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Review

Membrane recruitment of nNOSμ in microdystrophin gene transfer to enhance durability

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Abstract

Several gene transfer clinical trials are currently ongoing with the common aim of delivering a shortened version of dystrophin, termed a microdystrophin, for the treatment of Duchenne muscular dystrophy (DMD). However, one of the main differences between these trials is the microdystrophin protein produced following treatment. Each gene transfer product is based on different selections of dystrophin domain combinations to assemble microdystrophin transgenes that maintain functional dystrophin domains and fit within the packaging limits of an adeno-associated virus (AAV) vector. While domains involved in mechanical function, such as the actin-binding domain and β-dystroglycan binding domain, have been identified for many years and included in microdystrophin constructs, more recently the neuronal nitric oxide synthase (nNOS) domain has also been identified due to its role in enhancing nNOS membrane localization. As nNOS membrane localization has been established as an important requirement for prevention of functional ischemia in skeletal muscle, inclusion of the nNOS domain into a microdystrophin construct represents an important consideration. The aim of this mini review is to highlight what is currently known about the nNOS domain of dystrophin and to describe potential implications of this domain in a microdystrophin gene transfer clinical trial.

1. Gene therapy approach for the treatment of DMD

Duchenne muscular dystrophy (DMD) is a progressive and fatal neuromuscular disease that typically presents clinically in boys during early childhood as calf muscle pseudo-hypertrophy accompanied by motor difficulties including delayed motor milestones, frequent falls, and easy fatigability [1]. The disease mainly affects skeletal and cardiac muscle as it is caused by mutations in the *DMD* gene that result in a lack of functional dystrophin protein [2]. Dystrophin is a key component of skeletal muscle responsible for physically linking the intracellular actin cytoskeleton to the extracellular matrix through the establishment of the dystrophin glycoprotein complex (DGC) [3]. This complex stabilizes the muscle membrane during contraction, preventing injury to muscle fibers. Due to the important role of dystrophin in stabilizing both structural proteins and molecular signaling pathways at the muscle sarcolemma, its absence leads to chronic muscle degeneration. As a result, dystrophic muscle fibers are replaced with fibrotic tissue, leading to diminished muscle function over time [4]. Importantly, absence of dystrophin leads to respiratory muscle decline, resulting in dependence on assisted ventilation, and cardiomyopathy. Since there is currently no cure, treatment requires a multi-disciplinary approach using a variety of interventions including polytherapy, physiotherapy, nutritional and psychosocial support, and orthopedic, respiratory, and cardiac care [1]. Although the lack of dystrophin protein has been identified for decades as the cause of DMD, direct replacement currently remains an inviable therapeutic option due to the large size of the gene and protein and the systemic nature of the disease [5,6].

To account for these challenges, gene therapy utilizing adeno-associated virus (AAV) has emerged as one of the most promising approaches for DMD to restore functional dystrophin to dystrophic muscles [7,8]. AAV gene therapy confers major advantages over alternative approaches as it

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can impact all muscles throughout the body through systemic injection with a muscle specific promoter to drive gene expression only in target tissues, thus preventing off-target effects. However, due to the extremely large size of the dystrophin coding sequence, significant work was required to better characterize the dystrophin protein with the overall goal of generating microdystrophin variants that maintain function but could still fit within the packaging capacity of the virus [9,10]. As a result, shortened but functional forms of dystrophin, termed "microdystrophin," were generated utilizing varying combinations of the full-length dystrophin coding sequence.

Three clinical trials that aim to deliver a functional microdystrophin to DMD patients are underway (Clinical Trial #NCTT03362502, #NCT03375164, #NCT03368742). Of the three programs, a microdystrophin gene therapy that contains the R16/17 region of dystrophin, termed the 'nNOS domain,' is currently being evaluated for safety and efficacy in ambulant and non-ambulant DMD boys (INGITE-DMD, Solid Biosciences, Clinical Trial #NCT03368742). This construct was developed by Dr. Jeffrey Chamberlain and shown to have good preclinical efficacy [11,12]. Generally, inclusion of the nNOS domain in microdystrophin is predicted to provide benefit to patients, but its actual effect is currently unknown. The aim of this mini review is to gain a better understanding of the nNOS domain in the context of microdystrophin gene transfer. This is accomplished through reviewing what is currently known about the nNOS domain in dystrophic muscle, identifying the potential functions that the nNOS domain may impart by using Becker muscular dystrophy (BMD) patients as a model, and integrating this knowledge set to develop a hypothesized role for the nNOS domain in the context of microdystrophin gene therapy.

2. Designing a functional microdystrophin

Characterization of dystrophin was first studied from a clinical perspective as mutations in the *DMD* gene are also found in the allelic condition Becker muscular dystrophy (BMD). Although there is wide heterogeneity among patients, BMD often manifests as a milder phenotype because mutations are compatible with production of a semi-functional protein containing the relevant N- and C-terminal domains and variable intermediate components, albeit usually in reduced quantity compared to dystrophin content of normal skeletal muscle [13]. Clinical observations of a small subset of BMD subjects who remained ambulant beyond the 4th decade of life, or did not develop muscle weakness at all despite expressing 30–60% of dystrophin quantity on muscle biopsy, fueled the idea that restoring at least a partially functional protein can ameliorate the disease course of DMD [14]. Definitive conclusions could not be made on this small subset, but the observations launched laboratory studies which characterized the protein structure of dystrophin and identified the most critical domains for functionality. Studies showed that dystrophin is comprised of several distinct domains that collectively contribute to protein function [15]. Protein domains include the N-terminal actin-binding domain, a central rod domain containing 24 spectrin-like repeats and four hinge regions, a cysteine-rich domain, and a carboxyl-terminus domain [16]. The largest domain that comprises the majority of the protein structure is the central rod domain, which is thought to confer stability and flexibility to the muscle membrane during contraction due to its size and the presence of hinge regions [17]. The central rod domain, the cysteine-rich domain, and carboxyl-terminus domain also importantly contain distinct binding sites for proteins such as β-dystroglycan, the syntrophins, and recruitment of neuronal nitric oxide synthase (nNOS) [18–20]. These binding events are required for the formation of the DGC and maintaining the nitric oxide (NO) signaling pathway at the sarcolemma. As a result, they are considered critical components for dystrophin protein function [15,21,22]. Through these studies, it was determined that a functional microdystrophin must contain: (a) at least one actin binding domain [23]; (b) at least two hinges [24,25]; (c) at least 4 spectrin repeat domains [26]; and (d) the dystroglycan binding domain [27,28]. More recently, studies have suggested that the nNOS domain may also be a critical dystrophin component due to its role in regulating the NO signaling pathway in muscle [11,19].

3. Nitric oxide signaling in dystrophic muscle

The NO signaling pathway in muscle is regulated by nNOS, which is an enzyme encoded by *NOS1* that synthesizes nitric oxide from L-arginine. NO signaling is important to skeletal muscle as it regulates multiple processes, including muscle development, blood flow, metabolism, force, fatigue resistance, inflammation, and fibrosis [29]. Notably, these processes are controlled by a variety of nNOS splice variants that have distinct functions and are contained within different compartments of the muscle [30]. The most prominent splice variant in skeletal muscle is nNOSμ, which primarily displays sacrolemmal localization, but is also found in neuromuscular junctions [31–35]. The role of membrane localized nNOSμ is important as it preserves functional sympatholysis, a process that maintains blood flow in active muscle by counteracting α-adrenergic sympathetic vasoconstriction, leading to improved muscle perfusion [36–38]. This pathway is controlled through the nNOS domain located within R16/17 of the dystrophin protein, encoded by exons 42–45 of the *DMD* gene, and allows for membrane localization of nNOS [19,20]. Upon recruitment to the membrane, nNOSμ induces production of NO, which acts as a signaling molecule to increase blood supply and meet the metabolic demands of an actively contracting muscle [39–42]. Importantly, this membrane recruitment requires the dystrophin nNOS domain, is not direct binding as it is facilitated by α-syntrophin [34,43].

In dystrophic skeletal muscle, both nNOSμ protein levels and localization are dysregulated due to the absence of dystrophin [31,44]. This results in a failure to promote vasodilation in response to increased demand, leading to subsequent functional ischemia, edema, and myofiber necrosis [19,36,37,42]. Because of the role of the dystrophin nNOSμ

recruitment domain, defects in functional sympatholysis have been reported in both dystrophin deficient mice and dogs [37,45]. Importantly, these defects are only restored when the nNOS domain of dystrophin is present in exercised muscle [19,46]. Due to the specificity of this association, there are few published studies that categorically explore the relationship between nNOS recruitment and dystrophin, highlighting the need for more controlled and standardized methods to draw definitive conclusions regarding its importance.

Despite this, the literature generally suggests that both nNOS mRNA and protein are downregulated in dystrophic skeletal muscle [29,31,44,47–50]. Further, overexpression of nNOS in the mdx background resulted in an improved phenotype [51], while nNOS depletion heightened inflammation and muscle damage [52]. This sparked the hypothesis that increases in nNOS could lead to subsequent improvements in vasodilation. This hypothesis was tested in several preclinical and clinical trials with the aim of enhancing the NO signaling pathway within the muscle [46,53–60]. However, there were varying results, which potentially demonstrated that upregulation of NO signaling by itself is likely insufficient to modify the disease course. Further, this is echoed by preclinical studies showing that increases in nNOSμ expression to the cytoplasmic pool can worsen disease state in mdx mice [61], suggesting that the presence of the nNOS domain may be required under instances when NO signaling is enhanced.

Several preclinical studies have attempted to parse out the distinction between nNOSμ expression versus localization, but often no differences are reported in resting muscle [19,61,62]. Instead, significant findings are only seen in active, exercised muscle. Not only has exercise been shown to increase nNOSμ expression, but also function, especially in prolonged periods of activity [63]. Additionally, mdx mice expressing microdystrophin that lacked the nNOS domain showed restored force, but still displayed an exaggerated fatigue response after exercise [62]. This is paralleled by studies showing that cytoplasmic pools of nNOSμ can lead to further muscle damage by promoting muscle atrophy [64]. Therefore, sacrolemma-localized nNOSμ is thought to play an important part in exercise capacity and recovery with the nNOS domain as a requirement to regulate nNOSμ activity for optimal function.

4. BMD: An approach for defining the clinical importance of membrane localized nNOS

It has been described that the stabilization of nNOS at the muscle fiber membrane is important to improve blood flow during periods of increased energy demand, lessen focal ischemia, and decrease muscle necrosis, leading to sustainment of healthy muscle fibers. Therefore, inclusion of the nNOS domain in microdystrophin can be considered critical because of its role in this process. Several clinical studies have attempted to correlate functional impairments with expression of the nNOS domain, but often findings vary, illustrating the complexities of the pathway and how difficult it can be to interrogate. However, substantial evidence exists to supports its role in counteracting muscle ischemia, particularly in actively contracting muscle [19,36,42,46,53,56,58]. The strongest clinical evidence that exists to support this claim involves data collected from BMD subjects that participated in a well-controlled, age-matched study that examined differences in muscle ischemia [46]. In this study, BMD subjects were stratified according to mutations that either preserved or eliminated the nNOS domain. Functional sympatholysis was directly measured and compared to healthy controls. When the authors correlated mutation status with functional outcomes, a close relationship was reported between BMD mutation, sarcolemmal anchoring of nNOS, and functional sympatholysis [46]. Importantly, BMD subjects with mutations that disrupted the nNOS domain not only displayed mislocalization of nNOS, but also had greater impairments in vasoconstriction when compared to patients with an intact domain [46]. Similar impairments of vasoconstriction were also replicated preclinically when examining the effect of the nNOS domain in a head-to-head study of transgenes with and without the domain [19]. Importantly, rescue of these impairments when using gene therapy and exercise was only observed when the nNOS domain was present in microdystrophin [19].

The remainder of the clinical studies published that explore the potential significance of the nNOS domain in the presence of a truncated but functional dystrophin protein are retrospective, making findings challenging to interpret. In one such study, BMD subjects with mutations predicted to completely lack the nNOS domain were compared to BMD subjects with mutations predictive to preserve this domain. It was reported that muscle biopsies of subjects with intact domain had increases in relative nNOS staining intensity when compared to those lacking the domain. Not only were these histological findings assoicated with mild and asymptomatic phenotypes, but nNOS expression was significantly higher in asymptomatic when compared to mild phenotypes [65].

The association between localization of nNOS staining (cytoplasmic, membrane bound or both) with clinical readouts such as quantitative muscle testing, age at first motor symptoms and difficulty with walking stairs, has been explored; however, findings are variable [66,67]. These inconclusive results may be explained by the limited number of subjects in each group, poor tissue quality in some cases, and the nature of available clinical readouts. In addition, the population recruited in these studies had different levels of dystrophin, which could have a more significant impact on their phenotypic differences.

Other markers that have been characterized in the dystrophin-nNOS pathway are microRNAs, which are thought to be regulated by nNOS activity through modulation of muscle homeostasis via specific HDAC2 target genes that influence differentiation [68]. In patients lacking the nNOS domain, lower muscle microRNA levels (miR-29c, miR-1) were reported. Further, BMD subjects with the nNOS domain

present or slightly reduced were associated with better clinical phenotypes [68]. It is currently unknown how well these markers are predictive of nNOS-dystrophin function in DMD.

Overall, these findings need to be presented under the caveat that visible levels of properly localized nNOS have been found in a subset of BMD patients that have part of their nNOS domain deleted [66,68]. Further, this was observed in two separate cohorts of patients, but is hard to interpret due to extremely low sample sizes. Despite this, it may suggest that there are other factors in BMD that dictate nNOS localization at the sarcolemma other than the nNOS domain present in dystrophin, but no conclusive clinical evidence to support this claim has been presented. Additionally, trying to fully characterize the effect of nNOSμ on dystrophic muscle remains challenging due to the presence of *NOS1* splice variants and additional nNOS isoforms (*NOS2*, *NOS3*) that participate in NO signaling and may confound specificity [47,52,61,69,70]. Further, α-syntrophin expression has been shown to be altered in dystrophic muscle and how microdystrophin restoration will impact its expression is currently unknown [34,71,72].

Given the complexities that contribute to nNOS signaling in dystrophic muscle, it is not surprising that the association between phenotype, dystrophin abundance, and nNOS expression to clinical outcomes is not well characterized. In the majority of published studies, samples are often collected at varying timepoints and stages within disease, which could further compound variability that is characteristic of both intra- and inter-patient biopsy data [73]. Further, the effects of nNOS are difficult to measure clinically due to sample availability and the lack of well characterized biomarkers. Therefore, placing clinical findings in the context of gene therapy becomes difficult as many of the clinical studies have been performed retrospectively without access to standardized functional outcomes across different studies, resulting in the loss of consistent phenotypic groupings. Despite these challenges, the literature generally suggests that inclusion of the nNOS domain in BMD subjects results in a milder phenotype when compared to patients lacking the domain [65,67,68].

5. The hypothesized importance of the nNOS domain in microdystrophin gene transfer

Clinical data collected from BMD patients suggest that the presence of the nNOS domain in dystrophin has a role in promoting increased muscle function and health. This is further strengthened by preclinical work, which allows for direct head-to-head comparison of constructs with and without the nNOS domain [19]. Based on these studies, it is hypothesized that nNOS is a critical domain as it protects muscle fibers against exercise intolerance, muscle ischemia and subsequent death. This is mainly supported by the observation that the degree of sympatholysis was shown to be regulated by the presence of the nNOS domain in BMD subjects [46]. This hypothesis could prove to be vital to gene therapy trials for several reasons. First, AAV gene therapy is currently a one-time treatment so durability of transgene expression is of the utmost importance. The clinical differences in durability when comparing different microdystrophin constructs is currently unknown, but prevention of muscle cell death is key as necrosis will deplete the transgene. Interestingly, expression of microdystrophin, regardless of whether it contains the nNOS, should lead to an early increase in muscle activity. As a result of microdystrophin function, DMD boys are likely to become more active, which will in turn increase the relative hypoxia of DMD muscle; therefore, any additional vasodilation should prove to be beneficial. At this time, it is unknown how well microdystrophin-expressing fibers will tolerate the anticipated increase in activity levels over time. The long-term sustainability of these effects may be jeopardized by the lack of the nNOS domain as exercise is known to upregulate nNOSμ expression. To preserve muscle in these instances, both clinical and preclinical studies suggest that active muscle is protected from exercise-induced fatigue and ischemia due to the nNOS domain. Preventing exercise-induced fatigue will reduce ischemia and cell death and lead to improvements in durability. A microdystrophin construct lacking the nNOS domain cannot recruit nNOS and therefore may not be protected from ischemia and could lead to muscle cell death, depletion of microdystrophin, and reduced efficacy over time. Despite this hypothesis, the impact of the nNOS domain in a microdystrophin gene therapy in the clinic remains to be determined. Currently, a microdystrophin gene therapy that does contain the nNOS domain is being tested in a Solid Bioscience sponsored clinical trial in ambulant and non – ambulant DMD patients (for more information please visit the clinical trial.gov, Ignite DMD- Clinical Trial #NCT03368742), but it is unknown how well it restores nNOS localization to the sarcolemma and downstream NO signaling in a clinical setting. Despite this, it has been shown to recruit nNOS to the membrane in preclinical models [11,12], but a limitation involves measuring the impact of the domain as no validated methodology currently exists. There are published studies as far back as 2004 that involve the measurement of muscle perfusion using blood oxygenation level dependent magnetic resonance imaging (MRI-BOLD), arterial spin labeling (ASL), and functional near-infrared spectroscopy (fNIRS) [74–79], but all measures remain exploratory and have not been accepted as valid measurements for muscle perfusion in DMD. Moving forward, further characterization and validation of methods that attempt to measure muscle perfusion in DMD muscle are needed.

6. Future developments arising from clinical characterization of the nNOS domain in microdystrophin gene transfer

Currently, gene transfer of microdystrophin remains one of the most promising therapeutics for treatment of DMD. As patients begin to express microdystrophin, the hope is that muscle function will stabilize or perhaps even

improve. Given these potential increases in muscle activity, it becomes important to preserve and protect microdystrophin expressing fibers. Inclusion of the nNOS domain may help to maintain microdystrophin positive fibers for a longer period of time.

Declaration of Competing Interest

All authors are employees of Solid Biosciences Inc. Solid is the sponsor of the Clinical Trial Ignite DMD (#NCT03368742)

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