletion junction of our mouse model, leading to the design of an sgRNA targeting a region in intron 54 for knock-in of exons 52-54. I have assembled an exon 52-54 HMEJ knock-in construct with 800 bp flanking arms homologous to the genome at the intron 54 cut site. In testing my HMEJ knock-in strategy, myoblasts will be isolated from Δ52-54 DMD mice and matured into myotubes in culture. AAV9s packaged with Cas9, the appropriate sgRNA, and my HMEJ construct will be introduced to these myotubes in vitro. Quantitative PCR (qPCR) will assess HMEJ knock-in efficiency at the genomic level. Dystrophin restoration at protein level will be determined via Western blot and immunofluorescence, and at the transcript level using qPCR. Reassembly of the DGC will also be evaluated through immunofluorescence.

2) Upregulate nNOS in vitro using dsgRNA and Cas9 in cultured Δ52-54 mouse myotubes

As nNOS appears to be a powerful and beneficial modifier of the DMD phenotype, dsgRNAs will be designed to target various regions of the nNOS promoter. Typically, three tiled dsgRNAs are needed to achieve significant upregulation. Cas9 will be fused to the transcriptional activator VP64, and together with the various dsgRNA triplets on plasmids, delivered by AAV9s to mature Δ52-54 DMD mouse

myotubes in culture. Levels of nNOS transcript, protein, and activity will be evaluated to identify the best performing dsgRNA triplets.

3) Systemic in vivo delivery of AAV9 to simultaneously upregulate nNOS and perform HMEJ knock-in of exons 52-54 in Δ52-54 DMD mice

A dual AAV9 approach will be utilized. One AAV9 will contain Cas9 fused to VP64 under control of a muscle specific promoter. The second AAV9 will contain the exon 52-54 HMEJ knock-in construct as well as the intron 54 sgRNA and three nNOS upregulating dsgRNAs under a single hU6 promoter. Using pre-tRNA stuffer sequences between each sgRNA and dsgRNA, a single transcript can be processed into the individual sgRNA and three dsgRNAs.

Injection of both AAV9s into Δ52-54 DMD pups will enable simultaneous upregulation of nNOS and knock-in of exons 52-54. While the improved muscle pathology from the increased levels of nNOS may assist in delivery of the AAV9 knock-in construct, this might be further enhanced by taking advantage of restored sympatholysis. Putting the treated mice on a mild, short-term exercise regimen following systemic injection should increase muscular blood flow and possibly AAV9 delivery.

Future Directions

Short term

To design dsgRNAs that target the nNOS promoter. A large region upstream of the first nNOS exon contains the promoter. Determining the best area within this region to localize Cas9-VP64 to will require screening a variety of dsgRNAs.

Additionally, I am focusing on producing AAV9s containing my HMEJ construct and Cas9. Our AAV9 production is typically outsourced, but it can take over a month to receive the packaged virus. My experimental timeline for in vitro knock-in and upregulation tests will be shortened if AAV9s of a high enough titer can be made in the necessary amounts in lab.

Finally, the microvasculature, blood supply, and sympatholysis have not been characterized in our Δ52-54 DMD mice. I will utilize various techniques from the literature that have been verified to assess these properties in the context of DMD.

Long term

Phosphodiesterase (PDE) inhibitors, such as tadalafil, induce vasodilation by inhibiting PDE-mediated cGMP degradation[9]. As cGMP is downstream in the NO sympatholysis pathway, PDE inhibition enhances the vasodilatory response from NO[9]. In DMD patients and mdx mice, a single dose of tadalafil rapidly restores sympatholysis to wild-type functionality[13,19]. I would like to investigate a combined gene editing-pharmacological DMD treatment, as sympatholysis should be recovered quicker through PDE inhibition than nNOS upregulation alone, potentially allowing an earlier improvement in AAV9 delivery.

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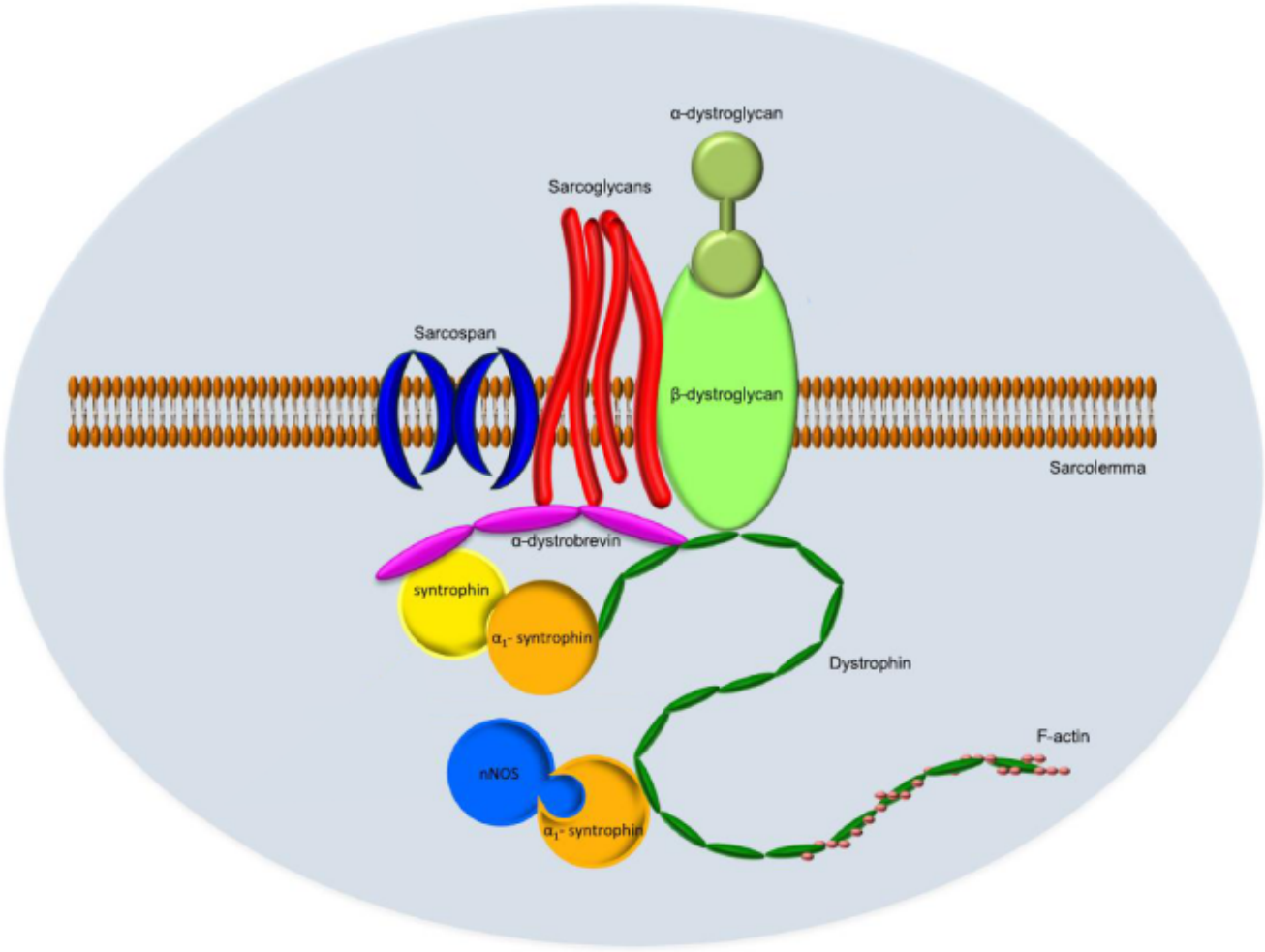

Figure 1. The dystrophin glycoprotein complex[9]. The dystrophin glycoprotein complex (DGC) is located at the sarcolemma of skeletal and cardiac muscle. Dystrophin pays a significant role in stabilizing the DGC. The adaptor protein syntrophin localizes nNOSμ to the mid-rod domain of dystrophin

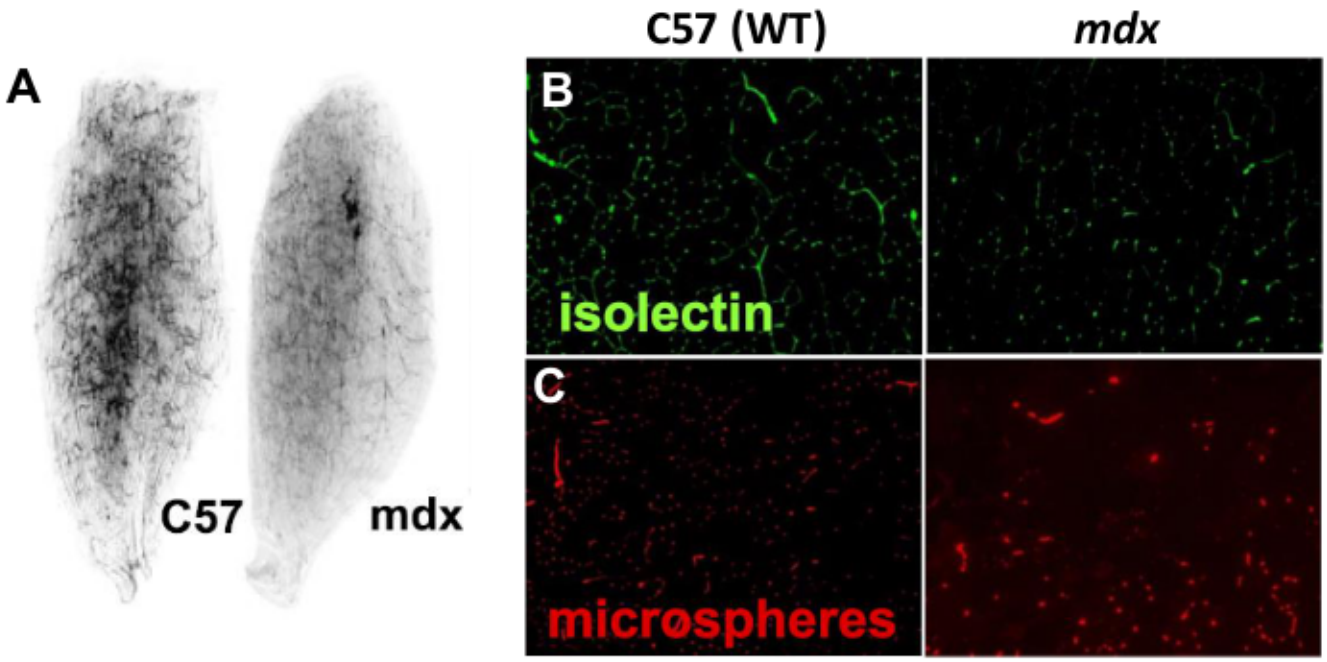

Figure 2. Impaired microvasculature in DMD[18]. The density of microvascular networks in mdx mice are significantly reduced. Vascular mapping of whole-mount mdx tibialis anterior (TA) by Microfil perfusion (A) and immunofluorescence staining of capillaries (B) shows loss of vessel density relative to C57 wild-type mice. (C) Injection of fluorescent microspheres into circulation marks only functional vessels. In mdx mice, there is a significant drop in the TA blood supply.

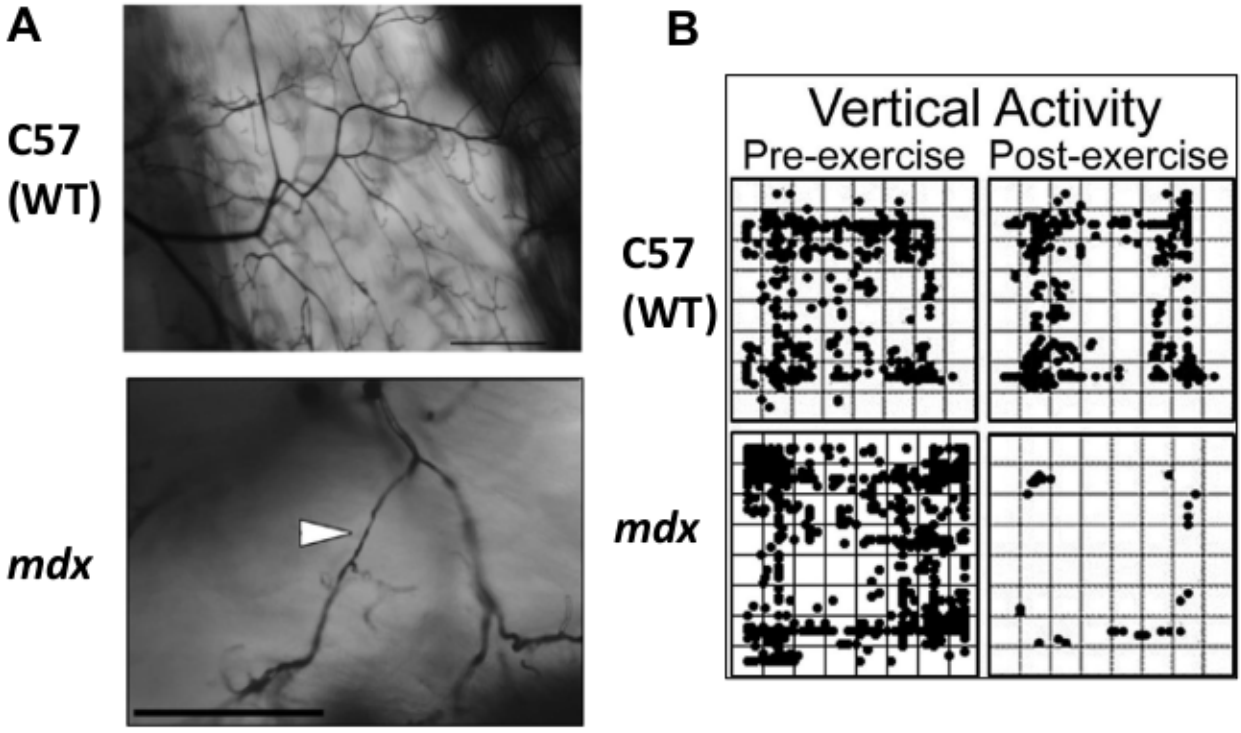

Figure 3. Ablation of sympatholysis in DMD[13]. (A) Post-exercise, C57 mice demonstrate increased capillary perfusion with no significant vascular narrowing. However, exercised mdx mice lack capillary perfusion with evident vascular narrowing. (B) C57 mice retain a similar amount of vertical activity pre- and post-exercise when measured in an open-field test. A significant decline of vertical activity occurs post-exercise in mdx mice, evident of an increased susceptibility to fatigue.

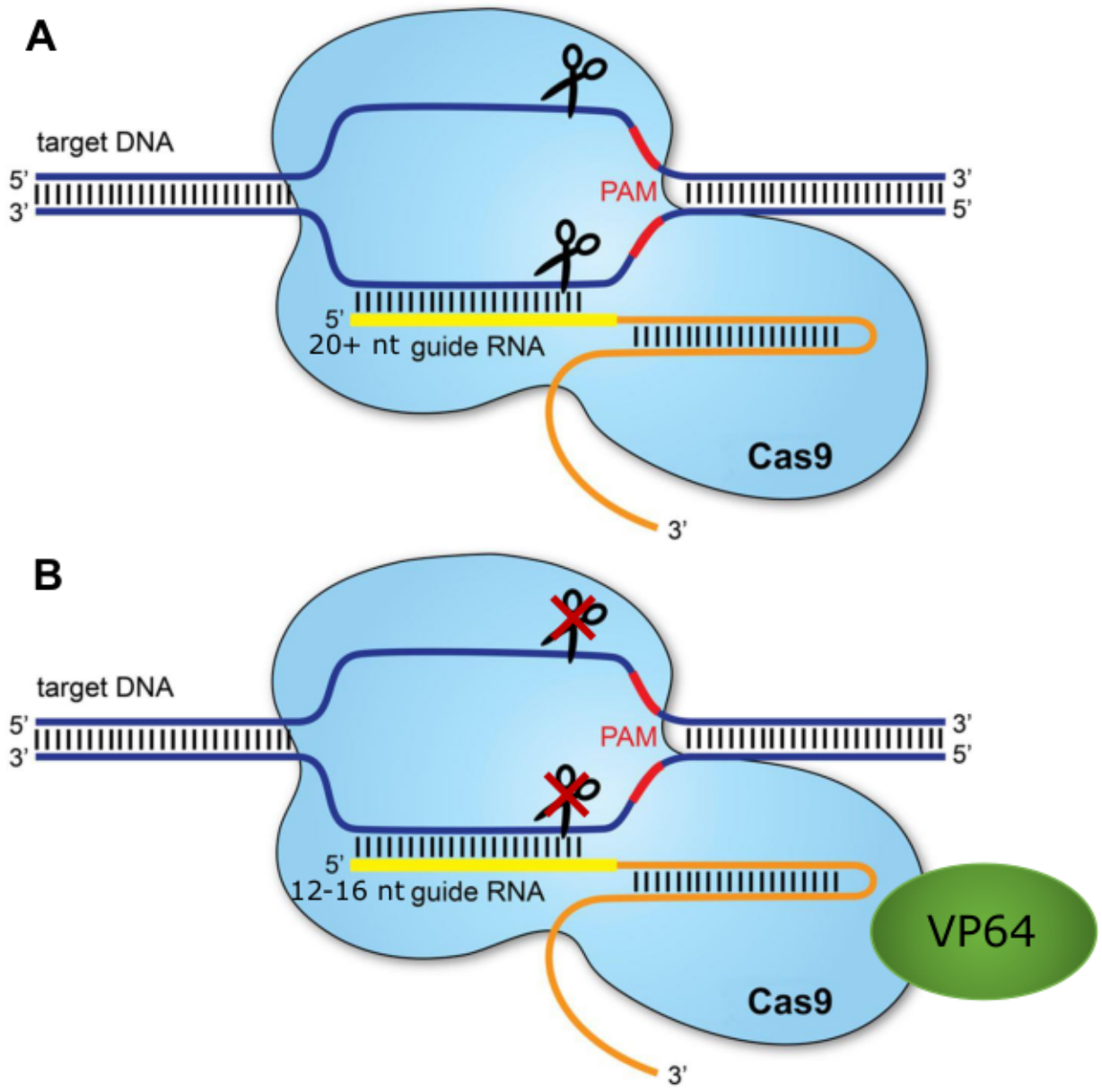

Figure 4. DNA cleavage and gene upregulation using the CRISPR/Cas9 system[12,17]. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a powerful technology for the manipulation of genomic material. Hybridized crRNA (yellow) and tracrRNA (orange) make up the single-guide RNA (sgRNA), which contains a sequence homologous to a region of the DNA immediately upstream of the protospacer adjacent motif (PAM). Association of Cas9 with sgRNA allows targeting to the homology region and separation of the DNA strands (A) With a 20+ nucleotide (nt) sgRNA homology region Cas9 will cut both DNA strands using its two endonuclease domains, generating a double strand break that can be utilized for a variety of gene editing processes. (B) With a 12-16 nt sgRNA homology region, Cas9 will still bind the targeted DNA region but not cut. Therefore, using shortened sgRNAs, transcriptional activators (such as VP64) can be localized to specific DNA regions when fused to Cas9.

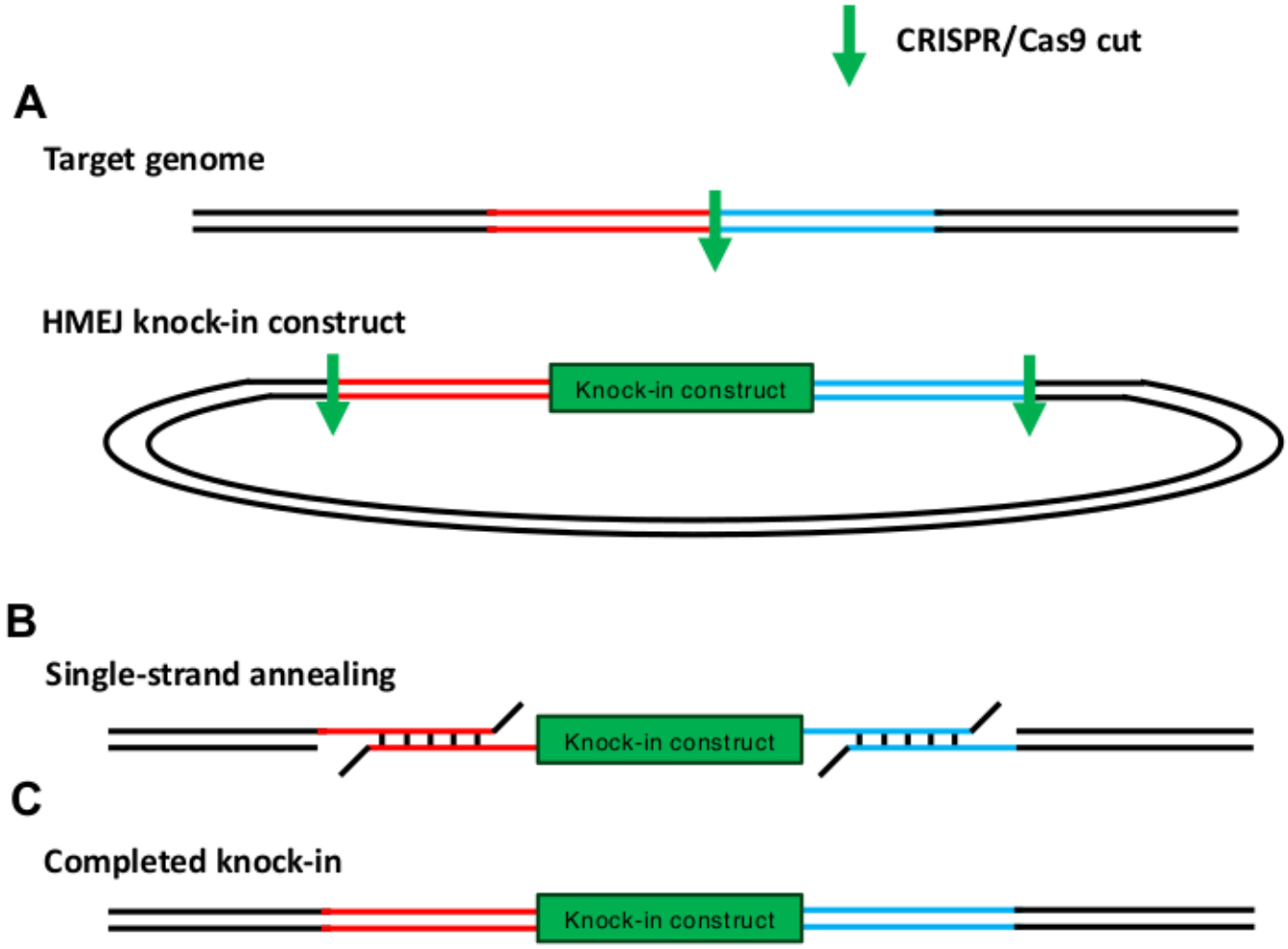

Figure 5. Current predicted mechanism of homology-mediated end joining (HMEJ) knock-in with CRISPR/Cas9[23]. Presented is a schematic of the current understanding of Cas9- mediated HMEJ knock-ins. The blue and red DNA are 800 base pairs long and homologous to regions of the same colour. The green arrows represent CRISPR/Cas9 cleavage sites using the same sgRNA. A) First, the desired insertion site in the genome is cut by CRISPR/Cas9. Simultaneously, the HMEJ knock-in construct is excised from its delivery vector by CRISPR/Cas9 cleavage at the ends of the flanking homology arms. B) Exonuclease activity degrades a single strand from the homology regions in the genome and knock-in construct. Annealing between complementary single-stranded homology regions of the genome and knock-in construct follows. C) Unannealed flaps are removed, gaps are filled by DNA polymerase, and ligase seals the DNA backbone to complete the HMEJ knock-in of the desired construct.

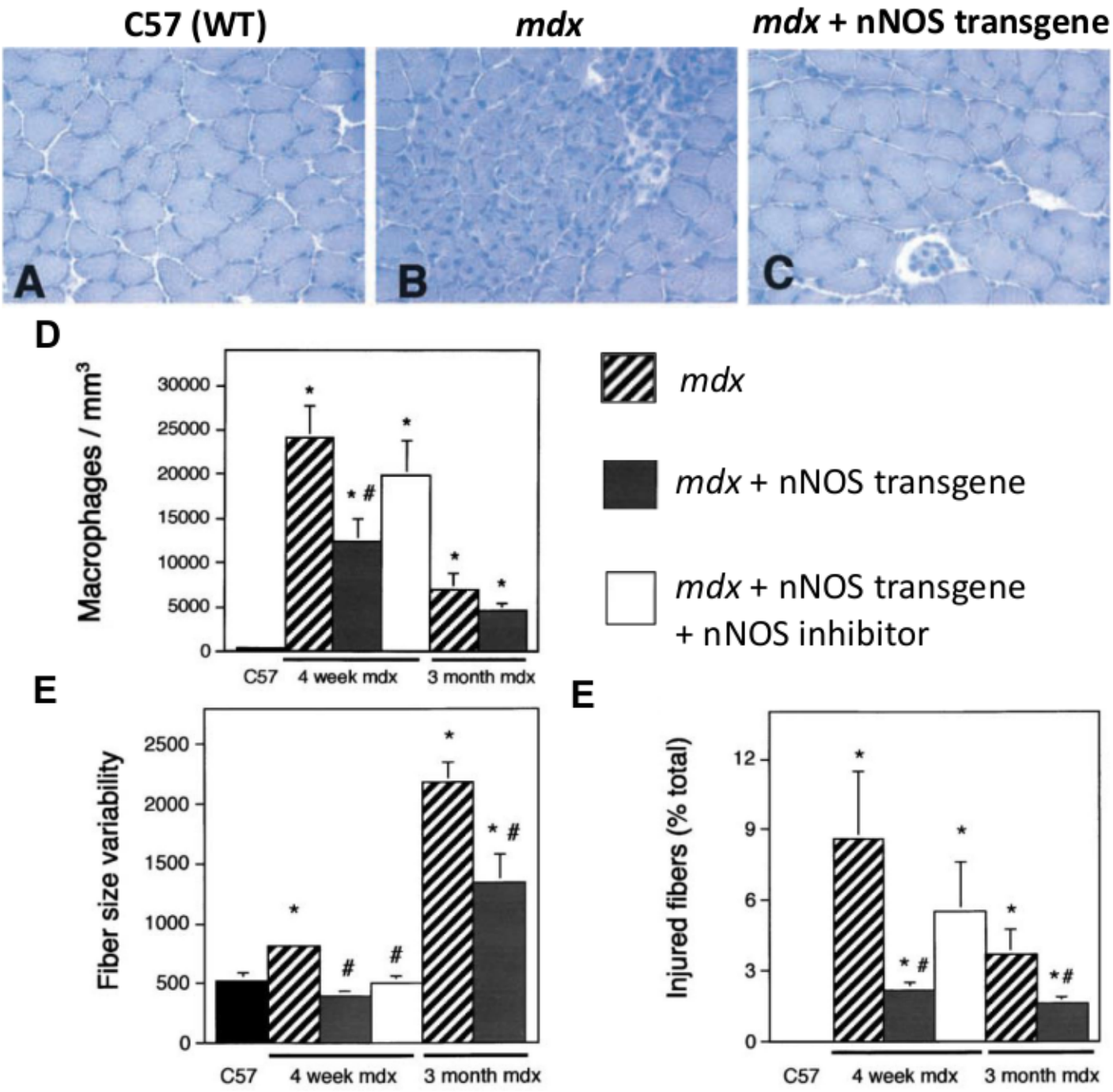

Figure 6. DMD pathogenesis in mdx mice is improved with expression of an nNOS transgene[22]. When mdx mice were bred to express an nNOS transgene, there was a significant improvement in soleus muscle histology at 4 weeks (A-C). Quantification of several DMD pathological hallmarks demonstrated improvement with nNOS expression, including reduced inflammation (D), reduced fiber size heterogeneity (E), and a reduced proportion of damaged muscle fibers (F). Application of an nNOS inhibitor significantly attenuated the impact of nNOS expression on inflammation and muscle injury (D, F).

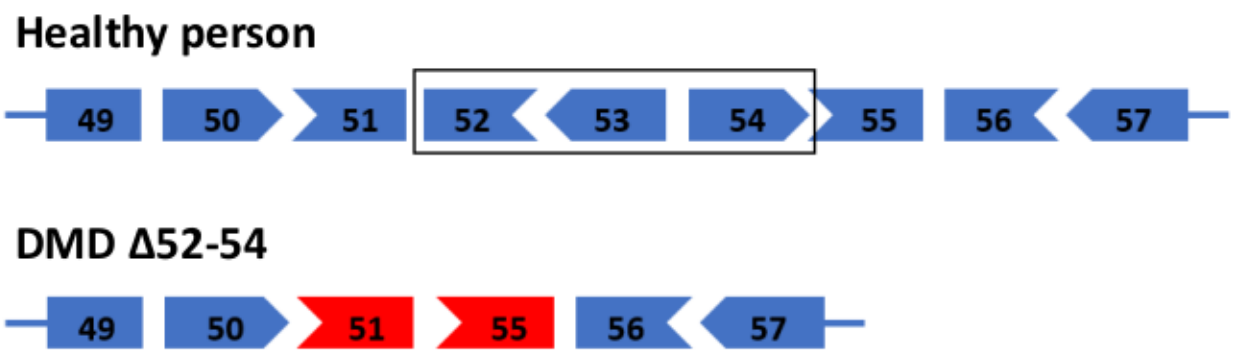

Figure 7. Deletion of exons 52-54 of dystrophin results in DMD. The DMD patient deletion I will be aiming to correct through an HMEJ/Cas9 knock-in is the exon 52-54 deletion (Δ52-54). This mutation disrupts the open reading frame of the dystrophin transcript, resulting in a truncated and non-functional protein. Exons remaining in frame are indicated in blue, whereas out of frame exons are denoted in red.