CURRENT INSIGHTS INTO LAMA2 DISEASE

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CURRENT INSIGHTS INTO LAMA2 DISEASE

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EDITORIAL
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Editorial: Current Insights Into LAMA2 Disease

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Keywords: laminin, mouse model, clinical aspect, therapy, pathogenesis

Editorial on the Research Topic

Current Insights Into LAMA2 Disease

Merosin Deficient Congenital Muscular Dystrophy type 1A (MDC1A; OMIM*156225), or LAMA2-Congenital Muscular Dystrophy (LAMA2-CMD) or LAMA2-Related Disease (LAMA2-RD), is an autosomal recessive disorder due to mutations in the *LAMA2* gene, which codes for the α2 chain of laminin-211. Laminin-211 (also called merosin) is an heterotrimer composed of α2, β1, and γ1 chain, and forming a key element in the formation of the basement membrane of skeletal muscle, peripheral nerve, and brain. As a consequence of *LAMA2* mutations, laminin-α2 containing heterotrimers are not assembled or are expressed at very low levels, causing progressive tissue degeneration. The disease has an estimated prevalence in UK and Italy of 0.6–0.7/100,000, and is characterized by a severe wasting muscular dystrophy, dysmyelinating neuropathy and brain abnormalities. Thus, it represents a multi-organ disorder with a preponderance of the muscle pathology and a wide range of severities from severe early-onset forms causing death in the first decade of life, to milder late-onset forms. Severe and milder forms are largely distinguished by the residual amount of laminin-211 expressed, and are both responsible of high social and economic costs due to chronic medications and hospitalizations in the absence of any effective therapy.

This Research Topic has generated a very informative collection of articles covering several aspects of LAMA2 disease, including molecular pathomechanisms, main clinical findings, lessons from animal models, development of potential treatments on the basis of mechanistic understanding, and the identification of potential biomarkers of the disease.

Sarkozy et al. introduce the clinical aspects of the disease, describing severe and milder forms, including typical hallmarks of early-onset forms, such as hypotonia, axial weakness, inability to achieve independent ambulation, and elevated creatine kinase (CK) levels in the blood. They also point out the presence of joints' contractures, feeding difficulties, respiratory dysfunction, central and peripheral nervous system involvement, and possible cardiac abnormalities. They finally provide indication for diagnosis and management of the disease, and discuss what is currently available on natural history studies and disease biomarkers to guide future clinical trials.

Previtali and Zambon dissert on the current knowledge of peripheral neuropathy in LAMA2 disease, summarizing all the findings reported in humans and animal models. They also present molecular mechansims responsible for LAMA2 neuropathy describing its pathological hallmark, the formation of bundles of unsorted axons, as a consequence of defective nerve development. Finally, they discuss the consequence of the disease on nerve regeneration, neuromuscular junctions, and potential therapeutic strategies.

Gawlik and Durbeej present a comprehensive overview of all the mouse models reproducing LAMA2 disease, describing their clinical aspects and lifespan, motor behavior, muscle pathology, and respiratory and heart function. They also show how clinical and pathological findings change

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with mouse age and discuss the molecular mechanisms sustaining the disease. Finally, they summarize pharmacological approaches to the disease in these mouse models.

Similar findings are reported by Fabian and Dowling in a different animal model, the zebrafish. They introduce this model as an excellent tool to investigate human muscle disorders in general, and present differences and similarities with the *LAMA2*-deficient zebrafish. Finally, results on drug screening and drug therapy in these animal models and potential caveats are discussed.

Arreguin and Colognato explore all the known findings on brain dysfunction in LAMA2 disease. They summarize data on laminin-211 expression in the central nervous system and its role in regulating the blood-brain-barrier, synaptic plasticity, axonal growth and pathfinding, neural stem cells and myelination. Moreover, they describe neurological, pathological, and imaging findings in human and mouse LAMA2 disease.

Accorsi et al. describe the central role of fibrosis and inflammation in the pathogenesis of LAMA2. The most significant driver of fibrosis is TGF-beta and its chronic dysregulation affects myofibroblast transdifferentiation and myogenesis. Finally, biomarkers of fibrosis and potential therapeutic strategies are discussed.

Yanay et al. dissert on the impairment of muscle regeneration in LAMA2 disease. They describe mechanisms of muscle regeneration and consequences of lack of laminin-211 impacting regeneration and repair. Finally, they present an overview of the signaling pathways involved and possible therapeutic strategies by interfering with these pathways to enhance regeneration.

The last two articles focus on specific therapeutic strategies to treat LAMA2 disease. In the article of Barraza-Flores et al., the authors discuss on the role of laminin-111 to ameliorate LAMA2 disease. They first present the effects of integrin-laminin interaction to regulate muscle structure and function, the role of laminin-211 and how laminin-111 can substitute it. They present data of the therapeutic effects of endogenous or exogenous laminin-111 protein on LAMA2 disease.

Finally, Yurchenco and McKee present findings of the use of linker proteins to repair LAMA2 basement membrane and thus improve LAMA2 disease. Following a detailed description of basement membrane composition and molecular players, they describe how mini-agrin and LNNd proteins can recruit laminin heterotrimers, other than laminin-221, to be polymerized and linked to laminin-211 receptors, in order to rebuild a proper and functional basement membrane in LAMA2 tissues and to ameliorate the disease.

We expect that the articles included in this Research Topic will expand knowledge and interest in the field of LAMA2 disease, in order conclude the characterization of the molecular mechanisms responsible of the disease, to develop extensive natural history studies and, finally, to achieve efficacious therapy(ies).

AUTHOR CONTRIBUTIONS

SP, RC, and MR edited the topic and wrote the editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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Linker Protein Repair of LAMA2 Dystrophic Neuromuscular Basement Membranes

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An understanding of basement membrane (BM) assembly at a molecular level provides a foundation with which to develop repair strategies for diseases with defects of BM structure. As currently understood, laminins become anchored to cell surfaces through receptor-mediated interactions and polymerize. This provisional matrix binds to proteoglycans, nidogens and type IV collagen to form a mature BM. Identification of BM binding domains and their binding targets has enabled investigators to engineer proteins that link BM components to modify and improve their functions. This approach is illustrated by the development of two linker proteins to repair the LAMA2-deficient muscular dystrophy (LAMA2-MD). Dystrophy-causing mutations of the *LAMA2* gene product (Lmα2) disrupt the BM molecular architecture, destabilizing it. In a mild ambulatory type of the dystrophy, α2LN mutations in laminin-211 prevents polymerization. In the more common and severe non-ambulatory type (MDC1A), an absent Lmα2 subunit is replaced by the naturally occurring Lmα4 subunit that is normally largely confined to the microvasculature. The compensatory laminin, however, is a poor substitute because it neither polymerizes nor binds adequately to the anchoring receptor α-dystroglycan. A chimeric laminin-binding protein called αLNNd enables laminins with defective or absent αLN domains to polymerize while another engineered protein, miniagrin (*mag*), promotes efficient α-dystroglycan receptor-binding in otherwise weakly adhesive laminins. Alone, αLNNd enables Lm211 with a self-assembly defect to polymerize and was used to ameliorate a mouse model of the ambulatory dystrophy. Together, these linker proteins alter Lm411 such that it both polymerizes and binds αDG such that it properly assembles. This combination was used to ameliorate a mouse model of the non-ambulatory dystrophy in which Lm411 replaced Lm211 as seen in the human disease. Collectively, these studies pave the way for the development of somatic gene delivery of repair proteins for treatment of LAMA2-MD. The studies further suggest a more general approach of linker-protein mediated repair in which a variety of existing BM protein domains can be combined together to stabilize BMs in other diseases.

Keywords: gene-therapy, self-assembly, adeno-associated virus, muscle, peripheral nerve, extracellular matrices

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INTRODUCTION

Basement membranes (BMs) of skeletal muscle and peripheral nerve are cell surface-anchored extracellular matrices (ECMs) that contain laminins, nidogens, type IV collagen, and the heparan sulfate proteoglycans agrin and perlecan as key structural elements. In muscle, the sarcolemmal BM (sBM) coats and protects myofibers from contraction-induced damage. In peripheral nerve, the endoneurial Schwann cell (SC) BM enables and supports the myelination of axons. A key component of these BMs is laminin-211 (Lm211), a heterotrimeric glycoprotein consisting of α2, β1 and γ1 subunits. Lm211 is required for proper muscle and SC BM assembly. Mutations that ablate expression of the α2 subunit, or that adversely alter Lm211 polymerization, result in a muscular dystrophy that is accompanied by peripheral nerve deficiencies. This disorder is referred to as LAMA2-MD (LAMA2 gene Muscular Dystrophy) and also as MDC1A (Muscular Dystrophy Congenital type 1A). Less common than X-linked Duchenne dystrophy, this autosomal recessive disorder is nonetheless the most prevalent of the congenital muscular dystrophies. Of note, LAMA2-MD is one of several disorders arising from protein-altering mutations now thought to disrupt linkages extending from the stromal-BM interface through laminin to receptors and cytoskeleton that collectively maintain the stability of the sarcolemmal zone (Kanagawa and Toda, 2006).

The muscle symptoms and signs of LAMA2-MD comprise the major component of what is actually a combined muscle-nerve disorder with the disease ranging from severe to mild, depending upon the type of mutation. Patients with complete absence of the laminin α2 subunit, often resulting from nonsense mutations of the LAMA2 gene, are seen to have hypotonia and extremity weakness at birth such that independent ambulation is never achieved. Children with this presentation display a hypotonic posture with splayed legs, facial and extremity weakness, flexed fingers, and foot contractures (Jimenez-Mallebrera et al., 2005; Bonnemann et al., 2014). Difficulty with respiration is common with death occurring in almost a third of patients in the first decade of life if the condition is left untreated. Patients with mutations causing a slight to modest decrease in laminin α2 expression develop a milder form of the dystrophy in which ambulation is achieved. Seen in no more than 5% of patients, the mutations are generally found to be missense or short in-frame deletion mutations. Many of these mutations localize to the N-terminal LN domain and adjacent supporting LE domains of the α2 subunit (Yurchenco, 2015; Yurchenco et al., 2018). It is worth noting that in mice, laminin α2-deficiency is characterized by a prominent sciatic nerve neuropathy as well as a muscular dystrophy. The peripheral nerve-induced atrophy is superimposed on the dystrophic myofiber loss, regeneration and fibrosis of the muscle, with the corresponding nerve phenotype of weakness and paralysis obscuring phenotypic improvements resulting from muscle-specific repairs in the hindlimbs.

In this article, we have set out to review how an understanding of BM assembly at the domain level of interaction can be used for the engineering of linker protein strategies to repair the BM defects of LAMA2-MD. Further, the outcomes of transgenic repair in mouse models have not only advanced the possibility of a new treatment of the human disease, but support important aspects of the model of BM assembly and provide verification of the use of biochemical and cell biological approaches to predict outcomes *in vivo*. The last conclusion is relevant for the approach of engineering of new linker proteins to repair different BM structural defects such as those found in other muscular dystrophies and in Pierson and Alport syndromes. Thus we suggest that the predictive *in vitro* studies and confirmatory mouse outcomes have broader clinically relevant implications.

BASEMENT MEMBRANE ASSEMBLY

Laminins are a family of heterotrimeric glycoproteins (**Figure 1**) that are essential for BM assembly [reviewed in Yurchenco and Patton (2009) and Yurchenco (2011)]. Most laminins have three short arms while a few have only two short arms. This difference is important for understanding the pathogenesis of the common severe form of laminin-deficient muscular dystrophy and how it may be treated.

A body of biochemical, cell and mouse data support a general model of *de novo* BM assembly and resulting structure [reviewed in Yurchenco (2011) and Hohenester and Yurchenco (2013)]. This model (**Figure 2**) explains how BMs are generated through binding interactions of laminins with cell surface receptors, with themselves, and with other secreted structure-forming components largely through a process of self-assembly. Lm111 (α1-β1-γ1 subunit composition), a well-studied representative of this group similar in domain structure and most interactions to neuromuscular Lm211, is characterized by a series of receptor binding activities that map to the distinct C-terminal moiety. These are the LG1-3 domains that along with the C-terminus of the coiled-coil serves as the key ligand complex for integrin binding (Nishiuchi et al., 2006; Yamada and Sekiguchi, 2015) and the LG4-5 domains in addition to LG1-3 that bind to the mannosyl carbohydrate containing glucuronate-xylose repeats of α-dystroglycan (αDG) (Gee et al., 1993; Hohenester et al., 1999; Smirnov et al., 2002; Briggs et al., 2016). In the case of Lm211 in muscle, it is the α7β1 integrin that provides integrin binding. The receptor αDG has been found to play a more substantial role in mediating BM anchorage to the myofiber as compared to integrin (Han et al., 2009), a difference reflected by the greater severity of the "LARGE" (myd) phenotype compared to the Itga7-mutant genes in mice (Mayer et al., 1997; Holzfeind et al., 2002; Reed et al., 2004; Levedakou et al., 2005).

As laminin molecules attach to receptors of the cell surface, they become concentrated as a two-dimensional layer, favoring polymerization through a process of receptor-facilitated assembly (Colognato and Yurchenco, 2000). This helps create a dense carpet of laminin that enables the binding to, and hence recruitment of, of nidogens-1 and -2, perlecan, agrin and type IV collagen. In this polymerization, the β1 and γ1 LN domains bind to each other followed by addition of the α2 LN domain to form the "polymer node," a repeating inter-laminin LN complex of the sheet-like laminin network on the cell surface (Yurchenco et al., 1985, 1992; Yurchenco and Cheng, 1993;

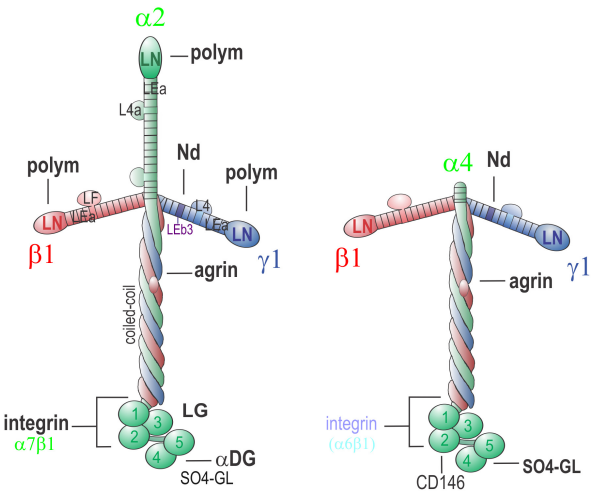

FIGURE 1 | Neuromuscular Laminins. Laminin-211 **(left)** is the principal laminin of the skeletal muscle sarcolemma. Polymerization activity maps to the three LN domains, nidogen-binding to γ1-LEb3, agrin-binding to the γ1 subunit of the coiled-coil, integrin α7β1 to LG1-3 and γ1E1607, α-dystroglycan (αDG) and (in SC BMs) sulfated glycolipids to α1-LG4-5. The LG interactions provide anchorage to the cell membrane and underlying cytoskeleton. Polymerization creates a sheet-like polymer. Nidogen-binding enables strong linkage to collagen-IV while agrin-binding permits additional binding to αDG. In the peripheral nerve BM of the SC sheath that insulates axons, laminin-411 **(right)** co-exists with laminin-211. Lm-411 lacks the short arm of the α2 subunit and hence is unable to polymerize. It also binds weakly to αDG binding and integrins (notably α6β1). In SCs, interactions with CD146 (MCAM) have been described. Laminin-411 is the principal compensatory laminin in muscle in LAMA2-MD.

McKee et al., 2007; Purvis and Hohenester, 2012). Laminins that lack a full complement of LN domains, notably the α4-laminins, are unable to polymerize (Reinhard et al., 2017). This is significant in that Lm411 is the principal compensating laminin expressed in the absence of the Lmα2 subunit (Moll et al., 2001; Reinhard et al., 2017). The nidogens (nidogen-1 is the principal nidogen in muscle and nerve) serve as high-affinity bridges between laminins and type IV collagen and between laminins and perlecan (Fox et al., 1991; Kohfeldt et al., 1998; Hopf et al., 2001). Type IV collagen separately polymerizes into a network through N-terminal (7S), C-terminal (NC1 domain) and weaker lateral interactions (Timpl et al., 1981; Yurchenco and Furthmayr, 1984; Yurchenco and Ruben, 1987; Vanacore et al., 2009; Bhave et al., 2012). Covalent stabilization occurs in the 7S and NC1 domains, while the lateral associations force the collagen polymer into a tight network. Agrin (A0B0 muscle isoform) binds to the γ1 subunit in the laminin coiled-coil domain via its N-terminal NtA domain and to αDG via its C-terminal moiety LG/EGF-like domain complex (Brancaccio et al., 1995; Yamada et al., 1996; Denzer et al., 1998; Kammerer et al., 1999).

Myofiber and Schwann Cell Basement Membranes

Laminin 211 (α2-β1-γ1) is common to both the muscle and SC BMs (Patton et al., 1997; Holmberg and Durbeej, 2013). However, SC BMs differ from those of the sarcolemma in that (a) Lm411 normally co-exists with Lm211 (Yang et al., 2005), (b) SC adhesion is mediated by several laminin-binding integrins (including α7β1, α6β1, α3β1, αvβ3) compared to only α7β1 in myofibers, (c) sulfatides, capable of adhering to both Lm411 and Lm211 are present on SC surfaces but not detected in muscle (Li et al., 2005), and (d) dystroglycan plays a more central adhesion/anchoring role in muscle (Han et al., 2009), while β1-integrins serve as the principal receptors required for cell polarization and myelination in nerve (Berti et al., 2011).

Dystrophic Muscle and Peripheral Nerve Basement Membranes

The BM ultrastructure of Lama2-deficient dystrophic mouse muscle (**Figure 3**) reveal focal attenuations (thinning) and denuded regions of the *lamina densa* (electron dense portion of the BM), particularly in the more severe dystrophy of the dy^W/dy^W and dy^{3K}/dy^{3K} mice (Gawlik et al., 2004; McKee et al., 2017; Reinhard et al., 2017). Adjacent to the BM there is an increase in interstitial collagen fibrils, normally sparsely and thinly distributed, that increasingly separate one muscle fiber from the next. An increasing number of myofibers undergo apoptosis and degeneration, inducing chronic inflammation and its sequelae of fibrosis.

With a reduction or absence of the Lmα2 subunit, there is a persistence of expression of laminin subunits that are normally present at only earlier developmental stages. The state of the severely laminin-α2-deficient sarcolemmal BM has been described for *dy/dy*, dy^W/dy^W, and dy^{3K}/dy^{3K} mouse models and is similar among them (Xu et al., 1994; Miyagoe et al., 1997; Reinhard et al., 2017). In the dy^W/dy^W and dy^{3K}/dy^{3K} mice, the laminin α2 epitopes are nearly completely to completely absent while those of the β1 and γ1 are present. In these mice, Lmα4 is substantially increased and laminin-α5 moderately increased (Moll et al., 2001; Reinhard et al., 2017). In the dy^{2J}/dy^{2J} mice, in which the α2-LN polymerization domain is altered but the remaining protein still expressed, there is a relatively small decrease in the Lmα2 epitope and a modest increase in Lmα4 with minimal changes in Lmα5, accompanied by more modest alterations of BM ultrastructure (McKee et al., 2017). These differences suggest the repair requirements for the mild and severe forms of LAMA2-MD are not identical.

During peripheral nerve development (extending from several days before birth to several weeks of age in mice), SC precursor cells express Lm211 and Lm411, with lesser levels of Lm221 and Lm421, and undergo a laminin- and integrin-dependent process of "radial axonal sorting" in which SC lamellipodial processes extend into, envelop and bundle naked axons followed by their sorting into a 1:1 axon: SC ratio with subsequent myelination (Webster, 1971, 1993). This process is dependent on laminins, collagen-IV, and β1 integrins (Benninger et al., 2007; Nodari et al., 2007; Pereira et al., 2009; Berti et al., 2011). SC BMs also bind to αDG and α6β4 integrins that also contribute to myelination, particularly at later stages of development. Expression of both laminin

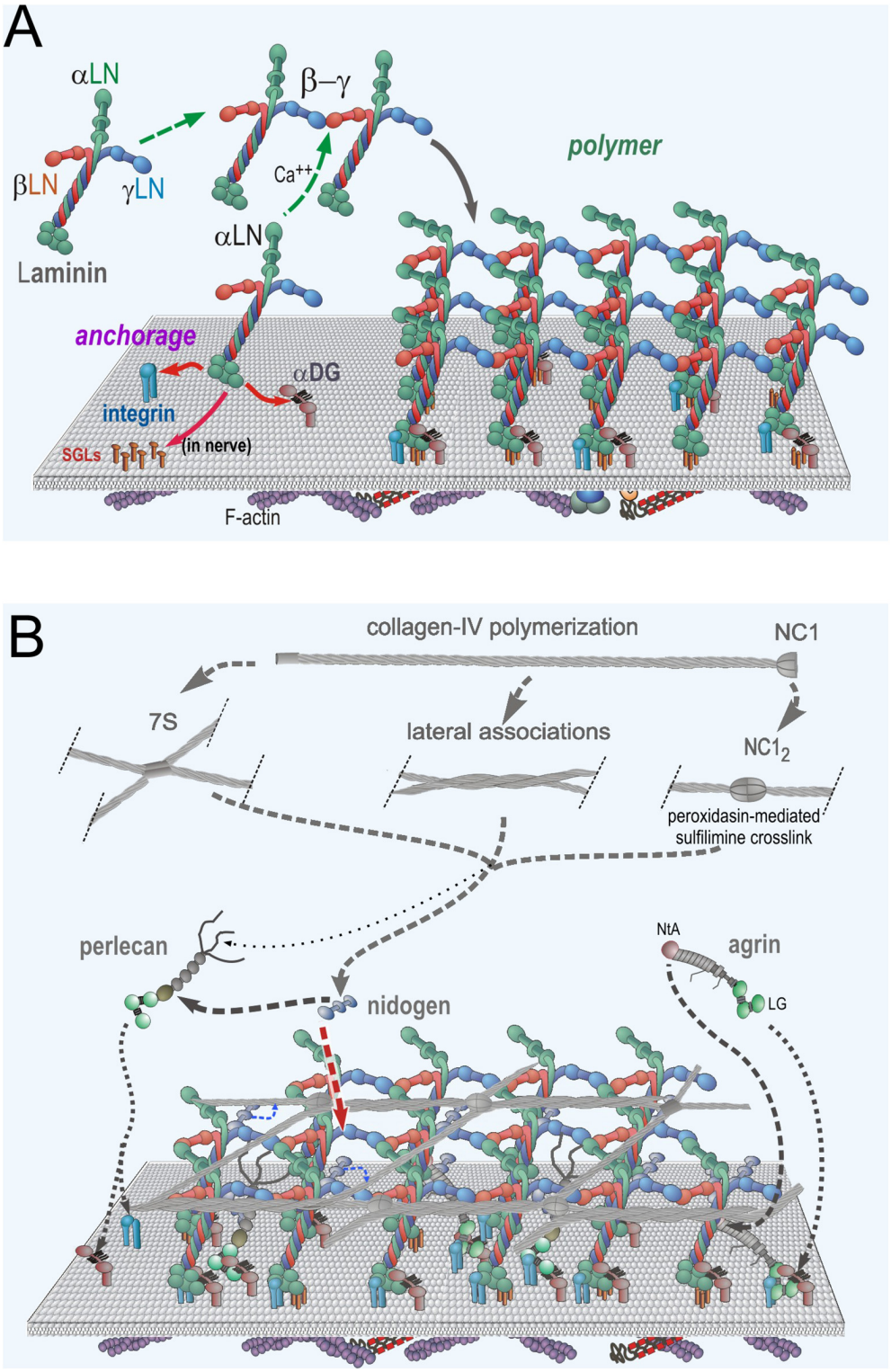

FIGURE 2 | Neuromuscular Basement Membrane Assembly Steps. **(A)** Laminin becomes anchored to the cell surface by binding to integrins (α7β1 in muscle and several laminin-class integrins on Schwann cells) and α-dystroglycan (αDG) receptors. In Schwann cells, there are additional attachments mediated by sulfated glycolipids (SGLs). The three different LN domains of laminins bind to each other to form a planar polymer, creating an initial matrix scaffolding. Lm411 (a normal components of SC BMs) is unable to polymerize. **(B)** The G3 domains of nidogen-1 and -2 bind to the Lmγ1-LEb3 domain. Agrin binds to the coiled-coil domain of laminin and to αDG. Perlecan, another HSPG, binds to the nidogen G2 domain and to αDG. Collagen-IV binds to the G2 nidogen domain and forms a second polymer through covalent N- (7S) and C-(NC1) terminal domain bonds. Non-covalent lateral associations force the collagen into a tighter network.

α-subunits appears to be important for normal myelination of the fibers, suggesting a need for balanced expression may be important (Yang et al., 2005). Absence of the α2 LN subunit (dy^{2J} mouse) was found to result in a prominent radial axonal sorting defect while absence of the α4 subunit was found to result in a less pronounced reduction of sorting with poly-axonal myelination, and absence of both subunits resulted in a particularly severe amyelination. Laminin polymerization, nidogen-binding, and receptor anchorage through β1-integrins were all found to contribute to myelination in organ culture of LamC1-null mouse embryonic dorsal root ganglia (DRG) treated with recombinant laminins (McKee et al., 2012). Of note, the polymerization-dependent deficit of myelination was largely reversed with a linker protein, described ahead, that restores polymerization.

The sciatic nerve and its branches are affected in dystrophic mice to a much greater degree than shorter nerves such as those of the brachial plexus. The myelination phenotype in mice therefore primarily presents as a hindlimb defect of increasing paralysis and extensor contractions that become permanent. The peripheral nerve defect in humans, detected principally as a reduction of conduction velocity, is less severe and sporadic compared to that in mice. An emerging impression is that the peripheral nerve consequences of amyelination are more likely to be recognized in patients with the milder ambulatory form of the dystrophy (Chan et al., 2014).

Linker Proteins and Repair of Dystrophic Basement Membranes

There are a variety of treatments that are under consideration for amelioration of LAMA2-MD ranging from structural repairs to the inhibition of apoptosis, fibrosis, and inflammation [reviewed in Durbeej (2015), Mohassel et al. (2018), and Yurchenco et al. (2018)]. It seems self-evident that the ideal treatment would be one that completely corrects the structural defect present in the BM early in the disease. One possible approach is to increase the expression of a laminin with properties similar to that found with α2-laminins. It was reported several years ago that transgenic expression of the laminin α1 subunit in dy^{3K}/dy^{3K} mice substantially rescued both the muscle and peripheral nerve phenotypes (Gawlik et al., 2004, 2006). Furthermore, in a recent study, it was shown that activation of expression of the α1 subunit by CRISPR-dCas9-mediated upregulation of Lama1 improved the dystrophic phenotype of the dy^{2J}/dy^{2J} mouse (Kemaladewi et al., 2019). This represents a new approach to the treatment of LAMA2-MD.

Another approach, the topic of this review, is to modify the defective BM with proteins such that they restore the laminin activities lost in the disease. Two such proteins have been developed and evaluated (**Figure 4**). One, miniagrin, the first such protein reported (Moll et al., 2001), increases laminin binding to αDG, the key anchoring receptor. The other, αLNNd, enables laminins lacking an αLN domain to polymerize by providing a synthetic short arm carrying a functional αLN domain (McKee et al., 2009). These proteins are sufficiently small in size such that their DNA can be packaged in adeno-associated viruses (AAVs) with appropriate promoters for expression in dystrophic muscle or muscle and nerve. We will discuss αLNNd first and then miniagrin.

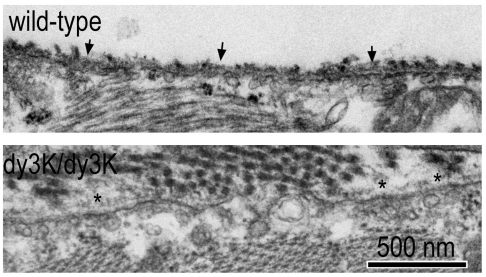

FIGURE 3 | Ultrastructure of normal and laminin-α2-null dystrophic muscle basement membranes. Wild-type and dy^{3K}/dy^{3K} skeletal muscle of mice at 3 weeks of age. Arrows indicate normal sarcolemmal BM with minimal overlying interstitial collagen fibrils. Asterisks indicate attenuated sarcolemmal BM with layer of overlying interstitial collagen fibrils.

αLNNd was engineered from the Lmα1 and nidogen-1 subunits and initially used as a tool to dissect laminin polymerization (McKee et al., 2009). It consists of the Lmα1 LN and four adjacent LEa domains fused to the more distal G2 through G3 domains of nidogen-1. The G3 domain binds to the Lmγ1 LEb3 domain near the intersection of the short arms, the LN domain mediates polymerization by attaching to β1 and γ1 LN domains, and the G2 domain enables binding to collagen-IV and also perlecan. The intervening LE (EGF-like domains with 8 rather than 6 cysteines) between LN and G2 and EGF-like domains between G2 and G3 are spacers between the globular domains and hence between bound ligands and, in the case of LEa1-2, enable proper folding and secretion of the LN domain. The expected interactions of αLNNd were confirmed by biochemical characterizations that included direct binding assays, polymerization assays of αLNNd coupled to non-polymerizing versions of recombinant laminin-111, rotary shadow electron microscopy to visualize molecular organization, and cell surface BM assembly experiments on Schwann cells and myotubes (McKee et al., 2009, 2017). The solid phase binding assays revealed that αLNNd bound to immobilized laminin-111 with the same apparent dissociation constant as nidogen-1, that αLNNd bound to immobilized collagen-IV with a dissociation constant slightly greater than that of nidogen-1, and that αLNNd linked laminin to collagen-IV. αLNNd, when coupled to Lmα1ΔLN-L4b, a laminin with only two short arms, appeared as a three short arm laminin in Pt/C rotary shadowed replicas by electron microscopy (McKee et al., 2007). This complex was found to polymerize with a polymerization slope and critical concentration similar to that of wild-type (WT) Lm111, whereas the truncated laminin lacking αLNNd did not polymerize. αLNNd similarly enabled Lmα1ΔLN (which lacks only the αLN domain) to polymerize. The linker-laminin complexes assembled on SC and myotubes (**Figure 5**) surfaces at levels comparable to wild-type laminin-111 (similar to Lm211), and well-above the levels seen with the non-polymerizing laminins alone (McKee et al., 2009, 2017).

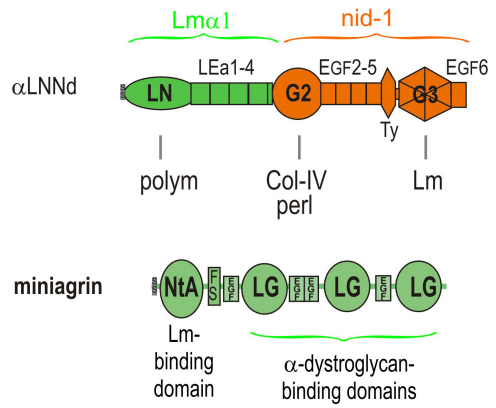

FIGURE 4 | Domain structure of αLNNd and miniagrin. αLNNd is a chimeric protein that consists of laminin α1 LN and adjacent LEa domains fused to the G2 through EGF6 domains of nidogen-1. The G3 domain allows the protein to be attached to a laminin at the nidogen-binding locus located near the origin of the short arms. When attached to a laminin lacking either the native αLN domain or the short arm containing that domain, the αLN domain of αLNNd binds to βLN and γLN domains present on the laminins, enabling laminin polymerization. The G2 domain binds to both collagen-IV and perlecan, activities normally present in nidogen. Miniagrin consists of domains present in non-neural (A0B0) agrin in which the NtA domain and adjacent follistatin domain has been fused to the LG and EGF-like domains of agrin. The NtA domain binds miniagrin to the coiled-coil of laminins while the LG domains bind miniagrin to the α-dystroglycan receptor.

Miniagrin (mag) was derived from the non-neural (A0B0) splice variant of agrin that is normally found in muscle at low levels (Moll et al., 2001). The protein consists of the laminin-binding N-terminal globular domain (NtA) followed by the first follistatin repeat fused to the C-terminal complex of three LG and four EGF-like domains that anchor agrin to the cytoskeleton largely through αDG. The N-terminal NtA globular domain of miniagrin binds to the upper segment of the coiled-coil domain of laminins through a sequence present in the Lmγ1 subunit (Kammerer et al., 1999). The miniagrin-laminin complex was visualized in Pt/C rotary shadowed replica, revealing that miniagrin bound to the coiled-coil domain of the long arm (McKee et al., 2009). LG-αDG binding interactions exist with laminins, agrins and perlecan and are mediated by O-mannosyl carbohydrate chains containing a xylose-glucuronate repeat that are attached to the neck region of αDG (Inamori et al., 2012; Briggs et al., 2016; Hohenester, 2019). This critical interaction creates a link from BM to actin cytoskeleton: αDG is coupled to transmembrane βDG, βDG binds to cytoskeletal dystrophin, and dystrophin binds to the actin-rich cytoskeleton (Matsumura et al., 1993). There are also miniagrin interactions to sulfatides and the α3β1 integrin: however, it is unclear if these interactions are relevant for dystrophy repair (McKee et al., 2007, 2012).

The effect of miniagrin on laminin assembly was evaluated in Schwann cell and myotube tissue culture models of BM assembly (McKee et al., 2009; Reinhard et al., 2017). In SCs, Lm111 lacking all LG domains were unable to adhere to cell surfaces and hence unable to assemble. If this laminin was coupled to miniagrin, the complex accumulated on SC surfaces to a similar degree as WT Lm111 and with a similar concentration dependency. The difference was striking. Miniagrin, expressed as a muscle-specific transgene, was shown to ameliorate the dystrophy of the dy^W/dy^W mouse (Moll et al., 2001; Reinhard et al., 2017). Improvements were seen at the histological and ultrastructural level, at the level of laminin expression and resistance to extraction, and at the level of survival, weights, and strength.

In separate studies, the miniagrin gene has been packaged in adeno-associated virus (AAV) and expressed in muscle and nerve and found to ameliorate the dystrophy of the dy^W/dy^W mouse (Qiao et al., 2005, 2018). Improvements were seen at the level of tissue histology and in animal performance and survival. The first AAV study revealed selective improvement of muscle. The second study, employing a different AAV serotype and promoter organization, enabled sufficient expression for improvements in both muscle and nerve. Together, these studies demonstrate that AAV can serve as a function-enhancing delivery gene system for a linker protein.

So far we have largely discussed the linker proteins as separate modifiers of BMs. However, single linker alterations of Lm411 in the severe (non-ambulatory) form of LAMA2-MD as a treatment represents an insufficient repair approach given our understanding of the BM requirements of both polymerization and anchorage and the degree of repair observed in mice. If we wish to alter Lm411 so it behaves more similarly to the absent Lm211, further modification is needed. Since both miniagrin and αLNNd proteins and DNA constructs in hand, it seemed logical to use both in a synergistic fashion to affect a change in the activities of the compensatory laminin. SC and myotube BM assembly experiments demonstrated that, at least *in vitro*, the two proteins could bind to the laminin and enable both polymerization and binding to αDG (McKee et al., 2009; Reinhard et al., 2017). The combination of linker proteins was considerably more effective than either alone as promoters of BM assembly on myotube surfaces, approaching levels achieved with Lm211 (**Figure 6**). The two transgenes were bred with dy^W/+mice to generate animal carrying the different alleles for analysis (Reinhard et al., 2017). The two transgenes, each driven by a muscle-specific promoter, were found to improve weights, survival, strength, and muscle histology to a much greater degree than each alone. Importantly, the improvements with the two transgenes effectively phenocopied the benefits observed *in vitro*, ones that could be explained at a mechanistic level in terms of known assembly interactions (**Figure 7**).

FUTURE STUDIES

We have discussed a model of neuromuscular BM assembly that led to hypotheses of how BM defects could be repaired. We traced the path of two engineered laminin-binding linker proteins from biochemical and cell culture analysis to studies conducted in mice following transgenic expression of these proteins. The data resulting from these studies suggest that the human disease the models represent can be ameliorated by similar expression of linker proteins. To do so, the next

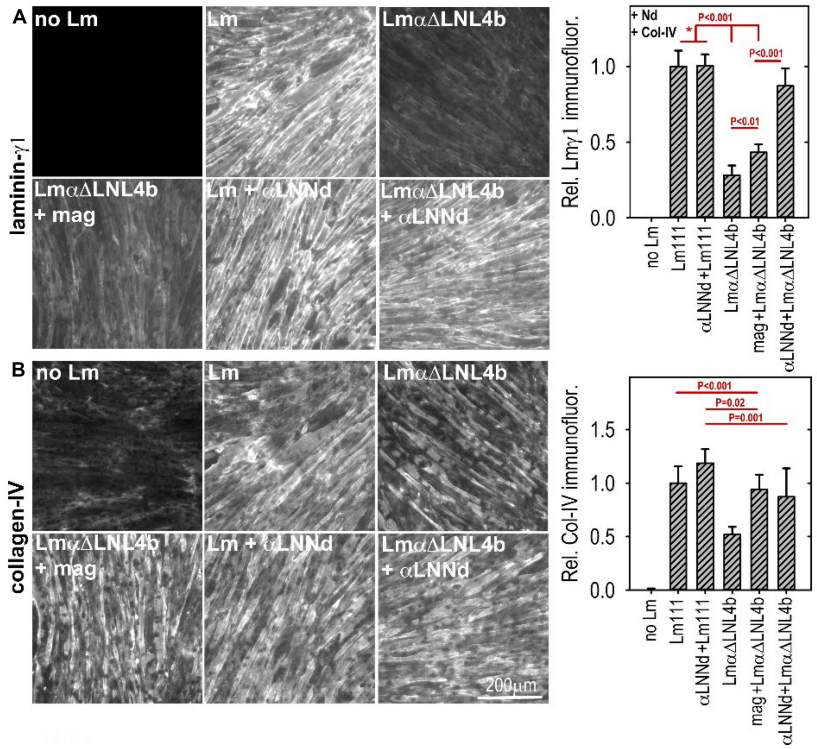

FIGURE 5 | (A,B) Laminin assembly on cultured myotubes. Laminin, linker protein and nidogen-1 were incubated at 28 nM separately or together in the presence of 14 nM collagen-IV. αLNNd, bound to the non-polymerizing Lmα1ΔLN-L4b, accumulated on myotubes to a much greater extend compared to that seen in the absence of the linker protein of laminin + miniagrin. Collagen-IV accumulation, which only requires limited amounts of surface-bound laminin, remained high for all linker-laminin complexes (McKee et al., 2017). In a comparison of dy^{2J}/dy^{2J} laminin without and with αLNNd transgene, it was found that αLNNd increased laminin present in the matrix fraction (McKee et al., 2017).

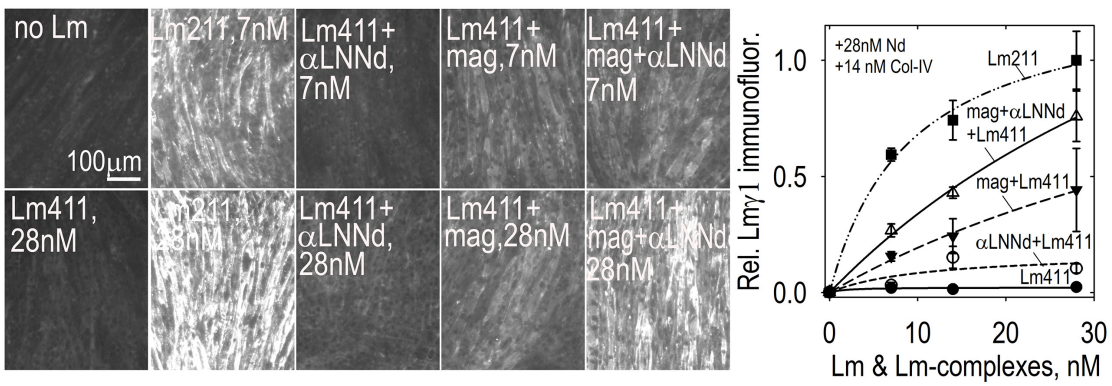

FIGURE 6 | Lm411-linker assembly on myotubes. Lm 411 was incubated without or with bound linker protein(s) at different concentrations in the presence of nidogen-1 (28 nM) and collagen-IV (14 nM). Accumulation on myotubes was assessed by Lmγ1-antibody immunofluorescence. Lm211 produced the highest level of laminin accumulation while Lm411 produced the lowest level. Each linker protein alone elevated Lm411 accumulation. The combined linker elevated accumulation to a level approaching that of Lm211 (Reinhard et al., 2017). An analysis of laminins in the dy^{W}/dy^{W} mice without and with miniagrin and miniagrin plus αLNNd transgenes revealed that transgene expression improved Lm411 levels and retention within matrix fractions in muscle (Reinhard et al., 2017).

step is to employ a somatic gene delivery system. AAV is DNA vector delivery system that has shown much promise for the treatment of Duchenne muscular dystrophy (Mendell et al., 2012). The size of the DNAs for αLNNd and miniagrin are sufficiently small to be packaged along with the required viral inverted repeats, promoter and poly(A) tail in the AAV capsid. These viruses, with appropriate choice of serotype and promoter, can be used for combined somatic gene therapy for evaluation of efficacy of repair. It is hoped that such studies will prove fruitful.

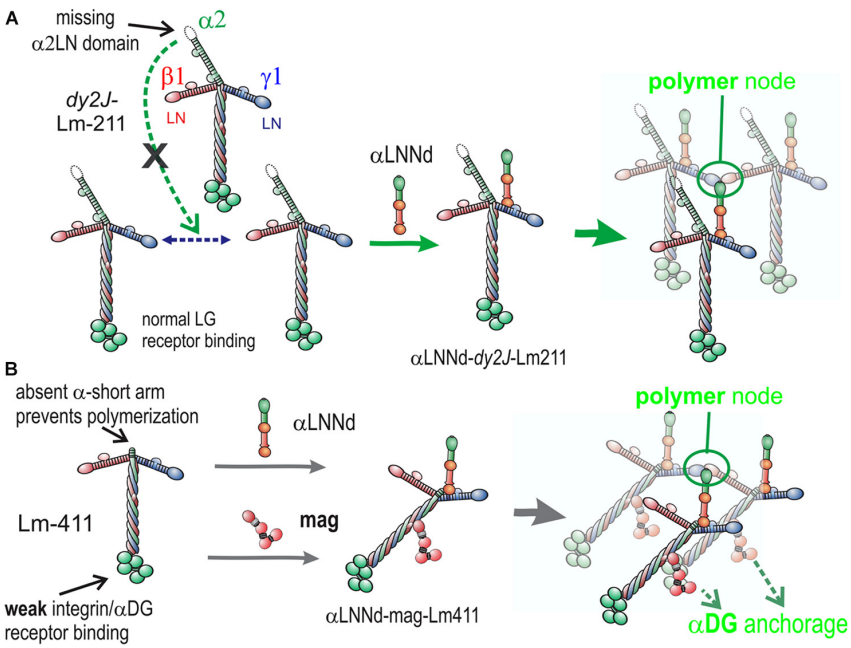

FIGURE 7 | Model of linker protein modification of laminin interactions in models of ambulatory and non-ambulatory laminin α2-deficiency (Yurchenco et al., 2018). **(A)** In the dy^{2J}/dy^{2J} mouse, Lm211 is unable to polymerize as a result of in-frame deletion within the α2LN domain leading to its degradation (Colognato and Yurchenco, 1999). αLNNd binds to the nidogen-binding locus (replacing nidogen), thus "restoring" polymerization. **(B)** In the Lama2 null mice, the compensatory Lm411 neither polymerizes nor binds to the key αDG receptor of muscle. Miniagrin binds to the coiled-coil (replacing native agrin) while αLNNd binds to the nidogen-binding locus. The combination of linker proteins enables polymerization and greatly enhances cytoskeletal anchorage such that the modified laminin behaves in a manner similar to Lm211.

Finally, given what is known about BM interactions, it seems reasonable that novel linker proteins can be engineered to repair of a variety of BM disorders in which structure has been compromised. These include other extracellular matrix-based muscular dystrophies, Pierson syndrome (glomerulopathy with ocular disease), and Alport syndrome (glomerulopathy with hearing and later visual losses). While studies are at early stages or even at a speculative stage, several possibilities can be outlined.

Since the discovery of dystrophic mutations in the genes coding for collagen-VI, collagen-IV, laminin-α2, the glycosylation enzymes required for αDG binding, and integrin α7 in addition to those affecting dystrophin, a unifying hypothesis has evolved, i.e., extracellular matrix proteins, their receptors and cytoskeletal partners maintain the sarcolemmal zone through a series of lateral (polymer) and transverse linkages extending from matrix above the BM to the cell cytoskeleton. Mutations adversely affecting these linkages can cause a muscular dystrophy through loss of mechanical stability and dependent signaling (Jimenez-Mallebrera et al., 2005). With this in mind, it may be possible to engineer a variety of proteins to replace lost linkages, e.g., to enhance collagen-VI stability in Bethlem myopathy by crosslinking the collagen to itself or to collagen-IV with a dimerized binding protein. However, one limitation of the linker approach worthy of mention concerns the generation of proteins that bind to the α7β1 or α6β1 integrins to enhance compensatory laminin adhesion. This restriction stems from the finding that integrin binding requires parts of all three laminin subunits, i.e., the distal coiled-coil and LG1-3 (Pulido et al., 2017; Taniguchi et al., 2019).

Other BM diseases can be addressed as well with this approach. In a subset of Pierson syndrome patients, the disease results from laminin β2LN mutations that cause a failure of laminin polymerization (Funk et al., 2017). Polymerization can be restored by βLNNd, a homolog of αLNNd that enables polymerization of laminins with βLN mutations (McKee et al., 2018). In Alport syndrome, there is a reduction of disulfide covalent crosslinked collagen due to loss of α3/α4/α5-collagen-IV (Gunwar et al., 1998). Here it may be possible to stabilize the BM by crosslinking the residual α1/α2 collagen-IV network with a dimerized collagen-binding protein domain such as nidogen G2. Finally, new insights into linker proteins will likely arise as we continue to elucidate how the BM and its stromal and receptor partners interact.

AUTHOR CONTRIBUTIONS

PY wrote the initial draft of the manuscript. KM carried out most of the αLNNd studies reported therein.

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REVIEW
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Fibrogenesis in *LAMA2*-Related Muscular Dystrophy Is a Central Tenet of Disease Etiology

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LAMA2-related congenital muscular dystrophy, also known as MDC1A, is caused by loss-of-function mutations in the alpha2 chain of Laminin-211. Loss of this protein interrupts the connection between the muscle cell and its extracellular environment and results in an aggressive, congenital-onset muscular dystrophy characterized by severe hypotonia, lack of independent ambulation, and early mortality driven by respiratory complications and/or failure to thrive. Of the pathomechanisms of MDC1A, the earliest and most prominent is widespread and rampant fibrosis. Here, we will discuss some of the key drivers of fibrosis including TGF-beta and renin–angiotensin system signaling and consequences of these pathways including myofibroblast transdifferentiation and matrix remodeling. We will also highlight some of the differences in fibrogenesis in congenital muscular dystrophy (CMD) with that seen in Duchenne muscular dystrophy (DMD). Finally, we will connect the key signaling pathways in the pathogenesis of MDC1A to the current status of the therapeutic approaches that have been tested in the preclinical models of MDC1A to treat fibrosis.

Keywords: congenital muscular dystrophy, laminin, fibrosis, integrin, TGF-beta, myofibroblast, renin–angiotensin system

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CONGENITAL MUSCULAR DYSTROPHY

Congenital muscular dystrophies (CMDs) are a highly heterogeneous group of early-onset neuromuscular disorders characterized by delayed motor development, severe hypotonia, and extreme muscle wasting (Schessl et al., 2006; Collins and Bonnemann, 2010). Although the first forms of CMD were recognized as early as 1903, it took decades to link these conditions to specific gene defects. CMDs can arise from mutations in many muscle proteins but the most prevalent forms of this disease arise from deficiencies in proteins at the interface of the muscle cell membrane (sarcolemma) and its associated extracellular matrix (ECM). Like in most cells, the interaction between the ECM and the sarcolemma is critical for muscle cell survival, homeostasis, and basic organ function (Tome et al., 1994; Jimenez-Mallebrera et al., 2005; Schessl et al., 2006; Lisi and Cohn, 2007; Collins and Bonnemann, 2010; Bertini et al., 2011). It is therefore conceivable that disruption in the link between these two structures often leads to rather severe forms of muscular dystrophies.

Pathology can arise from mutated gene products within the ECM itself. For example, defects in one of the alpha chains of type VI collagen cause Ullrich CMD or Bethlem myopathy, and disruption of the alpha2 chain of Laminin-211 leads to *LAMA2*-deficient congenital muscular

dystrophy (aka MDC1A). Alternatively, CMDs can arise from mutations in one of the many glycosyltransferases that add sugar moieties on alpha-Dystroglycan, an important receptor of the dystrophin–glycoprotein complex (DGC) that links the cell to the ECM. Abnormalities of *O*-mannosyl glycosylation of this key receptor result in a decrease or complete loss in the ability of alpha-dystroglycan to bind laminin, a ligand in the ECM (Tezak et al., 2003; Jimenez-Mallebrera et al., 2005; Schessl et al., 2006; Lisi and Cohn, 2007). While disruption of the DGC complex is also a consequence in Duchenne muscular dystrophy (DMD), a disease that is caused by mutations in the dystrophin gene (Lapidos et al., 2004), presentation, and clinical manifestations of CMDs differ from DMD in many respects. Patients with CMDs show signs of muscle and, in many cases, nervous system pathology at time of birth indicating a critical developmental role of the proteins involved. Additionally, children with CMD present with an early episode of severe pathology followed by a stabilization period. This is in contrast with DMD, which has a later onset but is a consistently progressive disease (Geranmayeh et al., 2010; Brogna et al., 2019). Pathological processes such as inflammation, fibrosis, and aberrant regenerative capacity appear to be conserved across all muscular dystrophies. However, they could play a much more etiological role in CMDs compared to DMD.

As previously stated, CMDs result from a large number of different genetic defects and encompass a wide range of pathophysiological processes, making it challenging to discuss them as a whole. Here we will review MDC1A, the second most prevalent form of CMD. We will particularly explore the clinical features and molecular signature of the fibrotic pathophysiology of MDC1A and discuss the therapeutic approaches that are currently being investigated to treat fibrosis in this devastating, incurable disease.

LAMA2-RELATED MUSCULAR DYSTROPHY

LAMA2-related muscular dystrophy (MCD1A) is caused by mutations in the *LAMA2* gene, located on chromosome 6q22-q23 in humans. It encodes the alpha2 chain of laminin-211 (composed of alpha2, beta1, and gamma1 subunits) that is an essential, multi-functional ECM protein. The laminin superfamily of matrix proteins plays an integral role in multiple cellular processes such as proliferation, differentiation, migration, and cell adhesion. Laminin-211 is primarily expressed in the basal membranes of skeletal muscle and Schwann cells, as well as in capillaries between the astrocyte foot processes and vessels of the brain. Other tissues expressing laminin-211 include heart, kidney, lung, stomach, placenta, and testis. It is expressed as early as week 7 in human embryos and E11 in mice, underscoring its critical role during skeletal muscle development (Leivo et al., 1989; Quijano-Roy et al., 1993; Patton et al., 1997; Nakagawa et al., 2001; Tezak et al., 2003; Jimenez-Mallebrera et al., 2005; Holmberg and Durbeej, 2013).

Laminin-211 has a host of binding partners both in the ECM and in the cell membrane. Specifically, it interacts with the extracellular alpha subunit of dystroglycan, a transmembrane dimeric protein belonging to the DGC. Alpha-dystroglycan connects to the intracellular cytoskeleton by interacting with its beta subunit. Beta-dystroglycan binds dystophin, thus connecting the ECM to the contractile apparatus. Yet another important partner of laminin-211 is integrin-alpha7beta1, a transmembrane protein complex that links the ECM with the underlying cytoskeletal actin network, possibly by interacting with intermediate proteins such as talin and/or integrin-linked kinase. The laminin–alpha-dystroglycan interaction has been shown to activate the PI3K/Akt pathway and the binding of laminin with integrins results in activation of the focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) pathways. In addition to these cell surface partners, laminin-211 also has many binding partners in the ECM such as agrin, nidogen, perlecan, and collagen IV (Yurchenco and O'Rear, 1994; Straub et al., 1997; Talts et al., 1999; Lapidos et al., 2004; Tzu and Marinkovich, 2008; Gumerson and Michele, 2011).

Pathophysiology of MDC1A was first described by Tome et al. (1994) and follows a strong genotype–phenotype correlation (Geranmayeh et al., 2010). Mutations that allow for at least partial expression of the alpha2 chain of laminin display a milder phenotype when compared to severe pathology that results when there is a complete loss of this protein. Children with MDC1A show profound muscle weakness and hypotonia, either at birth or soon after. They also develop spinal deformities as well as contractures at their elbows, knees, and ankles. Serum creatine kinase levels are high in these patients but not as high as DMD patients. Although some affected children may achieve the ability to sit and stand with support, most will never ambulate independently. Brain MRIs from these patients show white matter hypodensity, though impaired brain function is not part of the MDC1A pathology. In most cases of MDC1A, affected children without palliative care die prematurely due to either respiratory complications or failure to thrive (Quijano-Roy et al., 1993; Philpot et al., 1999; Allamand and Guicheney, 2002; Tezak et al., 2003).

FIBROSIS AND INFLAMMATION

Chronic inflammation and widespread fibrosis in the interstitial space are pathological signatures of laminin-211-deficient muscles. Unlike DMD, where fibrosis develops later during the disease progression, it may play a more etiological role in driving MDC1A pathology. In children with MCD1A, there is a massive early surge of inflammation in the months soon after birth. However, after the initial inflammatory episode, fibrosis sets in and seems to be the main driver of laminin-deficient pathology rather than chronic inflammation. Limited patient data are available that show fibrosis as an early driver of MDC1A pathology; however, a study by Taniguchi et al. (2006) provided, for the first time, the data to support early dysregulation of ECM proteins in the human disease. Taniguchi et al. (2006) reported that muscle tissue from MDC1A patients exhibited extensive interstitial connective tissue and a lack of regenerating fibers as early as 20 days of age. One potential

problem of very early fibrosis is its effect on myogenesis. An altered myomatrix could modify the myogenic potential of satellite cells and thus deleteriously impact postnatal muscle growth (Thomas et al., 2015). This was further supported by gene expression data in MDC1A patients which revealed overexpression of several ECM components and downregulation of muscle structural components. A similar gene expression profile was also seen in biopsies from Fukuyama muscular dystrophy, another CMD resulting from the loss of laminin–dystroglycan interaction (Taniguchi et al., 2006). Further, it has been shown that these ECM genes are not only expressed by interstitial fibroblasts but also by the muscle fibers themselves. This suggests that myofibers undergo an environment-driven transition into fibrotic effector cells, further deleteriously affecting myogeneis and driving the etiology of the disease (Pessina et al., 2015). While increased expression of ECM-related genes is also seen in DMD biopsies, they appear much later in the disease progression as a result of many failed rounds regeneration and thus are likely a secondary consequence rather than a primary disease driver as seen in CMDs. However, this needs to be confirmed with a more comprehensive study in a larger and longitudinal data set.

A number of laboratories, including our own, have corroborated MDC1A patient data in various mouse models of laminin-alpha2 deficiency, further implicating fibrosis and inflammation as critical drivers of this disease. There are several mouse models for MDC1A (dy^{2J}/dy^{2J}, dy/dy, dy^{W}/dy^{W}, dy^{3K}/dy^{3K}) that display moderate to severe CMD phenotypes directly correlating with the levels of laminin-alpha2 expressed (reviewed in Gawlik and Durbeej, 2011). It is worth noting that the level of fibrosis in these models also appears to correlate with severity. Our laboratory worked primarily with the dy^{W}/dy^{W} (*DyW*) model that was generated by crossing heterozygous B6.129 $Lama2^{dy-W/+}$ mice, which carry a targeted *DyW* mutation in the *Lama2* gene (originally generated and kindly shared by Dr. Eva Engvall, Burnham Institute, La Jolla, CA, United States). Like humans, mice that are homozygous for the *DyW* allele present with severe disease pathology characterized by accelerated muscle wasting, limited or no regenerative capacity, inflammation, and widespread fibrosis. This pathology can be observed as early as 1 day of age where there is a clear, widespread disruption of muscle architecture and increased endomysial connective tissue (**Figure 1**). At this time point, these muscles also show a large mononuclear cell population likely made up of some combination of unfused myoblasts, macrophages, fibroblasts, and/or other infiltrating cells, mimicking the very early rampant inflammatory response observed in children with MDC1A (Mehuron et al., 2014).

There are several pathways that could play a role in driving the fibrotic phenotype of MDC1A. We will, however, focus this review on the biology of fibrosis that is dictated by TGF-beta and renin–angiotensin system (RAS) signaling pathways since these pathways have been extensively characterized by us and others in the context of laminin-deficiency. We will also touch upon the biology of integrin-alphaV as it is intimately involved in the release of TGF-beta from its latent complex in the ECM and has been established in facilitating fibrosis and matrix remodeling in many organs.

TGF-BETA AS A DRIVER OF FIBROSIS

The most significant driver of fibrosis in many diseases, including muscular dystrophies, is TGF-beta. This pro-fibrotic cytokine is synthesized as a precursor protein in the endoplasmic reticulum and assembled as a non-covalently bound complex of a short C-terminal disulfide-linked homodimer (the mature cytokine) and a longer N-terminal disulfide-linked homodimer that called the latency associated peptide (LAP). This small latent complex (SLC) is further non-covalently linked to another set of proteins in the endoplasmic reticulum called latent TGF-beta binding proteins (LTBPs) to form the large latent complex (LLC). The LLC is secreted from cells, and the LTBPs tether to the ECM. This latent complex confines TGF-beta to an inactive form by hiding the TGF-beta receptor binding domains, allowing for tight regulation of signaling. Activation of TGF-beta can occur through multiple routes including proteolysis, thrombospondin-1, reactive oxygen species, or pH to dissociate the LAP from the mature TGF-beta peptide (Saharinen et al., 1999; Annes et al., 2003; Shi and Massague, 2003; Leask and Abraham, 2004; Pohlers et al., 2009).

Another way in which TGF-beta can become activated/freed from the LLC is through the actions of integrins. Integrins comprise a family of 18 alpha and 8 beta proteins that come together to form 24 distinct heterodimeric, membrane-spanning proteins consisting of one alpha and one beta subunit. The alpha subunit imparts ligand specificity and the beta subunit is the effector of downstream signaling. These proteins often serve as receptors for laminins and other extra/matricellular proteins and participate in a wide array of cellular functions including migration, signal transduction, and cell stability. In particular, integrin-alphaV, along with its beta dimer partners -beta1, -beta3, -beta5, -beta6, and -beta8, has been shown to be intricately linked to TGF-beta signaling dysregulation in the progression of many diseases including cancer, heart disease, and the fibrosis of various organs (Munger and Sheppard, 2011; Worthington et al., 2011; Mamuya and Duncan, 2012; Conroy et al., 2016).

Activated TGF-beta binds to TGF-beta receptor II to form a heterotetrameric complex with TGF-beta receptor I, initiating a signaling cascade ending with activation of the transcription factor Smad2/3 and a canonical gene expression program. This program includes TGF-beta itself as well as extra/matricellular proteins including integrins, collagen isoforms, fibronectin, osteopontin, periostin, thrombospondins, and other matrix remodeling proteins including matrix metalloproteases (MMPs) (Ignotz and Massague, 1986; Leask and Abraham, 2004; Pohlers et al., 2009). This process has been shown to be upregulated in mouse models of MDC1A. Indeed, Mehuron et al. (2014) showed that phosphorylated smad2/3 was increased during the early development of *DyW* mice. This very early onset is similar to that observed in MDC1A patients. In addition to increased activity of the phosphorylated smad2/3, there was a parallel downregulation of inhibitory smad7, suggesting even further amplification of

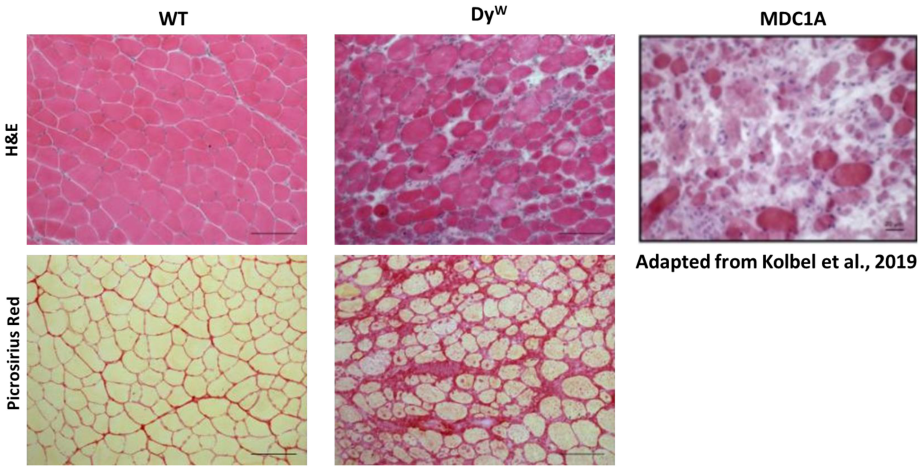

FIGURE 1 | Histological analysis shows an extensive amount of fibrosis in muscles from *DyW* mice, a model for MDC1A, during early development. Tibialis anterior muscle isolated from 4 weeks old wild-type (WT) and *DyW* are stained with Hematoxylin and Eosin **(top)** and Picrosirius Red **(bottom)**. Representative images show established muscle pathology with fiber size variability, infiltrating cells, and increased interstitial space in the DyW tissue compared to the healthy WT sections **(top)**. Picrosirius red staining of the TA muscle reveals extensive deposition of collagen by 4 weeks of age in these mice. For reference, we have included an image of a muscle biopsy from a seven-year-old boy stained with Hematoxylin and Eosin (Kolbel et al., 2019).

the TGF-beta signaling pathway. Downstream genes encoding extra/matricellular proteins were also upregulated including fibronectin, osteopontin, periostin, and collagen I (Mehuron et al., 2014). Increased TGF-beta signaling as well as matricellular protein expression was persistent throughout the entire postnatal development of *DyW* mice, thus demonstrating a chronically dysregulated matrix remodeling process that may be etiological to the pathology following loss of laminin-alpha2 (Accorsi et al., 2015). This is consistent with previously mentioned clinical data from MDC1A and Fukuyama muscular dystrophy patients showing significant upregulation of ECM genes indicative of active fibrosis at very early stages of disease (Taniguchi et al., 2006), supporting the hypothesis that fibrosis is at the center of CMD pathology.

CONSEQUENCES OF CHRONICALLY DYSREGULATED TGF-BETA SIGNALING: MYOFIBROBLAST TRANSDIFFERENTIATION

A potential consequence of chronically dysregulated TGF-beta signaling and matricellular protein upregulation is the transdifferentiation of various cell lineages into myofibroblasts. Myofibroblasts are contractile, hyper-secretory fibroblastic cells that are activated following tissue injury and are part of the normal wound healing process. Their secretions and contractile properties are critical to facilitate migration of inflammatory and tissue-specific stem cells to the site of injury as well as to induce wound closure. In the context of normal wound healing, myofibroblasts undergo mass apoptosis or de-differentiate back to their original cell types in response to decreased matrix stiffness. However, in pathological scenarios, these cells can persist and significantly exacerbate fibrotic pathology (Hinz, 2007, 2010; Hinz et al., 2007; Klingberg et al., 2013).

Multiple cell types have been shown to transdifferentiate into myofibroblasts via TGF-beta signaling via induction of epithelial-to-mesenchymal transdifferentiation (EMT)-related transcription factors (Slug, Snail, and Twist) (**Figure 2**). While fibroblasts are the most prominent cell type to transdifferentiate into myofibroblasts, hepatic stellate cells, smooth muscle cells, and bone marrow-derived progenitors like pericytes and fibrocytes have also been shown to undergo this transition (Mamuya and Duncan, 2012). Interestingly, myoblasts are also capable of transdifferentiating to myofibroblasts. It has been shown that C2C12 myoblasts can undergo this process in a TGF-beta, sphingosine-1 phosphate receptor3 (S1P3), Rho/Rho kinase (ROCK)-dependent pathway (Cencetti et al., 2010). Results in chronic injury models suggest that the process of myofiboblast transdifferentiation occurs in skeletal muscle and drives disease pathology in part due to dysregulation and accumulation of fibroadipogenic progenitors (FAPs). It has been shown that PDGFRalpha- and Tcf4-positive cells in denervated or mdx mice co-label with the myofibroblast marker alpha-smooth muscle actin that coincides with excessive fibrosis (Contreras et al., 2016). Additionally, it has been shown that excessive TGF-beta signaling prevents apoptosis of pro-fibrogenic FAPs during chronic injury thereby promoting a fibrotic environment (Lemos et al., 2015). These results suggest that in scenarios of chronic dysregulation of a pro-fibrotic cascade, such as in muscular dystrophies, persistent cellular identity changes away from a myogenic and toward a fibrogenic phenotype could be playing major roles in driving disease pathology.

This phenomenon has also been shown in other models of muscle fibrosis. In end-stage *mdx* mice, chronic TGF-beta signaling results in a loss of myogenic cell identity and a push toward a fibrogenic mesenchymal cell identity (Pessina et al.,

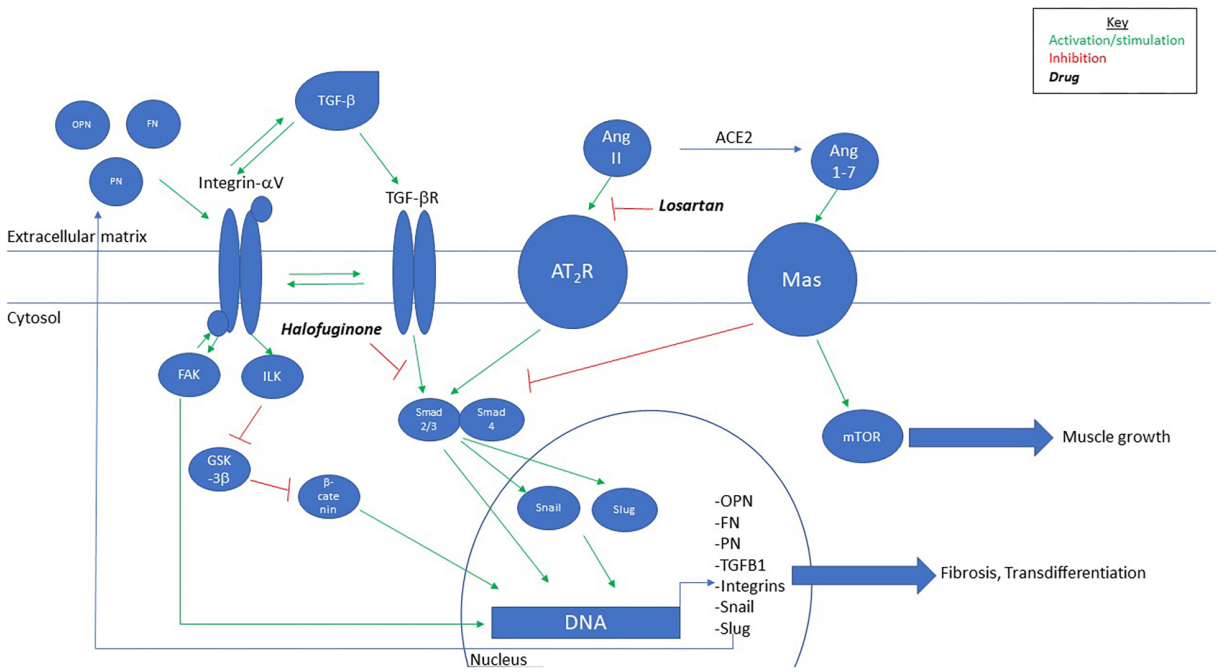

FIGURE 2 | Proposed model for signaling pathway of fibrosis in MDC1A. TGF-beta, through integrin-alphaV-mediated activation, leads to upregulation of extra-/matricellular proteins that feedback to activate integrin-alphaV and further activation of TGF-beta. Chronic signaling leads to myofibroblast differentiation and fibrosis. Intervention with Losartan, Halofuginone, or Angiotensin-(1–7) inhibits TGF-beta-mediated fibrosis. OPN, osteopontin; FN, fibronectin; PN, periostin.

2015). Likewise, we observed expression of integrin-alphaV on matured myofibers from *DyW* mice (Accorsi et al., 2015). This protein is typically only expressed in cells of a mesenchymal lineage in adult skeletal muscle (Sinanan et al., 2008; Murray et al., 2017). It is interesting to note that a similar phenomenon has been observed in aged human skeletal muscle where increased TGF-beta signaling and acetylation of the 27th residue of histone H3 (H3K27ac) on ECM genes pushes satellite cells away from myogenic fates and toward fibrogenic fates (Zhou et al., 2019). Taken together, this data demonstrate that satellite cells have the ability to become fibrotic effector cells in response to chronic TGF-beta signaling.

CHRONIC DYSREGULATION OF TGF-BETA SIGNALING LEADS TO DEFECTIVE MYOGENESIS

The consequences of chronically dysregulated TGF-beta signaling in muscle result in myomatrix remodeling/stiffening (Hinz, 2015) ultimately leading to impaired myogenesis and muscle function. Increased matrix stiffness has been shown to inhibit myogenesis in C2C12 myoblasts plated on stiff surfaces (Engler et al., 2004; Romanazzo et al., 2012). Additionaly, *in vivo* atomic force microscopy (AFM) measured a 50% increase in overall stiffness due to fibrosis in *mdx* mice compared to age-matched wild-type controls (Puttini et al., 2009). These studies have not been performed in models of MDC1A.

The transcription factors downstream of TGF-beta signaling that drive myofibroblast transdifferentiation have been shown to inhibit myogenic differentiation. Snail/Slug normally blocks MyoD binding to differentiation enhancer elements to prevent precocious differentiation. Overexpression of either protein, however, completely inhibits myoblast differentiation (Zhao et al., 2002; Soleimani et al., 2012). Twist has also been shown to play inhibitory roles in myogenesis. Overexpression of Twist during C2C12 differentiation induces downregulation of myogenin and reverses the course of differentiation (Mastroyiannopoulos et al., 2013). The marked increase of these transcription factors in MDC1A mouse models suggests not only that myofibroblast transdifferentiation is occurring, but that they are also playing active roles in inhibiting myogenesis.

CONTRIBUTIONS OF RENIN–ANGIOTENSIN SYSTEM

Dysregulation of the RAS also contributes to the pathogenic fibrotic process via crosstalk with the TGF-beta pathway. RAS plays a systemic role across the body by regulating blood pressure, maintaining fluid and electrolyte homeostasis, and stimulating the production of aldosterone in the adrenal cortex (Sparks et al., 2014).

Renin–angiotensin system signaling splits into two pathways which act in opposition to maintain homeostasis. In the classical pathway, renin cleaves angiotensinogen to form the inactive decapeptide angiotensin I [Ang-(1–10)]. Ang I is then

converted to Angiotensin II [Ang-(1–8)] by the angiotensin-converting enzyme (ACE). Ang II binds Angiotensin type 1 and type 2 receptors (AT_1R and AT_2R) to drive vasoconstriction, aldosterone synthesis, sodium retention, inflammation, fibrosis, and oxidative stress. Globally, the circulating levels of renin and angiotensinogen are produced by the kidney and liver, respectively (Stroth and Unger, 1999; Atlas, 2007; Sparks et al., 2014; Bavishi et al., 2016). However, individual tissues, including skeletal muscle, can generate local RAS signaling. Both global and local dysregulation of RAS signaling through Ang II leads to detrimental effects across different organ systems, contributing to cardiovascular diseases, diabetes, and kidney failure. Additionally, chronic activation of Ang II signaling is implicated in cardiac, renal, hepatic, lung, and skeletal muscle fibrosis (Kawano et al., 2000; Rodriguez-Vita et al., 2005; Cabello-Verrugio et al., 2012). Indeed, ACE, Ang II, and AT_1R are upregulated in DMD, BMD, and MDC1A patients as well as in mouse models of these muscular dystrophies (Sun et al., 2009; Mehuron et al., 2014). Ang II upregulates *TGFB1* and *SMAD2/3* expression levels and enhances nuclear translocation of phosphorylated Smad2/3 (Fukuda et al., 2000; Rodriguez-Vita et al., 2005; Carvajal et al., 2008). Together then, these pathways converge to drive myofibroblast transdifferentiation and activation contributing to the fibrotic remodeling of the ECM.

To balance the actions of Ang II signaling, it has been demonstrated that the RAS pathway can be shunted toward formation of the heptapeptide angiotensin-(1–7) [Ang-(1–7)], which acts antagonistically to Ang II. This pathway promotes vasodilation as well as anti-inflammatory, anti-fibrotic, and anti-proliferative pathways mediated by Mas receptor signaling. Ang-(1–7) can be synthesized through three different pathways: (1) Ang II conversion to Ang-(1–7) via angiotensin-converting enzyme 2 (ACE2), (2) Ang I conversion to Ang-(1–7) through enzymes neprilysin 24.11 (NEP), thimet oligopeptidase 24.15 (TOP), or prolyl oligopeptidase 21.26 (POP), and (3) Ang I conversion by ACE2 to form angiotensin-(1–9), which can then further be converted by ACE to form Ang-(1–7). Out of these three pathways, Ang II is the major substrate for synthesis of Ang-(1–7). Therefore, the profibrotic effects of Ang II could be combatted along the ACE2 axis through increased conversion of Ang II to Ang-(1–7).

Indeed, several studies have reported beneficial anti-fibrotic effects by inducing RAS signaling toward the ACE2/Ang-(1–7)/Mas receptor axis in the skeletal muscles of dystrophic mice. In work performed by Acuna et al. (2014), inhibition of the Mas receptor in the mdx model of DMD lead to impaired muscle histopathology due to increased TGF-beta signaling and fibrosis. Infusion of Ang-(1–7) via osmotic pumps had the opposite effects: delivery of the heptapeptide improved mdx skeletal muscle morphology, including decreased inflammation and fibrosis (Acuna et al., 2014). Additionally, Ang-(1–7) treatment protected wild-type mouse skeletal muscle from the TGF-beta-induced fibrosis (Abrigo et al., 2016). Likewise, Sabharwal et al. (2014) reported similar effects in a mouse model for delta-Sarcoglycanopathy. Early intervention with oral delivery of the Ang-(1–7) peptide (TXA127) decreased oxidative stress and fibrosis in skeletal muscle of *Sgcd*−/− mice (Sabharwal et al., 2014). Collectively, these data would suggest testing Ang-(1–7) as a single-mode therapy in preclinical models of MDC1A.

THERAPEUTIC STRATEGIES TARGETING FIBROSIS

As we have mentioned above, recent advances have elucidated that secondary pathomechanisms downstream of the primary genetic defects can become self-ruling disease drivers in their own right. As such, generating therapies targeted at arresting and/or reversing these secondary pathologies, such as fibrosis, can have a tremendous impact on the progression of the disease as well as quality of life. Despite extensive research into some of the major disease drivers, there remains no cure or treatment for MDC1A.

Most strategies that have been used to alleviate fibrosis directly or indirectly target TGF-beta signaling pathways. One such therapy that has been tested in different mouse models of MDC1A, showing remarkable amelioration of fibrosis, is Losartan. It is an FDA-approved AT_1R blocker (ARB) that is routinely used to control hypertension in adults but is also prescribed to children. It has been shown to be a potent anti-fibrotic and anti-inflammatory agent that works in part by reducing signaling along the Ang II/AT_1R axis, which indirectly attenuates dysregulated TGF-beta signaling. In 2012, Elbaz et al. (2012) and Meinen et al. (2012) independently published results showing Losartan treatment resulted in reduced TGF-beta signaling. In an earlier work, Cohn et al. (2007) showed in 2007 that Losartan treatment lowered the levels of thrombospondin-1 in *mdx* mice. They reasoned Losartan treatment led to prevention of thrombospondin-1-mediated activation of latent TGF-beta, likely by causing some conformational changes to the latent TGF-beta complex (Schultz-Cherry and Murphy-Ullrich, 1993). More recently, Accorsi et al. (2015) suggested that lowering of integrin-alphaV, a potent activator of TGF-beta, could be instrumental in mediating the anti-fibrotic effects of Losartan. We comprehensively showed that integrin-alphaV and its cognate beta partners were markedly downregulated in *DyW* mouse muscle treated with Losartan. More convincingly, we also showed the levels of active TGF-beta, but not its latent form, were reduced in response to Losartan treatment (Accorsi et al., 2015). These findings suggest a possible interplay of integrin-alphaV and Losartan; however, more work is warranted to establish this link. While it is less clear the relative role that thrombospondin-1 or integrin-alphaV play to activate TGF-beta in the context of fibrotic pathology in MDC1A, it is plausible that Losartan targets more than one pathway to strongly abrogate TGF-beta signaling.

While Losartan has been shown to reduce inflammation and fibrosis, it does not lead to increases in body or muscle weight in *DyW* or Dy^{2J} mice (Elbaz et al., 2012; Accorsi et al., 2016). Since failure to thrive is one of the most frequent complications in MDC1A, a successful therapeutic strategy needs to improve muscle growth (Philpot et al., 1999). Therefore, it is less likely that Losartan can be a single-mode therapy for MDC1A. Pairing the anti-inflammatory/anti-fibrotic effects of Losartan with the pro-myogenic effects of IGF-1/growth hormone indeed provided

a synergistic benefit as the dual therapy resulted in significant mitigation of inflammation and fibrosis that allowed for the pro-myogenic effects of IGF-1 to facilitate improved overall growth in *DyW* mice (Accorsi et al., 2016).

While we have found Losartan to be anti-myogenic in terms of terminal myotube differentiation, Ang 1–7 has the potential to actually induce muscle growth via Mas receptor-mediated mTOR activation (Morales et al., 2016), which is well known to promote protein synthesis and subsequent muscle growth. It has been shown in a model of muscle atrophy that treatment of Ang (1–7) prevents muscle mass and function loss due to disuse (Morales et al., 2016) further supporting the possibility of its role as a single-agent therapeutic to inhibit fibrosis and promote myogenesis but remains to be tested in lama2-related pathology. A pharmaceutical formulation of the Ang 1–7 peptide, TXA127, has been granted Orphan Drug designation for muscular dystrophies; however, Context Therapeutics (previously Tarix Orphan) has yet to initiate clinical trials for these indications.

Yet another compound that is known to inhibit the TGF-beta signaling pathway is Halofuginone (Juarez et al., 2017). It is a synthetic derivative of Febrifugine which is a naturally occurring alkaloid found in the roots of hydrangea plants (Zhu et al., 2009). It has been shown to decrease Smad2/3 phosphorylation and prevent fibroblast activation in the muscle tissue of Dy^{2j} mice (Nevo et al., 2010). It should be noted that a phase 1B/2A trial in DMD patients was conducted with HT-100 (a chemical formulation of Halofuginone) by Akashi Therapeutics (NCT01847573). Results have not yet been released.

Interventions that are not directly targeted toward attenuation of TGF-beta signaling have also resulted in amelioration of fibrosis. Both inhibition of a BCL2 family pro-apoptotic protein BAX (Yamauchi et al., 2013) as well as inhibition of GAPDH-Siah1-mediated apoptosis with Omigapil (a deprenyl analog) (Erb et al., 2009) attenuated fibrotic pathology to some extent in preclinical models of MDC1A. Santhera Therapeutics sponsored a single-center interventional trial at the NIH to establish pharmacokinetic (PK) profile and safety/tolerability of Omigapil (NCT01805024). The trial was completed in 2018 and successfully met the primary objectives. While the PK profile of Omigapil was found suitable for further development, the next steps have not yet been announced by the company.

Genetic ablation of matricellular proteins has also been shown to ameliorate fibrosis in models of muscular dystrophy. Deletion of periostin (Lorts et al., 2012) or osteopontin (Capote et al., 2016) have been shown to be protective in mouse models of DMD. Of note, deletion of osteopontin in the severe Dy^{3K} mouse model of MDC1A was not shown to be protective but rather exacerbated the pathology (Gawlik et al., 2017).

Finally, strategies such as AAV-mediated expression of mini-Agrin (Moll et al., 2001), CRISPR/CAS9-mediated over expression of laminin-alpha1 (Kemaladewi et al., 2019), or treatment with recombinant laminin-111 (Rooney et al., 2012) would be logical choices to compensate for the missing laminin-alpha2. *LAMA1* is similar to *LAMA2*, thus overexpression would compensate for lack of *LAMA2* function while minimizing the risk of an immune response to laminin-111. However, maximal translational impact might require a combination of targeting the genetic defect along with some of the secondary pathologies. This is particularly true for a disease like MDC1A because of the congenital onset of fibrosis. Therefore, it may be critical to treat fibrosis in parallel to any approach that will compensate for the missing gene. Proof of concept has been demonstrated in the context of volumetric muscle loss in mice. It has been shown that in order for regenerative therapies to be most effective, amelioration of the fibrotic signaling is required for maximal impact (Larouche et al., 2018).

NON-INVASIVE BIOMARKERS FOR FIBROSIS

Another critical aspect to development of therapeutics is the ability to measure changes in pathology in response to an intervention, ideally in a manner that does not involve the acquisition of muscle biopsies from patients, especially young children. This necessitates the development of non-invasisve biomarkers to measure disease. Numerous methodologies have been developed for this purpose including, but not limited to, serum biomarkers, magnetic resonance imaging (MRI), and electrical impedence myography (EIM).

In order for a biomarker to be effective, it needs to be indicative of disease process and be responsive to treatment. Serum biomarkers of fibrosis have been elucidated in models of lama2-deficient mice. Our lab showed that both latent and active levels of TGF-beta are increased in the serum of DyW mice but only levels of active TGF-beta were decreased in response to anti-fibrotic treatment (Losartan). We also showed that levels of Timp1 were overexpressed in DyW serum and were also decreased in response to Losartan (Accorsi et al., 2015).

Magnetic resonance imaging is also a robust measure of muscle mass, contractile area, inflammation, and fibrosis. We showed that pixel-by-bixel analyses of T2 MR maps were reduced in DyW mice compard to WT, indicative of fibrosis, but were rescued in response to anti-fibrotic therapy. These values correlated with changes in muscle fibrosis measured by Sirius red staining as well as collagen-1a gene expression. MR indices also validated the anti-inflammatory properties of Losartan as well as the lack of impact on muscle volume (Vohra et al., 2015).

Electrical impedance myography is another method for measuring intrinsic muscle properties and has been utilized as a pre-clinical and clinical non-invasive biomarker in numerous settings of muscle disease. Hakim et al. (2017) showed that EIM was able to measure significant difference in EIM parameters in DMD canines that significantly correlated with fibrotic build-up in the measured muscles. EIM has also been utilized in multiple clinical settings in FSHD (Rutkove et al., 2007; Mul et al., 2018), ALS (Rutkove et al., 2007, 2017), and DMD (Rutkove et al., 2017) where it was also shown to be responsive to intervention with corticosteroids. EIM was also recently measured in patients with Col6- and LAMA2-CMD showing significant changes in resistance in LAMA2-CMD patients suggestive of fibrosis (Nichols et al., 2018).

CONCLUSION

LAMA2-related muscular dystrophy has, at its root, chronic dysregulated remodeling of the myomatrix. This remodeling results in widespread fibrosis and expression of proteins that have the ability to change the course of muscle development. It is therefore important to understand further the natural disease progression and how these processes drive the etiology of MDC1A to allow elucidation of therapeutic windows and targets. While much of this is known in preclinical models of MDC1A, there is much to be learned about the clinical course of fibrosis, which drives the devastating pathology in patients with CMDs.

AUTHOR CONTRIBUTIONS

AA, MC, and MG: manuscript preparation.

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REVIEW
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Laminin and Integrin in LAMA2-Related Congenital Muscular Dystrophy: From Disease to Therapeutics

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Laminin-α2-related congenital muscular dystrophy (LAMA2-CMD) is a devastating neuromuscular disease caused by mutations in the LAMA2 gene. These mutations result in the complete absence or truncated expression of the laminin-α2 chain. The α2-chain is a major component of the laminin-211 and laminin-221 isoforms, the predominant laminin isoforms in healthy adult skeletal muscle. Mutations in this chain result in progressive skeletal muscle degeneration as early as neonatally. Laminin-211/221 is a ligand for muscle cell receptors integrin-α7β1 and α-dystroglycan. LAMA2 mutations are correlated with integrin-α7β1 disruption in skeletal muscle. In this review, we will summarize laminin-211/221 interactions with integrin-α7β1 in LAMA2-CMD muscle. Additionally, we will summarize recent developments using upregulation of laminin-111 in the sarcolemma of laminin-α2-deficient muscle. We will discuss potential mechanisms of action by which laminin-111 is able to prevent myopathy. These published studies demonstrate that laminin-111 is a disease modifier of LAMA2-CMD through different methods of delivery. Together, these studies show the potential for laminin-111 therapy as a novel paradigm for the treatment of LAMA2-CMD.

Keywords: LAMA2-CMD, Laminin, α7 integrin, muscular dystrophy, muscle, therapeutic

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INTRODUCTION

Laminin-α2-related congenital muscular dystrophy (LAMA2-CMD), also known as merosin deficient congenital muscular dystrophy type 1A (MDC1A), is a genetic disease caused by mutations in the LAMA2 gene encoding the laminin-α2 protein. Severely affected patients present with neonatal hypotonia and delayed motor milestones. Few children achieve independent ambulation, while most develop respiratory insufficiency, proximal joint contractures, and scoliosis. Patients show elevated serum creatine kinase (CK) and inflammatory cell infiltration in muscle biopsies (Konkay et al., 2016). There is currently no cure or effective treatment for LAMA2-CMD.

Laminins are ≈900 kDa heterotrimer glycoproteins composed of α, β, and γ chains. They are expressed in several tissues including skeletal muscle. The main isoforms of laminin expressed in healthy adult skeletal muscle are laminin-211 and 221. Laminin-α2 chain is essential for the assembly of these laminins (review on laminin in skeletal muscle; Holmberg and Durbeej, 2013). The laminin-α2 chain is a 380-kDa protein composed of a 300-kDa N-terminal fragment non-covalently bonded to an 80-kDA C-terminal fragment. The N-terminal domain nucleates the association between other laminin-α2 chains and components of the muscle

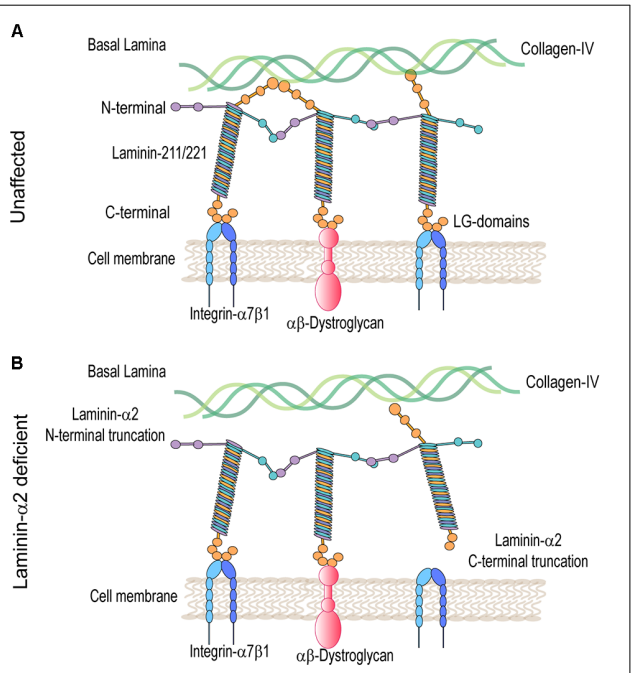

FIGURE 1 | Molecular model in unaffected and laminin-α2-deficient patient muscle. **(A)** Laminin 211/221 heterotrimeric proteins bind through their N-terminal domain to the collagen-IV-rich basal lamina and through their C-terminal domain to muscle cell receptors heterodimers integrin-α7β1 and αβ-dystroglycan protein complexes. **(B)** In LAMA2-CMD, laminins are unable to bind to collagen-IV and/or integrin-α7β1 and α-dystroglycan protein complexes disrupting communication between the basal and muscle cell membrane.

basal lamina including collagen IV and heparan sulfate proteoglycans (Timpl and Brown, 1996). The basal lamina, along with the fibrillar reticular lamina, is a component of the basal membrane or extracellular matrix (ECM; Sanes, 2003). The C-terminal domain is composed of five laminin G (LG) domains, important for cell receptor binding to, predominantly, integrin-α7β1 and the dystroglycan protein complex (DGC; Timpl and Brown, 1996; **Figure 1A**). These linkages modulate communication between the basal lamina of the ECM and muscle cell cytoskeleton. They also provide mechanical support and stabilization to the sarcolemma during muscle contraction.

Mutations in the LAMA2 gene result in various truncations of the laminin-α2 protein that can result in differential disease presentation and progression. Loss-of-function mutations in the LAMA2 gene are the most common cause of severe LAMA2-related congenital muscular dystrophy (LAMA2-CMD). These are mutations affecting the C-terminal domain, the N-terminal domain, or causing complete ablation of the laminin-α2 protein. Alternatively, variant missense mutations, in-frame deletions, and splice site mutations in the LAMA2 gene often result in a milder, limb-girdle-like, late-onset muscular dystrophy (Geranmayeh et al., 2010; Mohassel et al., 2018). Laminin-α2 deficiency causes disruption of the basal lamina, leading to increased susceptibility to mechanical stress and damage of the myofibers within the muscle. Loss of the laminin-211-rich microenvironment negatively impacts satellite cells (SC) and results in defects in muscle regeneration. Muscle damage results in an inflammatory cell infiltrate leading to replacement of functional skeletal muscle with fibrotic tissue, which exacerbates disease progression (Pegoraro et al., 1996; Nguyen et al., 2019).

Laminin-α2-deficient muscles in both mice and patients exhibit major transcriptome and proteome dysregulation. The major upregulated proteins are components of the ECM and proteins related to muscle regeneration (Taniguchi et al., 2006; van Lunteren et al., 2006; Yanay et al., 2019; Kölbel et al., 2019). The main laminin-α2 cell receptors, α-dystroglycan and integrin-α7β1, are also dysregulated in LAMA2-CMD (**Figure 1B**). Integrin-α7β1 plays an important role during SC activation, myoblast adhesion, and survival. Consequently, this integrin is important during embryonic development, regeneration, and repair of adult skeletal muscle. Integrin-α7β1 is found to be disrupted in laminin-α2-deficient muscle of multiple mouse models and human biopsies, and therefore, it was studied in LAMA2-CMD. Therapeutic approaches that aim to restore the basement membrane in LAMA2-CMD include LAMA2 gene replacement, engineered linker proteins mini-agrin and αLNND (Reinhard et al., 2017), and laminin-111 treatment. In this review, we will explore laminin-α2 and its receptor integrin-α7 in the LAMA2-CMD disease context, as well as laminin-111 as a disease modifier and therapeutic target for the treatment of LAMA2-CMD.

INTEGRIN AND LAMININ INTERACTION IN LAMININ-α2-DEFICIENT SKELETAL MUSCLE

Integrins are heterodimeric transmembrane glycoprotein receptors made of non-covalently bound α and β subunits (**Figure 2**). They are essential for cell attachment to the ECM, cell migration, and regulate cellular signal transduction. Integrin clustering and ligand binding to the ECM, including binding to fibronectin, laminins, and collagen, induce conformational changes in integrin, regulating cell adhesion, proliferation, migration, and differentiation (Boppart and Mahmassani, 2019).

Integrin-α7β1 plays a crucial role during embryonic development and in adult skeletal muscle repair by facilitating myoblast adhesion to laminin-111, -211, and -221 (Crawley et al., 1997). Integrin-α7 is encoded by the ITGA7 gene with multiple isoforms produced by developmentally regulated RNA splicing. Alternative RNA splicing of ITGA7 results in integrin-α7A and integrin-α7B cytoplasmic isoforms, as well as integrin-α7X1 and integrin-α7X2 extracellular domain isoforms. The cytoplasmicα7A domain is produced during terminal differentiation of myoblasts and is found in myofibers at the sarcolemma. The cytoplasmic domain α7B-integrin was originally identified during myoblast proliferation (Ziober et al., 1993). The extracellular domain isoform integrin-α7X1 binds strongly to laminin-411, laminin-511, and laminin-521, predominant during muscle embryonic development, while integrin α7X2 binds to laminin-111, expressed during early muscle development. Both integrin α7X1 and α7X2 isoforms bind to laminin-211. This suggests

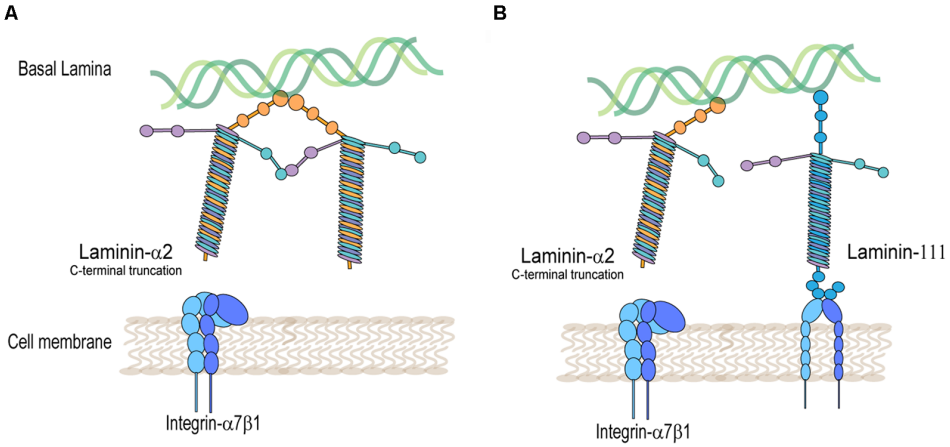

FIGURE 2 | Integrin-α7β1 protein complex is disrupted in laminin-α2-deficient muscle. **(A)** Decreased detection of integrin-α7β1 in the muscle sarcolemma in laminin-α2-truncated muscle. **(B)** Rescued expression of integrin-α7β1 in the muscle sarcolemma by expression of laminin-111 in laminin-α2-deficient muscle.

that integrin-α7X1 is involved during early embryogenesis, while integrin-α7X2 is involved at later stages of skeletal muscle development (von der Mark et al., 2002).

The main alternative isoforms for integrin-β1 include integrin β1A and β1D. Whereas integrins β1B and β1C are considered minor isoforms found only in humans, the integrin β1D subunit is expressed in skeletal muscle sarcolemma and associates with integrin-α7 subunit (Belkin et al., 1997).

Laminin-α2 is the main ligand for integrin-α7β1 in skeletal muscle. Integrin-α7 is localized at the neuromuscular and myotendinous junctions and throughout the muscle sarcolemma (Burkin and Kaufman, 1999). Targeted deletion of the integrin-α7 gene in mice results in a mild form of congenital muscular dystrophy and vascular defects (Mayer, 2003; Flintoff-Dye et al., 2005; Rooney et al., 2006). Histopathological studies of this mouse show variation in fiber size, a high percentage of centrally nucleated fibers, elevated CK, mononuclear cell infiltration, and little to no degeneration/regeneration cycles. Expression of laminin-α2 in this mouse, as well as expression of integrin-β1, is unaffected (Mayer et al., 1997). Consistent with this mouse model, patients identified as integrin-α7-negative and laminin-α2-positive present with mild myopathy from birth with no evidence of constituent muscle regeneration (Hayashi et al., 1998). This indicates that other integrin subunits that pair with integrin β1 can partially compensate for the loss of integrin-α7 in muscle.

On the other hand, mutations in the laminin-α2 protein disrupt the expression of integrin-α7β1 in skeletal muscle (**Figure 2A**). This was shown in several laminin-α2-deficient mouse models and human biopsies of LAMA2-CMD. Using immunofluorescence, studies typically show a decreased detection of integrin-α7B and integrin-β1 subunits in the sarcolemma of laminin-α2-deficient muscle. The dy/dy mouse model of LAMA2-CMD, which has low expression of full-length laminin-α2, exhibits reduced levels of integrin-α7A, integrin-β1D, and irregular integrin-α7B expression (Vachon et al., 1997). Similarly, the dy2J/dy2J mouse model of LAMA2-CMD, which has a mutation in the N-terminal domain, also shows a reduction in integrin-α7A and patchy expression in integrin-α7B (Hodges et al., 1997; Vachon et al., 1997; Cohn et al., 1999). Durbeej (2010) summarizes in a comprehensive review the different mouse models of LAMA2-CMD (Durbeej, 2010).

A case study using biopsies from congenital muscular dystrophy patients with laminin-α2 chain deficiency showed reduced integrin-α7B expression in the sarcolemma of six LAMA2-CMD patients. However, the level of laminin-α2 expression did not correlate with the level of integrin-α7β1 reduction. Restoration of laminin-α2 rescued expression of integrin-α7β1 in the sarcolemma (Vachon et al., 1997). Moreover, the integrin-α7 knockout mouse show no change in laminin-α2 levels (Mayer et al., 1997). Gawlik and Durbeej (2015) reported that loss of integrin-α7 and laminin-α2 in the dy3k/dy3k mouse model, did not result in more severe myopathy. This double knockout mouse presented similar levels of fibrosis, apoptosis, lifespan, and body weight. This same group had previously showed that loss of β-sarcoglycan or dystroglycan in laminin-α2-deficient dy3k/dy3k mice resulted in a dramatically worse muscle disease (Gawlik et al., 2014). This may indicate that the integrin-α7β1 complex has intersecting roles with laminin-α2, while the DGC complex, which includes β-sarcoglycan and dystrophin, may have non-redundant complementary roles. These observations indicate a strong dependence in the expression of integrin-α7β1 receptor to laminin-α2 ligand. This may also indicate that laminin-α2 ligand can compensate for its interaction with secondary integrin complexes.

Doe et al. (2011) showed improvements in myopathy in the dyW mouse model of LAMA2-CMD by transgenically overexpressing integrin-α7. The dyW model presents downregulated and N-terminally truncated levels of laminin-α2 along with severe, early myopathy (Guo et al., 2003). This study showed a threefold increase in lifespan, reduced CLN percent, and reduced macrophage infiltration (Doe et al., 2011). A previous study showed that overexpressing rat integrin-α7 in C2C12 myoblasts promoted adhesion to laminin and overall cell

survival, while it did not affect differentiation (Liu et al., 2008). Increased myoblast adhesion to other laminin isoforms may explain the improvements in integrin-α7 overexpressing dyW mouse; however, this remains to be fully explored *in vivo*.

Other integrin dysregulation may drive disease progression in laminin-α2-deficient muscle. Integrin-αV, integrin-α5, integrin-β1, and integrin-β3 were shown to be upregulated in the dyW model of LAMA2-CMD (Accorsi et al., 2015). Integrin-αV was proposed to have a role in TGF-β-mediated fibrosis. More research investigating aberrant disruption of integrin receptors in laminin-α2-deficient skeletal muscle is needed to further our understanding of their roles in LAMA2-CMD disease progression.

CELL-BASED LAMININ-111 TREATMENT OF SKELETAL MUSCLE

Skeletal muscle has the capacity to regenerate due to the presence of resident stem cells also known as SCs. These cells are located between the sarcolemma and the basement membrane of muscle fibers (Mauro, 1961). SCs are myogenic progenitors activated in response to muscle injuries and trauma. Upon activation, they proliferate, migrate, and differentiate into myotubes fusing into damaged muscle fibers or generating *de novo* fibers (Morgan and Partridge, 2003; Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). There are two types of SC division: apical and symmetrical. Apical or asymmetric division gives rise to two daughter cells of which one is located basally with respect to the other cell, while undergoing differentiation. Symmetrical division, on the other hand, will give rise to two daughter cells that undergo quiescence and maintain the SC pool (Yin et al., 2013).

The ECM, while essential for maintaining muscle fiber integrity (Grounds et al., 2005), also has a crucial function in supporting SC proliferation, migration, and differentiation (Öcalan et al., 1988; Sanes, 2003; Silva Garcia et al., 2019). Disruption of the ECM surrounding SCs, also known as the SC niche, has negative impact on SCs that can lead to failed myogenesis and impaired muscle regeneration (Thomas et al., 2015). The major protein components of the ECM in this niche include collagen (Gillies and Lieber, 2011) and laminin (Yurchenco and Patton, 2009). Laminin-211 and laminin-221, previously known as merosin and merosin-S, are the most abundant laminin isoforms in the adult skeletal muscle (Nissinen et al., 1991; Sasaki et al., 2002). Disrupted levels of laminin-α2 in LAMA2-CMD compromises the integrity of the SC niche, affecting the overall regenerative capacity of the skeletal muscle (Mohassel et al., 2018; Yurchenco et al., 2018). An *in vitro* study by Summers and Parsons (1981) showed that SCs isolated from the dy/dy mouse model of LAMA2-CMD, presented an 80% reduction in muscle colony formation compared to the wild-type controls at 5 months of age. This defect was not shown neonatally or at 1 week of age.

Another *in vitro* study using LAMA2-deficient embryonic stem cells showed that these cells had no defect in differentiating into cardiomyocytes, smooth muscle, and myotubes compared to the wild type. However, the myotubes formed were unstable, detached, collapsed, and degenerated (Kuang et al., 1998a). They found no proliferation or differentiation defects but potential ineffective/abortive development of myotubes (Summers and Parsons, 1981). This points at the role for laminin-α2 in the maintenance of fully differentiated and mature myofibers, essential for myotube stability and survival. An *in vivo* study in mice showed that homozygous LacZ insertion in the LAMA2 gene, effectively disrupting LAMA2 expression, results in incomplete muscle repair and presence of immature myofibers. This study also showed that the early expression of laminin-α2 is important in early embryonic and adult myogenesis (Kuang et al., 1999). Treatment with laminin-α2 protein rescued the instability of LAMA2-CMD myotubes isolated from patient samples (Vachon et al., 1996). An *in vivo* study found that laminin-α2 expression increased levels of SCs in dystrophic gastrocnemius and triceps of dy/dy mice. This provided evidence on the importance of laminin-α2 in the process of skeletal muscle early myogenesis and regeneration of adult muscle. LAMA2 gene replacement in dyW/dyW and dy2J/dy2J mice was capable of restoring the overall health and lifespan of these mouse models of LAMA2-CMD and could serve as a potential therapeutic target (Kuang et al., 1998b). However, immune response against exogenous and now foreign laminin-α2 protein in LAMA2-CMD could impair the benefits of this therapy and exacerbate disease severity.

To address this issue, other laminin isoforms are the target of interest for the treatment of LAMA2-CMD. Laminin-α4 and laminin-α5 are present and upregulated in laminin-α2-deficient muscle (Patton et al., 1997; Kölbel et al., 2019). These laminin isoforms are involved in myogenesis. However, their increased expression in laminin-α2-deficient muscle does not fully rescue its regenerative capacity. Moreover, upon SC activation, an increase in laminin-α4 and laminin-α5 expression is detected, followed by a transient deposition of laminin-α1 in the SC niche (Ishii et al., 2018; Rayagiri et al., 2018). Loss of laminin-α1 impairs SC proliferation and self-renewal leading to defective muscle regeneration indicating its role in SC cycle. Laminin-α1 effects on SC are mediated through integrin-α6 cell receptor and promote SC apical cell division (Rayagiri et al., 2018).

Laminin-α1 contains the highest amino acid homology to laminin-α2 compared to all other laminin chains, and therefore, it is a therapeutic target for LAMA2-CMD. The Laminin-111 heterotrimer differs only in homologous α1 subunit compared to laminin-211. Laminin-111 can be purified from the Engelbreth–Holm–Swarm (EHS) murine sarcoma cell line, where it was first discovered. Several groups reported that *in vitro* treatment with EHS laminin-111 promotes myoblast survival while promoting proliferation and migration (Silva-Barbosa et al., 2008; Goudenege et al., 2010). In a biomaterial study, it was shown that increasing concentrations of laminin-111 integrated into fibrin hydrogels presented a dose-dependent myogenic marker effect. C2C12 myoblasts seeded onto fibrin hydrogel conjugated with a low concentration of laminin-111 showed an increase in myogenin transcription factor, promoting differentiation. At a ninefold increased concentration of

laminin-111–fibrin hydrogel, C2C12s showed an increase in MyoD expression, promoting proliferation. This same study reported a dose-dependent increase in VEGF and decrease in IL-6 cytokine secretions effect after 4 days post-seeding onto laminin-111-supported fibrin gels (Marcinczyk et al., 2017). Another study developed hydrogels composed of poly-ethyl glycol conjugated with diacrylate (PEGDA) with and without 5% and 10% laminin-111 (PEGLM). The 5% PEGLM hydrogel showed a more porous structure compared to the other gels, indicating a more ECM-like structure. C2C12s were also seeded in each condition showing flat and spread out morphology in the 5% PEGLM ideal for myofiber formation, compared to rounded, multicellular clusters found in the other hydrogel conditions. A 5% PEGLM C2C12s showed a significant increase in EGF and IL-6 secretion as well as myogenin transcription factor (Ziemkiewicz et al., 2018).

Laminin-111–hydrogel studies show the potential for this biological to affect ECM morphology, alter myoblast differentiation and myokine secretion. Consistent with these studies, SCs expanded *in vitro* on laminin-111–hydrogel were later engrafted onto *mdx* mouse model of Duchenne muscular dystrophy (DMD) and more efficiently regenerated into the dystrophic muscle compared to cells expanded without laminin-111 (Ross et al., 2012). Further studies are necessary to characterize the SC niche and its role in normal muscle regeneration and in the context of LAMA2-CMD. These studies indicate the potential for laminin-111 to structurally support myogenic activity, activate cellular signaling pathways to promote survival, enhance SC engraftment, and promote regeneration in laminin-α2-deficient skeletal muscle.

LAMININ-111 PROTEIN THERAPEUTICS IN LAMA2-CMD MOUSE MODELS

Several groups tested the hypothesis that upregulation of endogenous laminin-α1 or exogenous treatment with laminin-111 protein can alleviate disease progression in mouse models of DMD and LAMA2-CMD (**Table 1**). A study by Rooney et al. (2009) treated the *mdx* mouse model of DMD with weekly intraperitoneal doses of EHS laminin-111. Their results showed an increase in protein levels of integrin-α7 in mice and human DMD myoblasts. They also showed reduced CK, Evans Blue dye-positive fibers, and centrally located nuclei fibers, indicating an increase in sarcolemma stability in dystrophin-deficient muscle (Rooney et al., 2009). These studies were followed by intramuscular treatments with EHS laminin-111 in the golden retriever muscular dystrophy (GRMD) dog model of DMD. The GRMD dog model more closely recapitulates disease progression in DMD compared to the *mdx* mouse model (Kornegay, 2017). These studies showed an increase in muscle regeneration and repair and *in vivo* force measurements in the dog's hindlimbs (Barraza-Flores et al., 2019).

Similarly, Rooney et al. (2012) treated the dyW mouse model of LAMA2-CMD, with weekly intraperitoneal injections of EHS laminin-111. These studies reported an increase in life-span and a decrease in centrally located nuclei fibers, fibrosis, and immune infiltration. In a subsequent study in dyW mouse, intramuscular injections with EHS laminin-111 showed improvements in muscle repair post cardiotoxin treatment of hindlimbs. Treated muscles showed increased integrin-α7β1 complex, increased myofiber size, and number (van Ry et al., 2014).

Other groups explored the transgenic overexpression of laminin-α1 in several mouse models of LAMA2-CMD and DMD. Overexpression of laminin-α1 results in proper assembly of laminin-111 and translocation to the sarcolemma as demonstrated by several transgenic mice using immunofluorescence. Gawlik et al. (2011) showed that the transgenic overexpression of laminin-α1 in the *mdx* mouse model of DMD, while increasing levels of integrin-α7β1, had little to no effect in myopathy. This could mean that the presence of endogenous laminin-211/221 in the *mdx* mice may reduce the efficacy of laminin-α1 transgenic expression. These studies may also indicate that in dystrophin-deficient muscle, exogenous treatment can repair sarcolemmal integrity, perhaps through signaling stimulated exclusively through an external laminin-111 receptor binding.

Transgenic overexpression of laminin-α1 was also achieved in two different models of LAMA2-CMD. Transgenic expression of laminin-α1 in dy3K mice showed a drastic increase in survival, weights, decreased centrally located nuclei, and alleviated myopathy (Gawlik et al., 2004). This model was also used to investigate the role integrin-α7β1 plays in laminin-α1 treatment of LAMA2-CMD. They found that the non-transgenic dy3K mouse already shows significantly increased synthesis of integrin-α7β1 (via transcript and protein levels) compared to the wild type. However, the integrin-α7 subunit increase was not detected in the skeletal muscle sarcolemma, indicating that it was not translocated from the cytoplasm to the sarcolemma. Interestingly, when laminin-α1 was transgenically overexpressed, integrin-α7 was detected in the sarcolemma of the muscle (Gawlik et al., 2006b) perhaps due to available ligand-binding receptors (**Figure 2B**). This provides a potential mechanism of action in which overexpression of integrin-α7 is able to prevent muscle disease in LAMA2-CMD. Additionally, aged dy3K mice overexpressing laminin-α1 had a lifespan of up to 1.5–2 years of age, and all non-transgenic dy3K mice died. These older laminin-α1 transgenic dy3K mice had improved survival; however, they had significantly lower weights, muscle strength, and exhibited muscle fibrosis compared to the wild type (Gawlik and Durbeej, 2010).

A new transgenic mouse able to express laminin-α1 in the peripheral nervous system of the dy3K mouse was reported. Results showed rescued peripheral neuropathy such as improved Schwann cell basement membrane and nerve conduction (Gawlik et al., 2006a). In order to show that laminin-111 is a disease modifier of LAMA2-CMD, laminin-α1 was overexpressed in a model with a different LAMA2 mutation, the dy2J. These studies provided a comparison between the most severe, laminin-α2 negative, and the less severe, laminin-α2-reduced mouse models. Transgenic dy2J mouse also showed laminin-α1 expression in the heart, skeletal muscle, and sciatic nerve. Functional assessments showed increased grip strength and activity, while histopathology revealed reduced central

TABLE 1 | Laminin-111 therapy articles in muscular dystrophy animal models.

References	Method	Model	MD*	Results
Gawlik et al. (2004)	Transgenic over expression of laminin-α1	Dy^{3K} mouse model	LAMA2-CMD	- Laminin-α1 expressed in skeletal muscle - Rescued myopathy - Decreased CLN, increased survival and weights
Gawlik et al. (2006a)	Transgenic over expression of laminin-α1	Dy^{3K} mouse model	LAMA2-CMD	- Laminin-α1 expressed in peripheral nerves - Delayed myopathy - Restored peripheral neuropathy
Gawlik et al. (2006b)	Transgenic over expression of laminin-α1	Dy^{3K} mouse model	LAMA2-CMD	- Translocation of integrin α7β1 protein complex to the sarcolemma in LAMA1-overexpressing mice
Rooney et al. (2009)	i.p. EHS laminin 111	Mdx mouse model	DMD	- Increased sarcolemma stability - Increased integrin α7β1 protein complex
Gawlik and Durbeej (2010)	Transgenic overexpression of laminin-α1	Dy^{3K} mouse model	LAMA2-CMD	- 1.5- to 2-year old LAMA1 overexpressing mice shows decreased myopathy - Small differences in weights, fibrosis, CK levels compared to wild-type controls
Gawlik et al. (2011)	Transgenic overexpression of laminin-α1	Mdx mouse model	DMD	- No changes in CLN, fiber size, sarcolemma damage, grip strength, CK - Increased integrin α7β1 protein complex
Rooney et al. (2012)	i.p. EHS laminin 111	dy^{W} mouse model	LAMA2-CMD	- Increased survival - Decreased fibrosis, immune infiltration, and CLN
Ross et al. (2012)	Laminin-111-treated myoblast engraftment	Mdx	DMD	- Myoblasts expanded on laminin-111 contributed more to muscle regeneration in *mdx* mouse model compared to fibronectin-expanded cells
van Ry et al. (2014)	i.m. EHS laminin 111	dy^{W} mouse model	LAMA2-CMD	- Laminin-111 restores muscle regeneration in cardiotoxin-treated muscle
Perrin et al. (2017)	CRISPR/dCas9 overexpression of laminin-α1	C2C12 myoblasts and Mdx mouse model	DMD	- Laminin-α1 expression in C2C12 and *mdx* mouse model
Gawlik et al. (2018)	Transgenic overexpression of laminin-α1	Dy^{2J}	LAMA2-CMD	- Decreased immune cell infiltration, fibrosis - Increased activity and female weights
Barraza-Flores et al. (2019)	i.m. EHS laminin 111	GRMD dog model	DMD	- Increased regeneration, *in vivo* force measurements, muscle weight - Decreased CLN
Kemaladewi et al. (2019)	CRISPR/dCas9 overexpression of laminin-α1	Dy2J mouse model	LAMA2-CMD	- Laminin-α1 expression in skeletal muscle - Increased functional and strength measurements - Improved nerve conduction

*Note. Articles are in chronological order indicating the animal model, related human disease, and a summary of most impactful results. *MD, muscular dystrophy; LAMA2-CMD, laminin-α2-related congenital muscular dystrophy; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; EHS, Engelbreth–Holm–Swarm; CLN, centrally located nuclei; CK, creatine kinase; CRISPR, clustered regularly interspaced short palindromic repeats.*

nucleation and fibrosis (Gawlik et al., 2018). Together, these studies indicate that laminin-111 can modify muscle disease progression in LAMA2-CMD independent of LAMA2 mutation and is a therapeutic target for LAMA2-CMD.

Ubiquitous expression of laminin-α1 in dystrophic skeletal muscle could be achieved through gene replacement using adeno-associated virus (AAV) delivery. However, this approach is currently limited by the ∼10-kb size of the LAMA1 and LAMA2 cDNAs. Production of micro-laminin-α2 retains the activity if the full protein is a possibility, but challenges include producing constructs that successfully associate with laminin-β1, laminin-β2, and laminin-γ1 chains and are functional.

Recent advancements in CRISPR/Cas9 technology showed the capacity for catalytically inactive dCas9 (or dead Cas9) nuclease to upregulate genes of interest by activating to gene promoters. A recent study in the dy2J mouse model used the *Streptococcus pyogenes* dead Cas9 (sadCas9) and fused it to eight copies of the transcriptional activator VP16. Five guide RNAs (gRNAs) were designed to target upstream LAMA1 promoter. CRISPR and VP64–sadCas9 along with gRNAs were packaged into AAVs and delivered *in vitro*. A combination of gRNAs was found optimal for the upregulation of LAMA1 and tested *in vivo*. Laminin-α1 was successfully upregulated in the skeletal muscle and sciatic nerve of dy2J mice *via* LAMA1 promoter activation. Histopathology and functional studies showed reduced fibrosis, increased fiber diameters, and myelination of sciatic nerves, as well as improvements in activity, force measurements, and nerve conduction after a single injection of AAV-packaged CRISPR/VP64–sadCas9 (Kemaladewi et al., 2019).

Potential problems of this approach include off-target activities, regulating CRISPR/VP64–sadCas9 activity so laminin-α1 expression is more precisely controlled, assessing the long-term impacts of CRISPR/Cas9 system in human muscle, potential requirement for multiple dosing, and immune response to the AAV delivery system. Nonetheless, once these limitations are addressed, this approach represents an exciting new frontier for the treatment of LAMA2-CMD.

FUTURE PERSPECTIVES

Laminin-211/221 is the main ligand for muscle cell receptors: the dystroglycan complex (DGC) and integrin-α7β1 heterocomplex. Therefore, mutations in laminin-α2 lead to disrupted communication between the basal lamina and muscle cell receptors. These interactions are detrimental for mechanical support during muscle contraction. Overexpression of integrin-α7 in a LAMA2-CMD mouse model was reported to reduce muscle disease progression in a mouse model of LAMA2-CMD. The observations indicate that overexpression of integrin-α7 can reestablish the linkage between muscle cells and laminin isoforms in laminin-211/221-deficient muscle. Possible approaches to restore integrin-α7β1 to the sarcolemma of LAMA2-CMD patients include AAV–ITGA7 expression. These studies were performed in two models of DMD and shown to improve muscle pathology and physiology (Heller et al., 2013, 2015). Problems with an AAV–ITGA7 approach include regulating ITGA7 expression in muscle and immune response to AAV delivery systems. Another approach is using small molecules that target an increase in integrin-α7. A muscle cell-based screen has recently identified SU9516 and a structural analog Sunitinib (an FDA-approved compound), which increase the α7β1 integrin in the muscle of *mdx* mice (Sarathy et al., 2017; Fontelonga et al., 2019). These small molecules could be used to restore the integrin-α7β1 linkage system to the sarcolemma and reduce muscle disease progression in LAMA2-CMD. However, this approach remains to be explored.

Laminin-111 was shown to be an effective protein replacement therapy for LAMA2-CMD by multiple groups (**Table 1**). EHS-derived laminin-111 was studied in multiple animal models with different forms of muscular dystrophy. Treatment with laminin-111 in LAMA2-CMD mouse model increased life expectancy, muscle function, and muscle regeneration and repair. Additionally, laminin-111 protein therapy may provide the unique ability to deliver different concentrations of the biological throughout the body. High local concentration of laminin-111 may enhance myoblast differentiation while maintaining their survival through myokine signaling. Low local laminin-111 concentrations may induce myogenic proliferation. However, the effects of laminin-111 on SCs and myogenic proliferation and differentiation require further investigation.

Translation of laminin-111 protein therapy from the bench to the bedside will require production of recombinant human laminin-111 protein. Human laminin-111 has 70% amino acid homology compared to the mouse isoform. Cross-species differences in amino acid sequence and/or glycosylation patterns may trigger an immune response in mice or humans. Treatment using EHS mouse laminin-111 in the GRMD dog model did stimulate an immune response, but appeared not to have any adverse effects in this large animal model of DMD. Potential problems for laminin-111 therapeutics include: (1) production of a 900-kDa recombinant human laminin-111 in sufficient quantity and purity to treat LAMA2-CMD patients; (2) potential immune response since multiple injections will be required throughout the lifespan of the patient; (3) care in administering the recombinant laminin to ensure it does not polymerize during intravenous treatments; and (4) determining dosing requirements and drug efficacy. Once these problems are resolved, then laminin-111 could serve as a potent therapy for LAMA2-CMD and other muscular dystrophies.

Finally, another approach to produce laminin-111 in skeletal muscle is *via* endogenous upregulation of laminin-α1 protein chain. Since the LAMA1 cDNA is ∼10 kb, then delivery using AAV is not feasible. A potential solution may be upregulation *via* LAMA1 promoter stimulation using CRISPR/sadCas9–VP64 technology. Treatment with CRISPR/sadCas9–VP64 in the more severe laminin-α2 negative mouse model, the dy3k, is needed to assess the efficacy of this approach with congenital onset. Although CRISPR/sadCas9–VP64 may be the strongest potential approach to upregulate laminin-α1 in patients, delivery challenges may include immune response against AAV.

In the development of several approaches based on integrin-α7β1 regulation, laminin- and cell-based therapies, laminin-111 protein replacement therapy, or laminin-α1 enhancement, each represents novel approaches for LAMA2-CMD. Each approach has unique strengths and weaknesses that will require extensive testing and assessment before entering clinical trials. Finally, a combinatorial approach using each of these approaches may serve as the most effective approach for treating this devastating muscle disease.

AUTHOR CONTRIBUTIONS

PB-F conceptualized and designed the article. PB-F, CB, and AO-S drafted the article. DB critically revised the article.

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Conflict of Interest: The University of Nevada, Reno, has a patent on the therapeutic use of laminin-111 and its derivatives. This patent has been licensed to Prothelia Inc., and the University of Nevada, Reno has a small equity share in this company.

frontiers in Molecular Neuroscience

REVIEW
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A Family of Laminin α2 Chain-Deficient Mouse Mutants: Advancing the Research on LAMA2-CMD

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The research on laminin α2 chain-deficient congenital muscular dystrophy (LAMA2-CMD) advanced rapidly in the last few decades, largely due to availability of good mouse models for the disease and a strong interest in preclinical studies from scientists all over the world. These mouse models continue to provide a solid platform for understanding the LAMA2-CMD pathology. In addition, they enable researchers to test laborious, necessary routines, but also the most creative scientific approaches in order to design therapy for this devastating disorder. In this review we present animals belonging to the laminin α2 chain-deficient "*dy/dy*" mouse family (*dy/dy*, dy^{2J}/dy^{2J}, dy^{3K}/dy^{3K}, dy^{W}/dy^{W}, et al.) and a summary of the scientific progress they facilitated. We also raise a few questions that need to be addressed in order to maximize the usefulness of laminin α2 murine mutants and to further advance the LAMA2-CMD studies. We believe that research opportunities offered by the mouse models for LAMA2-CMD will continuously support our efforts to find a treatment for the disease.

Keywords: muscular dystrophy, laminin, transgene, knockout, basement membrane, animal model

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INTRODUCTION

The use of animals for scientific purposes goes back to ancient Greece where philosophers and scientists such as Alcmaeon of Croton, Aristotle, and Erasistratus dissected animals for anatomical studies. Today, the use of animals is a general practice for studying human biology and disease as the genetic similarity between humans and mice is very high. Also, the rapid development of methods for creating genetically modified animals increases their scientific value and stimulates general interest in preclinical research in society. One of the mouse models for laminin α2 chain-deficiency, the spontaneous *dy/dy* mutant, was described in 1955. It was the first reported mouse strain representing the pathological criteria that were characteristic for muscular dystrophy. The hereditary pattern of the defects suggested an autosomal recessive disorder and the causative mutation was called *dystrophia muscularis*, designated by the symbol *dy* (Michelson et al., 1955). Understandably, at that time it was not linked to any specific inherited muscle disease but in

1994 two independent research groups suggested that deficiency of laminin α2 chain might be the primary defect in *dy/dy* mice (Sunada et al., 1994; Xu et al., 1994a). Yet, until this date the mutation in the *Lama2* gene in the *dy/dy* mouse has not been identified.

OVERVIEW OF LAMA2-CMD MOUSE MODELS

More than 300 mutations in the *LAMA2* gene have been identified so far in patients (Oliveira et al., 2018), which result in a large clinical heterogeneity of the disease. Realistically, the whole range of human mutations cannot be mimicked in mice, but currently available mouse models mirror well the broad spectrum of the human LAMA2-CMD condition (**Table 1**). This creates an opportunity for more efficient preclinical testing and conclusive preclinical studies.

The three most commonly used animal models for LAMA2-CMD are dy^{2J}/dy^{2J} (Xu et al., 1994b; Sunada et al., 1995), dy^{3K}/dy^{3K} (Miyagoe et al., 1997), and dy^{W}/dy^{W} mice (Kuang et al., 1998b; **Table 1** and **Figure 1**). Skeletal muscle and peripheral nerve are the tissues with the most evident pathology in LAMA2-CMD mutants (Yurchenco et al., 2017). The dystrophic symptoms and general muscle pathology at the advanced stages of the disease have been fairly well characterized in all three models (Xu et al., 1994b; Miyagoe et al., 1997; Kuang et al., 1998b; Moll et al., 2001; Guo et al., 2003; Girgenrath et al., 2004, 2009; Gawlik et al., 2010, 2018; Carmignac et al., 2011a; Kumar et al., 2011; Pasteuning-Vuhman et al., 2018). Yet, we still need to learn more about mechanisms driving the disease progression in mouse. Only recently the importance of the embryonic, pre-symptomatic stages and early pathogenesis in the different mouse models for LAMA2-CMD has been emphasized (Gawlik et al., 2014, 2019; Mehuron et al., 2014; Nunes et al., 2017; Moreira Soares Oliveira et al., 2018). Likewise, associated symptoms in non-muscle tissues (peripheral and central nervous system, cardiorespiratory system) have gained attention (Hager et al., 2005; Qiao et al., 2005, 2018; Yang et al., 2005; Gawlik et al., 2006a, 2018, Homma et al., 2011; Menezes et al., 2014; Willmann et al., 2017; Pasteuning-Vuhman et al., 2018; Rabie et al., 2019).

Below we present a concise overview of the pathology in the three mouse models.

Phenotype of dy^{2J}/dy^{2J} Mice

Dy^{2J}/dy^{2J} mice carry a splice site mutation in the LN domain, which results in production of a shorter laminin α2 chain lacking the N-terminal portion of the molecule (Xu et al., 1994b; Sunada et al., 1995). The truncation hinders polymerization of laminin matrices and formation of basement membranes (Colognato and Yurchenco, 1999; Yurchenco et al., 2004; Yurchenco and Patton, 2009), but the phenotype of dy^{2J}/dy^{2J} mice is relatively mild (Guo et al., 2003) (phenotype overview: **Figures 1**, **2** and **Tables 2**, **3**). Their survival extends over 6 months of age (Xu et al., 1994b). Although the majority of dystrophic features are similar between dy^{2J}/dy^{2J} males and females (dystrophic features in different muscle types) (Pasteuning-Vuhman et al., 2018), one has to take into consideration that gender-related phenotype differences (weight gain, creatine kinase levels (CK), water intake, muscle strength), exist in this mouse model (Fontes-Oliveira et al., 2018; Moreira Soares Oliveira et al., 2018; Pasteuning-Vuhman et al., 2018), which thus far has not been demonstrated for other LAMA2-CMD mutants. While these differences do not impact the overall disease presentation (they are rather subtle and a more severe phenotype cannot be attributed to any gender), they could significantly influence the outcomes of therapeutic strategies (Fontes-Oliveira et al., 2018). Hence, analysis of the treatment effects in dy^{2J}/dy^{2J} mice should take into account both genders separately.

The pre-symptomatic stages of the disease in the dy^{2J}/dy^{2J} mouse have not been delineated. The earliest time-point analyzed is 3 weeks of age and at this stage dy^{2J}/dy^{2J} animals show normal muscle strength and collagen content in muscle is not changed (Moreira Soares Oliveira et al., 2018). However, muscle damage has already taken place as demonstrated by elevated CK levels, pronounced inflammatory response and occurrence of regenerating fibers (**Figure 2**; Kemaladewi et al., 2018; Moreira Soares Oliveira et al., 2018). Additionally, 3-week-old dy^{2J}/dy^{2J} males weigh less than wild-type male littermates. One week later, other dystrophic hallmarks (central nucleation, decreased muscle strength) are established and fully developed pathology can be observed at 6–8 weeks of age, including increased production of several extracellular matrix components, muscle atrophy and decreased body weights in both genders (Nevo et al., 2010; McKee et al., 2017; Fontes-Oliveira et al., 2018; Moreira Soares Oliveira et al., 2018; **Tables 2**, **3**). Reduced muscle strength and mobility between 4–10 weeks of age has been clearly documented in several studies (Dadush et al., 2010; McKee et al., 2017; Fontes-Oliveira et al., 2018; Gawlik et al., 2018; Moreira Soares Oliveira et al., 2018; Pasteuning-Vuhman et al., 2018). The follow-up of the dy^{2J}/dy^{2J} mouse condition up to 34 weeks of age did not reveal significant muscle pathology progression compared to 6-week-old mice (Pasteuning-Vuhman et al., 2018), except for the additional body weight loss between week 6–10 (Fontes-Oliveira et al., 2018). In contrary, the CK levels were normalized with age (Holmberg et al., 2014; Pasteuning-Vuhman et al., 2018) and challenging the mice with functional tests throughout the course of the disease (between week 6 and 34) did not worsen the dystrophic symptoms (Pasteuning-Vuhman et al., 2018). It is also important to mention that different muscles display variation in the severity of the phenotype (gastrocnemius and tibialis anterior are more affected than triceps; diaphragm shows mild pathology) and distinct patterns of pathology development (for example different timing and degree of inflammation) (Gawlik et al., 2018; Kemaladewi et al., 2018; Pasteuning-Vuhman et al., 2018).

Due to its prolonged survival time compared to other mouse models, the dy^{2J}/dy^{2J} mouse is widely used for studies of peripheral neuropathy associated with laminin α2 chain-deficiency (Yang et al., 2005; Gawlik et al., 2018; Rabie et al., 2019). The first signs of peripheral neuropathy (hind limb clasping when lifted by tail) are visible 4 weeks after birth and aggravate with age. Temporary hind limb paralysis (symptoms ceasing and relapsing, often in one limb only) occurs already around week 6 and permanent hind limb paralysis by 3 months

TABLE 1 | Summary of available LAMA2-CMD mouse models.

Mouse	Mutation	Laminin α2 expression	Phenotype#	Time of death	References
dy/dy	Spontaneous, unknown	Reduced expression of normal sized laminin α2	Moderate muscular dystrophy Peripheral neuropathy	Before 6 months of age	Sunada et al., 1994; Xu et al., 1994a
dy^{2J}/dy^{2}	Spontaneous splice site mutation in LN domain	Slightly reduced expression of truncated laminin α2 missing LN domain	Mild muscular dystrophy Peripheral neuropathy	After 6 months of age	Xu et al., 1994b; Sunada et al., 1995
dy^{6J}/dy^{6J}	Spontaneous, unknown	Unknown	Moderate (?) muscular dystrophy Peripheral neuropathy	Before 6 months of age	https://www.jax.org/strain/003589
dy^{7J}/dy^{7J}	ENU-induced missense mutation in LN domain	Normal levels of normal sized laminin α2	Mild muscular dystrophy Peripheral neuropathy	After 6 months of age	Patton et al., 2008
dy^{W}/dy^{W}	Knock-out	Severely reduced expression of truncated laminin α2 missing LN domain	Severe muscular dystrophy Peripheral neuropathy	5–12 weeks of age	Kuang et al., 1998b; Willmann et al., 2017
dy^{3K}/dy^{3K}	Knock-out	Complete deficiency	Very severe muscular dystrophy Peripheral neuropathy	3 weeks of age	Miyagoe et al., 1997
dy^{8J}/dy^{8J} (extinct)	Spontaneous, unknown	Unknown	Severe (?) muscular dystrophy Peripheral neuropathy	3–4 weeks of age	https://www.jax.org/strain/009692
dy^{Pas}/dy^{Pas} (extinct)	Spontaneous, retrotransposal insertion between exon 34 and 35	Complete deficiency	Severe muscular dystrophy Peripheral neuropathy	Before 13 weeks of age	Besse et al., 2003

The symbol "#" denotes description of muscular dystrophy severity (mild, moderate, severe, and very severe). The severity is based on age of onset, histopathological severity and time of death. The symbol "?" denotes characterization of mice remains to be published. Description of severity is based on information on https://www.jax.org/.

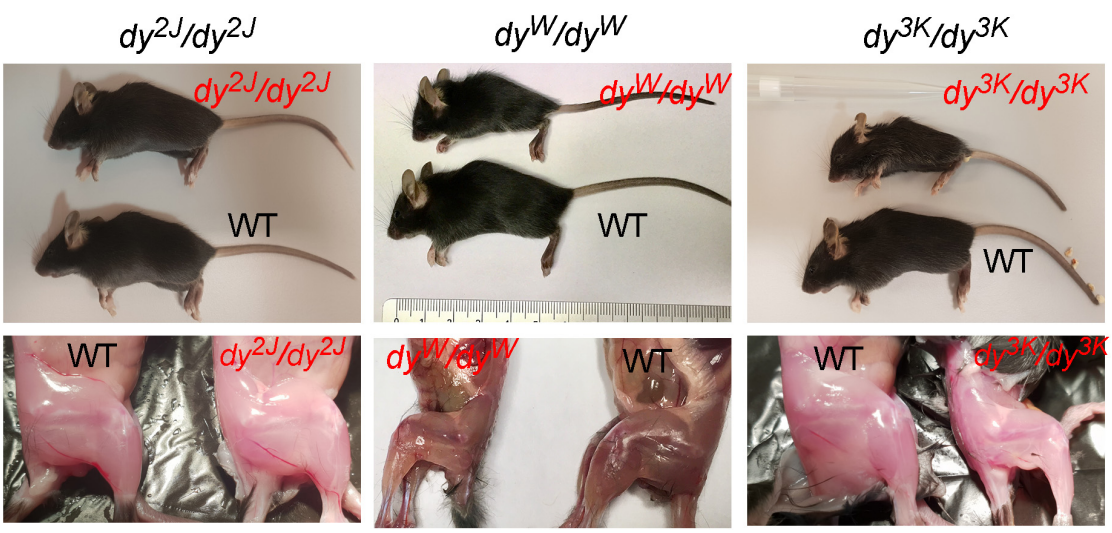

FIGURE 1 | General phenotype of dystrophic LAMA2-CMD mice (dy^{2J}/dy^{2J}, dy^{W}/dy^{W} and dy^{3K}/dy^{3K}) and their normal littermates (WT) at 3 weeks of age. Muscle wasting and weight loss are evident in dy^{W}/dy^{W} and dy^{3K}/dy^{3K} mice.

of age (Yang et al., 2005) (it is noteworthy that forelimbs, at least outwardly, are not affected). These symptoms are caused by impaired axonal sorting and dysmyelination (Yang et al., 2005; Gawlik et al., 2018), particularly in motor nerves (ventral roots), but also in dorsal roots (Yang et al., 2005; Rabie et al., 2019). Accordingly, motor nerve conduction velocity in the sciatic nerve is reduced (Domi et al., 2015; Rabie et al., 2019). It is important to mention that peripheral nerve defects cause neurogenic atrophy of muscle fibers (Gawlik et al., 2004; McKee et al., 2012) and contribute to dystrophic phenotype of skeletal muscle. Interestingly, although peripheral neuropathy is pronounced in all LAMA2-CMD mouse models (Guo et al., 2003), it is rarely manifested in patients (Yurchenco et al., 2017).

Respiratory function in dy^{2J}/dy^{2J} mice has been assessed with the whole-body plethysmography. The respiratory rate and amplitude were significantly impaired in dy^{2J}/dy^{2J} mice and the respiration rate further declined with age (Yu et al., 2013; Pasteuning-Vuhman et al., 2018). Cardiac manifestations are

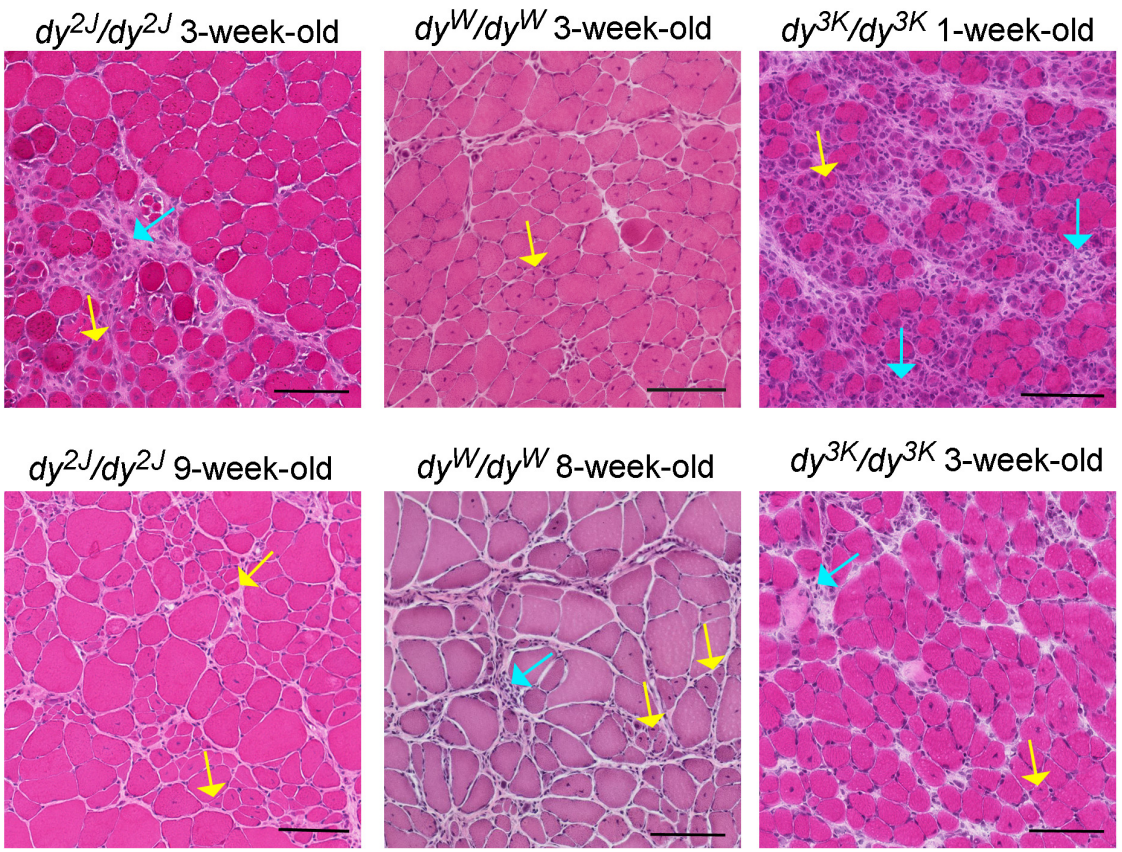

FIGURE 2 | Different stages of muscle pathology in three LAMA2-CMD mouse models (dy^{2J}/dy^{2J}, dy^{W}/dy^{W}, and dy^{3K}/dy^{3K}). Limb muscles (rectus femoris for dy^{2J}/dy^{2J} and dy^{3K}/dy^{3K}, triceps for dy^{W}/dy^{W}) were stained with hematoxylin and eosin. Yellow arrows point to regenerating fibers with centrally located nuclei; turquoise arrows depict areas with inflammatory cells. Bars, 100 μm.

TABLE 2 | An overview of time-points describing the disease progress in LAMA-CMD mouse models (dy^{2J}/dy^{2J}, dy^{W}/dy^{W}, dy^{3K}/dy^{3K}) based on muscle pathology.

	Disease progress (age)			
Mouse model	**No symptoms**	**Onset**	**Early disease**	**Established disease**
dy^{2J}/dy^{2J}	Time-point not determined	Time-point not determined	Week 3–4	Week 6 onward
dy^{W}/dy^{W}	E10.5–E17	E17.5–E18.5, smaller muscles	Week 1–2	Week 3–15
dy^{3K}/dy^{3K}	E18.5 (earlier time-points were not analyzed)	Postnatal day 1, apoptosis	Week 1	Week 2–3, despite improvements of muscle morphology (plateau)

Onset: the first signs of muscle pathology. Early disease: muscle damage, inflammation and regeneration. Established disease: fibrosis, atrophy in addition to hallmarks mentioned earlier. Please note that only a few muscles were included in most of the studies and often a limited number of phenotyping methods was applied.

associated with some cases of LAMA2-CMD (mostly attributed to complete absence of laminin α2 chain) (Nguyen et al., 2019). Cardiomyopathy has not been observed in dy^{2J}/dy^{2J} mice as assessed by histological means (8-week-old animals) (Gawlik et al., 2018) and echocardiography (12–15 and 30–33 week-old animals) (Yu et al., 2013). Yet, the heart rates are increased in dy^{2J}/dy^{2J} mutants (Yu et al., 2013) and hearts from 10-week-old dystrophic mice tend to weigh less (Fontes-Oliveira et al., 2018) than wild-type hearts. In the light of growing evidence that cardiac defects in patients (also with partial deficiency of laminin) could be underreported in literature (Nguyen et al., 2019), it is possible that histological features of cardiomyopathy may be manifested in hearts from older dy^{2J}/dy^{2J} animals, despite no clear changes in electrophysiological parameters.

Phenotype of dy^{W}/dy^{W} Mice

The dy^{W}/dy^{W} mouse was generated by homologous recombination in embryonic stem cells and intended to represent the *Lama2*-null mutant (Kuang et al., 1998a,b). It was revealed later that the mouse is not completely devoid of laminin α2 chain, but shows strongly reduced expression of the truncated molecule

TABLE 3 | A summary of the overall health and muscle phenotype of LAMA2-CMD mouse models (dy^{2J}/dy^{2J}, dy^{W}/dy^{W}, dy^{3K}/dy^{3K}) at the different stages of development.

	Developmental stage				
Mouse model	**Embryonic**	**Neonatal, early postnatal (1–7 day old)**	**Postnatal (1–3 week-old)**	**Early adulthood (3–6 week-old)**	**Adulthood (older than 6 weeks)**
dy^{2J}/dy^{2J}	Not determined.	Not determined.	Week 1–2 not determined. Pronounced inflammation and increased CK at week 3.	Central nucleation, inflammation, muscle fiber size variation, weight loss, CK variable, impaired muscle function. Phenotype clearly visible outwardly.	Fibrosis, central nucleation, fiber size variation. Impaired neuromuscular function.
dy^{W}/dy^{W}	Normal back muscle morphology, smaller muscle size from E18.5, more detailed studies needed.	Smaller back muscles, limb muscles normal size. Morphology abnormalities, apoptosis, inflammation.	Atrophy (smaller limb muscles, muscle fiber loss), apoptosis, central nucleation, inflammation, fibrosis onset. Phenotype clearly visible outwardly.	Apoptosis, inflammation, central nucleation, muscle fiber loss, fibrosis, increased CK, muscle function impairment.	Apoptosis, inflammation muscle fiber loss, atrophy, fibrosis, increased CK, muscle function impairment.
dy^{3K}/dy^{3K}	Normal limb muscle, more detailed studies needed.	Apoptosis, muscle degeneration, muscle fiber loss, inflammation, central nucleation, weight loss. Phenotype clearly visible outwardly.	Muscle repair, atrophy (smaller muscle fibers), apoptosis, inflammation, increased extracellular matrix deposition. Impaired motor function. Abnormalities of masticatory muscles. Increased CK, death at week 3.	Currently NA (death at age of 3 weeks). In previous studies when dy^{3K}/dy^{3K} mice survived up to 5 weeks: Apoptosis, inflammation, central nucleation, muscle fiber loss, fibrosis, increased CK, muscle function impairment.	NA (death at age of 3 weeks).

NA – not applicable.

(lacking the LN domain) (Guo et al., 2003). The phenotype is severe and dy^{W}/dy^{W} mice die typically 5–12 weeks after birth (Kuang et al., 1998b) (phenotype overview: **Figures 1**, **2** and **Tables 2**, **3**), but some do not survive post-weaning[1].

Early timepoints of the disease pathology have been fairly well characterized in this mouse model (Mehuron et al., 2014; Nunes et al., 2017), including embryonic stages (E10.5–E18.5) (Nunes et al., 2017). In fact, myogenesis defects *in utero* have been pinpointed in dy^{W}/dy^{W} mice (Nunes et al., 2017). While no obvious defects of muscle development have been demonstrated in LAMA2-CMD before (Kuang et al., 1998a; Gawlik and Durbeej, 2011), myofibers are smaller at birth and muscle degeneration/regeneration occurs shortly after birth in patients (Hayashi et al., 2001; Voit and Tome, 2004). Additionally, reduction of fetal movements has been reported (Jones et al., 2001). Hence, it is reasonable to consider muscle developmental abnormalities in LAMA2-CMD (even if they are subtle).

Although myotomal (E10.5), primary (E11.5–E13.5) and the first stages of secondary myogenesis (E14.5–E16.5) proceed normally in dy^{W}/dy^{W} mice, the final stages of fetal myogenesis (E17.5–E18.5) are marked with a few abnormalities (studied in epaxial muscles). The developmental defects include decreased number of Pax7 and myogenin positive cells (secondary myogenesis, E17.5 onward), smaller muscles (E18.5 onward), which results in muscle growth impairment that is not recovered during postnatal development (Nunes et al., 2017; **Tables 2**, **3**).

A few discrepancies concerning early postnatal development emerge from two separate studies: Nunes et al. (2017) reported no obvious morphology change in 2-day-old dy^{W}/dy^{W} muscle, but alteration of muscle size (epaxial muscles), whereas Mehuron et al. (2014) observed morphology defects in 1-day-old muscle with clearly reduced number of myofibers, but no change in muscle size (tibialis anterior). This suggests that the characteristics of pathological events could differ between muscles and one has to be careful to draw generalized conclusions when studying one muscle. Accordingly, it has been shown in older mice that tibialis anterior is more affected than triceps (Reinhard et al., 2017), soleus and extensor digitorum (Vohra et al., 2015).

The natural history of the disease studied in dy^{W}/dy^{W} limb muscles between 1 and 4-weeks of age reveals appearance of a broad range of dystrophic hallmarks (**Figure 2** and **Tables 2**, **3**. One-week old dy^{W}/dy^{W} muscle is characterized with muscle fiber loss, extensive apoptosis, initiation of the inflammatory response together with the myogenic program as well as increased production of fibronectin, osteopontin and matrix remodeling proteins (Wardrop and Dominov, 2011; Mehuron et al., 2014). A decreased number of myofibers does not correlate with loss of body weight, muscle weight, and reduction of muscle size (Mehuron et al., 2014), although slight body weight differences between wild-type mice and dy^{W}/dy^{W} mutants have been reported for 1-week-old animals in another study (Wardrop and Dominov, 2011). Upregulation of fibrillar collagens accompanies the pathology progression at week 2. Decrease in limb muscle

[1]https://www.jax.org/strain/013786

weight coincides with the delay of the whole-body weight gain at week 3 (Mehuron et al., 2014). CK is significantly elevated in serum from 1-month-old dy^W/dy^W mice (Kuang et al., 1998b) and fibrotic lesions are a signature of advanced pathology in dy^W/dy^W muscle (Meinen et al., 2012; Accorsi et al., 2015; Accorsi et al., 2016). Interestingly, apoptosis is less pronounced in 3–4 week-old mice compared to younger mutants (Mehuron et al., 2014). Basement membranes have patchy appearance in dy^W/dy^W muscle (Moll et al., 2001). The dystrophic changes described above correlate with impaired muscle function (Moll et al., 2001; Doe et al., 2011; Willmann et al., 2017).

Peripheral neuropathy is a prominent feature of the disease also in the dy^W/dy^W mouse model (Kuang et al., 1998b), just like in all mouse models for LAMA2-CMD (Yurchenco et al., 2017). Several studies have explored the phenotype of peripheral nervous system in dy^W/dy^W mutant, showing myelination defects in sciatic nerve and ventral roots, smaller cross sectional area of sciatic nerve, as well as motor dysfunction and sensorimotor gating deficits at age of 4–6 weeks (Homma et al., 2011; Qiao et al., 2018).

The respiratory function in dy^W/dy^W mice has also been evaluated by whole-body plethysmography. However, the breath rate did not appear to change over time and may not be predictive of pathology in dy^W/dy^W animals (Willmann et al., 2017). Analysis of cardiac muscle in this mouse model is required.

Phenotype of dy^{3K}/dy^{3K} Mice

The dy^{3K}/dy^{3K} mouse is the only available LAMA2-CMD mouse model void of laminin α2 subunit (Miyagoe et al., 1997; Guo et al., 2003). The complete loss of laminin α2 chain conceivably contributes to the most severe phenotype and much earlier death of the dy^{3K}/dy^{3K} mouse compared to other models (currently 3 weeks of age, they used to survive up to 5 weeks, but their phenotype has gradually worsened over the years in our colony at Lund University) (phenotype overview: **Figures 1**, **2**, **Tables 2**, **3**, and **Supplementary Video 1**). The cause of death is most probably linked to malnutrition and respiratory difficulties (Gawlik et al., 2019).

No obvious development abnormalities have been detected in embryonic dy^{3K}/dy^{3K} muscle (E18.5, calf muscles, thigh muscles) (Gawlik et al., 2019). Yet, more detailed studies are warranted in a wide range of muscles to confirm the current observations and comprehend myogenic events in dy^{3K}/dy^{3K} embryos. One-day-old dy^{3K}/dy^{3K} muscles display normal morphology (fiber size, number of fibers). Inflammation and abnormal expression of the extracellular matrix components have not been detected. Yet, occasional occurrence of apoptotic fibers marks the pathogenesis onset in 1-day-old dy^{3K}/dy^{3K} muscle (Gawlik et al., 2019). This suggests that dy^{3K}/dy^{3K} muscles are prone to damage already at the neonatal stage, when the mouse movements are limited to sporadic limb waddling. Muscle damage progresses quickly and already 4-day-old muscles show inflammatory response to injury. At day 7 the body weight is significantly reduced coinciding with pronounced loss of muscle fibers and inflammation (**Figure 2**; Miyagoe et al., 1997; Gawlik et al., 2019). The extracellular matrix components are massively upregulated, which is associated with muscle repair. Importantly, the severe phenotype observed at day 7 is partially ameliorated with age: at day 14 and 21 dy^{3K}/dy^{3K} muscles show recovery of muscle fiber number, reduced inflammation (**Figure 2**) and downregulation of fibronectin and collagen III (Gawlik et al., 2019). Yet, apoptotic fibers and regenerating fibers are present at these stages (Miyagoe et al., 1997; Gawlik et al., 2019). Despite normalization of the number of myofibers, there is a decrease in fiber size and muscle mass (Carmignac et al., 2011a; Gawlik et al., 2019). Likewise, although the abundance of interstitial extracellular matrix is diminished in older mutants, the levels of collagen III and fibronectin remain elevated compared to a healthy age-matched muscle (**Table 3**). CK levels are also increased in serum at that stage compared to wild-type mice (Gawlik et al., 2010; Holmberg et al., 2014). Basement membranes surrounding muscle fibers are discontinuous throughout the disease course (Miyagoe et al., 1997; Gawlik et al., 2004). Similarly to other LAMA2-CMD mouse models, dy^{3K}/dy^{3K} limb muscles display variety of phenotype presentation: vastus lateralis, medialis, gastrocnemius are more affected than rectus femoris, triceps and tibialis anterior (Gawlik et al., 2019). However, one has to be aware that complete quantitative analyses of dystrophic features in a wide range of muscles over time are missing for all mouse models.

Interestingly, the muscle function tests performed on dy^{3K}/dy^{3K} mice at different ages do not necessarily correlate with muscle phenotype. For example, 7-day-old dy^{3K}/dy^{3K} animals do not display significant difficulties when performing functional tests. On the contrary, 14-day-old mice show significant impairments of motor function (Gawlik et al., 2019).

Non-limb muscles have been studied in the dy^{3K}/dy^{3K} mouse (Nystrom et al., 2006; Hager and Durbeej, 2009; Gawlik et al., 2014, 2019). The principal extraocular muscle and intrinsic laryngeal muscles appear spared (Nystrom et al., 2006; Hager and Durbeej, 2009), whereas accessory extraocular muscles show signs of myopathy (Gawlik et al., 2014). Esophagus shows mild muscular dystrophy (day 21); diaphragm, intercostal muscles, tongue, and temporalis demonstrate moderate dystrophic changes (studied at day 14 and 21). The most severe phenotype has been observed in masseter muscle (day 7, 14, and 21) (Gawlik et al., 2014, 2019).

Heart muscle has not been shown to be affected in dy^{3K}/dy^{3K} mouse, based on histology analysis (Gawlik et al., 2004; Gawlik and Durbeej, 2015). It might be that dy^{3K}/dy^{3K} animals die too early to develop cardiac manifestations. Alternatively, the presence of laminins containing laminin α4 chain that are expressed in dy^{3K}/dy^{3K} heart (Durbeej lab, unpublished data) might be sufficient to prevent cardiomyopathy in laminin α2-chain deficient mice. However, it is not excluded that cardiac function could be impaired and this needs to be verified by electrophysiology studies.

It is interesting that death of dy^{3K}/dy^{3K} mouse coincides with the improvement of limb muscle phenotype, which points toward severe defects in other tissues, importance of masticatory muscles for proper nutrition, and perhaps importance of respiration maintenance. Respiratory function is yet to be analyzed in dy^{3K}/dy^{3K} mice.

Demyelination of axons in sciatic nerve and in particular in ventral and dorsal roots, as well reduced size of axons (sciatic nerve) have been reported in dy^{3K}/dy^{3K} mice (Nakagawa et al., 2001; Gawlik et al., 2006a). These defects result in reduced motor nerve conduction velocity of sciatic nerve (Nakagawa et al., 2001). Despite that, hind limb paralysis is not clearly manifested, probably due to early death of dy^{3K}/dy^{3K} animals. Additionally, epilepsy episodes have been observed (Durbeej lab, unpublished observations).

Other Mouse Models

The moderately affected *dy/dy* mouse expresses reduced amounts of full-length laminin α2 subunit (Sunada et al., 1994; Xu et al., 1994a). Patients showing a similar expression defect exist (Nissinen et al., 1996; Voit and Tome, 2004). Therefore, *dy/dy* animals constitute an important addition to dy^{2J}/dy^{2J}, dy^{3K}/dy^{3K}, and dy^{W}/dy^{W} murine models, especially because cardiomyopathy in *dy/dy* mouse has been clearly demonstrated (fibrotic lesions at week 8, hypertrophic cardiomyopathy as evidenced by echocardiography at week 18) (Rash et al., 1998; Gawlik et al., 2010). *Dy/dy* mice live between 12 and 24 weeks (or longer), weigh much less already at the age of 3 weeks and remain very weak throughout the disease course (Connolly et al., 2001). They also display peripheral neuropathy (Matsumura et al., 1997). Initially this mouse model greatly facilitated experimental studies of the molecular pathology of the disease (Sunada et al., 1994; Xu et al., 1994a; Ringelmann et al., 1999). Unfortunately, the unidentified mutation makes the model more difficult to study (genotyping is not possible, which negatively impacts breeding strategies). Hence, the use of *dy/dy* mice in research has diminished.

A point mutation (Arg to Cys) disrupting a conserved paired cysteine motif in dy^{7J}/dy^{7J} mice results in mild pathology and normal laminin α2 levels (Patton et al., 2008). In contrast to the other LAMA2-CMD mouse models, basement membrane is thickened in dy^{7J}/dy^{7J} muscle and normal in peripheral nerve. Yet, muscle degeneration and severe amyelination of nerve roots affect the dy^{7J}/dy^{7J} mouse (Patton et al., 2008). This mouse model provides opportunities to investigate underlying mechanisms and treatments for mild forms of LAMA2-CMD and to study additional factors that could play major roles in modulating laminin-related interactions in basement membrane (Patton et al., 2008). In particular, dy^{7J}/dy^{7J} mutant overthrows the dogma that the presence of basement membrane *per se* is necessary for prevention of the dystrophic phenotype.

Yet another mouse model, dy^{6J}/dy^{6J}, is available at Jackson Laboratories. The mutation remains to be identified but mice show progressive weakness and hind limb paralysis is visible at about 3 weeks of age[2]. Lastly, two nowadays extinct mouse models have also been described; dy^{8J}/dy^{8J} and dy^{Pas}/dy^{Pas}. The former was identified at Jackson Laboratories[3] and the latter spontaneously arose at the Pasteur Institute in Paris (Besse et al., 2003).

[2]https://www.jax.org/strain/003589

[3]https://www.jax.org/strain/009692

MOLECULAR ASPECTS OF PATHOGENESIS STUDIED IN LAMA2-CMD MODELS

It is difficult to study the pathogenesis of laminin α2 chain-deficiency *in vivo* in patients (ethical considerations, limited access to human material, large heterogeneity of patients). Mouse models for LAMA2-CMD help to overcome these obstacles and are excellent tools to explore molecular mechanisms of the disease. Over the years a tremendous amount of studies describing different aspects of the LAMA2-CMD pathology in mice has been collected. These include: (1) description of cellular and molecular events in laminin α2 chain-deficient muscle, (2) transgenic and gene therapy strategies to prevent the dystrophic phenotype (**Table 4**), (3) knockout strategies to prevent the dystrophic phenotype and/or understand the mechanisms of the disease (**Table 5**), and (4) pharmacological approaches to develop clinical treatment options (**Table 6**).

Cellular and Molecular Events in Murine Laminin α2 Chain-Deficient Muscle

Loss/reduction/truncation of laminin α2 subunit triggers secondary molecular changes in skeletal muscle. These changes lead to disruption of muscle homeostasis, but some of them may also constitute compensatory alterations. For example, a compensatory upregulation of laminin α4 and α5 subunits in LAMA2-CMD mouse models has been described (Ringelmann et al., 1999; Moll et al., 2001; Gawlik et al., 2004, 2018; McKee et al., 2017). Laminin α4 and laminin α5 chains do not bind equally well to α-dystroglycan compared to laminin α1 and α2 subunits (Talts et al., 1999, 2000; Ferletta et al., 2003) and laminin α4 chain is also devoid of a domain that is essential for laminin polymerization (Yurchenco and Patton, 2009). Therefore, this upregulation only partially prevents a more acute disease progression (Voit and Tome, 2004). Ablation of laminin α4 in dy^{3K}/dy^{3K} and dy^{2J}/dy^{2J} mice apparently worsened the phenotype (dy^{3K}/dy^{3K} double mutant reported to die before 2 weeks of age) but the skeletal muscle phenotype remains to be characterized (Miner et al., 2004). Hence, the role of laminin α4 chain in LAMA2-CMD is not entirely clear. Yet, it is possible to enhance polymerization and muscle cell binding of compensatory laminin chains and this strategy has been used to boost the phenotype of laminin α2 chain-deficient mice (Moll et al., 2001; Meinen et al., 2007; McKee et al., 2017; Reinhard et al., 2017) (mini-agrin or agrin overexpression that re-establishes connection between laminin α4 chain and dystroglycan; overexpression of laminin/nidogen chimeric protein that provides polymerization domain and combinatorial approach using both mini-agrin and the chimeric protein, see also section "Laminin α2 Chain-Deficient Mice Overexpressing Transgenes or Lacking Other Genes").

The laminin α2 chain receptor integrin α7 (forming a dimer with the β1 subunit) is lost from the sarcolemma of laminin α2 chain-deficient muscle (Vachon et al., 1997; Hodges et al., 1997; Cohn et al., 1999; Gawlik et al., 2004, 2006b; Doe et al., 2011). Dystroglycans, on the other hand (both α and β), are

TABLE 4 | Summary of LAMA2-CMD mice overexpressing transgenes or AAV/lentivirus.

Approach	Transgene/AAV	Mouse model	Muscle condition and overall health	References
Extracellular matrix and receptor modulation	Laminin α2	dy^W/dy^W	Improved	Kuang et al., 1998b
		dy^{2J}/dy^{2J}	Improved	
	Laminin α1	dy^{3K}/dy^{3K}	Improved	Gawlik et al., 2004, 2006a, 2018;
		dy^{2J}/dy^{2J}	Improved	Gawlik and Durbeej, 2010
	Truncated laminin α1	dy^{3K}/dy^{3K}	Improved	Gawlik et al., 2010
	AAV carrying dCas9, VP64 transactivators and single-guide RNAs targeting *Lama1* promoter.	dy^{2J}/dy^{2J}	Improved	Kemaladewi et al., 2019
	AAV carrying Cas9, and single-guide RNAs correcting the splicing defect	dy^{2J}/dy^{2J}	Improved	Kemaladewi et al., 2017
	Mini-agrin	dy^W/dy^W	Improved	Moll et al., 2001;
		dy^{3K}/dy^{3K}	Improved	Bentzinger et al., 2005
	Mini-agrin AAV1 and 9	dy^W/dy^W	Improved	Qiao et al., 2005, 2018
	Mini-agrin + Bcl-2	dy^W/dy^W	Improved	Meinen et al., 2011
	Mini-agrin + αLNNd	dy^W/dy^W	Improved	Reinhard et al., 2017
	Agrin	dy^W/dy^W	Improved	Meinen et al., 2007
	Agrin-perlecan	dy^W/dy^W	Improved	Meinen et al., 2007
	αLNNd	dy^{2J}/dy^{2J}	Improved	McKee et al., 2017
	Integrin α7	dy^W/dy^W	Improved	Doe et al., 2011
	Galgt2/galgt2 AAV	dy^W/dy^W	Improved	Xu et al., 2007
Apoptosis inhibition	Bcl-2	dy^W/dy^W	Improved	Girgenrath et al., 2004
Promoting regeneration	IGF-1	dy^W/dy^W	Improved	Kumar et al., 2011
	IGF-1 + Bax null	dy^W/dy^W	Improved	Yamauchi et al., 2013
	ADAM12	dy^W/dy^W	No difference	Guo et al., 2005
Polyamine modulation	Smox or Amd1 lentivirus	dy^{2J}/dy^{2J}	Improved	Kemaladewi et al., 2018

TABLE 5 | Summary of LAMA2-CMD mice lacking other genes.

Approach	Deletion	Mouse model	Muscle condition and overall health	References
Extracellular matrix and receptor modulation	Integrin α7	dy^{3K}/dy^{3K}	No difference	Gawlik and Durbeej, 2015
	Dystrophin	dy^{3K}/dy^{3K}	Worsened	Gawlik et al., 2014
	β-sarcoglycan	dy^{3K}/dy^{3K}	Worsened	Gawlik et al., 2014
	Laminin α4	dy^{3K}/dy^{3K} dy^{2J}/dy^{2J}	Overall phenotype worsened but skeletal muscle condition remains to be reported	Miner et al., 2004
Apoptosis inhibition	Bax	dy^W/dy^W	Improved	Girgenrath et al., 2004
Modulation mitochondrial permeability transition	Cyclophilin D	dy^W/dy^W	Improved	Millay et al., 2008
Inflammation and fibrosis modulation	Complement C3	*dy/dy*	Improved	Connolly et al., 2002
	Galectin-3	dy^{3K}/dy^{3K}	No difference	Gawlik et al., 2017
	Osteopontin	dy^{3K}/dy^{3K}	Slightly worsened	Gawlik et al., 2017
	miR-21	dy^{3K}/dy^{3K}	No difference	Moreira Soares Oliveira et al., 2017
		dy^{2J}/dy^{2J}	No difference	
Muscle growth	Myostatin	dy^W/dy^W	No difference	Li et al., 2005

TABLE 6 | Summary of pharmacological approaches in LAMA2-CMD mice.

Approach	Compound	Mouse model	Muscle condition and overall health	References
Extracellular matrix modulation	Laminin-111 protein	dy^W/dy^W	Improved	Rooney et al., 2012
Apoptosis inhibition	Doxycycline	dy^W/dy^W	Improved	Girgenrath et al., 2009
	Omigapil	dy^W/dy^W	Improved	Erb et al., 2009; Yu et al., 2013
		dy^{2J}/dy^{2J}	Improved	
	Omigapil in combination with mini-agrin transgene	dy^W/dy^W	Improved	Meinen et al., 2011
Proteasome inhibition	MG-132	dy^{3K}/dy^{3K}	Improved	Carmignac et al., 2011a
	Bortezomib	dy^{3K}/dy^{3K}	Improved	Korner et al., 2014; Korner and Durbeej, 2016
		dy^{2J}/dy^{2J}	No difference	
Autophagy inhibition	3-methyladenine	dy^{3K}/dy^{3K}	Improved	Carmignac et al., 2011b
Inflammation and fibrosis modulation	Losartan	dy^{2J}/dy^{2J}	Improved	Elbaz et al., 2012; Vohra et al., 2015
		dy^W/dy^W	Improved	
	Losartan derivative	dy^W/dy^W	Improved	Meinen et al., 2012
	Losartan and IGF-1 transgene	dy^W/dy^W	Improved	Accorsi et al., 2016
	Losartan and growth hormone	dy^W/dy^W	Improved	Accorsi et al., 2016
	Halofuginone	dy^{2J}/dy^{2J}	Improved	Nevo et al., 2010
	Glatiramer acetate	dy^{2J}/dy^{2J}	Improved	Dadush et al., 2010
	Prednisolone	*dy/dy*	Improved	Connolly et al., 2002
Muscle growth	Clenbuterol	*dy/dy*	Improved	Hayes and Williams, 1998
	IPLEX	dy^W/dy^W;*Bax-/-*	Improved	Yamauchi et al., 2013
Calcium modulation	Caldecrin	*dy/dy*	Improved	Tomomura et al., 2011
Metabolism modulation	Metformin	dy^{2J}/dy^{2J}	Improved (in females)*	Fontes-Oliveira et al., 2018
Oxidative stress inhibition	N-acetyl-L-cystein, vitamin E	dy^{2J}/dy^{2J}	Improved	Harandi et al., 2020
Exon skipping	Phosphorodiamidate morpholino oligomer targeting exon 4 of *Lama2*	dy^{3K}/dy^{3K}	n.d.	Aoki et al., 2013

**Some minor beneficial effects were also noted in males. n.d., not determined.*

upregulated (Gawlik et al., 2006b). It is not excluded that other, yet unidentified, laminin receptors in muscle are also perturbed.

Impaired interactions between laminin α2 and its binding partners instigate alternations of signaling pathways. Mouse models for LAMA2-CMD have provided an excellent platform for studies of signaling events. Signaling cascades associated with apoptosis, inflammation, metabolism, regeneration, protein turnover, and fibrosis (GAPDH-Siah1-CBP/p300-p53, Akt, TGF-β, NFκB, p53, JAK/STAT, to mention a few) have been shown to be affected in laminin α2 chain-deficient murine muscle (Girgenrath et al., 2004, 2009; Erb et al., 2009; Carmignac et al., 2011a,b; Kumar et al., 2011; Meinen et al., 2012; Elbaz et al., 2015; de Oliveira et al., 2014; Mehuron et al., 2014; Accorsi et al., 2015; Fontes-Oliveira et al., 2017; Gawlik et al., 2017, 2019; Nunes et al., 2017; Pasteuning-Vuhman et al., 2018; Yoon et al., 2018). Additionally, microarray, RNA-sequencing and proteomic technologies were applied to study murine LAMA2-CMD dystrophic muscle and provided a global overview of the gene and protein expression changed upon laminin α2 chain-deficiency (van Lunteren et al., 2006; Hager et al., 2008; de Oliveira et al., 2014; Kemaladewi et al., 2017; Moreira Soares Oliveira et al., 2018; Yanay et al., 2019). Such findings are essential to capitalize on opportunities given by preclinical studies and advance toward treatment design.

Analysis of cellular events in dystrophic muscle comes hand in hand with the studies of molecular interactions. A lot of attention has been given to regeneration and cells that could repair damaged muscle (satellite cells, myoblasts, non-muscle cells with myogenic potential). The subset of proliferating pro-regenerative cells has been shown to be diminished in dy^W/dy^W muscle (Girgenrath et al., 2005), resulting in myogenesis impairment and differentiation delay in laminin α2 chain-deficient muscle (Kuang et al., 1999; Kumar et al., 2011; Mehuron et al., 2014). Consequently, cell therapy approaches have been implemented in *dy/dy*, dy^W/dy^W, and dy^{3K}/dy^{3K} mice to support muscle renewal (myoblast, bone marrow and CD90-positive cells transplantation, manipulated mesoangioblasts) (Vilquin et al., 1996, 1999; Hagiwara et al., 2006; Fukada et al., 2008; Domi et al., 2015). Further studies exploring the properties of adult stem cells in laminin α2 chain-deficient muscular dystrophy are warranted, especially in the light of the enormous impact of the basement membranes on the stem cell niche remodeling in muscle (Rayagiri et al., 2018).

Muscle regeneration, inflammation and fibrosis are tightly connected in muscular dystrophy and this venue has, to some extent, been explored in mouse models for LAMA2-CMD. There is a thin line between correct tissue repair and uncontrolled fibrosis. It is all about "getting the balance right" and the

balancing factor is inflammation (Perdiguero et al., 2012; Kharraz et al., 2013; Serrano and Munoz-Canoves, 2017). Since fibrosis is a signature of LAMA2-CMD (Mrak, 1998; Voit and Tome, 2004; Elbaz et al., 2012; Mehuron et al., 2014; Accorsi et al., 2015), inflammation should take a central spot in aiming at disease prevention. Yet, not that much is known about the inflammatory response in LAMA2-CMD, but probably it is crucial for the initial wave of muscle repair (Gawlik et al., 2017, 2019). Monocytes, macrophages and neutrophils, the elements of innate immune response, constitute inflammatory infiltrates at the site of muscle damage in laminin α2 chain mutants (Connolly et al., 2002; Kumar et al., 2011; Wardrop and Dominov, 2011; Meinen et al., 2012; Gawlik et al., 2017). Also, cytokines de-regulation has been demonstrated in laminin α2 chain-deficient murine muscle (Wardrop and Dominov, 2011; Gawlik et al., 2017). Much less is known about adaptive immunity and role of lymphocytes in laminin α2 chain-deficiency, although a few T lymphocytes have been identified in laminin α2 chain-deficient muscle (Rooney et al., 2012). Even if fibrosis has a destructive impact on condition of dystrophic muscle, myofibroblasts and fibroblasts have not been studied in LAMA2-CMD mouse models.

In summary, more effort should be dedicated to decipher interactions between cells involved in muscle regeneration, inflammation and fibrosis in LAMA2-CMD mouse models. Such studies could provide answers to mechanisms of human pathology and identify molecular targets for therapy of muscle wasting diseases.

Laminin α2 Chain-Deficient Mice Overexpressing Transgenes or Lacking Other Genes

The different LAMA2-CMD mouse models have been vital tools for the identification of disease driving mechanisms and for developing therapeutic approaches. *Dy/dy*, dy^{2J}/dy^{2J}, dy^{W}/dy^{W}, and dy^{3K}/dy^{3K} mice have been genetically manipulated to overexpress or knockout specific genes that were hypothesized to impact disease pathogenesis or serve as suitable therapy candidates (**Tables 4**, **5**). Moreover, successful gene editing by CRISPR-Cas9 has been performed to correct the splicing defect in dy^{2J}/dy^{2J} animals (Kemaladewi et al., 2017; **Table 4**). Correcting the primary underlying abnormality, which is loss of laminin α2 chain, and amending the subsequent disruption of the linkage between the basement membrane and the cytoskeleton, is probably the most attractive therapeutic goal for LAMA2-CMD. Consequently, laminin α2 and α1 transgenes, respectively, have been overexpressed in dy^{W}/dy^{W}, dy^{3K}/dy^{3K}, and dy^{2J}/dy^{2J} mice, conferring excellent amelioration of the dystrophic phenotype (Kuang et al., 1998b; Gawlik and Durbeej, 2010; Gawlik et al., 2004, 2018; **Table 4**). More recently, overexpression of laminin α1 chain in dy^{2J}/dy^{2J} mice was achieved with an adeno-associated virus carrying a catalytically inactive Cas9 with VP64 transactivators and single guide RNAs that target the *Lama1* promoter (Kemaladewi et al., 2019; **Table 4**).

The primary defect of the disease was also targeted through clever molecular strategies that aimed at restoring the linkage between the extracellular matrix and cytoskeleton without the necessity of introducing the whole laminin α2 or α1 chain. Transgenic or AVV-mediated overexpression of mini-agrin alone (Moll et al., 2001; Qiao et al., 2005) or in particular mini-agrin transgene in combination with αLNNd transgene (a laminin/nidogen chimeric protein) (Reinhard et al., 2017) resulted in superb skeletal muscle restoration in dy^{W}/dy^{W} mice (**Table 4**). Mini-agrin together with the anti-apoptotic Bcl-2 transgene also profoundly reduced muscular dystrophy in dy^{W}/dy^{W} mice (Meinen et al., 2011; **Table 4**). Moreover, mini-agrin overexpression has been evaluated in dy^{3K}/dy^{3K} mice (very good muscle restoration) (Bentzinger et al., 2005) but not the more attractive combination of mini-agrin and αLNNd. Additionally, αLNNd overexpression alone was shown to correct muscular dystrophy in dy^{2J}/dy^{2J} animals (McKee et al., 2017; **Table 4**).

Xu et al. (2007) tested another way to modulate the extracellular matrix in dy^{W}/dy^{W} mice. Cytotoxic T cell GalNAc transferase is an acetylgalactosaminyl-transferase that creates a CT-carbohydrate on selected glycoproteins and glycolipids. When overexpressed extrasynaptically in dy^{W}/dy^{W} muscle, muscular dystrophy was reduced (**Table 4**).

Genetic manipulations have also been used to evaluate the roles of the laminin α2 chain receptor integrin α7 and the members of the dystrophin-glycoprotein complex (DGC) in the LAMA2-CMD disease pathology in the different mouse models. Overexpression of integrin α7 (that is absent from the sarcolemma in LAMA2-CMD muscle) reduced muscular dystrophy in dy^{W}/dy^{W} mice (Doe et al., 2011; **Table 4**). Deletion of integrin α7, on the other hand, did not aggravate the disease symptoms in dy^{3K}/dy^{3K} mice, indicating that laminin α2 chain and integrin α7 have complementary functions in skeletal muscle (Gawlik and Durbeej, 2015; **Table 5**). In contrast, deficiency of dystrophin and β-sarcoglycan, respectively, severely worsened the phenotype of dy^{3K}/dy^{3K} mice (Gawlik et al., 2014; **Table 5**). These results suggested non redundant roles of laminin α2 and the DGC and a key impact of laminin-DGC axis on muscle homeostasis. At the same time, studies with dy^{3K}/dy^{3K} mice overexpressing laminin α1 chain with preserved integrin α7 binding domains but lacking dystroglycan binding sites emphasized the significance of both linkages in rescuing the dystrophic phenotype in muscle (Gawlik et al., 2010; **Table 4**).

As the dystrophic LAMA2-CMD pathology is very complex, a great deal of effort has been aimed at elucidating the secondary pathogenic mechanisms. For example, augmented apoptosis, proteasomal activity and autophagy as well as impaired mitochondrial function, excessive inflammation and pathological fibrosis are all major disease drivers in LAMA2-CMD (see also section "Cellular and Molecular Events in Murine Laminin α2 Chain-Deficient Muscle"). Accordingly, genetic approaches that target some of these secondary pathologies have been assessed in the different mouse models. Both transgenic overexpression of the anti-apoptotic protein Bcl2 and deletion of the pro-apoptotic protein Bax reduced muscular dystrophy in dy^{W}/dy^{W} mice (Girgenrath et al., 2004; **Tables 4**, **5**) and removal of the mitochondrial calcium regulator cyclophilin D, which regulates mitochondrial permeability transition pore, attenuated muscular dystrophy in dy^{W}/dy^{W} mice (Millay et al., 2008; **Table 5**).

As LAMA2-CMD skeletal muscle is characterized by early acute inflammation and subsequent fibrosis, a few studies

have investigated the roles of certain pro-inflammatory and pro-fibrotic molecules in disease pathogenesis. Deletion of osteopontin and galectin-3 (both involved in inflammatory and fibrotic processes), respectively, did not reduce muscle pathology in dy^{3K}/dy^{3K} mouse. In fact, removal of osteopontin slightly worsened the phenotype indicating that osteopontin might be a beneficial immunomodulator in LAMA2-CMD (Gawlik et al., 2017). Similarly, absence of pro-fibrotic miR-21 in dy^{3K}/dy^{3K} and $dy^{2J}dy^{2J}$ mice did not improve muscular dystrophy (Moreira Soares Oliveira et al., 2017). Complement 3-deficiency, on the other hand, prolonged survival in *dy/dy* mice (Connolly et al., 2002; **Table 5**). Although genetic manipulations of osteopontin, galectin-3 and miR-21 in LAMA2-CMD mice did not reveal any major impact on development on fibrosis, pharmacological treatment with compounds that target inflammation and fibrosis has successfully been employed in dy^{W}/dy^{W} and dy^{2J}/dy^{2J} mice (these compounds and other pharmacological strategies will briefly be described below in section "Pharmacological Approaches in the Mouse Models for LAMA2-CMD").

Moreover, several attempts to boost regeneration in LAMA2-CMD mouse models have been performed. For example, transgenically overexpressed IGF-1 very well improved the outcome of dy^{W}/dy^{W} mice (Kumar et al., 2011; **Table 4**). In contrast, overexpression of ADAM12 or removal of myostatin, respectively, did no reduce muscular dystrophy in dy^{W}/dy^{W} mice (Guo et al., 2005; Li et al., 2005; **Tables 4**, **5**).

Finally, Kemaladewi et al. (2018) recently identified imbalanced polyamine metabolism in dy^{2J}/dy^{2J} tibialis anterior muscle and developed a strategy to increase the polyamine level by lentiviral-mediated overexpression of adenosylmethionine decarboxylase (Amd1) and spermine oxidase (Smox) (**Table 4**).

Pharmacological Approaches in the Mouse Models for LAMA2-CMD

The genetic interventions described above are undoubtedly important for the development of therapeutic approaches for LAMA2-CMD. Yet, the translation of several of these lines of attack into clinical practice remains challenging. For this reason, a number of pharmacological approaches have been investigated in the different mouse models and could ultimately permit clinical treatment possibilities. The approaches include targeting both the primary gene deficiency as well as the secondary disease drivers. Burkin and co-workers have elegantly demonstrated that laminin-111 protein therapy reduces muscular dystrophy and improves muscle repair in dy^{W}/dy^{W} mice (Rooney et al., 2012; Van Ry et al., 2014). Apoptosis inhibition has also been evaluated with pharmacological compounds. Doxycycline and omigapil, respectively, decreased muscle pathology in dy^{W}/dy^{W} mice and omigapil also had beneficial effects in dy^{2J}/dy^{2J} mice (Girgenrath et al., 2004; Erb et al., 2009; Yu et al., 2013). Similarly, proteasome inhibition with MG-132 and bortezomib, respectively, and autophagy inhibition with 3-methyladenine amended some of the pathological features in dy^{3K}/dy^{3K} mice (Carmignac et al., 2011a,b; Korner et al., 2014). Additionally, compounds that modulate inflammation (glatiramer acetate, prednisolone) (Connolly et al., 2002; Dadush et al., 2010; Rabie et al., 2019) and anti-fibrotic compounds (halofuginone, losartan, and losartan derivative) have been shown to diminish muscle pathology in *dy/dy*, dy^{2J}/dy^{2J}, and dy^{W}/dy^{W} mice (Nevo et al., 2010; Elbaz et al., 2012; Meinen et al., 2012; Vohra et al., 2015). Lastly, compounds that modulate muscle growth (clenbuterol), calcium levels (caldecrin), metabolism (metformin), oxidative stress as well as exon skipping with phosphoroamidate morpholino oligomers have all shown a positive influence on the muscle phenotype in the different LAMA2-CMD mouse models (Hayes and Williams, 1998; Tomomura et al., 2011; Aoki et al., 2013; Fontes-Oliveira et al., 2018; Harandi et al., 2020; **Table 6**).

Considering that many different cellular functions are dysregulated in LAMA2-CMD, there have been a few reports describing strategies that simultaneously target diverse processes. Indeed, it was demonstrated that amelioration of pathology was greater with a combination of mini-agrin and Bcl-2 transgenes than single mode therapies. Similarly, a combination of mini-agrin and omigapil resulted in excellent muscle condition (Meinen et al., 2011; **Tables 4**, **6**). Girgenrath and co-workers have also evaluated combinatorial treatment and demonstrated that a combination of IGF-1 transgene and removal of Bax profoundly ameliorated disease pathology in dy^{W}/dy^{W} mice and so did removal of Bax in combination with systemic recombinant human IGF-1 (IPLEX) (Yamauchi et al., 2013; **Tables 4–6**). Moreover, a combinatorial treatment utilizing transgenic IGF-1 in conjunction with losartan led to remarkable reduction of muscular dystrophy in dy^{W}/dy^{W} mice and also growth hormone enhanced losartan treatment in dy^{W}/dy^{W} mice (Accorsi et al., 2016; **Table 6**).

DISCUSSION

Mouse models for LAMA2-CMD have facilitated our understanding of the disease for over two decades. During that time the research community has tried to address important disease-related questions: from the basic phenotype description, through strategies to prevent the disorder by the most obvious transgenic means (introducing the laminin α2 and α1 transgenes) or the more sophisticated molecular manipulations (combination of engineered transgenes, genome editing with CRISPR/Cas9), through pharmacological approaches to target the disease symptoms.

We are now facing a number of questions that need to be answered in order to advance the research on LAMA2-CMD. What is next? How can we use the available animal models in the best possible way? What should we focus on? Would it be justified and feasible to mimic the disease in a bigger animal? Should we increase our interest in Drosophila, nematodes, zebrafish, frogs, and newts? Indeed, Peter Currie's work in zebrafish has substantially contributed to understanding of pathogenic mechanisms of the disease (Gupta et al., 2012; Sztal et al., 2012; Hall et al., 2019; Wood et al., 2019).

Is there a need for creating additional mouse models for LAMA2-CMD? For example, mice with a tissue-specific deletion of the *Lama2* gene would surely become an asset in LAMA2-CMD research. Perhaps one important task would be to identify

mutations in existing mouse models (*dy/dy* and dy^{6J}/dy^{6J}). Additionally, we should further explore the new methods and technologies that in a robust way contribute to reliable evaluation of preclinical treatment outcomes. For example, magnetic resonance imaging, electrical impendence myography and identification of biomarkers could complement the classical histopathology evaluations and functional tests (Accorsi et al., 2015; Vohra et al., 2015; Moreira Soares Oliveira et al., 2018; Nichols et al., 2018).

The reproducibility of data and well-grounded comparison of outcomes are a key to good science. One of the crucial tasks that we need to dedicate more energy to is a seemingly trivial, low-status assignment to create the standard operating procedures (SOPs) for all mouse models that all researches agree upon. Another task is to. . . stick to them: it is still a common practice that researchers follow their own protocols despite SOPs availability. SOPs have been generated for some of the mouse models for various muscular dystrophies/muscle diseases[4]. Some of these protocols can be easily adapted to LAMA2-CMD mouse models (Gawlik et al., 2019). It is also encouraging that several SOPs have been described for dy^{W}/dy^{W} mouse model[5]. Hence, we head toward the right direction, but the researchers in the LAMA2-CMD field need to step up and focus further on this issue. For example, SOPs for dy^{3K}/dy^{3K} and dy^{2J}/dy^{2J} mouse models should be created.

Variability in growth rates and overall survival of dy^{W}/dy^{W} animals from different laboratories have been noted (Willmann et al., 2017) and such discrepancies are likely to occur for the other mouse models as well. Therefore, it may be highly relevant to replicate the different therapeutic strategies in different mouse models and in different laboratories (even though such studies will be difficult to publish). It is also essential to thoroughly analyze the onset of pathological features in the variety of muscles in LAMA2-CMD mouse models in order to choose optimal time-points for preclinical interventions (despite the fact that most patients today may not be treated before the disease onset in the corresponding clinical set up) and select the most relevant muscles for treatment evaluation.

Thus far we face an incomplete picture of pathology in different muscle types. Considering that only in the hind limb and pelvis of a mouse there are 39 muscles (Charles et al., 2016), very few muscles have been analyzed in LAMA2-mouse models. What is more, only selected dystrophic features (for example, only fibrosis) and a limited range of time-points have been described in most publications (see references in **Tables 4–6**). Therefore, assessments of muscle phenotypes lack a quantitative aspect and are often based on general morphology and a researcher's perception. Again, potential studies that aim at filling those gaps do not align with current publishing and funding policies. As a result, they are rarely prioritized. Coordinated effort between different laboratories should be undertaken to focus on quantitative methods, time points and muscles of choice. In general, natural history studies, standardization of outcome measures, reporting of negative findings and importance of data validation should be further emphasized. Notably, the researchers are not on their own in these efforts. Patients' organizations (for example, Cure CMD[6]) understand the significance of such studies and greatly support basic research.

Exploring treatment strategies in a preclinical setup is a challenging and attractive goal for scientists that work with animal models. However, it has become clear that deeper understanding of pathogenic mechanisms underlying the disease development needs to be focused on. In particular, signaling pathways and involvement of different cell types in LAMA2-CMD pathology could be crucial for therapy design. Apart from well-known abnormalities in the central and peripheral nervous system (that are manifested both in LAMA2-CMD patients and mouse models) (Chun et al., 2003; Quijano-Roy et al., 2012; Bönnemann and Voermans, 2012; Bonnemann et al., 2014; Menezes et al., 2014), more subtle extramuscular defects are evident in mice (e.g., hearing loss, impaired spermatogenesis, and aberrant development of thymocytes and odontoblasts) (Magner et al., 2000; Pillers et al., 2002; Yuasa et al., 2004; Hager et al., 2005). These aspects of LAMA2-CMD together with the central nervous system manifestations have been poorly studied. Similarly, other tissues that normally express laminin α2 chain (heart, smooth muscle) have not been fully evaluated.

Altogether, animal models are an attractive platform to investigate the variety of the disease symptoms and treatment strategies. Nevertheless, it is important to bear in mind that relatively few preclinical findings will be successfully translated to humans as mice do not always truthfully model human disease pathology. This diminishes the predictive value of animal-based discoveries for future clinical studies (Gawlik, 2018). But if only one sole finding is positively verified and could be implemented in humans, it would be a huge achievement. It all boils down to cumulated effort to properly validate preclinical results and carefully choose targets for clinical trials. That is especially important for LAMA2-CMD where a limited pool of patients for clinical test is available. The take-home message is that we have to keep going: even if the alternatives for animal research become more and more useful (induced pluripotent stem cells, human cell-based assays), they suffer from various limitations: they cannot mimic multiple interactions between various tissues, organs and immune system. In addition, pharmacokinetic/pharmacodynamic modeling is not feasible using these methods. Consequently, they are not robust enough to provide reliable verification platform for such a complex disease. Hence, the mouse models still offer the best chance for important discoveries and preclinical studies for muscular dystrophies like LAMA2-CMD will certainly remain fully dependent on animals.

CONCLUSION

In summary, we believe that research opportunities on the mouse models for LAMA2-CMD will continue to inspire us,

[4]https://treat-nmd.org/research-overview/preclinical-research/

[5]https://treat-nmd.org/research-overview/preclinical-research/sops-for-cmd-animal-models/

[6]www.curecmd.org

scientists, and spur our joint effort to design effective treatment for the disease.

AUTHOR CONTRIBUTIONS

KG and MD wrote the manuscript and secured funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.00059/full#supplementary-material

VIDEO S1 | A 3-week-old dy^{3K}/dy^{3K} mouse and a wild-type littermate. The dy^{3K}/dy^{3K} mouse is not active when placed in a new cage.

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frontiers in Molecular Neuroscience

REVIEW
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LAMA2 Neuropathies: Human Findings and Pathomechanisms From Mouse Models

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Merosin deficient Congenital Muscular Dystrophy (MDC1A), or LAMA2-related muscular dystrophy (LAMA2-RD), is a recessive disorder resulting from mutations in the *LAMA2* gene, encoding for the alpha-2 chain of laminin-211. The disease is predominantly characterized by progressive muscular dystrophy affecting patient motor function and reducing life expectancy. However, LAMA2-RD also comprises a developmentally-associated dysmyelinating neuropathy that contributes to the disease progression, in addition to brain abnormalities; the latter often underappreciated. In this brief review, we present data supporting the impact of peripheral neuropathy in the LAMA2-RD phenotype, including both mouse models and human studies. We discuss the molecular mechanisms underlying nerve abnormalities and involved in the laminin-211 pathway, which affects axon sorting, ensheathing and myelination. We conclude with some final considerations of consequences on nerve regeneration and potential therapeutic strategies.

Keywords: *LAMA2 gene*, neuropathy, human, mousemodel, laminin

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INTRODUCTION

LAMA2 encodes the α2 chains of the laminin-211 (also known as merosin), a major component of the basal lamina of Schwann cells and skeletal muscles (Ehrig et al., 1990). Indeed, loss of function mutations of the *LAMA*2 gene in humans, and *the Lama*2 gene in rodents, results in muscular dystrophy, dysmyelinating neuropathy, and brain abnormalities. This results in Merosin-deficient Congenital Muscular Dystrophy (MDC1A, OMIM #607855) also known as LAMA2-related muscular dystrophy (LAMA2-RD).

LAMA2 NEUROPATHY IN HUMAN BEING

Shortly after the identification of the *LAMA2* gene in 1995 (Helbling-Leclerc et al., 1995), abnormalities in the nerve conduction studies of children affected by LAMA2-RD were reported (Shorer et al., 1995). Over two decades later, the clinical significance and pathophysiology of such alterations are yet to be clarified.

The first neurophysiological studies conducted on genetically confirmed patients outlined the high prevalence of mild-to-moderate motor demyelinating neuropathy: deep peroneal nerve motor conduction velocity ranged from 27 to 42 m/s in patients older than 2 years (normal values >42 m/s; 43–57 m/s; Shorer et al., 1995). Although initial reports suggested that compound motor action potential (CMAP) amplitudes and sensory fibers were both preserved in LAMA2-RD, subsequent studies disproved these findings.

Neurophysiological evidence of demyelinating sensorimotor neuropathy can be present as early as 1–6 months of age. With growth, conduction velocities may progressively decrease (Mercuri et al., 1996; Quijano-Roy et al., 2004) along with a reduction of CMAP amplitudes, consistent with a combined axonal and demyelinating polyneuropathy (Brett et al., 1998; Fujii et al., 2011; Verma et al., 2018). Conversely, conduction blocks have not been reported in other studies (Di Muzio et al., 2003; Quijano-Roy et al., 2004; Verma et al., 2018). Although the presence of residual merosin in muscle usually correlates with a milder clinical phenotype and lesser muscle involvement, there is no proven relation with peripheral nerve damage. This may be due to either discordant expression of the laminin-α2 chain in the basement membrane surrounding myofibers and Schwann cells, or the role of compensatory tissue-specific laminin isoforms (see paragraphs below on animal models; Vainzof et al., 1995; Mora et al., 1996; Prelle et al., 1997; Di Muzio et al., 2003).

Muscle and skin biopsies of patients affected by LAMA2-RD display absence of laminin α2 in intramuscular motor nerves (a finding not observed in patients with secondary merosin deficiency), and in skin neural structures, respectively (Tomé et al., 1994; Hayashi et al., 1995; Osari et al., 1996; Marbini et al., 1997; Sewry et al., 1997; Chan et al., 2014).

Morphological data from sensory sural nerve biopsies have been scarcely described in the literature. The few studies available show a reduced number of fibers, especially those of larger caliber (>6–7 μm), and variable myelin diameter. In particular, both focally thickened myelin (tomacula like), and thinner and uncompacted myelin have been reported; the former predominantly in small fibers and possibly at paranodes. Associated findings were shorter internodes and wider nodes of Ranvier (>5 μm), suggesting a disorder in myelinogenesis that resembles murine models (Shorer et al., 1995; Mercuri et al., 1996; Deodatoa et al., 2002; Di Muzio et al., 2003; Quijano-Roy et al., 2004; North et al., 2014). Whilst demyelination and onion bulbs were not observed in sural nerve biopsies, post-mortem pathology of the cauda equina showed clear evidence of ongoing segmental demyelination and remyelination in one case (Hissong et al., 2016). Moreover, one case report described a marked reduction in the number of myelinated axons together with naked axons and increased collagen deposition on electron microscopy (Brett et al., 1998).

It is still not clear to which extent peripheral neuropathy contributes to muscle weakness in patients affected by LAMA2-RD. Absent deep tendon reflexes, distal muscle atrophy and weakness, neurophysiology, and neuropathology studies are consistent with a predominantly dysmyelinating sensory-motor polyneuropathy with some axonal involvement (Mora et al., 1996; Deodatoa et al., 2002; Di Muzio et al., 2003; Verma et al., 2018). However, studies are reporting the preservation of CMAP amplitudes and the absence of neurogenic changes on electromyography (EMG), suggesting that axonal degeneration may be negligible in some patients (Quijano-Roy et al., 2004). It is of course possible that neurogenic defects are somehow masked in these patients by the predominant muscular dystrophy phenotype, or that nerve involvement is prevalent in some mutation types.

Neurophysiology revealed reduced sensory action potentials (SAP) in a few cases (Di Muzio et al., 2003; Quijano-Roy et al., 2004). However, tactile sensation, proprioception, and vibration are usually normal (Chan et al., 2014) or mildly altered (Mora et al., 1996) at clinical assessment.

Overall, the main neuropathophysiological feature of LAMA2-RD seems to be an abnormal maturation of myelin sheets accompanied by segmental demyelination. In murine models, the secondary axonal loss has been extensively described; yet, this is less commonly reported in humans (Brett et al., 1998). Neuropathy could be still a potentially detrimental contributor to disease burden, particularly in patients with partial deficiency where the overall clinical picture is not overshadowed by severe muscle involvement. Finally, although we lack consistent data suggesting progressive axonal loss and clinically significant neuropathy in patients with LAMA2-RD, future therapies might reveal new phenotypes.

LAMA2 NEUROPATHY IN MOUSE MODELS

The prototype of LAMA2-RD was first reported in mice of the Bar Harbor 129 Re strain in 1955 (*dystrophic* mice *Lama2*$^{dy/dy}$); this was however limited to the recessive inherited muscular dystrophy phenotype (Michelson et al., 1955). In the 1970s, nerve conduction studies showed functional abnormalities of the peripheral nerve (Papapetropoulos and Bradley, 1972; Bradley, 2008), which was confirmed by subsequent morphological studies. In 1971, Harris observed a reduced number of intramuscular myelinated fibers in *Lama2*$^{dy/dy}$ mice (Harris and Wilson, 1971). This data was confirmed and quantified in the tibialis nerve in 1972 (Harris et al., 1972), and further extended to other nerves and roots by Bradley and Jenkison (1973). Similar findings were described a year later in *Lama2*$^{dy2J/dy2J}$ (Biscoe et al., 1974). The genetic characterization of *dystrophic* mice was achieved two decades after this, when mutations of the Laminin chain α2 gene (*Lama2*) were reported (Xu et al., 1994; Sunada et al., 1995). Recently, mouse engineering by homologous recombination generated further mutants that have almost complete (*Lama2*$^{dyW/dyW}$; Kuang et al., 1998) or a complete lack (*Lama2*$^{dy3K/dy3K}$; Miyagoe et al., 1997) of laminin-211 expression. Finally, a further mutant was generated by ENU-induced point mutation C79R, called *Lama2*$^{dynfm417/dynfm417}$ (Patton et al., 2008). This mouse mutant shows typical *Lama2* muscle and nerve pathology in the presence of normal expression (but not function) of laminin-211. This is likely due to defective high-level organization (3D interactions between different laminin-211 heterotrimers; see

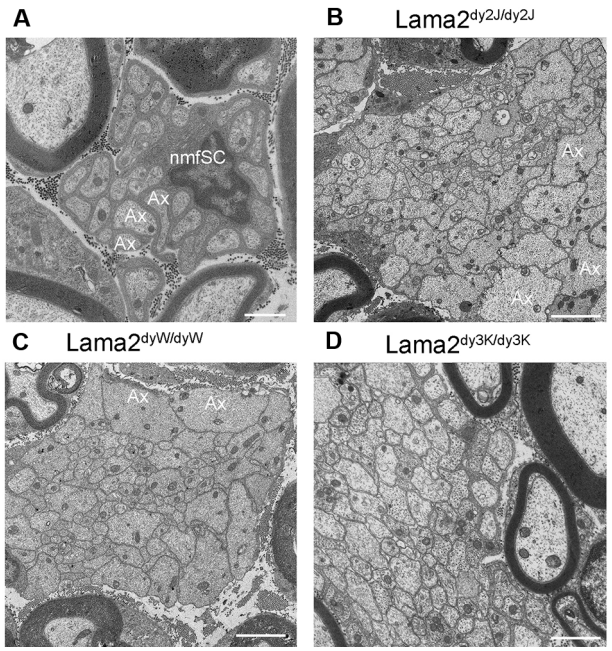

FIGURE 1 | Bundle of unsorted axons in *Lama2* mutants. **(A)** Normal Remak fiber, formed by a non-myelin forming Schwann cell (nmfSC) with unmyelinated axons (Ax); axons are smaller than 1 μm and are well separated and ensheathed by a single non-myelin forming Schwann cell. **(B–D)** Bundle of unsorted, non-ensheathed, and tightly packed axons of different caliber (some are larger than 1 μm, as Ax in figure **B** and **C**), are similarly present in Lama2$^{dy2J/dy2J}$ **(B)**, Lama2$^{dyW/dyW}$ **(C)** and Lama2$^{dy3K/dy3K}$ **(D)** mice. Bar = 1 μm in **(A)**, 2 μm in **(B,C)**, 1 μm in **(D)**.

Yurchenco and McKee, 2019) and missing interaction with other specific molecular partners.

All of these *Lama2* mutants are characterized by progressive muscle wasting and consequent motor impairment, ranging from a less severe phenotype and almost normal lifespan of *Lama2*$^{dy2J/dy2J}$ (and likely *Lama2*$^{dynfm417/dynfm417}$) to the most severe form *Lama2*$^{dy3K/dy3K}$, which die within 3–4 weeks after birth. Peripheral nerves display typical morphological features that are considered prototypic abnormalities of *Lama2* neuropathy. The morphological hallmark is the presence of defective axonal sorting during nerve development resulting in bundles of "naked" unsorted axons (**Figures 1**, **2** and **Supplementary Table S1**). This finding is spanning from spinal roots (more consistently) to peripheral nerves, including cranial nerves (Biscoe et al., 1974). These bundles contain several axons of mixed caliber, as well as those larger than 1 μm that should be sorted out and myelinated. Axons are tightly packed and often completely unsheathed or only partially surrounded by Schwann cell protrusions (**Figure 3**). Axonal sorting is a process that in rodents is completed within a few weeks, starting a couple of weeks before birth and ending around postnatal day 10 (P10). A similar process likely occurs in all vertebrates. In this process, immature Schwann cells surrounding bundles of mixed caliber axons start a physiological process of axon "docking" and "locking" to sort them out of the bundle, as well described and illustrated by Henry Webster in the 1970s and recently reviewed (Feltri et al., 2016). Only axons with a diameter larger than 1 μm are sorted, although the molecular mechanism that sustains their selection (docking) and engagement (locking) is still mostly unclear. In parallel, immature Schwann cells proliferate to match the axon number and to organize a continuous basal lamina around them (Webster et al., 1973; Jessen and Mirsky, 2005). In *Lama2* models, many axon bundles are devoid of Schwann cell processes, although in other Schwann cell processes are visible between axons (Stirling, 1975;

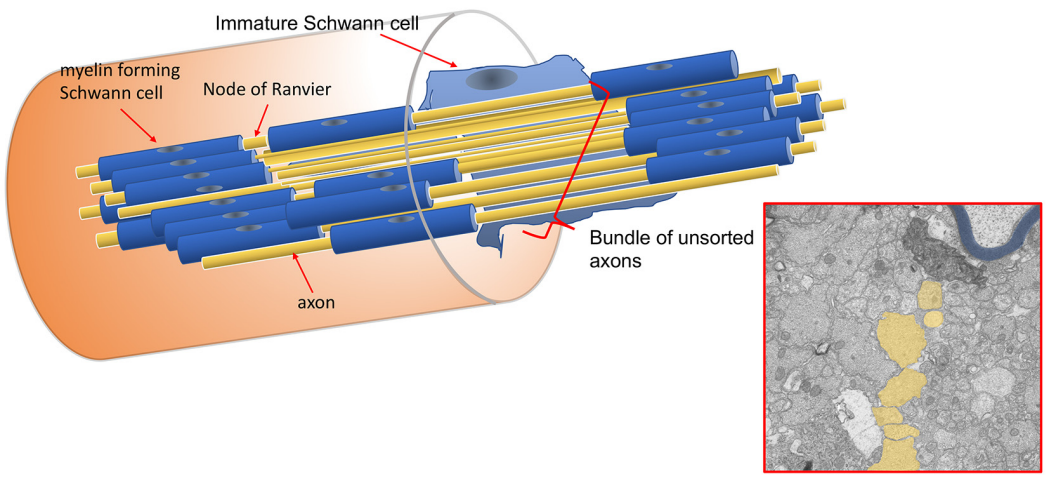

FIGURE 2 | Schematic representation of unsorted axons in *Lama2* nerves. Each axon along its length can be myelinated at one internode and belong to a bundle of unsorted axons in the subsequent one. The electron microscope (E.M.) constitutes a hypothetical cross-section of the nerve fibers in the scheme; unsorted axons are pseudo-colored in yellow, myelinated fibers in blue.

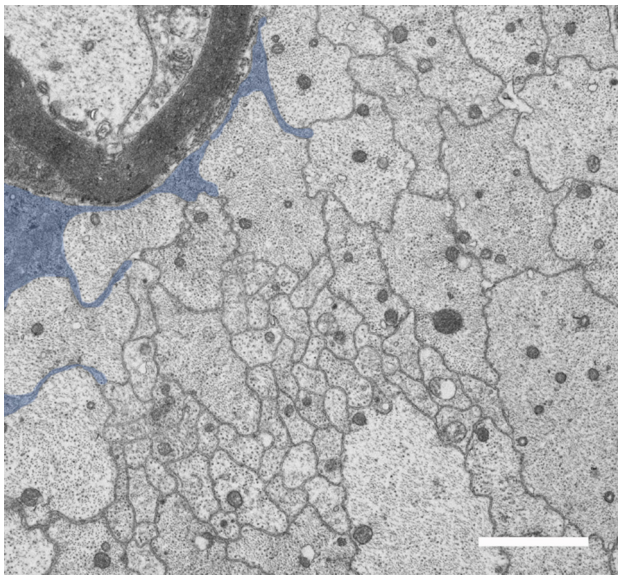

FIGURE 3 | *Lama2* Schwann cells fail to sort axons. Unsorted axons are surrounded by immature Schwann cells that fail to sort them. In this E.M. photograph from Lama2$^{dy2J/dy2J}$ sciatic nerve, the axon protrusions of one immature Schwann cell are pseudo-colored in blue. Bar = 1 μm.

Yu et al., 2001; Yang et al., 2005). This also reflects the mouse age as they tend to reduce in size and number with age (at least in long-living *Lama2*$^{dy2J/dy2J.}$ *mice*), suggesting that radial sorting may last longer in mutant mice (Yang et al., 2005 and S. C. Previtali personal observation).

Other nerve abnormalities include a reduced number of myelinated fibers as already reported in the original description of *Lama2*$^{dy/dy}$ mice, including ventral and dorsal roots, tibial and sciatic nerves (Harris et al., 1972; Bradley and Jenkison, 1973; Salafsky and Stirling, 1973). Axon diameters are significantly reduced in *Lama2*$^{dy2J/dy2J}$ (Gawlik et al., 2006) along with a reduced number of myelinated fibers in *Lama2*$^{dy3K/dy3K}$ mice (Yu et al., 2001). Occasional degenerating axons are described in *Lama2*$^{dy/dy}$ mice (Bradley and Jenkison, 1973).

Myelin thickness is reported normal or reduced in many myelinated fibers of *Lama2*$^{dy/dy}$ and *Lama2*$^{dy2J/dy2J}$ mice (Gawlik et al., 2006), in which few fibers (usually of small diameter) may have thicker myelin sheath (Ghidinelli et al., 2017). In *Lama2*$^{dy3K/dy3K}$ nerves, fibers are significantly thinner (Yu et al., 2001).

Nodes of Ranvier are reported wider in *dystrophic Lama2*$^{dy/dy}$ and *Lama2*$^{dy2J/dy2J}$ (Madrid et al., 1975; Jaros and Bradley, 1979; Occhi et al., 2005), whereas they are narrowed in *Lama2*$^{dy3K/dy3K}$ (Yu et al., 2001; Gawlik et al., 2006). Internodes are diffusely shorter in almost all the *Lama2* subtypes (Court et al., 2009 and E. Porrello and S.C. Previtali, unpublished results).

Finally, sensory nerves are sometimes reported as morphologically less affected than motor nerves (Jaros and Jenkison, 1983), although our experience revealed similar findings in motor and sensory roots and nerves (S.C. Previtali, unpublished results).

With regards to other laminin isoforms described in peripheral nerves of *Lama2* mice, the α1 chain (generating laminin-111) is not expressed in wild type nerves, whereas it is reported to be upregulated in sciatic nerves of *Lama2*$^{dy2J/dy2J}$ mice (Previtali et al., 2003b), but absent in *Lama2*$^{dy3K/dy3K}$ (Gawlik et al., 2006). The α4 chains, generating the main laminin isoform in embryonic nerve development laminin-411, is reported to be upregulated in the nerves of Lama2$^{dy/dy}$, *Lama2*$^{dy2J/dy2J}$ and *Lama2*$^{dy3K/dy3K}$ mice (Patton et al., 1997; Ringelmann et al., 1999; Yu et al., 2001; Gawlik et al., 2006; Domi et al., 2015). The α5 chain (generating laminin-511) is also modestly upregulated in nerves of *Lama2*$^{dy/dy}$, *Lama2*$^{dy2J/dy2J}$ and *Lama2*$^{dy3K/dy3K}$ mice (Patton et al., 1997; Ringelmann et al., 1999; Gawlik et al., 2006; Domi et al., 2015). Moreover, β1 and γ1 chains, necessary to generate all the laminin isoforms, are normally expressed in all the *Lama2* mutants (Patton et al., 1997; Gawlik et al., 2006).

Accordingly, minor defects in radial sorting are described when Laminin-411 is deleted (Yang et al., 2005), whereas a severe defect is observed when both α2 and α4 chains are ablated (Yang et al., 2005). Similarly, targeted deletion of γ1 chain, impeding the formation of all the above-mentioned laminin isoforms (laminin-111, -211, -411 and -511) results in the most severe and widespread neuropathy characterized by complete lack of myelination and sorting defect (Chen and Strickland, 2003).

MOLECULAR MECHANISM OF *LAMA2* NEUROPATHY

Several laminin-211 receptors have been described so far in peripheral nerves, which activate downstream signaling pathways necessary for proper nerve development, function, and maintenance. Among them, the expression of integrins α6β1, α7β1, α6β4 and dystroglycan has been reported as timely and spatially regulated (Previtali et al., 2003b; Berti et al., 2006), and their effective function confirmed or denied by specifically targeted gene disruption. The G-protein-coupled receptor 126 (GPR126) has been more recently included in this list (Petersen et al., 2015).

Expression studies (Previtali et al., 2003b) showed that β1 integrins are the first ones expressed in nerve development, since early embryonic stages, likely associated with the α6 subunits. Dystroglycan appears later on, around the time of birth. Finally, α7β1 and α6β4 appear in post-natal development. Accordingly, the deletion of the integrin β1 chain resulted in severe neuropathy with axonal sorting defects and dysmyelination (Feltri et al., 2002), thus confirming the role of β1 integrins in this pathway and the pathogenesis of the neuropathy. The Schwann cell deletion of dystroglycan, instead, only mildly impacted nerve development (Saito et al., 2003; Berti et al., 2011), whereas the deletion of both β1 integrins and dystroglycan almost completely impaired axonal sorting and myelination (Berti et al., 2011). Not surprisingly, loss of β4 integrin did not affect nerve development, while it may confer stability to mature myelin in peripheral nerves (Nodari et al., 2008).

More intriguingly, neither loss of α6 nor α7 integrin chains in Schwann cells affected nerve development and function (Previtali et al., 2003a; Pellegatta et al., 2013). While the loss of α7 could be compensated by the presence of other (redundant) β1 integrins (likely α6β1) in post-natal development, it was unexpected that deletion of α6, expressed since early embryonic nerve development, did not affect nerve formation. It was shown that α7 is upregulated, thereby compensating for the absence of α6 during nerve development and that both α6 and α7 integrins should be deleted to impair the ability of Schwann cells to spread and bind laminin-211 or -411 (Pellegatta et al., 2013). However, double α6/α7 integrin mutants showed only a mild phenotype suggesting that other Schwann cell-β1 integrins might also contribute to radial sorting during peripheral nerve development (Pellegatta et al., 2013). Integrin β1 can couple with other α chains described in the peripheral nerve, such as α1, α2, α3, α4, α5, α8, α9, α10 and αv (Lefcort et al., 1992; Milner et al., 1997; Stewart et al., 1997; Previtali et al., 2003b). These β1-integrins bind other extracellular matrix components (i.e., collagen, fibronectin, vitronectin, tenascin, and partially laminins) and may participate in radial sorting of axons or partially compensate for the loss of α6 and α7 in mutant mice. Accordingly, loss of collagen XV aggravates radial sorting defects in laminin-411 null mice (Rasi et al., 2010).

Downstream signaling pathways originating from integrins and/or dystroglycan laminin-211 receptors have been widely, although not exhaustively, investigated. They involve molecules mainly regulating cytoskeleton rearrangement for proper cell polarization and formation of Schwann cell protrusions necessary for axon recognition, sorting, and ensheathment. Among others, they include integrin-linked kinase (ILK), focal adhesion kinase (FAK), Rho (Rac1, Cdc42) and Ras (RalA/B) -GTPases, Profilin, Merlin/neurofibromin (Nf2) and neuronal Wiskott-Aldrich syndrome protein (N-WASP; Benninger et al., 2007; Nodari et al., 2007; Pereira et al., 2009; Jin et al., 2011; Novak et al., 2011; Guo et al., 2013; Grove and Brophy, 2014; Montani et al., 2014; Ommer et al., 2019). Other laminin-211 downstream molecules are instead involved in regulating cell cycle and survival for proper matching of axon-Schwann cell units, such as Cdc42, FAK, Wingless-Integrated (Wnt)/α-catenin, and Jab1 (Benninger et al., 2007; Grove et al., 2007; Grigoryan et al., 2013; Porrello et al., 2014). Most of these molecules and associated pathways have been previously reviewed (Monk et al., 2015; Feltri et al., 2016).

More recently, a strict interaction between laminin-211 and neuregulin 1 type III (NRG1 III), the main signal for peripheral nerve myelination (Nave and Salzer, 2006), has been discovered (Ghidinelli et al., 2017). During nerve development, laminin-211 limits NRG1 III function through the inhibition of protein kinase A (PKA). Loss of laminin-211 would result in overactivation of the NRG1 III pathway resulting in defective radial sorting, inappropriate/premature myelination causing polyaxonal myelination or thicker myelin sheath (Ghidinelli et al., 2017). This would explain the occurrence of hypermyelinated small-caliber fibers in *LAMA2* patients (Shorer et al., 1995; Di Muzio et al., 2003) and mice (Ghidinelli et al., 2017). Whether this effect is mediated by different laminin-211 receptors, and possibly α6α4, remains elusive (Heller et al., 2014).

Finally, studies in zebrafish and mouse mutants showed that GPR126 is required in Schwann cells for myelin expression (Monk et al., 2009) and radial sorting of axons (Monk et al., 2011; Mogha et al., 2013). GPR126 acts as a collagen IV and laminin-211 receptor (Paavola et al., 2014; Petersen et al., 2015), whose interaction promotes receptor cleavage into N-terminal fragment (NTF) and seven-transmembrane containing C-terminal fragment (CTF; Langenhan et al., 2013). The NTF fragment is necessary to guide radial sorting of axons and is generated by the interaction of GPR126 with a sort of (more) "immature" laminin-211 (i.e., low polymerization state), thus keeping the GPR126 receptor "inactive" for myelination (Petersen et al., 2015). Laminin-211 maturation (i.e., polymerization and interaction with other ECM components) switches to GPR126 in "active" state and through CTF can promote cAMP elevation, PKA activation, and thus myelination (Petersen et al., 2015).

REGENERATION IN *LAMA2* NEUROPATHIES

Intact Schwann cell basal lamina and correct formation of regenerating tracks of transdifferentiated Schwann cells (known as Bungner bands) is a prerequisite to preserve Schwann cell-axon interaction in successful nerve regeneration after damage (Jessen and Mirsky, 2019). Thus, matrix components of the basal lamina, such as laminin-211, would constitute key elements for nerve regeneration. Accordingly, the expression of laminin-211 and -411 (either as protein or mRNA) are upregulated after nerve damage (Wallquist et al., 2002). Moreover, laminin-211 is well known to promote neurite growth and nerve regeneration (Anton et al., 1994), even as a substrate of artificial nerve graft (Seo et al., 2013).

It is therefore not surprising that *Lama2*$^{dy/dy}$ Schwann cells provide a poor environment for neurite growth *in vitro* (Uziyel et al., 2000). Accordingly, nerve or spinal root damage in *Lama2*$^{dy/dy}$ mice resulted in defective axon regeneration and remyelination (Bray et al., 1983; Uziyel et al., 2000). Defective reinnervation was also observed in *Lama2*$^{dy2J/dy2J}$ mice (Parry and Melenchuk, 1981; S.C. Previtali and E. Porrello, unpublished results), and have been described in mice with conditional inactivation of the laminin γ1 chain, disrupting both laminin-211 and -411 (Chen and Strickland, 2003). Finally, the deletion of the laminin α4 chain did not affect nerve regeneration, suggesting that only laminin-211 (not -411) is necessary for the nerve to regenerate (Wallquist et al., 2005).

In conclusion, although there is no direct evidence in human LAMA2-RD patients, data from animal models suggest that defective nerve regeneration may contribute to the progression of *LAMA2* neuropathy.

LAMININ α2 CHAIN AND NEUROMUSCULAR JUNCTIONS

At the basal lamina of neuromuscular junctions (NMJs), the laminin α2 chain assembles in trimers with β2 and γ1 forming laminin-221 (Sanes et al., 1990; Patton et al., 1997). Therein, other laminin isoforms are also present, including laminin-421 and -521 (Patton et al., 1997). These three laminin isoforms are essential in establishing and maintaining the structure of NMJs and the alignment of the presynaptic zone (Rogers and Nishimune, 2017). Thus, potentially, loss of laminin chain α2 might affect NMJ formation and function, contributing to the motor phenotype of *LAMA2*/*Lama2*.

There were no reports in the literature of NMJ abnormalities in *LAMA2* patients, neither in terms of symptoms nor as neurophysiological findings typical of the myasthenic syndrome. Single fiber EMG was reported in one case and described as unremarkable (Chan et al., 2014). *Lama2* mice have been investigated at NMJs. *Lama2* mutants (at least *Lama2*$^{dy/dy}$ and *Lama2*$^{dy2J/dy2J}$) develop smaller post-synaptic junctional folds, partial axon detachment and minor Schwann cell infiltration of the synaptic cleft (Gilbert et al., 1973; Banker et al., 1979; Law et al., 1983; Desaki et al., 1995). However, they normally assemble the presynaptic active zone and properly appose to the acetylcholine receptors (Gilbert et al., 1973). More severe effects on NMJs at pre and post-synaptic zone are instead a consequence of the deletion of laminin chain α4 and/or α5, and particularly in mice devoid of β2 chain (reviewed in Rogers and Nishimune, 2017).

Laminin chain α2 has been also described in the assembly and clustering of acetylcholine receptors, through the interaction with agrin, perlecan, and MuSK (Smirnov et al., 2005). However, acetylcholine receptors seem to be preserved in *Lama2*$^{dy/dy}$ and *Lama2*$^{dy2J/dy2J}$ mice (Banker et al., 1979) and are most likely regulated by laminin α4 and α5 chains (Nishimune et al., 2008).

Loss of laminin α2 in Lama2$^{dy/dy}$ does not affect α4 and α5 expression at the NMJ (Ringelmann et al., 1999). α4 and α5 was instead upregulated in Lama2$^{dy3K/dy3K}$ mice, which otherwise showed normal expression of other NMJ components such as neuronal cell adhesion molecule and utrophin (Miyagoe et al., 1997). Finally, NMJs of *Lama2*$^{dyNmf417/dyNmf417}$ mice showed normal expression of laminin chain α2 (as well as other components) suggesting normal assembly and possibly function (Patton et al., 2008).

All these data suggest that loss of laminin-221 at the NMJ is mostly compensated by laminin-421 and -521, although it cannot be excluded that minor abnormalities described in *Lama2* NMJs might contribute to the motor phenotype and axonal neuropathy in these mutants.

POTENTIAL TREATMENTS FOR *LAMA2*/*LAMA2* NEUROPATHY

Lack of the α2 chains of the laminin-211 in peripheral nerves is responsible for peripheral neuropathy in *LAMA2* disorder. The obvious mechanism to repair this genetic defect would involve gene replacing and/or gene editing, still not feasible therapeutically so far. Gene replacement is mainly limited by the size of the *LAMA2* gene (around 9 Kb), too large to be inserted in useful viral vectors. Gene editing, instead, has been successfully used to repair *Lama2* mutations with the contemporary rescue of the peripheral neuropathy (Kemaladewi et al., 2017). Here, the main limitation is related to the off-target effects of the technique (Tsai et al., 2015).

Other strategies have been used to counteract or prevent the neuropathy. One major finding was the observation that the expression of α1 chain in peripheral nerves improved the neuropathy in *Lama2*$^{dy2J/dy2J}$ mice, including axonal sorting and myelination (Gawlik et al., 2006). This was recently confirmed by employing the CRISPR/Cas9 technology targeting the *Lama1* gene promoter delivered by adeno-associated virus (AAV9; Kemaladewi et al., 2019). Laminin-111 protein was upregulated in muscle and nerves of *Lama2*$^{dy2J/dy2J}$ mice, and specifically, in peripheral nerve, it rescued myelination and nerve conduction velocities (Kemaladewi et al., 2019).

A further strategy for treating *LAMA2*/*Lama2* disorder is the use of linker proteins mini-agrin and αLNNd. The first one was able to reconnect orphan laminin-211 receptors to the other laminin isoforms expressed in muscle and nerves and the second one to allow laminin polymerization (Yurchenco et al., 2018). These proteins are sufficiently small to be packed into AAV vectors. Accordingly, mini-agrin delivery with AAV9 was able to reach the peripheral nerve promoting the amelioration of axonal sorting and myelination in *Lama2*$^{dyW/dyW}$ treated mice (Qiao et al., 2018). Cell delivery of mini-agrin by mesoangioblasts showed instead efficacy in skeletal muscle but not in peripheral nerves, as these cells could not enter the endoneurium and were stopped in the perineurium of treated mice (Domi et al., 2015). Finally, αLNNd was proven to be effective in promoting myelination in the presence of non-polymerizing laminin isoforms (McKee et al., 2012).

Apoptosis was shown to play a role in the pathology of Lama2 mice (Girgenrath et al., 2004; Dominov et al., 2005), while doxycycline, as well as other tetracycline derivatives, had been reported to inhibit apoptosis in mammalian cells (Davies et al., 2005). Thus, doxycycline was investigated in *Lama2*$^{dyW/dyW}$ mutants where it showed amelioration of muscle and nerve pathology (Girgenrath et al., 2009; Homma et al., 2011). Although the doxycycline mechanism of action in nerves of *Lama2* mutants remains vague, it might be linked to reduced cell death of immature Schwann cells and amelioration of Schwann cell differentiation (Homma et al., 2011). Moreover, is possible that doxycycline acts through different mechanisms in different tissues.

Glatiramer acetate (GA), an agent for immune modulation, has been shown to significantly improve mobility and muscle strength in the *Lama2*$^{dy2J/dy2J}$ mice (Dadush et al., 2010). Nerve conduction velocities were also reported significantly increased in these treated mice, suggesting a valuable effect of the drug on *Lama2* neuropathy (Rabie et al., 2019).

AUTHOR CONTRIBUTIONS

SP and AZ wrote the manuscript and prepared figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.00060/full#supplementary-material.

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REVIEW
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Impaired Regeneration in Dystrophic Muscle—New Target for Therapy

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Muscle stem cells (MuSCs), known as satellite cells (SCs) have an incredible ability to regenerate, which enables the maintenance and growth of muscle tissue. In response to damaging stimuli, SCs are activated, proliferate, differentiate, and fuse to repair or generate a new muscle fiber. However, dystrophic muscles are characterized by poor muscle regeneration along with chronic inflammation and fibrosis. Indications for SC involvement in muscular dystrophy pathologies are accumulating, but their contribution to muscle pathophysiology is not precisely understood. In congenital muscular dystrophy type 1A (LAMA2-CMD), mutations in *Lama2* gene cause either complete or partial absence in laminin-211 protein. Laminin-211 functions as a link between muscle extracellular matrix (ECM) and two adhesion systems in the sarcolemma; one is the well-known dystrophin–glycoprotein complex (DGC), and the second is the integrin complex. Because of its protein interactions and location, laminin-211 has a crucial role in muscle function and survival by maintaining sarcolemma integrity. In addition, laminin-211 is expressed in SCs and suggested to have a role in SC proliferation and differentiation. Downstream to the primary defect in laminin-211, several secondary genes and pathways accelerate disease mechanism, while at the same time there are unsuccessful attempts to regenerate as compensation for the dystrophic process. Lately, next-generation sequencing platforms have advanced our knowledge about the secondary events occurring in various diseases, elucidate the pathophysiology, and characterize new essential targets for development of new treatment strategies. This review will mainly focus on SC contribution to impaired regeneration in muscular dystrophies and specifically new findings suggesting SC involvement in LAMA2-CMD pathology.

Keywords: LAMA2-CMD, laminin-211, next-generation sequencing, satellite cells, dy^{2J}/dy^{2J} mouse model, muscular dystrophy

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INTRODUCTION

Skeletal muscle tissue is the most abundant tissue in the body. It is dynamic and in its healthy state adaptable to changes, such as exercise or injury. Skeletal muscle has the ability to fully regenerate in response to injury and to increase cell number or size accordingly. Muscle adaptive behavior is maintained by muscle stem cells (MuSCs), also known as satellite cells (SCs), located in a niche in

close proximity to the muscle fiber. In response to either intrinsic or microenvironment (extrinsic) signaling, SCs are activated, proliferate, and fuse in a very controlled manner to repair or create new muscle fiber.

Muscular dystrophies are characterized by progressive skeletal muscle weakness and atrophy. The clinical deterioration is caused by substitution of muscle by fibrotic and fatty nonfunctional tissues. With new advanced diagnostic methods, accumulating data are emerging regarding decreased SC function and number, leading to impaired regeneration as a contributory mechanism to the pathology in muscular dystrophy. In this review, we mainly focus on impaired regeneration and SC involvement in the pathology of muscular dystrophies and the new findings in LAMA2-CMD.

SKELETAL MUSCLE REGENERATION

Adult healthy skeletal muscles have an excellent regeneration capacity to undergo constant repair and create new muscle fibers owing to MuSCs, also known as SCs. SCs are located in a niche between the myofiber sarcolemma and basement membrane, near the vasculature, and thus can act very rapidly, migrating and proliferating upon muscle injury (Mauro, 1961), and are the primary source of muscle regenerative cells (Ciciliot and Schiaffino, 2010; Almeida et al., 2016). Many other mononuclear cells, such as bone marrow stem cells, mesenchymal stem cells, and pericytes accompanying the muscle microenvironment, also have a role in muscle homeostasis and repair (Ferrari et al., 1998; Corti et al., 2002; Lee et al., 2005; Muskiewicz et al., 2005; Dellavalle et al., 2007; Liu et al., 2007; Negroni et al., 2009; de la Garza-Rodea et al., 2011). Following muscle injury, necrosis of damaged myofibers is followed by inflammatory responses, including recruitment of neutrophils and macrophages, which secrete inflammatory cytokines activating the quiescent SC mononuclear population in order to regenerate muscle (Otis et al., 2014). Muscle regeneration is a controlled finely tuned process, very similar to muscle formation during embryonic development (Allbrook, 1981), and can be subdivided into three main stages: proliferation, differentiation, and fusion (Randolph and Pavlath, 2015; Chal and Pourquié, 2017; Yanay et al., 2019), as can be seen in **Figure 1A**.

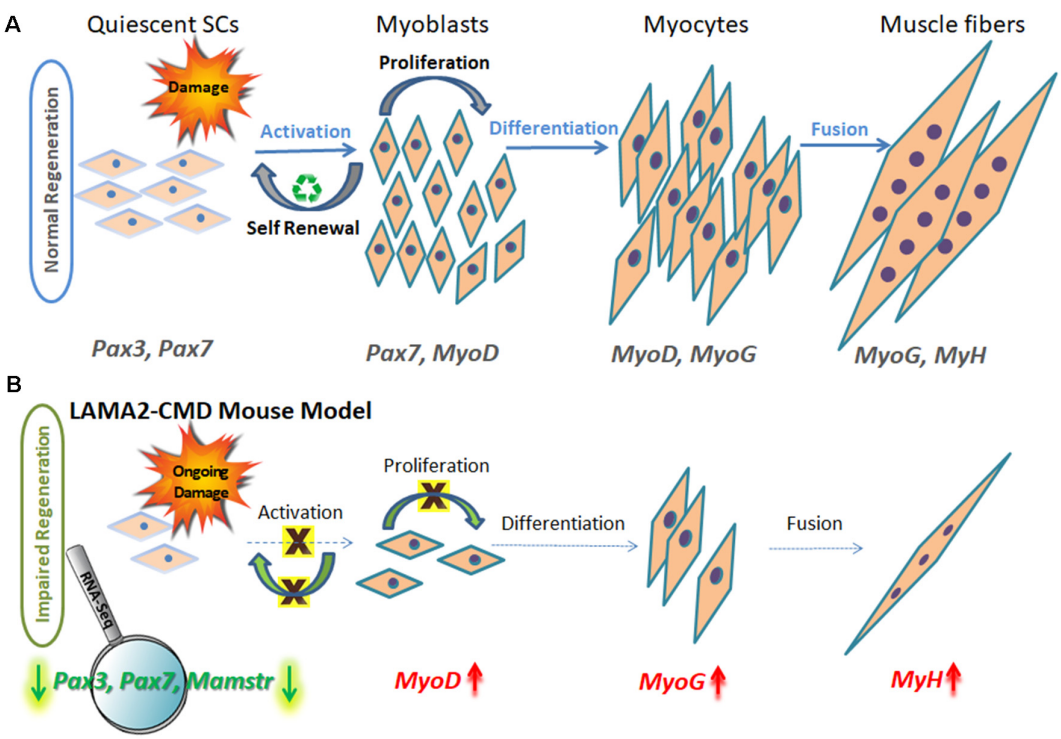

FIGURE 1 | Schematic model of muscle regeneration process in normal and LAMA2-CMD mouse model. **(A)** In healthy muscle, satellite cells (SCs) are in a quiescence state as a default, located in a specialized niche, between the sarcolemma and basal lamina. Quiescent SCs express Pax7, and a subpopulation expresses also Pax3 transcription factors; however, MyoD is not expressed at this stage. Upon injury, SCs are activated and undergo proliferation. SCs can perform asymmetric or symmetric divisions. Asymmetric division allows self-renewal and maintenance of SC pool, and symmetric division allows myogenesis and generating myoblasts. Following proliferation stage, myoblasts express MyoD transcription factor and exit the cell cycle to promote differentiation. Upon differentiation, myoblasts differentiate into elongated myocytes expressing myogenin (MyoG). Myocytes can fuse forming myotubes, which become myofibers, the contractile unit of muscle, and express developmental myosin-heavy chain marker (MyH). **(B)** In dystrophic muscle, SCs have limited ability to compensate for muscle damage. In LAMA2-CMD dy^{2J}/dy^{2J} mouse model, RNA-Seq data indicated little or even absent SC population, due to significant downregulation of Pax7 and Pax3 genes in quadriceps muscle of 8-week-old mice (Yanay et al., 2019). Absence of those transcription factors disable proper proliferation and self-renewal; thus, the entire regeneration process is impaired. MyoD and MyoG upregulation is postulated to be an attempt to compensate for the unbalanced process and the reduced repair of the damage.

Each stage of muscle regeneration can be classified by a variety of molecular markers. The most familiar marker of all muscle SC state is paired box transcription factor 7 (Pax7), being essential for SC function during postnatal growth both in regeneration of skeletal muscle and maintaining a renewable SC pool (Seale et al., 2000; Olguin and Olwin, 2004; Zammit et al., 2004; Lepper et al., 2009; von Maltzahn et al., 2013). Subsets of SCs express premyoblast marker paired box transcription factor 3 (Pax3), important in the initial steps of muscle formation (Relaix et al., 2005, 2006); however, Pax3's role in adult human muscle still remains to be established. The population of Pax3 and Pax7 double-positive stem cells expression is observed in the preliminary stage throughout embryonic and fetal development, as well as later in adult skeletal muscle SCs. Pax3 and Pax7 stimulate gene expression promoting proliferation and asymmetric divisions (known as self-renewal) and repress genes driving differentiation (Ben-Yair and Kalcheim, 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005, 2006).

Upon SC activation, proliferating myoblasts coexpress Pax7 and MyoD transcription factors. From this stage, the differentiation process begins and is characterized by MyoD and MyoG expression.

Proliferating myoblasts either down-regulate Pax7 to differentiate or down-regulate MyoD to undergo self-renewal process, which maintains the SC pool. Maintaining the balance between myoblast proliferation and differentiation is crucial for the appropriate muscle regeneration process (Yablonka-Reuveni, 2011).

Under dystrophic conditions, muscle is persistently injured and degenerates (Otis et al., 2014) in a way that muscle necrosis surpasses regenerative capacity, and muscle repair cannot effectively compensate for damage. The muscle undergoes replacement by fibrotic tissue and fat leading to severe loss of muscle mass and function. Recently, there are cumulative indications of SC involvement in different muscular dystrophies in which SCs apparently fail to repair muscle damage efficiently (Logan et al., 2011; Ross et al., 2012; Urciuolo et al., 2013; Di Gioia et al., 2017).

THE CONTRIBUTION OF SATELLITE CELL REGENERATION FAILURE TO MUSCULAR DYSTROPHY PATHOPHYSIOLOGY

Intrinsic and extrinsic defects may occur at each stage of this complex multipart muscle regeneration process. Several theories for the limited regenerative capacity of SCs have been proposed by Randolph and Pavlath (2015) and the 240th ENMC workshop on "The involvement of skeletal MuSCs in the pathology of muscular dystrophies" (Morgan et al., 2019). These theories can be divided into impairment of intrinsic and extrinsic factors:

Intrinsic Impairment in SCs

1. Reduction in SC pool. Defects in self-renewal, SC exhaustion, or senescence eventually lead to reduction in SC number. Self-renewal defects causing ineffective generation of SCs were demonstrated in sarcoglycanopathy LGMD2C and 2F patients (Higuchi et al., 1999), Col6A1 knockout mice (Urciuolo et al., 2013; Gattazzo et al., 2014a), Sepn1$^{-/-}$ murine model, and in selenoprotein-related myopathy (SEPN1-RM) patient muscle biopsies (Sacco et al., 2010; Castets et al., 2011). SC exhaustion caused by constant activation from ongoing cycles of degeneration and regeneration was demonstrated in *mdx* mouse model of Duchenne muscular dystrophy (DMD) and myoblasts isolated from aging DMD patients (Mouly et al., 2005; Sacco et al., 2010; Jiang et al., 2014). Lately self-renewal defect in SCs, in addition to exhaustion, was also observed in *mdx* mice indicating asymmetric division anomalies (Dumont et al., 2015). Senescence (premature aging) was demonstrated in both *in vitro* and *in vivo* models of LGMD2H mouse SCs (Kudryashova et al., 2012; Mokhonova et al., 2015) and myoblasts of human myotonic dystrophy type 1 (DM1) and 2 (DM2; Bigot et al., 2009; Beffy et al., 2010; Renna et al., 2014).
2. Defects in myoblast proliferation. Decreased proliferation of SCs has been observed in oculopharyngeal muscular dystrophy (OPMD) patients' myoblasts (Périé et al., 2006), Emery–Dreifuss muscular dystrophy (EDMD) patient's muscle tissue sections and cultured myoblasts (Meinke et al., 2015), muscle biopsies of a DM1 patient (Thornell et al., 2009), and in myoblasts of murine model of LGMD2O (Miyagoe-Suzuki et al., 2009). In patients and mouse model of Pompe disease, insufficient SC activation was noted, although their function and number seemed normal (Schaaf et al., 2018). Additionally, impaired SC transition from proliferation to differentiation has been demonstrated in biopsies from LGMD2A patients (Rosales et al., 2013).
3. Defect or delay in the differentiation stage was demonstrated for facioscapulohumeral muscular dystrophy (FSHD) in DUX4c-expressing C2C12 myoblasts (Bosnakovski et al., 2008). Defects in myoblast differentiation were also demonstrated in EDMD primary muscle and patient-derived myoblast cultures (Frock et al., 2006) and in C2C12 cells expressing mutated lamin A, representing the cellular model of AD EDMD (Favreau et al., 2004).
4. Defect or delay in the fusion stage was reported in FRG1 mouse model for FSHD primary myoblasts (Feeney et al., 2015) and limb-girdle muscular dystrophy type 2L (LGMD2L) *Ano5*$^{-/-}$ knockout mouse model (Whitlock et al., 2018).

Extrinsic Impairment in SC

1. Hostile microenvironment of dystrophic muscle may not be permissive for continued SC regeneration (Boldrin et al., 2012). Altered composition of the extracellular matrix (ECM), chronic inflammation (Wanschitz et al., 2013), defective autophagy (Tang and Rando, 2014), and fibrosis lead to a defect in SC niche and to cell senescence, as demonstrated in mouse models of dystroglycanopathy (Ross et al., 2012).
2. Alterations in signaling pathways as a result of the dystrophy may also underlie failure of SC regeneration. This was demonstrated in a limb-girdle muscular dystrophy patient with missense mutation in POGLUT1 (protein

O-glucosyltransferase 1), in whom a decrease in Notch signaling was associated with muscle degeneration and loss of SCs (Servián-Morilla et al., 2016).

Intrinsic as well as extrinsic defects can be caused by the disease's primary mutation.

In case that the mutation is in protein expressed within SCs, the primary mutation itself can impair SC function as demonstrated in DMD and LGMD2H mouse models (Kudryashova et al., 2012; Dumont et al., 2015). If not critical for SC function, the primary mutation may change structures of the ECM and basal lamina, altering SC niche, which may lead to microenviromental defects and ineffective SC activation (Gattazzo et al., 2014b).

Regeneration defects in more than one stage have been reported in several dystrophy types. However, the origin of these defects was elucidated in later studies, when new and powerful technologies enabled more accurate detection and analysis of the various components of the pathophysiology, beyond the primary mutation.

Until recently, the dystrophin gene, *DMD*, was considered to be expressed in myofibers and not SCs. Accordingly, the main contribution to DMD pathology was assumed to be limited to myofiber membrane fragility due to absence of dystrophin, leading to continuous necrosis with progressive fibrosis and muscle wasting (Morgan and Zammit, 2010). Hence, current therapies mainly focus on preventing dystrophy by targeting the myofiber. However, in a recent study, using RNA-sequencing (RNA-Seq) and microarray techniques, dystrophin expression was detected in a subset of SCs and postulated to have an important role in their polarity and asymmetric divisions. Therefore, a primary intrinsic SC dysfunction contributing to DMD pathogenesis has been suggested (Dumont et al., 2015). Evidences for active and unbalanced proliferation in DMD with no change in SC number compared to healthy muscle are available (Boldrin et al., 2009, 2015). However, this process of proliferation is impaired due to abnormal and uncontrolled SC divisions, impaired self-renewal, and eventual decline in the SC pool with time.

In accordance with these results, a recent study at our laboratory supports an intrinsic SC defect in muscle of the *mdx* mouse model of DMD. This study examined 8-week-old *mdx* mouse whole muscle transcriptome using RNA-Seq and found unsynchronized upregulation of all major regeneration transcription factors: Pax7, Myf5, MyoD, MyoG, and Mamster compared to WT muscle, indicating unbalanced active regeneration and SC uncontrolled activation, which may increase the proportion of abnormal cell divisions (Yanay et al., 2019).

CONGENITAL MUSCULAR DYSTROPHY TYPE 1A

Congenital muscular dystrophy type 1A (LAMA2-CMD), also known as merosin-deficient congenital muscular dystrophy type 1A (MDC1A), is a devastating incurable disease, caused by mutations in *Lama2* gene encoding the α-subunit of laminin-211. Mutations in laminin-211, a key anchor basement membrane protein, disrupt the link between cytoskeleton, basement membrane, and ECM, rendering the muscle fiber sarcolemma fragile.

Laminin 211 interacts with α-dystroglycan, a member of the dystrophin–glycoprotein complex (DGC; Spence et al., 2002). This complex links ECM to the actin cytoskeleton of the myofiber, thereby protecting skeletal muscle membrane against contraction-induced damage (Petrof et al., 1993).

Independently, laminin-211 interacts with β1 integrin, a part of the α7β1 integrin complex that gathers a large number of proteins and, like the DGC, functions as a structural link between ECM and actin cytoskeleton, hence similarly playing a significant role in protecting skeletal muscle against contraction-induced injury. Recent studies suggest that the DGC and integrin complex have compensatory abilities in maintaining sarcolemma integrity, as demonstrated by much more severe muscle pathology in double-mutant knockout mice for dystrophin and α7β1 integrin than mice lacking either dystrophin or α7β1 individually (Allikian et al., 2004; Guo et al., 2006; Rooney et al., 2006).

In LAMA2-CMD, either partial or complete absence of laminin-211 expression leads to increased muscle vulnerability to injury with ensuing severe clinical features in children, as well as in LAMA2-CMD mouse models.

Several useful mouse models of LAMA2-CMD are available, demonstrating correlation between laminin-211 expression and disease severity, thus representing heterogeneity in their clinical presentation. Very severe forms of muscular dystrophy are represented by dy^W/dy^W and dy^{3k}/dy^{3k} models. These models have a similar severe dystrophic phenotype, with life spans of only a few weeks of age, commensurate with almost absent or completely absent laminin-211 expression in dy^W/dy^W and dy^{3k}/dy^{3k} models, respectively. Moderate severity with reduced life span of 6 months of age represents the *dy/dy* mouse, which has reduced laminin-211 expression and primary mutation still unknown. Mild muscular dystrophy represents the dy^{2J}/dy^{2J} mouse model, with spontaneous mutation in the LN domain resulting in partial deficiency, hind limb paralysis by 3–4 weeks of age, and decreased life span compared to wild type (Xu et al., 1994; Sunada et al., 1995; Miyagoe et al., 1997; Gawlik and Durbeej, 2011; Durbeej, 2015).

IMPAIRED REGENERATION IN LAMA2-CMD

Previous studies have provided some clues that the proliferation stage in the regeneration process is impaired in LAMA2-CMD.

One clue for impaired regeneration in LAMA2-CMD, suggested by Kuang et al. (1999) is that a major contributor to muscle disease is abortive regeneration in the *dyW* mouse model. They reported immature myofibers and excessive mononuclear cell death in this model.

Next, in 2005, Girgenrath et al. (2005) showed in the same *dyW* model that mononucleated cells, and in particular muscle SC population, were decreased compared to WT and *mdx* mice,

and therefore an altered proliferation stage was assumed. Thus, they hypothesize that SC poor proliferation may be one of the mechanisms underlying the lack of successful regeneration in LAMA2-CMD muscle (Girgenrath et al., 2005).

Only a few studies exploring LAMA2-CMD transcriptome and proteome have been published (Taniguchi et al., 2006; van Lunteren et al., 2006; Moreira Soares Oliveira et al., 2018). As for human data, muscle transcriptome was studied in a single LAMA2-CMD patient at the age of 8 months, using microarray technique with costume chip representing a limited number of 5,600 genes expressed in muscle. Most of the upregulated genes in this patients' muscle were ECM components, which according to the author reflect active fibrosis and poor muscle regeneration (Taniguchi et al., 2006).

In mouse models, a single study demonstrating gene expression profiling in *dy/dy* using microarray technique found a limited number of genes differentially expressed in the diaphragm (van Lunteren et al., 2006). Genes with altered expression in the diaphragm belonged to cell motility, development, immune response, cellular adhesion, and collagen synthesis. In addition, a study by de Oliveira et al. (2014) described proteomic analysis in the dy^{3k}/dy^{3k} mouse model, showing approximately 100 differentially expressed proteins compared to WT, mainly involving metabolic processes, calcium binding, or ECM protein expression (fibrosis) in diaphragm and gastrocnemius muscles (de Oliveira et al., 2014).

Correlation between clinical phenotype and gene expression was mainly suggested for fibrosis in studies of LAMA2-CMD; thus, therapies so far have largely focused on amelioration or prevention of fibrosis (Nevo et al., 2010; Elbaz et al., 2012; Yu et al., 2013; Accorsi et al., 2016).

In our recent article, next-generation sequencing *via* RNA-Seq technique was applied in the dy^{2J}/dy^{2J} mouse (Yanay et al., 2019). Using this method, we detected a large number of novel significantly differentially expressed genes in quadriceps muscle of 8-week-old dy^{2J}/dy^{2J} mice.

The most significant finding was downregulation of three key myogenic stem cell factor genes: Pax3, Pax7, and Mamstr (**Figure 1B**). This demonstrated an abnormal regeneration process, which mainly points to impaired SC self-renewal in the dy^{2J}/dy^{2J} mouse model compared to WT.

These results are in agreement with ours and others histological results and previous clinical observation demonstrating poor proliferation and high degree of fibrosis in LAMA2-CMD patients and mouse models (Girgenrath et al., 2005; Yanay et al., 2019).

Earlier studies demonstrate that with absence of Pax3/Pax7 expression, cells undergo apoptosis or adopt alternative nonmuscle lineages (Soleimani et al., 2012), possibly explaining the mechanism underlining very early tissue replacement by fibrosis and fat in LAMA2-CMD models. Transient activation of Pax3 expression in cultures of primary myoblasts results in enhanced proliferation in these cells (Conboy and Rando, 2002; Kuang et al., 2006).

Not only the environment is important for SC functionality, but SCs may also have an impact on their own environment, as loss of SCs may also increase muscle fibrosis. Thus, the presence of normal SCs is required to maintain functional niches that support regeneration (Morrison and Spradling, 2008).

SC involvement in additional myopathies pathologies was reported for XL myotubular myopathy, SEPN1-related myopathies, and as a primary cause for EMARDD (early-onset myopathy with areflexia, respiratory distress, and dysphagia; Castets et al., 2011; Logan et al., 2011; Boyden et al., 2012; Lawlor et al., 2012; Di Gioia et al., 2017). Mutated genes expressed in myotubes as well as in SCs may alter myofiber maturation during embryogenesis, and early growth is postulated to be the cause for the early onset of pathology and weakness in these disorders. Similarly to these congenital myopathies, there are also indications for laminin-211 expression in LAMA2-CMD SCs (Schuler and Sorokin, 1995; Vachon et al., 1996; Morgan and Zammit, 2010). In addition to its roles in prevention of myofiber injury, laminin-211 has therefore been suggested to have a role in myoblast proliferation and SC differentiation and regulation (Girgenrath et al., 2005; Morgan and Zammit, 2010). SC dysfunction in LAMA2-CMD due to an intrinsic defect or extrinsic/microenvironment defects, or a combination of the two, should be further studied.

Understanding SC contribution to LAMA2-CMD muscle pathology may suggest new therapeutic strategies, as current therapies mainly target myofiber damage rather than the SC regeneration process.

SIGNALING PATHWAYS UNDERLYING MUSCLE REGENERATION

Multiple signaling pathways are involved in skeletal muscle regeneration, and each of them is tightly controlled and regulated to enable efficient muscle repair. Notch, Wnt, Janus kinase/signal transducers and activators of transcription (JAK/STAT), Mitogen-activated protein kinase (MAPK), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Transforming growth factor beta (TGF-β) signaling pathways, and others have been intensely studied, and their contribution to developmental and adult muscle regeneration confirmed (Brack et al., 2007; Bjornson et al., 2012; Parker et al., 2012; von Maltzahn et al., 2012a; Tierney et al., 2014). Each signaling pathway is critical for accurate regeneration, but above all, their synchronization and balanced cross-talk are most important for proper regeneration (**Figure 2**). For instance, in adult skeletal muscle, SCs express high levels of Notch to remain in a quiescent state and prevent Pax7 induced differentiation (Conboy and Rando, 2012; Wen et al., 2012; Fujimaki et al., 2018). On the other hand, with upregulation of the canonical Wnt signaling pathway, SC differentiation is activated (von Maltzahn et al., 2012a). Thus, a precise timing to switch from Notch to Wnt signaling is required for proper SC differentiation (Brack et al., 2007). While the canonical Wnt signaling drives differentiation of SCs, mainly through the ligand Wnt3a, noncanonical Wnt signaling is responsible for mediating self-renewal and migration of SCs, and also growth of muscle fibers through Wnt7a ligand (von Maltzahn et al., 2012a).

Examples for noncanonical Wnt signaling pathways include PCP (planar cell polarity), Wnt/Ca^{2+}, and PI3K/AKT/mTOR

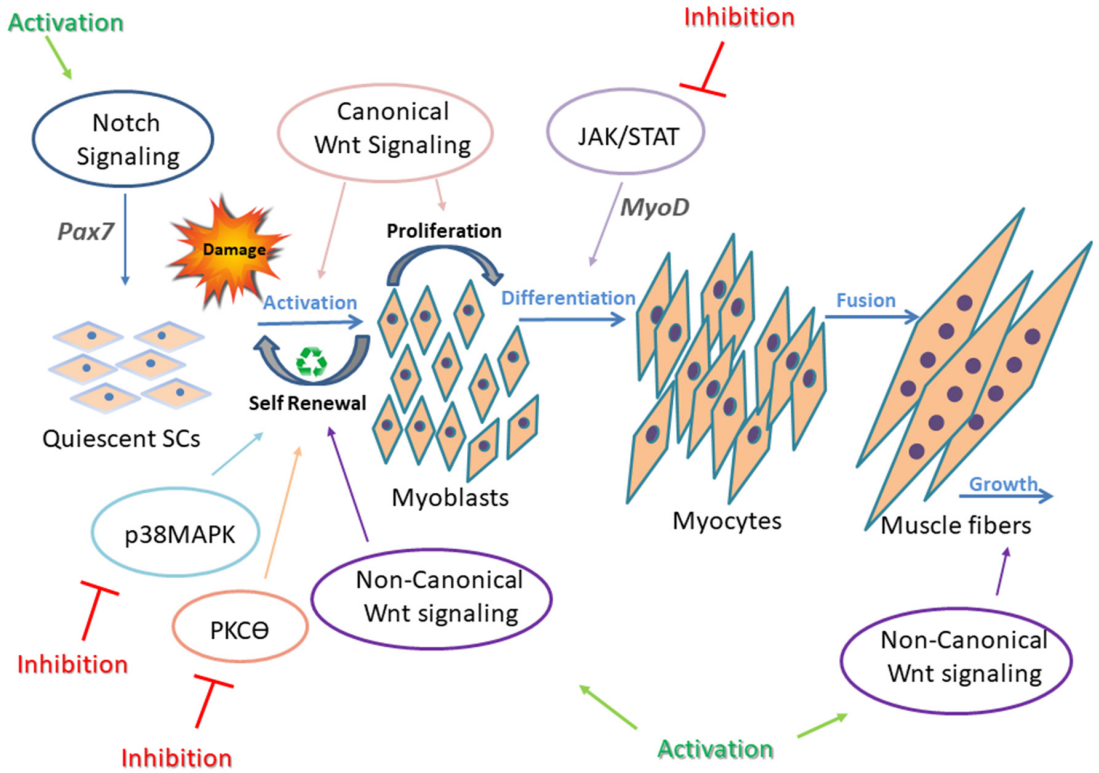

FIGURE 2 | Underlying mechanisms in impaired regeneration during myogenesis. Main regeneration signaling pathways in muscular dystrophy and the corresponding targets for intervention are presented. In adult healthy skeletal muscle, all signaling pathways are tightly controlled and highly synchronized for proper SC differentiation. This is well demonstrated by the interplay between Notch and Wnt signaling. Upregulation of Notch maintains SCs in a quiescent state and promotes self-renewal of activated SCs through upregulation of Pax7. Canonical Wnt signaling antagonizes the effects of Notch signaling, thus allowing the progression through differentiation. Also, canonical and non-canonical Wnt signaling oppose each other, as canonical Wnt signaling regulates the differentiation of muscle SCs, and noncanonical Wnt signals (through ligand Wnt7a) mediate self-renewal (symmetric divisions), migration of SCs, and growth of muscle fibers. p38MAPK pathway regulates toward asymmetric division and SC self-renewal. In dystrophic muscle, signaling is altered and unsynchronized, resulting in defective regeneration. Continuous intervention to activate or inhibit abnormal signaling in SCs failed to improve muscle regeneration. Thus, a dynamic/cyclic regulation of signaling is necessary to balance regeneration of SCs in order to improve long-term regenerative defects of dystrophic muscles (Jiang et al., 2014; Tierney et al., 2014). Cyclic administration of Wnt7a, Notch activators, and JAK/STAT, MAPK, and PKCθ inhibitors have shown beneficial effects on the regeneration process in dystrophic muscles.

signaling cascades. Interplay between these factors upon Wnt signaling results in activation of small GTPases, Rac, and Rho, leading to cytoskeletal remodeling, are essential for myoblast fusion.

Additional signaling pathways involved in SC regeneration include p38MAPK signaling, which is highly regulated to permit asymmetric division and SC self-renewal (Troy et al., 2012).

Also, JAK/STAT signaling pathway activates myogenic differentiation by regulating expression of specific genes such as MyoD (Wang et al., 2008).

IMPAIRED SATELLITE CELL REGENERATION SIGNALING PATHWAYS IN MUSCULAR DYSTROPHY

Unsynchronized SC signaling pathways resulting in defective muscle regeneration has been described in several muscular dystrophies.

Decrease number of SCs in dystrophic muscle has been associated with a reduced Notch signaling pathway (Jiang et al., 2014; Servián-Morilla et al., 2016).

Activation of Notch signaling was shown to improve self-renewal capacity of SCs in *mdx* mice (Jiang et al., 2014) and ameliorate DMD phenotype in Golden Retriever muscular dystrophy dogs (GRMD; Vieira et al., 2015).

Recently, Fiore et al. (2020) suggested that lack or pharmacological inhibition of protein kinase C theta (PKCΘ), which modulates several signaling pathways in muscle, leads to increased Notch signaling and improved muscle repair and SC self-renewal ability in *mdx* mice. Muscles from limb-girdle muscular dystrophy patients show decreased Notch signaling and a dramatic reduction in SC pool (Servián-Morilla et al., 2016). Significant rescue of the myogenesis was demonstrated in these patients by increasing Notch signaling (Servián-Morilla et al., 2016).

Canonical Wnt signaling dysregulation has been reported in multiple muscle pathologies, such as DMD (Trensz

et al., 2010), FSHD (Block et al., 2013), and OPMD (Abu-Baker et al., 2013).

Muscle lacking secreted factor, Wnt7a, a Wnt signaling ligand, exhibited a marked decrease in SC number following regeneration, whereas Wnt7a overexpression enhanced muscle regeneration and increased SC numbers. Wnt7a also induced myotube hypertrophy and a shift in fiber type toward slow-twitch in human primary myotubes (von Maltzahn et al., 2012b). Intramuscular treatment with Wnt7a increased activated SC number, myofiber size, and muscle force of *mdx* mice.

Inhibition of dysregulated p38MAPK signaling pathway in *mdx* mice improved SC self-renewal and mice phenotype (Smythe and Forwood, 2012; Wissing et al., 2014).

In addition, pharmacological inhibition of JAK/STAT signaling (by Calbiochem) increased numbers of SCs, enhanced muscle repair, and enhanced functional performance in aged healthy mice (Price et al., 2014).

Both canonical Wnt and TGF-β2 signaling are chronically elevated in *mdx* mouse muscle tissue (Biressi et al., 2014).

NF-κB and TGF-β signaling were overexpressed and involved in the pathophysiology of the dy^{2J}/dy^{2J} mouse (Elbaz et al., 2012, 2015). Also, it is well known that chronic activation of NF-κB signaling contributes to DMD pathology, promotes necrosis and inflammation, and inhibits muscle regeneration (Proto et al., 2015). NF-κB inhibition improves *mdx* dystrophic muscle regeneration by directly promoting lineage progression of muscle progenitor cells and by increasing progenitor cell survival (Proto et al., 2015).

Moreover, using canonical pathway analysis by Ingenuity Pathway Analysis, RhoA, NF-κB, epithelial–mesenchymal transition (EMT), TGF-β, and PKCθ were found to be altered in the LAMA2-CMD mouse model examined by RNA-Seq method (Yanay et al., 2019).

POTENTIAL THERAPIES TO ENHANCE REGENERATION

As previously mentioned, a fine-tuned balance between intrinsic signaling pathways and extrinsic factors is required to correctly control SC function. We therefore subdivide potential therapies to enhance regeneration in muscular dystrophy into those that affect intrinsic factors, extrinsic factors, or both (**Figure 3**).

As can be seen in **Figure 3**, pharmacological inhibitor or activator compounds balance and synchronize cell signaling of different intrinsic factors and may prevent SC loss and improve SC function.

Preliminary evidence for the use of SC transplantation as a potential therapeutic treatment was provided from the results of a phase II clinical trial in OPMD patients (www.clinicaltrials.gov NCT00773227; Périé et al., 2014). In that study, local autologous myoblast transplantation demonstrated significant improvement in patients' swallowing abilities with no adverse side effects. Because this procedure was accepted as generally safe with good tolerance, it has been extended to a larger cohort of OPMD patients. However, many obstacles to SC transplantation still exist, such as SCs' limited number, delivery methods, cell source (auto or allogeneic) as reviewed by Almeida et al. (2016), and need to be addressed before considering SC therapy for LAMA2-CMD patients or other muscular dystrophies with more widespread muscle involvement.

SC impaired genes can be targeted using viral vectors. Restoration of dystrophin expression using adeno-associated virus was shown to restore dystrophin in myotubes and restore dystrophin at low levels in SCs in the *mdx* mouse model (Tabebordbar et al., 2016). In addition, using lentiviral vector delivery of microdystrophin to neonatal SCs of *mdx* mice

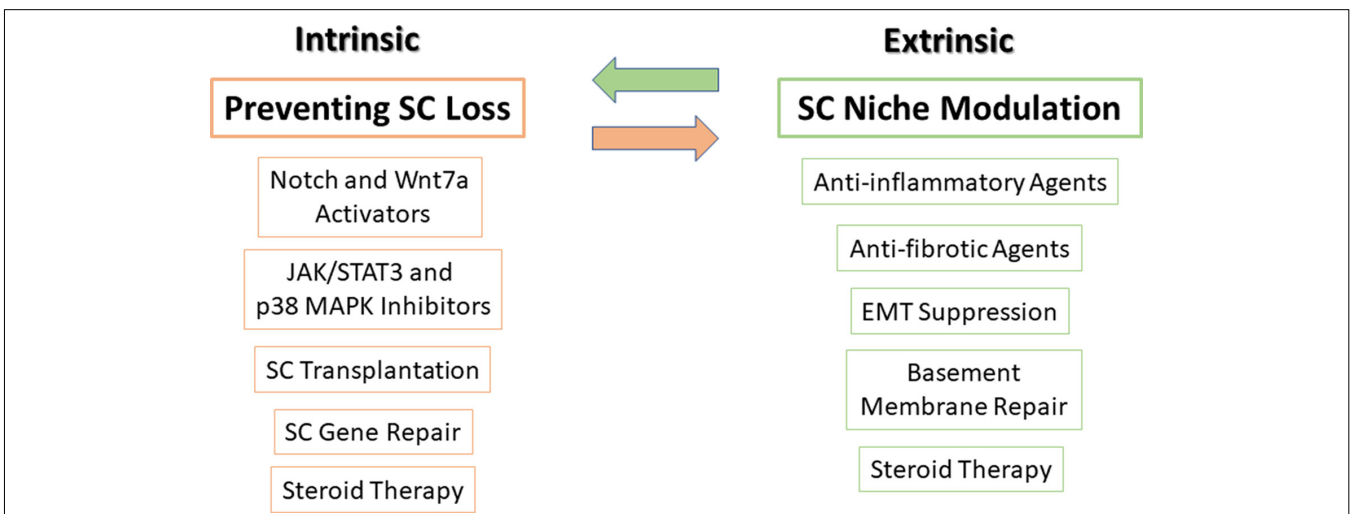

FIGURE 3 | Potential therapies to target SCs and their niche. Very tightly controlled interactions exist between SCs and their microenvironment niche, thus, in order to optimize treatment, targeting both SCs (intrinsic factors) along with their microenvironment niche (extrinsic factors) should be carried out simultaneously. On the left side, SC transplantation, modulation of major signaling pathways in SCs, mediation of gene delivery and repair, and steroid treatment can prevent SC loss and improve their function. On the right side, using anti-inflammatory and antifibrotic agents, suppression of epithelial–mesenchymal transition (EMT), basement membrane protein repair, and steroid treatment can restore SC niche. Steroid treatment is presented in both sides as it affects both SCs and satellite niche.

resulted in preservation for 2 years of dystrophin expression in myofibers, demonstrating stable transduction of SCs (Kimura et al., 2009).

Challenges in muscle formation may also be due to extrinsic factors in the microenvironment of SCs.

Host SC niche modulation should also be considered in order to produce efficient SC transplantation. Excess fibrosis and altered ECM composition alters SC functionality, resulting in impaired regeneration (Ross et al., 2012). A functional niche will provide an adjusted microenvironment for donor cells to be received by the hosts' tissue (Boldrin et al., 2012). Thus, preservation of the defective components of the SC niche is critical in order to optimize stem cell therapies.

Contribution of fibrosis and also chronic inflammation to the pathogenesis of LAMA2-CMD are well documented (Girgenrath et al., 2005; Taniguchi et al., 2006; Elbaz et al., 2012, 2015; Yanay et al., 2019). Improved regeneration can be achieved using anti-inflammatory and antifibrotic agents as demonstrated over the years (Dadush et al., 2010; Nevo et al., 2011; Elbaz et al., 2012; Accorsi et al., 2016). Immunosuppressant cyclosporin A treatment maintained SC number in Col6a1$^{-/-}$ mice following multiple bouts of induced injury (Gattazzo et al., 2014a).

Our recent RNA-Seq results support the significant role not only, as expected, of fibrosis, but also of the immune system contribution to LAMA2-CMD pathogenesis. Our findings also proposed that epithelial–mesenchymal transition (EMT) contributes to the dystrophic tissue fibrosis (Yanay et al., 2019). Thus, development of therapeutic interventions, either anti-inflammatory therapies at disease onset or suppression of EMT, may decrease fibrosis in muscular dystrophy in the mouse model.

Repair and preservation of the basement membrane could also support SC niche and functionality and restore muscle regeneration. Laminin-411 was found to be overexpressed in LAMA2-CMD as an attempt to compensate for the abnormal laminin-211 protein (Patton et al., 1997; Ringelmann et al., 1999). Laminin-411 upregulation was also confirmed in our RNA-Seq of *dy^{2J}/dy^{2J}*. However, laminin-411 lacks binding domains to laminin-211 receptors. In the study by Reinhard et al. (2017), laminin-411 was used as a scaffold protein to link mini-agrin and αLNNd (a chimeric protein that contains laminin-211 binding parts to a basement membrane component nidogen-1) in order to stabilize the muscle basement membrane. Transgenic expression of mini-agrin composed of laminin-211 binding sites to α-dystroglycan and αLNNd, together with an increased laminin-411 level, restored basement membrane stability, muscle function and size, and animal survival in the *dy^{W}/dy^{W}* mouse model.

Steroid therapy is the gold standard treatment in DMD, and its effect is associated with elevated regeneration and reduced numbers of inflammatory cells (Sklar and Brown, 1991; Hussein et al., 2006).

Prednisolone treatment was found to upregulate the Mamstr gene in dystrophin-deficient mouse muscle compared to untreated controls (Chadwick et al., 2016) and upregulate Myh and dystrophin in human DMD primary culture (Sklar and Brown, 1991). In a clinical report by Hussein et al. (2010), 6 months' prednisone therapy was associated with ultrastructural changes in dystrophic muscle and increased numbers of SCs, together with a decreased number of immune system cells (dendritic cells) and fibroblasts in DMD and BMD patients' biopsies.

SUMMARY

Recent findings point toward SC involvement in muscular dystrophy pathology.

The pathophysiology of LAMA2-CMD includes impaired SC regeneration in addition to muscle cell degeneration due to a fragile cell membrane, as a result of reduced laminin-211.

In support of this, we characterized in a recent study using RNA-Seq technique, a novel molecular signature of the contribution of specific key genes: *Pax3*, *Pax7*, and *Mamstr*, and signaling pathways, to the impaired muscle regeneration process in the *dy^{2J}/dy^{2J}* mouse model. These results suggest that the muscle phenotype in LAMA2-CMD may be ameliorated by therapies focused on recovering SC number and function.

In addition, preservation of the host SCs' niche is also required in order to provide an optimal microenvironment to improve SC function or SCs/stem cell transplantation efficacy.

The lack of Pax3 may point to very early, possibly prenatal dysfunction of SCs; thus, early treatment may be considered, before massive fibrosis accumulation and as a consequence establishment of hostile microenvironments, which presents further difficulty to SC-induced regeneration.

Confirmation of this mouse model data with additional LAMA2-CMD human data is required to advance the development of additional new therapies in LAMA2-CMD.

AUTHOR CONTRIBUTIONS

NY and YN wrote the manuscript with support of MR.

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REVIEW
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Zebrafish Models of *LAMA2*-Related Congenital Muscular Dystrophy (MDC1A)

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LAMA2-related congenital muscular dystrophy (CMD; LAMA2-MD), also referred to as merosin deficient CMD (MDC1A), is a severe neonatal onset muscle disease caused by recessive mutations in the LAMA2 gene. LAMA2 encodes laminin α2, a subunit of the extracellular matrix (ECM) oligomer laminin 211. There are currently no treatments for MDC1A, and there is an incomplete understanding of disease pathogenesis. Zebrafish, due to their high degree of genetic conservation with humans, large clutch sizes, rapid development, and optical clarity, have emerged as an excellent model system for studying rare Mendelian diseases. They are particularly suitable as a model for muscular dystrophy because they contain at least one orthologue to all major human MD genes, have muscle that is similar to human muscle in structure and function, and manifest obvious and easily measured MD related phenotypes. In this review article, we present the existing zebrafish models of MDC1A, and discuss their contribution to the understanding of MDC1A pathomechanisms and therapy development.

Keywords: laminin, LAMA2 gene, muscular dystrophy, zebrafish model, MDC1A

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INTRODUCTION

LAMA2-related congenital muscular dystrophy (CMD; LAMA2-MD), also called merosin deficient CMD or MDC1A, is the most common subtype of CMD (Schorling et al., 2017; Sframeli et al., 2017; Mohassel et al., 2018; Mercuri et al., 2019). MDC1A is an autosomal recessive neuromuscular disorder caused by mutations in laminin α2 (*LAMA2*, Helbling-Leclerc et al., 1995; Holmberg and Durbeej, 2013). Complete loss of LAMA2 protein leads to an early onset clinical phenotype featuring severe, diffuse muscle weakness and wasting, demyelinating peripheral neuropathy, and pauci-clinical central nervous system abnormalities, including white matter changes and, in some cases, structural brain lesions (Quijano-Roy et al., 1993; Menezes et al., 2014; Oliveira et al., 2018). The disease is associated with significant co-morbidities, including wheelchair dependence and respiratory failure, and early mortality in some cases (Dimova and Kremensky, 2018). A less common entity is partial merosin deficiency, a disorder caused by partial loss of LAMA2 expression/function

that is associated with a later onset, milder form of muscular dystrophy (Nguyen et al., 2019). Both MDC1A and partial merosin deficiency, as well as other rare clinical phenotypes associated with *LAMA2* mutations, are all classified as LAMA2-MD (Oliveira et al., 2018; Verma et al., 2018; Amin et al., 2019). Currently, there are no treatments for LAMA2-MD, and there is an incomplete understanding of disease pathogenesis.

Laminins

Laminins are high molecular weight glycoproteins expressed abundantly in the basal lamina, a specialized layer of the extracellular matrix (ECM; Aumailley, 2013). Laminins are multidomain heterotrimeric proteins comprised of α, β and γ polypeptide chains (Mohassel et al., 2018), which come in five (LAMA1–5), four (LAMB1–4) and three (LAMC1–3) genetic variants, respectively (Aumailley, 2013; Yurchenco et al., 2018). The polypeptide chains fold in a similar cross-shaped pattern, with distinct structural domains performing specific functions, such as facilitation of self-assembly of most laminins into large polymers by the globular laminin N-terminal (LN) domain (Hohenester, 2019). A few of the polypeptide chains, such as a4 lack the LN domain and therefore do not self-assemble (Aumailley, 2013). Based on the chain composition, more than 15 laminins have been identified in humans (Colognato and Yurchenco, 2000; Sztal et al., 2011). In zebrafish, 12 laminin-encoding genes have been found, out of which 10 have mammalian orthologs, with evolutionarily conserved function (Sztal et al., 2011), whereas two of them, *lamb1b* and *lamb2l*, have no mammalian orthologs (Sztal et al., 2011). Two human laminin-encoding genes, *LAMB3* and *LAMC2*, have not been found in zebrafish (Sztal et al., 2011). Laminins play essential roles in many tissues and organs during development (Yao, 2017). In zebrafish, laminins are involved in myriad developmental processes spanning multiple organ systems (**Table 1**).

Several laminin genes are expressed during skeletal muscle development in zebrafish. Some of these, like *lama2*, *lama4*, *lamb2*, and *lamc1*, are detected as early as 24 hpf (when myogenesis begins) and persist in the post-juvenile stages, whereas others (*lamb1*, *lamb4*, and *lamc3*) have only a brief-expression during early muscle development (Sztal et al., 2011). For example, during zebrafish early skeletal muscle development, lamb1 and lamc1 are required for fast muscle fiber elongation, orientation, and their attachment at the myotendinous junctions (MTJs), the primary site of force transmission (Snow et al., 2008; Snow and Henry, 2009). Zebrafish *lamb1* and *lamc1* mutants and morphants show delayed or impaired muscle fiber elongation, non-parallel orientation of fibers in the myotome, and defects in MTJ morphogenesis (Snow et al., 2008). Lama4 is essential for mechanical stability in zebrafish skeletal muscle (Postel et al., 2008). In *lama4* morphants, recruitment of focal adhesion proteins integrin-linked kinase (ilk) and paxillin at the MTJs is impaired, resulting in detachment of myofibers and their surrounding sarcolemma from the MTJ complex (Postel et al., 2008). Lama2 was also shown to be important for zebrafish muscle development and relevant studies on its role in this process are discussed below.

Laminin α2

The laminin α2 (*LAMA2*) gene encodes the alpha2 chain and constitutes a subunit of several laminin proteins (Tunggal et al., 2000; Aumailley et al., 2005): Laminin 211 (Laminin α2β1γ1, Laminin 2 or merosin; Durbeej, 2015; Barraza-Flores et al., 2020), Laminin α2β2γ1 (Laminin 221, Laminin 4 or S-merosin; Patton et al., 1997) and Laminin 213 (Laminin α2β1γ3, Laminin 12; Koch et al., 1999; Ido et al., 2008). LAMA2 is the major laminin isoform expressed in the vertebrate muscle system (Sztal et al., 2012).

The zebrafish *lama2* gene, representing the ortholog of human *LAMA2*, maps to chromosome 20 and is expressed in the nervous system, head, otic vesicle, adaxial cells, and skeletal muscle (Sztal et al., 2011). Mutations in zebrafish *lama2* results in a type of muscular dystrophy phenotypically similar to the human MDC1A (Hall et al., 2007), which identifies zebrafish as a suitable model for understanding this disease and for development of therapies.

ZEBRAFISH MODELS OF MUSCULAR DYSTROPHIES

Studies of animal models of muscular dystrophies have proven essential for a better understanding of the pathogenesis of these disorders and for developing disease-specific therapies (Saunier et al., 2016; Widrick et al., 2019). Research using mouse models for LAMA2-MD have identified potential therapeutic strategies, which, in turn, have led to improvements in murine disease pathology and survival (Miyagoe-Suzuki et al., 2000; Meinen et al., 2007, 2011; Vishnudas and Miller, 2009; McKee et al., 2017; Reinhard et al., 2017; Willmann et al., 2017; Mohassel et al., 2018; Yurchenco et al., 2018).

Recently, zebrafish have emerged as an excellent animal model for studying human muscle diseases, mainly due to their highly similar skeletal muscle, with conserved genetic, molecular and histological features (Telfer et al., 2010; Berger and Currie, 2012; Gibbs et al., 2013). Also, external fertilization, a large number of offspring, rapid embryonic development, optical transparency of embryos and larvae, combined with the ability to easily absorb pharmacological compounds, make zebrafish an excellent tool for studying disease pathomechanisms and identifying potential therapeutic targets (Zon and Peterson, 2005; Gibbs et al., 2013; Waugh et al., 2014; MacRae and Peterson, 2015; Cassar et al., 2020; Fazio et al., 2020). Importantly, readily available and easily applied experimental approaches allow for efficient and rapid assessment of structural and functional damage of the muscular system in the numerous zebrafish dystrophy models. For example, comprehensive phenotypic analysis of muscle damage can be easily done by using birefringence assay (**Figures 1A,A′**; Berger et al., 2012), histochemistry or immunohistochemistry staining (**Figures 1B,B′**), injections with fluorescently-tagged markers (**Figures 1C,C′**; Lombardo et al., 2012) or vital dyes, such as Evans Blue Dye (EBD;

TABLE 1 | Examples of developmental processes where laminins are involved.

Developmental process	Genes	References
Neuronal migration	*lama1*	Sittaramane et al. (2009)
Brain morphogenesis	*lamb1*, *lamc1*	Gutzman et al. (2008)
Axon-axon interactions, axon guidance	*lama1*	Paulus and Halloran (2006) and Wolman et al. (2008)
Notochord and blood vessel formation	*lama1*, *lamb1*, *lamc1*	Parsons et al. (2002) and Pollard et al. (2006)
Establishment of liver and pancreas left-right asymmetry	*lamb1a*	Hochgreb-Hägele et al. (2013)
Fin development	*lama5*	Webb et al. (2007)
Myocardial function	*lama4*	Knöll et al. (2007)
Retinal differentiation and maintenance	*lama1*, *lamb1*, *lamc1*	Biehlmaier et al. (2007)
Eye development	*lama1*, *lamb1*, *lamc1*	Semina et al. (2006), Zinkevich et al., 2006 and Lee and Gross (2007)

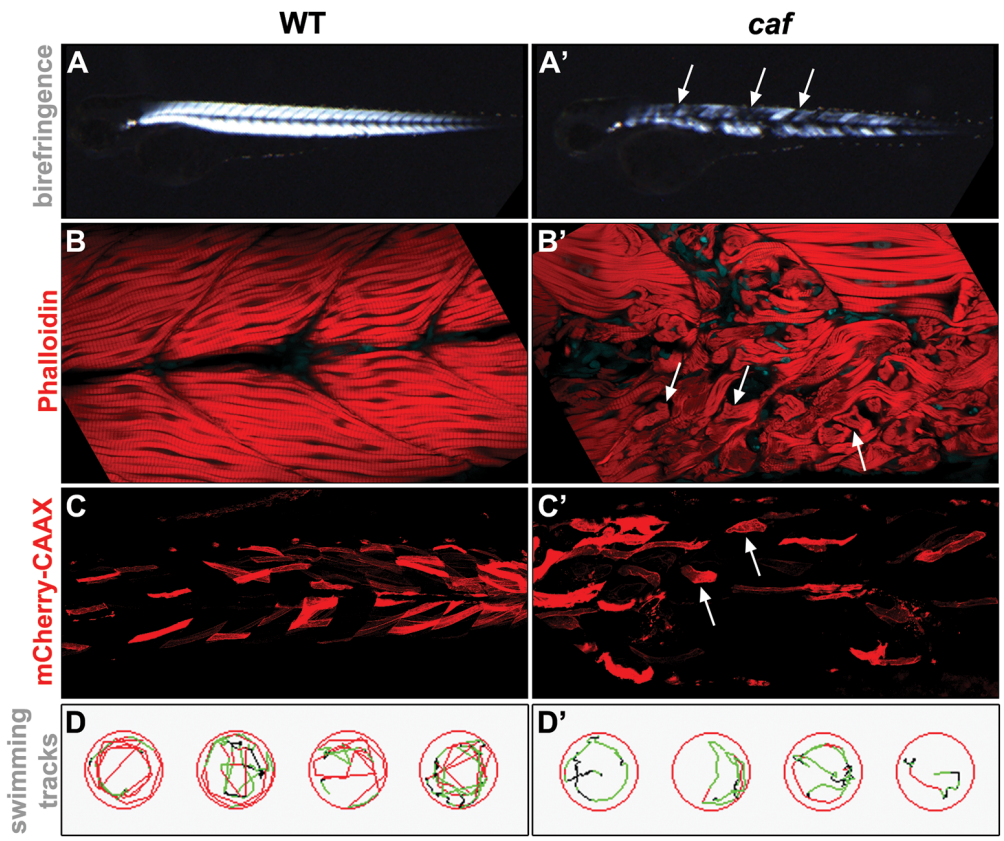

FIGURE 1 | Examples of experimental approaches used for the phenotypical analysis of the zebrafish LAMA2-related congenital muscular dystrophy (CMD, LAMA2-MD) model. **(A,A')** Birefringence assay. The organization of muscle fibers can be seen by polarized light. Detached muscle fibers in the *caf* mutant show up as dark regions in the muscle (arrows). **(B,B')** Whole-mount staining. Phalloidin stains the actin filaments in the muscle. Muscle fibers detached from the myotendinous junctions (MTJs) in the *caf* mutant can be easily identified (arrows). **(C,C')** Injected fluorescent marker. *unc53*:mCherry-CAAX-pA construct (Zhao et al., 2019), which marks muscle cells, was injected into 1-cell stage embryos and visualized by live imaging at 3 dpf. Detached fibers in the *caf* mutant can be easily identified (arrows). **(D,D')** Swimming assay. Swim behavior can be tracked and quantified using Viewpoint Zebrabox software (Viewpoint Life Sciences Inc.). Time spent moving, distance traveled and speed of movement are useful indicators of muscle function. Fewer tracks and lower speed (indicated by green tracks) are seen in the recorded *caf* mutants.

Smith et al., 2015) followed by live imaging, swimming assay to assess motor behavior (**Figures 1D,D'**; Zon and Peterson, 2005; Gibbs et al., 2013; Smith et al., 2013), and other equally useful techniques (Gibbs et al., 2013; MacRae and Peterson, 2015).

Zebrafish models have been developed for many human diseases, such as glioblastoma (Gamble et al., 2018), eye diseases (Moosajee et al., 2008; Bryan et al., 2016), cardiovascular disorders (reviewed in Gut et al., 2017), and kidney diseases (reviewed in Jerman and Sun, 2017), to name a few. Various human muscle disorders, such as Duchenne muscular dystrophy (DMD; Bassett and Currie, 2003; Bassett et al., 2003; Widrick et al., 2016), Laminin α2-associated muscular dystrophy (Jacoby et al., 2009), Ullrich CMD (Telfer et al., 2010),

dystroglycanopathies (reviewed in Hewitt, 2009; Lin et al., 2011; Bailey et al., 2019), facioscapulohumeral muscular dystrophy (Mitsuhashi et al., 2013; Pakula et al., 2019), X-linked myotubular myopathy (Dowling et al., 2009; Lawlor et al., 2016; Sabha et al., 2016), and nemaline myopathies (Telfer et al., 2012; Sztal et al., 2015), also have been modeled in zebrafish (reviewed in Nance et al., 2012; Gibbs et al., 2013; Lek et al., 2015; Li et al., 2017).

ZEBRAFISH MODELS FOR LAMA2-MD

To date, only a handful of zebrafish models for LAMA2-MD have been developed, even though there are more than three hundred *LAMA2* gene variants associated with human disease (Oliveira et al., 2018).

The first zebrafish model of LAMA2-MD was described by *Currie and colleagues* (Hall et al., 2007) and was identified through complementation studies between dystrophic mutants generated through an N-ethyl-N-nitrosourea (ENU) mutagenesis screen at the University of Tubingen, Germany (Granato et al., 1996). Homozygous mutant zebrafish carrying either *teg15a* or *tk209* recessive mutant allele, show impaired swimming behavior, severe muscle loss, and detached myofibers. Based on the specific shape of the detached fibers, which resemble cotton candy, they named this mutant *candyfloss* (*caf*). The two caf alleles, caf^{teg15} and caf^{tk209}, represent loss-of-function mutations in *lama2* gene, and both homozygous mutants exhibit a loss of lama2 protein expression, with similar degenerative muscle phenotype, death by 16 dpf in the majority of cases, and lack of progeny for the small percent of surviving mutants. The mutations have been mapped to the globular domain of *lama2*, which is required for binding to dystroglycan (Hall et al., 2007), a component of the dystrophin-associated glycoprotein complex (DGC) involved in attaching the muscle fibers to the ECM (Sztal et al., 2012). *caf* mutations are located within amino acid residues conserved in humans and mice where known human LAMA2-MD mutations have been identified (Hall et al., 2007).

Using a birefringence assay as a screening tool, it was shown that the muscle defects present in the *caf* zebrafish resemble those described in human patients with LAMA2-MD, namely a stochastic pattern of myofiber detachment from the MTJs. This detachment affects both slow and fast muscle fibers (Sztal et al., 2012), is muscle cell-autonomous, and is dependent on the motor activity of the muscle (Hall et al., 2007; Thomasi et al., 2018). Notably, even though the detachment of the damaged fibers happens rapidly, they maintain the integrity of their sarcolemma, in contrast to what is happening in the muscle of the DMD zebrafish model *sapje* (Bassett et al., 2003; Smith et al., 2015). This is well demonstrated through the use of EBD injections, with *caf* zebrafish showing limited uptake into the sarcoplasm (Hall et al., 2007; Smith et al., 2015). A similar finding of limited/reduced EBD uptake is observed in the *dy* mouse model of LAMA2-MD (Straub et al., 1997), suggesting that impairment of membrane integrity is less prominent in LAMA2-MD vs. other MDs, and also supporting the validity of the muscle phenotype of *caf* zebrafish.

Of note, through elegant *in vivo* time-lapse experiments using various fluorescently-tagged constructs, the specific properties of the *lama2*-deficient myofibers were characterized in detail (Hall et al., 2019). The authors showed these fibers are long-lived, and undergo extensive cellular remodeling by extending protrusions to re-attach to the ECM. They display the formation of new pre-myofibers and undergo nuclear fusion with nearby satellite cells, all processes that indicate that repair, regeneration, and survival mechanisms are activated in the *lama2*-deficient myofibers. Importantly, the authors showed this is not the case in dystrophin-deficient fibers (Hall et al., 2019).

More recently, another zebrafish model for LAMA2-MD has been characterized (Gupta et al., 2012; Smith et al., 2017). The $lama2^{cl501}$ mutant, also identified through an ENU mutagenesis screen (Gupta et al., 2011), carries a mutation in a highly conserved splice site located in the coiled-coil α-helical domain in the long arm of *lama2*, which is required for binding of LAMA2 to the other laminins in the heterotrimeric complex. This mutation results in a complete loss-of-function due to defective splicing of the *lama2* mRNA. The phenotype of $lama2^{cl501}$ mutants is essentially identical to that of *caf* zebrafish, with early-onset muscle degeneration due to detachment of fibers from the MTJs and death by 15 dpf (Gupta et al., 2012). Importantly, the detachment of the myofibers from the MTJs in $lama2^{cl501}$ happens without the loss of sarcolemmal integrity, similar to *caf* mutants. These mutants show reduced laminin expression in the basal membrane at the MTJs complexes, smaller myotomes indicative of growth defects, disorganized sarcomere structure, and increased number of necrotic fibers. Also, $lama2^{cl501}$ mutants exhibit brain and eye defects (Gupta et al., 2012). Pathogenesis of $lama2^{cl501}$ is similar to that of human patients with MDC1A, making this mutant another excellent animal model for identifying potential therapies for MDC1A.

Additional research looking at genetic interactions between *lama2* and other dystrophic genes has contributed to our understanding of the specific pathomechanism(s) by which the muscle damage occurs in LAMA2-MD (Sztal et al., 2012). LAMA2, as the major muscle isoform, regulates attachment of myofibers to the ECM either through the dystroglycan complex or through integrin pathways (Tunggal et al., 2000; Pozzi et al., 2017). However, proteins such as dystroglycan (Ervasti and Campbell, 1993; Lisi and Cohn, 2007), dystrophin (Bassett and Currie, 2004), integrin-α7 (Postel et al., 2008), or ilk (Postel et al., 2008) that interact directly or indirectly with LAMA2, play important roles in modifying the LAMA2-MD phenotype. Systematic epistatic experiments in this study (Sztal et al., 2012) showed that concomitant loss of *ilk and dmd* (dystrophin), or *ilk* and *DAG1* (dystroglycan) result in a more severe dystrophic phenotype than the loss of *lama2* or either one alone. Also, the authors show that the phenotype of *lama2/ilk*, *lama2/dmd*, or *lama2/dag1* double homozygous mutants is less severe than the one exhibited by the *ilk/dmd* or *ilk/dag1* mutants, implicating other laminins, in addition to *lama2*, in maintaining the attachment of myofibers to the ECM. Further, by injecting either *lama4* or *lama1* morpholino in *lama2* mutants, Sztal et al. (2012) showed that *lama1*, but not *lama4*, also plays a significant role in this process.

A key outcome of the studies using the *caf* and $lama2^{cl501}$ zebrafish models was the ability to discriminate between this

disorder and DMD, another severe form of muscular dystrophy (Bassett et al., 2003; Bassett and Currie, 2004; Widrick et al., 2016). In muscle from the DMD zebrafish model *sapje* the detached fibers show significant sarcolemmal damage, followed by rapid and increased apoptosis and/or necrosis (Bassett et al., 2003), whereas muscle from the *caf* and *lama2*^{cl501} zebrafish fully detaches without concomitant sarcolemmal damage (Hall et al., 2007; Gupta et al., 2012). Also, detached myofibers in *lama2* zebrafish show increased survival and regeneration due to the up-regulation of *lama4* in detached fibers (Sztal et al., 2012).

THERAPEUTIC STRATEGIES FOR LAMA2-MD—LESSONS FROM ZEBRAFISH

Several studies using *lama2* zebrafish models identified potential therapeutic strategies for LAMA2-MD (Sztal et al., 2012; Smith et al., 2017; Hall et al., 2019). Results from studies in other dystrophic zebrafish models can also be translated and applied to LAMA2-MD (Goody et al., 2012; Kawahara et al., 2014; Widrick et al., 2019; Wood et al., 2019).

Drug Screening and Drug Therapy

Studies by Smith et al. (2017) identified and characterized a very early coiling defect in the *lama2*^{cl501} fish, which can be used as a measurable and reliable phenotype for drug screening. The mutant fish complete significantly fewer tail coiling movements compared to their wild type siblings (Smith et al., 2017). Importantly, this phenotype manifests only in *caf* and *lama2*^{cl501}fish, not in DMD mutants. This early phenotype is consistent with the early perinatal changes observed in LAMA2-MD mouse models (Mehuron et al., 2014), and mirrors the congenital onset phenotype of patients. Therefore, this zebrafish model may be effectively used to identify drug therapies that act at early stages in the LAMA2-MD disease process, which then could be translated into mouse models and clinical trials.

Recent studies using an integrin beta1 zebrafish (*itgβ1*) showed that targeting LAMA2 binding partners, such as integrin, could also provide insights into putative drug therapies for LAMA2-MD (Wood et al., 2019). *itgβ1*-deficient fish displayed increased amounts of LAMA2 and collagen at the ECM, indicating that inhibition of itgβ1 in *lama2*-deficient models might ameliorate the LAMA2-MD phenotype. Injections of the peptide RGD, an itgβ1 inhibitor, led to increased myofiber stability at the basal lamina in *caf* zebrafish, by increasing the levels of lama2 at the ECM (Wood et al., 2019).

Additional insights into using zebrafish models for the development of drug therapies for muscular dystrophies were provided by studies modulating nicotinamide adenine dinucleotide (NAD+) biosynthesis in *dag1* and *itga7* dystrophic morphants (Goody et al., 2012). NAD+ synthesis, mediated by the muscle-specific nicotinamide riboside kinase 2b (nrk2b), was shown to be essential for lamc1 polymerization at the MTJs and identified additional regulators of muscle morphogenesis in the cell adhesion signaling pathway (Goody et al., 2010). Exogenous supplementation of NAD+ or overexpression of its downstream effector, paxillin, ameliorate the dystrophic phenotype, by increasing the MTJ-basement membrane organization through laminin augmentation (Goody et al., 2012).

Gene Therapy and Protein Replacement Therapy

Recent studies in the *caf*^{teg15} zebrafish model showed that expressing *lama2* or injecting lama2 rescues the LAMA2-MD dystrophic fiber phenotype (Hall et al., 2019). Generalized expression of *lama2* under a heat-shock promoter during embryonic development or muscle-specific overexpression of *lama2* in *caf* fish led to normal levels and correct distribution of laminin at the MTJs and complete rescue of the dystrophic phenotype (Hall et al., 2019). Driving the expression of *lama2* later in development, after the dystrophic phenotype is fully established, resulted in a significant decrease in the number of detached fibers, increased survival, remodeling, repair and reattachment of detached fibers (Hall et al., 2019). Intramuscular delivery of Laminin111, a laminin complex similar to Laminin211 shown to functionally replace Laminin211 in an MCD1A mouse model (Van Ry et al., 2014), increased the population of muscle stem cells and resulted in significant improvement of the *caf* phenotype (Hall et al., 2019). This is similar to what has been described for laminin replacement therapy in DMD and *alpha7 integrin*-null mouse models (Rooney et al., 2009a,b; Goudenege et al., 2010) and provides additional validation of the therapy, as well as of the model as a vehicle for discovery and development of therapies.

Caveats of Using Zebrafish as the LAMA2-MD Disease Model

The above studies describing LAMA2-MD zebrafish models, together with the increasing number of studies modeling other human diseases in zebrafish (Steffen et al., 2007; Wood and Currie, 2014), prove the amenability of zebrafish as an organism for advancing our understanding of pathogenic mechanisms and therapies development. However, we should mention that a few caveats should be taken into consideration when translating the results from the LAMA2-MD zebrafish to human patients with LAMA2-MD.

LAMA2-MD pathophysiology shows slight differences between human patients and zebrafish models. For example, in humans, the LAMA2-MD dystrophic phenotype is associated, besides other features, with increased atrophy and apoptosis, defective regeneration and repair, depletion of satellite cell pools, upregulated autophagy and abnormal proteasome-dependent degradation (Durbeej, 2015). These changes have yet to be thoroughly examined in zebrafish models of LAMA2-MD. Also, evaluating non-muscle phenotypes associated with LAMA2 mutations presents challenges in the zebrafish. Importantly, myelination is distinctly different in zebrafish compared to mammals, with peripheral myelin expressing myelin basic protein and not MPZ or PMP22. Thus, studying mechanisms related to white matter disease and peripheral neuropathy may not be feasible in zebrafish, and addressing the impact of therapeutic interventions on these features of disease not possible.

Designing drug screens for LAMA2-MD in zebrafish requires taking into consideration that detachment of myofibers in *caf* mutants is movement- and mechanical load-dependent (Hall et al., 2007). Therefore, it is necessary to ensure the drugs tested do not affect swimming behavior. Fish immobilized due to highly toxic drugs, for example, would lead to the identification of false-positive drug hits.

Overall, there is therefore the need to balance the advantage of the zebrafish with these shortcomings. The integration of observations in *caf* mutants with other *in vivo* models of LAMA2-MD should greatly aid in their translatability. In particular, several mutant mouse models accurately phenocopy key aspects of the human disease (Gawlik and Durbeej, 2020), and provide an opportunity to test and validate findings from the *caf* mutants and to determine their relevance to non-muscle systems. This is particularly true concerning therapy development, where a pipeline of large scale drug screening in zebrafish combined with testing and validation in the mouse may yield candidate therapeutics with the highest potential for successful translation to patients. The establishment of a similar pipeline crossing multiple species was recently reported for congenital muscle disease due to *RYR1* mutation (Volpatti et al., 2020).

CONCLUSIONS

Zebrafish models of human diseases contribute significantly to our understanding of underlying pathogenic mechanisms, characterization of signaling pathways regulating them, and development of therapeutic strategies. The main strengths of the zebrafish model are a large number of offspring, rapid embryonic development, and optical transparency of the embryos, which allow for successful screening approaches, from drug discovery to genome-scale CRISPR and genetic modifiers screening (reviewed in Gut et al., 2017).

Zebrafish is an excellent model organism to study LAMA2-MD, as they mirror the genetics and motor phenotypes of patients and carry important advantages for pathway analyses and drug discovery. Zebrafish are extremely amenable to high-throughput chemical screening to identify therapeutic drugs for LAMA2-MD (MacRae and Peterson, 2015). This approach has been successfully used in other zebrafish models of human disease (Bootorabi et al., 2017; Jardine et al., 2020; Gut et al., 2017; Matsuda et al., 2018). Furthermore, genome-editing technologies such as TALENs and CRISPR/Cas systems are easily applied to zebrafish and could be used to generate and study a large number of patient-specific mutations (Zhang et al., 2018; Giardoglou and Beis, 2019; Lek et al., 2020). Despite some limitations (Gut et al., 2017), these genome-editing approaches allow for the generation of a theoretically unlimited number of zebrafish mutants, which could ultimately enable scientists to systematically and comprehensively study full allele series for disorders such as LAMA2-MD. Lastly, performing genetic modifiers screens in *caf* zebrafish with methodologies including ENU mutagenesis and CRISPR gene editing (McGovern et al., 2015; Quattrocelli et al., 2017a,b; Rahit and Tarailo-Graovac, 2020; Volpatti et al., 2020) should enable the identification of genetic interactions and novel disease modifiers, data which would greatly advance our understanding of the pathomechanisms and phenotypic variability of LAMA2-MD.

AUTHOR CONTRIBUTIONS

JD: conception and final approval. JD and LF: design and critical revisions. LF: drafting the article.

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frontiers in Molecular Neuroscience

REVIEW
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Brain Dysfunction in LAMA2-Related Congenital Muscular Dystrophy: Lessons From Human Case Reports and Mouse Models

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Laminin α2 gene (LAMA2)-related Congenital Muscular Dystrophy (CMD) was distinguished by a defining central nervous system (CNS) abnormality—aberrant white matter signals by MRI—when first described in the 1990s. In the past 25 years, researchers and clinicians have expanded our knowledge of brain involvement in LAMA2-related CMD, also known as Congenital Muscular Dystrophy Type 1A (MDC1A). Neurological changes in MDC1A can be structural, including lissencephaly and agyria, as well as functional, including epilepsy and intellectual disability. Mouse models of MDC1A include both spontaneous and targeted LAMA2 mutations and range from a partial loss of LAMA2 function (e.g., dy^{2J}/dy^{2J}), to a complete loss of LAMA2 expression (dy^{3K}/dy^{3K}). Diverse cellular and molecular changes have been reported in the brains of MDC1A mouse models, including blood-brain barrier dysfunction, altered neuro- and gliogenesis, changes in synaptic plasticity, and decreased myelination, providing mechanistic insight into potential neurological dysfunction in MDC1A. In this review article, we discuss selected studies that illustrate the potential scope and complexity of disturbances in brain development in MDC1A, and as well as highlight mechanistic insights that are emerging from mouse models.

Keywords: LAMA2, MDC1A, brain development, congenital muscular dystrophy, dystroglycanopathies, laminin

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INTRODUCTION

Laminin Structure and Function

Laminins are developmentally regulated extracellular matrix (ECM) molecules comprised of α, β, and γ chains. Laminins play critical roles in the establishment and overall organization of basement membranes (BMs) as well as in controlling signaling pathways through interactions with cell surface receptors. All laminins exist as heterotrimers comprised of one α, one β, and one γ chain, assembled via a triple coiled-coil, and are named based on their composition, e.g., Laminin-111 contains the α1, β1, and γ1 chains. Five genes encode the α1–α5 chains (LAMA1 to LAMA5), four genes encode the β1–β4 chains (LAMB1-LAMB4) and three genes encode the γ1–γ3 chains (LAMC1–3; Yurchenco et al., 2018). In most laminin trimers, the three N-terminal regions are "free," i.e., they extend beyond the coiled-coil region, producing a cross-shaped molecule with N-terminal short arms. Laminins with a full complement of three N-terminal short arms can form an inter-laminin meshwork, or polymer, while laminins

without short arms (e.g., laminins with α3A or α4 subunits) do not polymerize (Durbeej, 2010; Yurchenco et al., 2018). Importantly, the C-terminal α-subunit of all laminins, which extends beyond the coiled-coil region, contains binding sites for the majority of laminin-binding receptors. These differing structural properties, in concert with varying abilities to bind cell surface receptors and other ECM proteins such as nidogen and perlecan, are thought to underlie the ability of different laminins to convey differing chemical and mechanical signals to cells.

Receptors for laminins include integrins, most prominently integrins α6β1 and α7β1. Integrins are transmembrane glycoprotein receptors that mediate cellular and ECM interactions as well as activate signal transduction pathways (Belkin and Stepp, 2000). α7β1 is a critical receptor of laminin in developing and adult skeletal muscle and loss of α7β1 leads to mild muscular dystrophy (Saher and Hildt, 1999). The interaction between LAMA2 loss and α7β1 is discussed in depth in another review of this special issue (Barraza-Flores et al., 2020). Laminin-binding integrins mediate signaling responsible for developmental processes in the central nervous system (CNS) such as axon outgrowth (Tomaselli et al., 1988) and cell migration (Desban and Duband, 1997). Other non-integrin binding partners include dystroglycan (Brancaccio et al., 1995; Colognato and Yurchenco, 2000) and heparan sulfate proteoglycans (Brown et al., 1994). Dystroglycan, a transmembrane receptor of the Dystrophin-Glycoprotein Complex (DGC), is comprised of α- and β-subunits that remain tightly associated (Moore and Winder, 2012). α-Dystroglycan, which is entirely extracellular, undergoes extensive glycosylation that is necessary for α-dystroglycan to directly interact with laminin-like G (LG) domain-containing ligands, which include laminins, agrin, and perlecan, while β-dystroglycan is transmembrane and interacts with multiple binding partners within the cell (Moore and Winder, 2012). For instance, laminin-binding to α-dystroglycan in skeletal muscle anchors the ECM to the actin cytoskeleton via the interaction of β-dystroglycan with the actin-binding protein, dystrophin (Yurchenco et al., 2018). Disturbances in α-dystroglycan glycosylation, which impair binding to LG domain-containing extracellular ligands including laminin, underlie a collection of genetic disorders known as α-dystroglycanopathies (Jimenez-Mallebrera et al., 2005).

LAMA2 encodes for the α2 chain and mutations in the LAMA2 gene can disrupt either the expression or the binding capacity of α2-containing laminins such as laminin-211 (Lm-211). This loss-of-function is best understood in skeletal muscle, where Lm-211, via interactions with dystroglycan and α7β1 integrin receptors, is critical for proper BM assembly and function, which in turn is needed for the stability of the muscle sarcolemma (Yurchenco et al., 2018). While muscle pathology is described in detail in other reviews of this issue (Accorsi et al., 2020; Barraza-Flores et al., 2020; Gawlik and Durbeej, 2020), in general these abnormalities include apoptosis, fibrosis, inflammation. However laminin-α2 deficiency in muscle also leads to an upregulation of Lm-411 (α4, β1, γ1; Patton et al., 1997), and Lm-511 (α5, β1, γ1), which normally disappear postnatally, remaining only at the neuromuscular junction (NMJ) in healthy adult muscle (Patton et al., 1997; Kölbel et al., 2019). Several lines of evidence suggest that these laminins may not functionally compensate for Lm-211. For instance, while Lm-511 can polymerize, Lm-411 cannot (Durbeej, 2010; Di Russo et al., 2017; Yurchenco et al., 2018); and, while Lm-411 and Lm-511 both bind to α3β1 and α6β1 integrins (Fujiwara et al., 2001), there is no direct evidence to suggest that Lm-411 and Lm-511 bind to α-dystroglycan strongly enough to provide a fully functional mechanical "ECM-to-cytoskeletal bridge" for contracting muscle (Yu and Talts, 2003; Reinhard et al., 2017). One study, using various combinations of knockout mouse models, found that only a double mutant ($Lama4^{-/-}$; $Lama5^{M/M}$), but not single mutants, resulted in less clustering of dystroglycan at the NMJ (Nishimune et al., 2008). In a different study that more directly assessed α4 and α5 binding to α-dystroglycan, both laminins were able to bind but with low affinity and only in the presence of linker proteins, including mini-agrin (Reinhard et al., 2017). Lastly, in a study that compared muscle integrity in a dystroglycan-deficient mouse ($Dag1^{-/-}$) vs. an α7β1 deficient mouse ($Itga7^{-/-}$), the BM only detached in the $Dag1^{-/-}$ mouse (Han et al., 2009), indicating the importance of the laminin-dystroglycan connection in the sarcolemmal BM. These experiments and others suggest that laminins that contain α4 and α5 chains are not able to fully compensate for the loss of laminin-α2 expression, especially regarding its binding to α-dystroglycan. However, a full understanding of the cell and tissue phenotypes that occur in the absence of normal LAMA2 expression remains challenging, as phenotypes may arise from a complex mixture of loss- and gain-of-functions.

The ability of receptors to interact with α2-containing laminins is also critical in the CNS, where the loss of laminin-α2 results in brain abnormalities that include neuronal migration defects that can result in lissencephaly. Interestingly, in severe cases of α-dystroglycanopathies, there are gross cortical lamination abnormalities, resulting in profound neurological deficits; these more extreme deficits are more rarely observed in congenital muscular dystrophy type 1A (MDC1A). These and other differences described ahead suggest that MDC1A and dystroglycanopathies may have some shared, but some distinct, cellular mechanisms underlying their respective CNS pathologies. As in muscle, LAMA2-related phenotypes in the brain are likely to be a complex mixture of functional loss coupled with an altered ECM landscape.

Congenital Muscular Dystrophy and LAMA2 Mutations

Congenital muscular dystrophies (CMD) are a collection of heterogeneous genetic disorders that largely result from mutations in genes required for the DGC. Subtypes of CMD include MDC1A, Walker–Warburg Syndrome (WWS), Muscle–Eye–Brain disease (MEB), and additional dystroglycanopathies (Johnson et al., 2018; **Figure 1**). CMD patients present with symptoms that include hypotonia, muscle weakness, and elevated serum creatine kinase (CK), of which onset occurs at birth or early infancy (Mackay et al., 1998). If these symptoms are present at birth, they are collectively called "floppy infant" syndrome, which is highly suggestive of

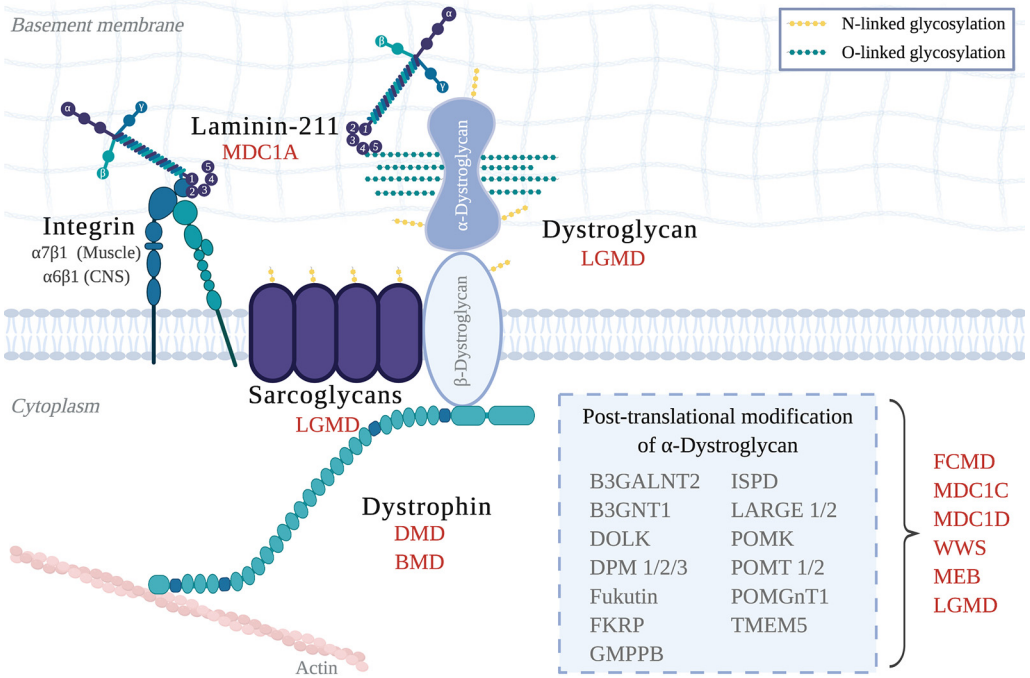

FIGURE 1 | The Dystrophin-Glycoprotein Complex (DGC) in skeletal muscle and its related muscular dystrophies. Abbreviations: MDC1A, Congenital Muscular Dystrophy Type 1A; LGMD, Limb-Girdle Muscular Dystrophy; DMD, Duchenne Muscular Dystrophy; BMD, Becker Muscular Dystrophy; FCMD, Fukuyama Congenital Muscular Dystrophy; MDC1C, Congenital Muscular Dystrophy Type 1C; MDC1D, Congenital Muscular Dystrophy Type 1D; WWS, Walker–Warburg Syndrome; MEB, Muscle–eye–brain disease.

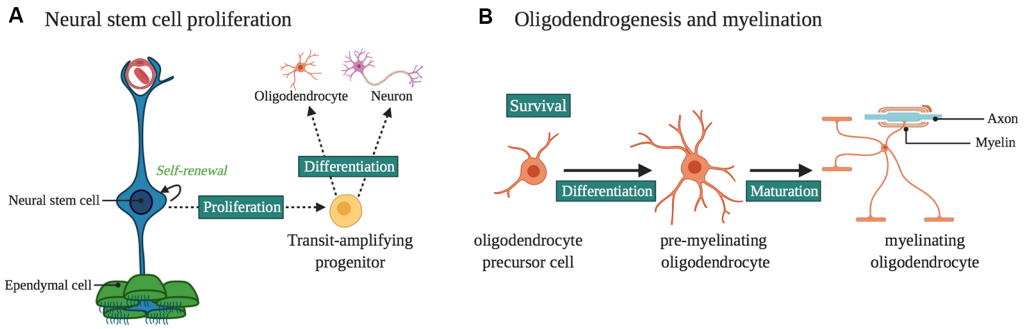

FIGURE 2 | Laminin α2 in the ventricular-subventricular zone (VZ-SVZ) neural stem cell (NSC) niche. **(A)** Laminin α2 influences NSC proliferation and differentiation. It influences neurogenesis and gliogenesis in the VZ-SVZ, as well as the proliferation and survival of progenitors for midbrain dopaminergic neurons. **(B)** Oligodendrogenesis and myelination are influenced by LAMA2 expression. In particular, LAMA2 expression influences the survival and differentiation of oligodendrocyte precursor cells (OPCs) as well as the maturation of oligodendrocytes.

neuromuscular disease (e.g., spinal muscular atrophy, CMD), and require further testing. Pathological features of all CMDs include extensive muscle wasting, necrosis, and fibrosis, and most, if not all, CMDs can also present with CNS involvement, which includes white matter abnormalities, structural brain abnormalities (e.g., cortical dysplasias; see **Figure 3**), and ocular involvement (Jimenez-Mallebrera et al., 2005).

While over 20 genes have been implicated in CMDs, and CMDs can share clinical characteristics, this review article will focus on features of MDC1A from human and mouse model studies, as well as compare MDC1A to CMDs arising from mutations in Lm-211 binding partners. MDC1A, while still classified as a rare disease, is the most common form of CMD in many regions (Allamand et al., 1997; Sframeli et al., 2017; Ge et al., 2019). MDC1A is considered the classical CMD as nearly half of all CMD patients lack or express reduced levels of laminin-α2 in muscle BM (Cohn et al., 1998). Patients with MDC1A (previously referred to as merosin-deficient CMD as merosin was the original name for α2-containing laminins) also represent a heterogeneous group. For instance, two large studies

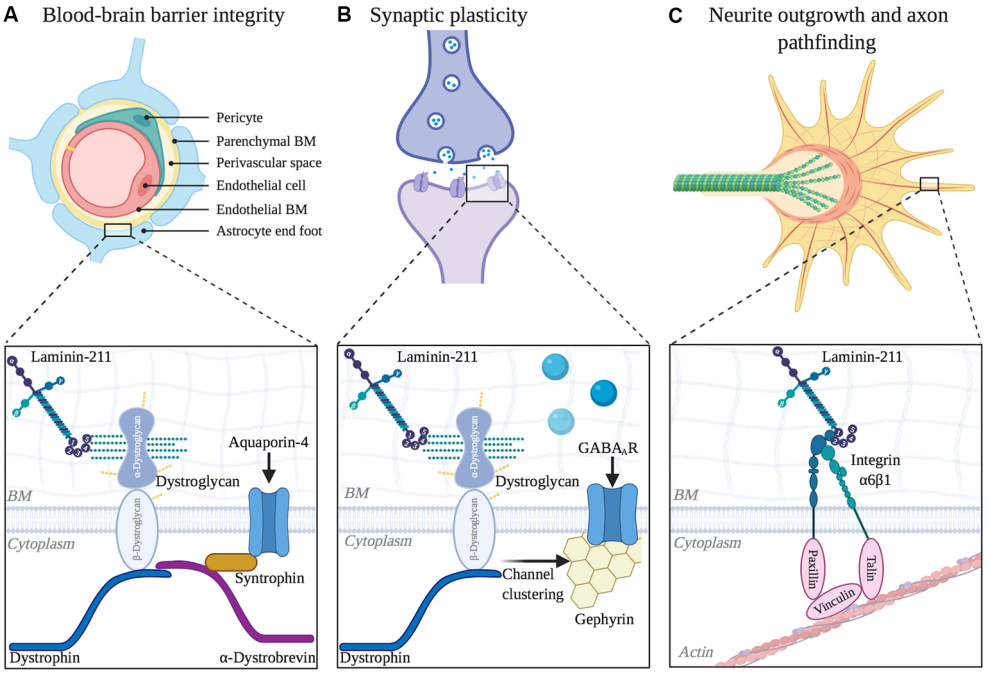

FIGURE 3 | Functional roles of laminin α2 in other areas of the central nervous system (CNS). **(A)** Blood-brain barrier integrity is regulated by LAMA2 expression. Inset depicts laminin interaction with dystroglycan, which helps to cluster aquaporin-4 channels at barrier astrocytic endfeet. **(B)** Laminin α2 influences long-term synaptic plasticity. Inset depicts laminin interaction with dystroglycan, which helps to mediate the clustering of $GABA_A$ receptors at inhibitory synapses. **(C)** Neurite outgrowth and axon pathfinding are influenced by α2-containing laminins. Inset depicts laminin interaction with integrin α6β1 in neuronal growth cones, which helps to couple extracellular matrix (ECM) and the actin cytoskeleton as growth cones extend.

have revealed a correlation between the severity of MDC1A and the degree of laminin-α2 expression (Allamand et al., 1997; Geranmayeh et al., 2010). In one study of 51 MDC1A cases, the *complete absence* of laminin-α2 correlated with worse clinical outcomes and earlier presentation of the disease when compared to a *partial loss* of laminin-α2 (Geranmayeh et al., 2010). This study highlights the importance of determining the status of laminin-α2 levels in patients with MDC1A, as laminin-α2 levels appear to correlate with the severity of the muscle phenotype. In the following sections, we will highlight both typical and atypical CNS findings in patients with MDC1A.

CENTRAL NERVOUS SYSTEM INVOLVEMENT IN MDC1A

Diffuse White Matter Changes by MRI

Although only a subset of MDC1A patients have profound neurological deficits, white matter changes (i.e., hypointensity on T1 weighted imaging) are routinely found in all MDC1A patients such that the presence of these findings increases the clinical suspicion of MDC1A (Allamand et al., 1997; Geranmayeh et al., 2010; Bönnemann et al., 2014). In a study of 20 patients with the clinical diagnosis of CMD, 13 patients had complete absence or deficiency of laminin-α2, with all 13 having white matter abnormalities upon MRI (Herrmann et al., 1996). Although genetic testing was not performed in these patients, the clinical suspicion for MDC1A is high because of the white matter abnormalities seen in these 13 patients. In another MDC1A study (referred to as classic CMD in the study) in which 9 out of 15 patients were laminin-α2 deficient, disease onset occurred in the first year of life, and all nine had substantial white matter changes that resembled periventricular white matter involvement seen in leukodystrophy (Lamer et al., 1998). Also, seven out of nine laminin-α2 deficient patients had pons hypoplasia and one had evidence of cortical atrophy, likely a result of failed cortical growth (Lamer et al., 1998). In a larger MRI study of 21 MDC1A patients, all but three had white matter changes (Geranmayeh et al., 2010). However, since the MRIs in these three patients were done neonatally, and MRIs of MDC1A patients can appear normal in the first 6 months of age but go on to become abnormal, it remains unclear if these patients were in fact truly lacking white matter changes. The same study reported that while 18 out of 21 patients with MDC1A had typical white matter changes, 13 out of 21 had additional brain abnormalities that included ventricular dilation, cortical dysplasia and atypical white matter changes (Geranmayeh et al., 2010). These numbers indicate that changes beyond the typical white matter changes in MDC1A are not rare, but instead are fairly common.

While it is largely known that MDC1A is accompanied by diffuse brain white matter changes, the underlying pathogenesis remains unclear. One hypothesis is that these white matter abnormalities are due to increased water content due to an impaired selective filtration caused by laminin-α2 deficiency

(Villanova et al., 1996, 1997; Menezes et al., 2014). A second hypothesis is that these white matter changes indicate structural changes in the white matter tracts themselves, since interactions between laminin-α2 containing laminins and integrins on developing oligodendrocytes (myelinating cells of the CNS) enhance the development of myelin membrane (Buttery and ffrench-Constant, 1999; Chun et al., 2003; Barros et al., 2009; Relucio et al., 2009, 2012). Ahead we will describe studies that support both hypotheses.

Reduced Fractional Anisotropy on Diffusion Tensor Imaging

Fractional anisotropy (FA) is a metric of diffusion that is sensitive to aligned obstacles such as cell membranes and myelin (Mori and Zhang, 2006). In the brain, a white matter region with a low FA reflects a lack of coherent fiber organization, which is often due to myelin deficits but can also be due to other structural problems such as an abnormal axon packing and organization. However, FA is used as a readout for white matter maturation, as the typical time course in both mice (Mori and Zhang, 2006) and humans (Keunen et al., 2018) demonstrates that during the perinatal period FA increases in white matter tracts as resident axons become increasingly myelinated. In one case report of a 6-year-old MDC1A patient with gross developmental delay, along with typical diffuse white matter hyperintensities on T2 weighted MRI, diffusion tensor imaging (DTI) revealed a reduced FA (Ip et al., 2012).

In a second case report of a 6-month-old MDC1A patient with hypotonia and delayed motor development, MRI at the age of 5 years old revealed the typical diffuse white matter abnormalities (Sijens et al., 2007). By 10 years old, the patient developed epilepsy, cognitive regression and behavioral problems which eventually required hospitalization at 15 years old, where DTI revealed reduced FA in both the gray and white matter (Sijens et al., 2007). Unfortunately, due to a lack of DTI studies in MDC1A patients, it is currently unclear whether a low FA metric, a hallmark of poor white matter maturation, is characteristic of the MDC1A or just found in these isolated cases.

Prevalence of Focal Cortical Dysplasia and Epilepsy in MDC1A

While not as universal as white matter abnormalities, epilepsy and cortical dysplasias (i.e., polymicrogyria and lissencephaly) also occur in patients with MDC1A (Herrmann et al., 1996; Brett et al., 1998; Vigliano et al., 2009; Geranmayeh et al., 2010; Gavassini et al., 2011; Marques et al., 2014). In one example, a case report describes a child with floppy infant symptoms at birth with absent laminin-α2 muscle staining and an abnormal brain MRI with diffuse white matter abnormalities and cortical dysplasia in the occipital lobe (Brett et al., 1998). Another report describes an infant who was diagnosed with MDC1A at 5 months, without any notable CNS involvement, but progressively developed CNS symptoms that began with accidental falls at 4 years old, and eventually was diagnosed with epilepsy at 6 years old (Vigliano et al., 2009). Focal cortical dysplasia is a developmental neuroanatomical malformation that frequently leads to refractory epilepsy, and an MRI study of this patient after the first seizure revealed occipital polymicrogyria. Three MDC1A patients with focal cortical dysplasias and refractory occipital epilepsies that began in early childhood were described in a separate report, with two patients having normal intellect and one patient having moderate intellectual disability (Pini et al., 1996).

Another report described five patients with LAMA2 mutations, of which two patients had epilepsy and three patients had more subtle neurological symptoms (Gavassini et al., 2011). One patient had refractory epilepsy, and interestingly only had "mild muscle weakness" (Gavassini et al., 2011). This case suggests that the severity of the CNS and muscular dystrophy phenotypes do not necessarily correlate. One possible explanation for this lack of correlation is that, depending on the tissue type, a laminin-α2 deficiency could be differentially compensated for, either by other laminins or by other mechanisms entirely.

Another example of the discrepancy between the CNS and muscle symptoms in MDC1A is a case report of a girl with a partial laminin-α2 deficiency with delayed motor development, moderate intellectual disability and severe epileptic seizures (Deodato et al., 2002). At 6 years of age, she underwent neurological evaluation and a CT scan revealed white matter changes typical of MDC1A (diffuse hypointensity on T1 weighted imaging). Examination also revealed peripheral neuropathy and bilateral reduction in amplitude and increased latency of visual evoked potentials (Deodato et al., 2002). At 19 years of age, her MRI revealed similar diffuse white matter changes and she still had severe epilepsy, which had become refractory (Deodato et al., 2002). Despite these relatively severe CNS symptoms for MDC1A, she had normal strength in all proximal muscle, and no muscle wasting or contractures, with only slight difficulty climbing stairs (Deodato et al., 2002).

In another case of an MDC1A patient with extensive CNS involvement, macrocephaly was detected before 1 year of age and refractory epilepsy developed by 6 years of age (Marques et al., 2014). MRI revealed agyria in the occipital cortex, along with white matter abnormalities and widening of frontal gyri, which is consistent with structural abnormalities observed in other cases of laminin-α2 deficiency (Marques et al., 2014). Remarkably, although on muscle biopsy there was irregular laminin-α2 immunofluorescence staining, the patient did not present with any neuromuscular or cardiac complaints, even upon follow-up at 21 years of age (Marques et al., 2014). Sequencing of the LAMA2 gene revealed two heterozygous missense mutations in the N-terminus, one in exon 5 (LN domain) and one in exon 18 (LEb domain). This is an additional case demonstrating that the severity of the neurological and the muscular symptoms do not have to correlate. It is important to note that in all of these MDC1A cases with a discrepancy in the severity of muscle vs. neurological symptoms, patients had reduced laminin-α2 expression (immunofluorescence and protein blots), but not a complete loss. Intriguingly these cases suggest

that certain LAMA2 mutations can present largely as a neurological disorder, thus expanding the clinical spectrum of MDC1A.

Epilepsy frequently affects patients with MDC1A and a more recent study found that 9 out of 25 MDC1A patients had epilepsy with a mean age onset of first seizure at 8 years of age (Natera-de Benito et al., 2020). MRI studies of 19 patients revealed that all had white matter abnormalities and that polymicrogyria was found in all epilepsy patients (Natera-de Benito et al., 2020). Although MDC1A-related white matter changes are typically seen in patients of at least 6 months of age, this study revealed that four patients under 6 months of age already had white matter changes, including two in the perinatal period (Natera-de Benito et al., 2020).

Neuronal Migration Defects

While moderate or subtle changes in CNS anatomy are common in MDC1A, gross structural abnormalities are less frequent, but do occur. In a brain imaging study of 14 patients with MDC1A, all had typical white matter changes consistent with laminin-α2 deficiency, while within the subset of patients that demonstrated gross structural brain abnormalities, on muscle biopsy all had complete loss of laminin-α2 immunofluorescence protein labeling (Philpot et al., 1999). Structural changes included hypoplasia of the cerebellar vermis (5 out of the 10 with complete loss of laminin-α2), cerebellar hemisphere (4 out of the 10) and the pons (3 out of the 10; Philpot et al., 1999). One patient had involvement in U fibers, which are connections between adjacent gyri (Philpot et al., 1999). Another patient, who had epilepsy and moderate intellectual disability, also demonstrated occipital agyria, also consistent with a neuronal migration defect (Philpot et al., 1999).

In another study, an MRI of a 6-week old patient revealed diffuse white matter hypointensity and left occipital lobe pachygyria and agyria (Mackay et al., 1998). An electroencephalogram (EEG) performed at 7 months was abnormal, with isolated sharp components and occipital lobe spike upon photic stimulation (Mackay et al., 1998). However, by 17 months of age, this patient did not have any evidence of seizures (Mackay et al., 1998). A second patient of 2 years of age also exhibited bilateral occipital lobe pachygyria and polymicrogyria, as well as gray matter heterotopia, which is a neuronal migration defect that results in the abnormal localization of neurons. Similar to the first patient, despite these structural defects and an abnormal EEG, this patient was seizure-free (Mackay et al., 1998). Lastly, another report (Sunada et al., 1995) described two cases of MDC1A with severe muscular dystrophy and brain structural abnormalities. The first patient had abnormal gyral formations of the occipital lobe, suggestive of polymicrogyria, and white matter abnormalities within the corpus callosum and centrum semiovale, a large white matter area beneath the cerebral cortex (Sunada et al., 1995). In the occipital lobe, the patient also had areas that resembled hamartomas, which are often due to neuronal migration or overgrowth defects (Sunada et al., 1995). The second patient had polymicrogyria of the posterior temporal, parietal, and occipital lobes and white matter abnormalities of the supratentorial white matter (Sunada et al., 1995). Overall these and other studies reveal that defects in neuronal migration are not uncommon in MDC1A, demonstrating the importance of laminin-α2 expression to brain development. Yet there remains an incomplete understanding of spatial and temporal LAMA2 expression in the developing brain. In the next section, we will review what is known regarding laminin-α2 expression in the CNS, highlighting areas with relevance to MDC1A CNS phenotypes.

LAMA2 EXPRESSION IN THE BRAIN AND SPINAL CORD

During CNS development, laminins are broadly expressed, influencing neurite outgrowth (Morissette and Carbonetto, 1995; Powell et al., 1998), synaptogenesis (Tian et al., 1997) and myelination (Buttery and ffrench-Constant, 1999; Chun et al., 2003; Relucio et al., 2009, 2012; De La Fuente et al., 2017). In the adult brain, the distribution and the expression of different laminins is restricted, for instance to regions such as the basal lamina of cerebral blood vessels and the retina (Morissette and Carbonetto, 1995; Toti et al., 1997; Villanova et al., 1997). **Table 1** summarizes the expression of laminin-α2 in the CNS. All of the studies included in **Table 1** used antibodies specific for the laminin-α2 chain. This is not likely to be an exhaustive list of the LAMA2 expression given that many studies (not included in **Table 1**) use antibodies that detect other laminin subunits that may be present in α2-laminin containing trimers. For example, many studies use antibodies that detect the laminin γ1 subunit, which is found in Lm-211 but also in a large subset of laminins, thus making it impossible to know which laminin trimer is present without further analysis. In the following section, we will describe the roles of laminins during brain development and discuss their lesser-known roles in the postnatal and adult brain. In some cases, laminin-α2 association has been clearly described, and in some cases, laminins more broadly as a family have been implicated.

Astrocytes and the Blood-Brain Barrier

Laminin-α2 is highly expressed by astrocytes (Sixt et al., 2001; Menezes et al., 2014; Yao et al., 2014; Hannocks et al., 2018) and pericytes (Yousif et al., 2013; Menezes et al., 2014; De La Fuente et al., 2017) in mouse brains. However, less is known about laminin-α2 at the blood-brain barrier (BBB) in human brains. It has been hypothesized that the absence of laminin-α2 leads to an impaired BM of the BBB, which in turn leads to a disruption of the BBB's selective transport and filtration properties (Villanova et al., 1996, 1997). In an electron microscopy immunolabeling study examining the localization of laminin-α2 protein in five adult brains of at least 27 years old, as well as in one newborn brain (1 day old), laminin-α2 protein exclusively localized to the basal lamina of all cerebral blood vessels and was not detected in the meningeal or choroid blood vessels (Villanova et al., 1997). However other studies went on to find laminin-α2 expression in other areas of the CNS (see ahead

TABLE 1 | Sites of laminin-α2 expression in the developing and adult central nervous system.

Location	Embryonic	Postnatal*	Adult**
Astrocytes at the blood-brain barrier	Not available	**Immunofluorescence** *Human*: - α2 chain mouse mAb, 80 kDa specific; α2 chain rat mAb, 300 kDa specific (Villanova et al., 1997). *Mouse*: - α2 chain rat mAb, N-terminus specific (Menezes et al., 2014) **Protein, western blotting** *Mouse*: - α2 chain rat mAb, N-terminus specific (Menezes et al., 2014)	**Immunofluorescence** *Human*: - α2 mouse mAb, 80 kDa specific, and α2 rat mAb, 300 kDa specific (Villanova et al., 1997). *Mouse*: - α2 chain rat mAb, N-terminus specific (Menezes et al., 2014) - α2 chain mouse mAb, 300 kDa specific (Hannocks et al., 2018) - α2 chain mouse mAb, 300 kDa specific (Sixt et al., 2001) - α2 chain rat mAb, N-terminus specific (Yao et al., 2014)
Cortical plate	**Immunofluorescence** *Mouse*: - α2 chain (Campos et al., 2004) - α2 chain rat, N-terminus specific (Lathia et al., 2007)	Not available	Not available
Choroid plexus	**RNA, northern hybridization** *Human*: - α2 chain, multiple nucleotide residues screened using previously reported cDNA (Vuolteenaho et al., 1994)	Not available	Not available
Cerebellum	**RNA, northern hybridization** *Human*: - α2 chain, multiple nucleotide residues screened using previously reported cDNA (Vuolteenaho et al., 1994)	**Immunofluorescence** *Mouse*: - α2 chain mouse mAb, N-terminus (Powell et al., 1998) **Protein, western blotting** *Mouse*: - α2 chain mouse mAb, N-terminus (Powell et al., 1998)	Not available
Hippocampus	**Protein, western blotting** *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997)	**Immunofluorescence** *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997)	**Immunofluorescence** *Monkey*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997) - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) **Protein, western blotting** *Mouse*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997) *Pig*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997) *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997) *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997)
Meninges	**RNA, northern hybridization** *Human*: - α2 chain, multiple nucleotide residues screened using previously reported cDNA (Vuolteenaho et al., 1994)	Not available	**Immunofluorescence** *Mouse*: - α2 chain rabbit polyclonal Ab, C-terminus domain G1–3 specific (Sasaki et al., 2002)

(Continued)

TABLE 1 | Continued

Location	Embryonic	Postnatal*	Adult**
	Immunofluorescence *Mouse*: - α2 chain rabbit polyclonal Ab, C-terminus domain G1–3 specific (Sasaki et al., 2002)		
Cortex (i.e., entorhinal and piriform cortices)	Not available	Not available	**Immunofluorescence** *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997) - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) **Protein, western blotting** *Pig*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Tian et al., 1997)
Olfactory bulb	**RNA, northern hybridization** *Human*: - α2 chain, multiple nucleotide residues screened using previously reported cDNA (Vuolteenaho et al., 1994)	Not available	**Immunofluorescence** *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997)
Pericytes	Not available	**Immunofluorescence** *Mouse*: - α2 chain rat mAb, N-terminus specific (Menezes et al., 2014) - α2 chain rat mAb, N-terminus specific (De La Fuente et al., 2017) **Protein, western blotting** *Rat* - α2 chain rat mAb, N-terminus specific (De La Fuente et al., 2017)	**Immunofluorescence** *Mice*: - α2 chain rat mAb, N-terminus specific (De La Fuente et al., 2017) **RNA, in situ hybridization** *Rat* - α2 chain rat mAb, N-terminus specific (De La Fuente et al., 2017)
Retina	**RNA, northern hybridization** *Human*: - α2 chain, multiple nucleotide residues screened using previously reported cDNA (Vuolteenaho et al., 1994)	Not available	Not available
Spinal cord	**Immunofluorescence** *Mouse*: - α2 chain rabbit polyclonal Ab, C-terminus domain G1–3 specific (Sasaki et al., 2002) Human: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific; α2 chain mouse mAb, 80 kDa fragment specific (Liesi et al., 2001)	Not available	Not available
Thalamus and hypothalamus	Not available	Not available	**Immunofluorescence** *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997)
Tanycytes	Not available	Not available	**Immunofluorescence** *Rabbit*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997) *Rat*: - α2 chain rabbit polyclonal Ab, C-terminus domain G4/5 specific (Hagg et al., 1997)
Ventricular-subventricular zone	**Immunofluorescence** *Mouse*: - α2 chain (Campos et al., 2004)	**Immunofluorescence** *Mouse*: - α2 chain (Campos et al., 2004)	Not available

(Continued)

TABLE 1 | Continued

Location	Embryonic	Postnatal*	Adult**
	- α2 chain rat mAb, N-terminus specific (Lathia et al., 2007) *Rat*: - α2 chain (Campos et al., 2004)	- α2 chain rat mAb, N-terminus specific (Relucio et al., 2012) *Rat*: - α2 chain (Campos et al., 2004)	

*Note: *postnatal day 0–20. **postnatal day 21 or greater*

sections; Hagg et al., 1997; Tian et al., 1997; Liesi et al., 2001; Colognato and Tzvetanova, 2011; Nascimento et al., 2018; Sato et al., 2019). The inability to detect laminin-α2 protein in other areas of the CNS may have been due to the limited capacity to detect laminins by immuno-EM from human post-mortem brain tissue. Another limitation of this study is the small sample size and the use of only one neonatal brain, as laminin expression is more abundant during the embryonic and neonatal period than in adulthood. However, given that meningeal and choroid blood vessels do not have a BBB, while cerebral blood vessels do, the expression pattern in this human study is consistent with the hypothesis that laminin-α2 is a key component at the BBB (Villanova et al., 1997).

Hippocampal Dendritic Spines

Similar to muscle, peripheral nerve, and placental tissue, laminin-α2 protein is also found in the cortex and dendritic spines of hippocampal neurons, where it can be separated by SDS-PAGE into two fragments of 80 and 300 kDa (Tian et al., 1997). The 300 kDa fragment contains the short arm and coil-coil region of laminin-α2 protein, while the 80 kD contains a large part of the G domain, which has the interaction sites for dystroglycan and α7β1 integrin (Ehrig et al., 1990). The 300 kDa fragment only appeared in hippocampal extracts and was not detected in synaptosomes and neuronal cultures, which suggests the 300 kDA fragment could be non-neuronal or possibly that laminin-α2 was preferentially degraded in particular locations (Tian et al., 1997). Lysates from the hippocampus and cortex also revealed high levels of 160 and 140 kDa laminin-α2 protein fragments, which were at higher levels during active synaptogenesis. In the adult brain, only an 80 kDa laminin-α2 protein fragment was present. Upon a cortical lesion, which results in denervation and subsequent reinnervation, laminin-α2 immunohistochemical staining closely corresponded to the immunolabeling of the synaptic marker synaptophysin, which is loss immediately after injury and is recovered during reactive synaptogenesis (Tian et al., 1997). The dynamic expression pattern of laminin-α2 associated with synapses and dendrites suggests its likely role in synaptogenesis and/or synaptic plasticity (Anderson et al., 2005), both of which could contribute to a subset of the CNS deficits seen in MDC1A patients, such as a high incidence of epilepsy.

Cerebellar Neurons

Laminin-α2 is spatiotemporally regulated in the cerebellum, as different laminins demonstrate distinct temporal patterns (Powell et al., 1998). Cell bodies of migrating granule cells of the cerebellum stained with laminin-111 antibodies at postnatal days 1 and 6 and less pronounced by postnatal day 12 (Powell et al., 1998). Conversely, laminin-α2 immunohistochemical staining only faintly labeled granule neurons at postnatal day 1 and then was prominently found in cell bodies of Purkinje cells at postnatal days 6 and 12 (Powell et al., 1998). Cerebellar granule cells cultured on Lm-111 and Lm-211 both stimulate neurite outgrowth, so the functional significance of differential regulation of laminin isoforms during cerebellar development remains unclear (Powell et al., 1998).

Neurons and Glial Cells of the Spinal Cord

Immunofluorescence labeling of the BM surrounding the mouse spinal cord reveals α2-containing laminin(s) protein at 11.5 days in embryonic development (Sasaki et al., 2002). In addition, Liesi et al. (2001) used immunofluorescence, western blotting, and RT-qPCR to reveal both laminin-α2 protein and mRNA in the human fetal brain and spinal cord. While lysates from spinal cord neuronal and mixed glia cultures contained laminin-α2 protein, appearing as a 300 kDa band and a 220 kDa doublet, only mixed glial cultures contained laminin-α2 as a single 300 kDa band (Liesi et al., 2001). Interestingly, the 300 kDa α2 chain protein fragment was also previously reported as non-neuronal in hippocampal and cortical extracts (Tian et al., 1997). Why there are different laminin-α2 banding patterns by SDS-PAGE in different tissue locations remains unclear, but it has been speculated as possibly arising from either differential glycosylation, differential proteolysis, or even different splicing isoforms. Further analysis is needed to better understand the expression and processing of laminin-α2 in the brain and spinal cord.

Laminin-α2 Expression in the Postnatal and Adult Brain

Aside from the BBB, laminin-α2 expression in the postnatal and adult brain occurs in locations associated with adult neuro- and gliogenesis, and in regions associated with ongoing progenitor cell migration, such as the olfactory bulb. In the adult CNS, neurogenesis is largely confined to the dentate gyrus of the hippocampus and the ventricular-subventricular zone of the lateral ventricles (VZ-SVZ), as well as in other ventricle-associated regions. For example, laminin-α2 immunofluorescence labeling is found in the dentate gyrus and CA3 and CA4 of the adult rat hippocampus and limbic structures (Hagg et al., 1997). In the adult rabbit brain, laminin-α2 is found in similar regions although more pronounced than in rats (Hagg et al., 1997). Laminin-α2 staining was described as noticeably labeling neuronal and dendritic processes and possibly synapses as in certain areas (i.e., thalamus and hypothalamus) the staining appeared bouton-like (Hagg et al., 1997). Consistent

with this pattern, another study reported laminin-α2 protein in mouse synaptosomes from the hippocampus (Tian et al., 1997). Also, laminin-α2 protein immunofluorescence staining was reported to be prominent in tanycytes (specialized ependymal cells) of the third ventricles and ensheathing cells (specialized glia) of the olfactory bulb (Hagg et al., 1997). In the adult VZ-SVZ, laminin-α2 protein is found in fractones, which are considered an atypical BM-like ECM structure close to the ventricular surface, in close proximity to both neural stem cells (NSCs) and ependymal cells (Nascimento et al., 2018). Indeed laminin-α2 is localized to both the embryonic (Lathia et al., 2007) and early postnatal VZ-SVZ NSC niche (Relucio et al., 2012), where it has been shown to influence NSC output (Loulier et al., 2009; Relucio et al., 2012) which will be discussed in the next section.

Laminin-α2 Expression in the Adult Brain: Changes in Response to Injury

Laminin-α2 protein is found in the adult hippocampus (Hagg et al., 1997; Tian et al., 1997), and evidence suggests that its expression is upregulated in the hippocampus after injury (Tian et al., 1997). In the developing rat hippocampus, laminin-α2 immunoreactivity increases during synaptogenesis, and upon a cortical lesion, which results in denervation and subsequent reinnervation, laminin-α2 immunoreactivity closely corresponds to the immunolabeling of the synaptic marker synaptophysin (Tian et al., 1997). These findings suggest that an α2-containing laminin has roles in synaptogenesis and synaptic plasticity, which we will discuss ahead when describing synaptic plasticity deficits in an MDC1A mouse model.

Pericytes, cells that help regulate the BBB by regulating microvascular blood flow, were found to respond to a demyelinating injury by secreting laminin-α2, which in turn promoted oligodendrocyte precursor cell (OPC) differentiation and the ability to repair myelin (De La Fuente et al., 2017). In the perinatal period OPCs are responsible for producing pre-myelinating oligodendrocytes, which later mature and become myelinating oligodendrocytes (van Tilborg et al., 2018). Since mesenchymal stem cell-conditioned medium is known as a strong inducer of OPC differentiation (Jadasz et al., 2013), and pericytes share similar features as mesenchymal stem cells (MSCs), pericyte conditioned medium was assessed and found to similarly promote OPC differentiation (De La Fuente et al., 2017). OPCs were subsequently cultured in pericyte conditioned medium pre-incubated with a laminin-α2 blocking antibody, which attenuated its effect on OPC differentiation (De La Fuente et al., 2017). Thus, a model was proposed in which pericytes produce α2-containing laminins as paracrine factors, similar to what occurs in MSCs. In a similar assessment, pericytes were found to influence the ability of NSCs to generate progenitors of an oligodendrocyte fate by producing α2-containing laminin proteins (Silva et al., 2019). Such a role for laminin-α2 in oligodendrocyte lineage development is consistent with findings from several mouse models of MDC1A demonstrating defective or delayed developmental myelination, to be discussed ahead (Chun et al., 2003; Relucio et al., 2009, 2012).

THE ROLE OF LAMA2 IN THE CNS: INSIGHTS FROM MDC1A MOUSE MODELS

Using a variety of MDC1A mouse models, laminins have been found to regulate NSC proliferation (impacting both neurogenesis and gliogenesis), neuronal migration, axon outgrowth, synaptogenesis, and retinal development (**Figures 2**, **3**).

MDC1A Mouse Model Overview

Several mouse models of MDC1A exist, with distinct Lama2 expression properties and phenotypes (**Table 2**; reviewed in this issue; Gawlik and Durbeej, 2020). *dy/dy* mice have a spontaneous mutation in a non-coding region of the Lama2 gene that results in substantially reduced laminin-α2 levels, causing muscular dystrophy, nervous system involvement, and premature death (Michelson et al., 1955). dy^{2J}/dy^{2J} mice have a point mutation that causes a splicing change, resulting in a truncated laminin-α2 chain that lacks the N-terminal LN domain (Xu et al., 1994; Sunada et al., 1995), a domain that is critical for mediating laminin-laminin polymer interactions (Colognato and Yurchenco, 1999; Yurchenco et al., 2018). Similar to *dy/dy* mice, dy^{2J}/dy^{2J} mice have muscular dystrophy and a shortened lifespan, although dy^{2J}/dy^{2J} mice are healthier and live longer than do *dy/dy* mice. The dy^{w}/dy^{w} mouse was generated by homologous recombination in embryonic stem cells and was initially thought to be a full Lama2 knock-out mouse, but later found to express low levels of laminin-α2. The dy^{w}/dy^{w} mouse exhibits severe muscular dystrophy and survival is reduced to a range of 5–16 weeks (Willmann et al., 2017). Lastly, dy^{3k}/dy^{3k} mice are Lama2 knockout mice, having a complete loss of laminin-α2 expression, which leads to severe muscular dystrophy and reduced life expectancy (∼5 weeks; Miyagoe et al., 1997).

dy/dy mice exhibit myelination defects of both the peripheral nervous system (PNS; Harris et al., 1972) and CNS (Chun et al., 2003; Relucio et al., 2009), impaired sodium channel clustering at NMJs (Occhi et al., 2005), and aberrant neural stem cell function (Loulier et al., 2009). Similar to dy/dy mice, dy^{3k}/dy^{3k} mice have impaired oligodendrogenesis (Relucio et al., 2012). dy^{3k}/dy^{3k} mice also have BBB dysfunction and increased permeability (Menezes et al., 2014). Lastly, there is currently no information regarding the CNS in dy^{w}/dy^{w} mice. The CNS findings from the *dy/dy*, dy^{2J}/dy^{2J}, and dy^{3k}/dy^{3k} mice will be expanded upon in the following section.

Laminin-α2 Regulates Synaptic Plasticity

Despite the occurrence of epilepsy in many MDC1A patients, there is currently limited knowledge regarding the role of laminin-α2 in CNS synapse function. However, dy^{2J}/dy^{2J} mice were found to have disruptions in long term neuronal plasticity, despite having no change in basal synaptic transmission and paired-pulse stimulation (Anderson et al., 2005). Using cerebellar slice preparation to invoke long term depression, over half of the cells examined in dy^{2J}/dy^{2J} mice displayed long term depression that was significantly reduced compared to that in control mice. Furthermore, nearly 1/3 of the sampled cells exhibited aberrant

TABLE 2 | LAMA2 mouse models.

Lama2 Mouse	Mutation	Laminin-α2 levels	CNS Involvement		
			Myelination deficits	BBB Deficits	Other
dy/dy (Michelson et al., 1955)	Spontaneous mutation	Substantially reduced	Impaired oligodendrogenesis and myelination (Chun et al., 2003; Relucio et al., 2009).	Unknown	Elevated audiometric threshold response, degeneration of cochlear and vestibular structures (Pillers et al., 2002). Atrophy of motor neurons and abnormal neurotrophic factor expression in CNS (Sakuma et al., 2002)
dy^{2J}/dy^{2J} (Meier and Southard, 1970)	Spontaneous mutation; abnormal splicing and subsequent instability leads to a truncated protein that lacks the N-terminus	Expressed but lacking LN domain; modest reduction in levels	Unknown	Unknown	Long term plasticity was disrupted (Anderson et al., 2005).
dy^{W}/dy^{W} (Kuang et al., 1998)	Targeted knock-out; truncated protein	Very low to absent	Unknown	Unknown	
dy^{3k}/dy^{3k} (Miyagoe et al., 1997)	Targeted knock-out	Absent	Impaired oligodendrogenesis and myelination (Relucio et al., 2012).	BBB dysfunction and increased permeability (Menezes et al., 2014).	Impaired NSC proliferation and attachment within the ventricular zone (Loulier et al., 2009; Relucio et al., 2012).

long-term potentiation, which was not seen in controls. Since there was not a difference in pre-synaptic mediated short-term plasticity, this study proposed that the differences in long-term plasticity originate post-synaptically.

There is additional support for the DGC being involved in synaptic plasticity. It is already known that the DGC is necessary for stabilizing the clustering of acetylcholine receptors at the NMJ (Jacobson et al., 2001; Nishimune et al., 2008). In the hippocampus, dystroglycan co-localizes with almost all $GABA_A$ receptor α1 clusters (Pribiag et al., 2014). Disruption of the DGC by either loss of dystrophin, which anchors the complex to the actin cytoskeleton, or mutations that result in hypoglycosylation of α-dystroglycan, result in fewer dystroglycan and α1 clusters in the hippocampus (Kueh et al., 2011; Pribiag et al., 2014). Furthermore, dystroglycan is required to recruit additional $GABA_A$ receptors at the postsynaptic site during homeostatic scaling up (Pribiag et al., 2014). Homeostatic scaling up of inhibitory synaptic strength is a critical physiological mechanism to maintain balance between excitation and inhibition of neuronal activity, and the loss of this response by $GABA_A$ receptors contributes to the development of epilepsy (Chuang and Reddy, 2018). Lastly, the loss of inhibitory synaptic protein 1 (InSyn1), which binds to both the DGC and to gephyrin, a GABA receptor anchoring protein, also leads to poor dystroglycan and $GABA_A$ receptor α1 clustering at the postsynaptic site in the hippocampus (Uezu et al., 2019).

Together these findings support the hypothesis that laminin and the DGC impact inhibitory synapses and that mutations affecting either can alter neuronal activity.

Laminin-α2 Regulates the Attachment and Proliferation of Neural Stem Cells

Laminins are expressed in the developing (Campos et al., 2004; Lathia et al., 2007), postnatal (Relucio et al., 2012; Nascimento et al., 2018) and adult ventricular-subventricular zone (VZ-SVZ; Shen et al., 2008; Tavazoie et al., 2008; Nascimento et al., 2018; Sato et al., 2019). In embryonic development, NSCs of the VZ-SVZ express laminin-α2 receptors such as α6β1 integrin and dystroglycan during a critical time when NSCs undergo expansion via asymmetrical division for cortical neuronal development (Lathia et al., 2007). During this time, laminin-α2 protein is also present in the VZ-SVZ (Lathia et al., 2007). dy^{3k}/dy^{3k} mice show impaired NSC proliferation, cellular position, and attachment within the VZ-SVZ, a phenotype that is similar to what occurs when β1 integrin functions are blocked by antibodies injected into the lateral ventricle (Loulier et al., 2009). Laminin-α2 also has a role in the postnatal VZ-SVZ. In both laminin α2-deficient *dy/dy* mice and laminin α2-absent dy^{3k}/dy^{3k} mice, defects in oligodendrocyte production and maturation were observed (Chun et al., 2003; Relucio et al., 2009, 2012). In the case of dy^{3k}/dy^{3k} mice, increased death of glial progenitors was observed in the neonatal VZ-SVZ, suggesting a lack of appropriate survival cues (Relucio et al., 2012). Laminin-α2 also has a role in the midbrain dopaminergic neuron progenitor niche (Ahmed et al., 2019). In the ventral midbrain, laminin-α2 interactions with integrins regulate the proliferation and survival of progenitors (Ahmed et al., 2019). In the dy^{3k}/dy^{3k} mouse the absence of laminin-α2 results in increased apoptosis and depletion of the progenitor pool, leading to a reduction

in later-born ventral tegmental area (VTA) neurons (Ahmed et al., 2019). In this study (Ahmed et al., 2019) it was hypothesized that the loss of VTA neurons, which normally innervate the hippocampus and prefrontal cortex (Morales and Margolis, 2017), provides a mechanism for the hypoplasia of the brainstem seen in some MDC1A patients. They also propose (Ahmed et al., 2019) that loss of laminin-mediated interactions in this neurogenic niche could contribute to the autism-like behaviors seen in some related muscular dystrophies such as DMD (Ricotti et al., 2016), although to date there are no reports of increased incidence of autism-like behaviors in MCD1A itself.

Laminin-α2 Regulates Neurite Outgrowth and Axonal Pathfinding

The detection of laminin-α2 in the developing visual pathways is spatiotemporal, occurring as retinal ganglion cell (RGC) growth cones extend their projection into the brain (Cohen and Johnson, 1991; Morissette and Carbonetto, 1995). In vitro, embryonic RGC neurite outgrowth is mediated by α6β1 integrin receptors and α2-containing laminin (Cohen and Johnson, 1991). Both RGCs and astrocytes from developing optic nerves are capable of synthesizing α2-containing laminin, although thus far this capability has only been directly demonstrated in culture (Morissette and Carbonetto, 1995). After the development of the retina and optic nerve, laminin-α2 protein levels are reduced but still detectable by immunofluorescence in the adult mouse (Morissette and Carbonetto, 1995).

Primary oligodendrocyte cultures from mice demonstrated a similar neurite outgrowth-like response to Lm-211(Buttery and ffrench-Constant, 1999; O'Meara et al., 2013; Michalski et al., 2016). Depletion of integrin-linked kinase (ILK), an adapter protein that interacts with β1-integrin to regulate cytoskeletal dynamics in growth cone extension, results in impaired oligodendrocyte process extension and ability to form myelin membrane upon axonal contact (O'Meara et al., 2013). When cultured oligodendrocytes from $ILK^{-/-}$ mice are grown on Lm-211, $ILK^{-/-}$ oligodendrocytes have less branching and severely stunted branches when compared to control oligodendrocytes, which are highly branched (Michalski et al., 2016). These results suggested that coordination between the ECM (i.e., Lm-211 binding) and oligodendrocyte cytoskeleton dynamics (i.e., β1 integrin and ILK) is necessary for oligodendrocyte process extension. Oligodendrocytes that lack expression of the Lm-211 ligand, dystroglycan, also have deficits in oligodendrocyte branching (Eyermann et al., 2012), suggesting either coordination or redundancy in the receptors required for the ability of Lm-211 to regulate process dynamics in the developing CNS.

Laminin-α2 Regulates the Integrity of the Blood-Brain Barrier

Laminin-α2 is found in BMs of the blood-brain barrier, or BBB (Villanova et al., 1997), whose correct function requires contributions from cerebral blood vessels, astrocytes, and pericytes. In the absence of laminin-α2, there is increased permeability of the BBB dysfunction as well as several cellular and molecular changes associated with BBB dysfunction (Menezes et al., 2014). For instance, dy^{3k}/dy^{3k} mice have reactive astrogliosis, altered gliovascular morphology, and decreased pericyte coverage along the cerebral vasculature throughout postnatal development (Menezes et al., 2014). The Lm-211 receptor, dystroglycan, is expressed in BBB astrocytes where it anchors aquaporin channels (AQP4) at astrocytic endfeet; this localization is crucial to water homeostasis at the BBB (Lien et al., 2012). dy^{3k}/dy^{3k} cerebral cortices have decreased AQP4 immunoreactivity along astrocytic endfeet, although overall AQP4 levels were not significantly affected (Menezes et al., 2014), suggesting that in the absence of α2-containing laminins, AQP4 fails to appropriate localize. Consistent with BBB findings in dy^{3k}/dy^{3k} mice, the loss of laminin γ1 chain in pericytes, which will indirectly prevent the expression of Lm-211, results in a similar phenotype with BBB disturbances (Gautam et al., 2016).

Loss or Deficiency of Laminin-α2 Impairs Oligodendrogenesis and Myelination

Laminin-α2 protein is found in the postnatal ventricular/subventricular zone (VZ-SVZ; Campos et al., 2004), the largest neuro/gliogenic niche of the postnatal brain. In the complete absence of laminin-α2 (dy^{3k}/dy^{3k} mice), there is a reduction in the thickness of the dorsal VZ-SVZ, suggesting either decreased cell division, increased cell death, or a combination thereof (Relucio et al., 2012). In both the embryonic (Loulier et al., 2009) and postnatal (Relucio et al., 2012) VZ-SVZ, dy^{3k}/dy^{3k} mice have impaired NSC arrangements, presumably due to failed or altered cellular attachments. In the VZ-SVZ at postnatal day 1, NSC densities are unchanged but there are fewer OPCs in dy^{3k}/dy^{3k} mice compared to control littermates, accompanied by increased OPC death (Relucio et al., 2012). This deficit seems to be time-sensitive, as at postnatal day 8 dy^{3k}/dy^{3k} mice rebound and have *more* OPCs than control mice, along with a return to normal levels of cell death (Relucio et al., 2012). Beyond the NSC niche of the VZ-SVZ, laminin-α2 also regulates the development of neuronal and glial progenitor cells. Similar to how laminin-α2 promotes the survival of dopaminergic progenitors (Ahmed et al., 2019), laminin-α2 regulates the number of OPCs in the developing corpus callosum (Relucio et al., 2012), the nearest white matter tract to the dorsolateral VZ-SVZ germinal niche. Laminin-α2 also promotes OPC differentiation in the corpus callosum and other white matter regions. For example, despite increased OPC densities during early cortical myelination in dy^{3k}/dy^{3k}, these mice have significantly fewer mature oligodendrocytes as well as delayed myelination (see ahead). Together these data suggest that α2-containing laminins are important during a critical stage of oligodendrocyte development.

Laminin-α2 protein immunoreactivity is transiently found in axon tracts undergoing myelination (Milner and Ffrench-Constant, 1994; Colognato et al., 2002) and myelinating oligodendrocytes express the laminin-α2 receptor integrin

α6β1 (Milner and Ffrench-Constant, 1994; Colognato et al., 2002). In the *dy/dy* mouse (sharply reduced laminin-α2 levels) there is a reduction in both mature oligodendrocytes and myelin content in the corpus callosum, a major white matter tract. Ultrastructural analysis using electron microscopy, the gold standard for examining myelin structure, revealed an increased g-ratio (i.e., thinner myelin) in the axons in the corpus callosum, optic nerve, brainstem and the cerebellum, but not from the spinal cord (Chun et al., 2003; Relucio et al., 2009). In addition to thinner myelin, *dy/dy* mice have other indications of impaired myelination such as regions of noncompacted myelin (Chun et al., 2003; Relucio et al., 2009). This suggests that the loss of laminin-α2 either diminishes the capacity of OPCs to differentiate into mature myelinating oligodendrocytes or alternatively, OPCs differentiate but oligodendrocytes are unable to proceed with normal myelination, i.e., have impaired myelination capacity. Given that dy^{3k}/dy^{3k} mice have fewer mature oligodendrocytes the former is a likely component but the latter has not been ruled out as a contributing factor. In agreement with findings in dy^{3k}/dy^{3k}, *dy/dy* mice have an increase in OPC markers and a decrease in mature oligodendrocyte markers, determined by both immunohistochemical staining and western blotting (Relucio et al., 2009).

While it not entirely clear how α2-containing laminins regulate oligodendrocyte development, some mechanistic details have emerged. Disturbances in the regulation of Fyn, a Src family kinase required for myelination, were reported in *dy/dy* mice (Relucio et al., 2009). The level of phosphorylated Fyn at its Y529 site is significantly increased in the cerebral cortex of *dy/dy* mice, as are levels of Csk, a negative regulator of Fyn that phosphorylates Fyn at the Y529 position to render it inactive. Thus, it appears that disturbances in Fyn activity are a strong contender to contribute to delayed oligodendrocyte maturation and myelination in laminin-α2 deficiencies (Relucio et al., 2009). This hypothesis is supported by work using cultured wildtype OPCs, in which α2-containing laminins accelerate OPC development into oligodendrocytes (Buttery and ffrench-Constant, 1999), an effect that was later shown to be blocked by PP2, a Src kinase inhibitor (Relucio et al., 2009). In addition, changes in oligodendrocyte numbers are not likely due to increased oligodendrocyte death since no differences in oligodendrocyte death were observed in *dy/dy* mice (Relucio et al., 2009). In agreement with this observation, while increased death of OPCs occurred in germinal zones, no change in oligodendrocyte death was observed in dy^{3k}/dy^{3k} mice (Relucio et al., 2012).

Lastly, although peripheral myelination is not the primarily focus of this review, it is important to note that Lm-211 is also a major component of the BM in the PNS. Schwann cells, the myelinating cells of the PNS, express laminin receptors such as dystroglycan and integrins, which interact with Lm-211 to anchor the outer myelin membrane to the ECM and also activate pathways involved in Schwann cell maturation and myelination of peripheral nerves (Nakagawa et al., 2001; Patton et al., 2008; Court et al., 2009; Homma et al., 2011; Heller et al., 2014; Petersen et al., 2015; Ghidinelli et al., 2017). Defects associated with loss of LAMA2 expression in the PNS are detailed further in a review in this issue (Previtali and Andrea Zambon, 2020).

MDC1A: LESSONS FROM RELATED DEVELOPMENTAL DISORDERS

Collectively, muscular dystrophies are characterized by dystrophic muscle fibers: hypercontracted and degenerating fibers, increased fiber size variability, and increased connective tissue infiltration. While CMDs present in the first months to years of life, other muscular dystrophies such as limb-girdle muscular dystrophy have later onsets. CMDs can furthermore present with neurological symptoms such as ventricular enlargement, abnormalities in brain morphology and white matter changes, which can also be highly heterogeneous. It should be noted that non-MDC1A CMD patients may also have reduced laminin-α2 levels, as a reduction in laminin-α2 protein levels may be from primary deficiency, i.e., MDC1A, or from secondary reduction caused by mutations not in the LAMA2 gene. Secondary reductions are thought to reflect the interplay between α2-containing laminin proteins and members of the DGC, where the loss of one binding partner in the DGC has been observed to cause changes in the localization or levels of other complex members (Muntoni et al., 1998; Brockington et al., 2001). Ahead we will discuss key features of other neurodevelopmental disorders that share similar CNS clinical features as MDC1A, and how these similar features could point to shared cellular and molecular mechanisms.

Selected Dystroglycanopathies

Neuronal migration defects are seen in several CMDs that result from mutations impairing dystroglycan function (**Figure 4**). Dystroglycan mRNA is found in selected radial glial cells in the VZ-SVZ, while dystroglycan protein is mostly localized in the radial glial endfeet, where it mediates radial glial anchoring to the pial BM (Myshrall et al., 2012). Loss of dystroglycan function in radial glia results in the over-migration of neurons due to disruptions in the pial BM, a phenotype known as cobblestone lissencephaly (Myshrall et al., 2012). Primary dystroglycanopathies resulting from mutations in the DAG1 gene that encodes dystroglycan are quite rare, however secondary dystroglycanopathies, of which there are multiple types, occur due to mutations in multiple genes involved in the glycosylation of α-dystroglycan (selected examples discussed ahead).

Fukuyama congenital muscular dystrophy (FCMD) is caused by mutations in the FKTN gene, which encodes for fukutin. Closely related is Congenital Muscular Dystrophy Type 1C (MDC1C), resulting from mutations in FKRP, which encodes the fukutin-related protein. Both Fukutin and FKRP are Rbo5P transferases responsible for the post-translational modification of α-dystroglycan (Kanagawa et al., 2016). Impaired glycosylation of α-dystroglycan prevents its binding to extracellular ligands including Lm-211 but also to other ECM proteins that contain LG domains (e.g., perlecan, neurexin; Kanagawa et al., 2016). Similar to MDC1A, both FCMD and MDC1C are multisystem disorders that present with delays in neurodevelopment, cortical

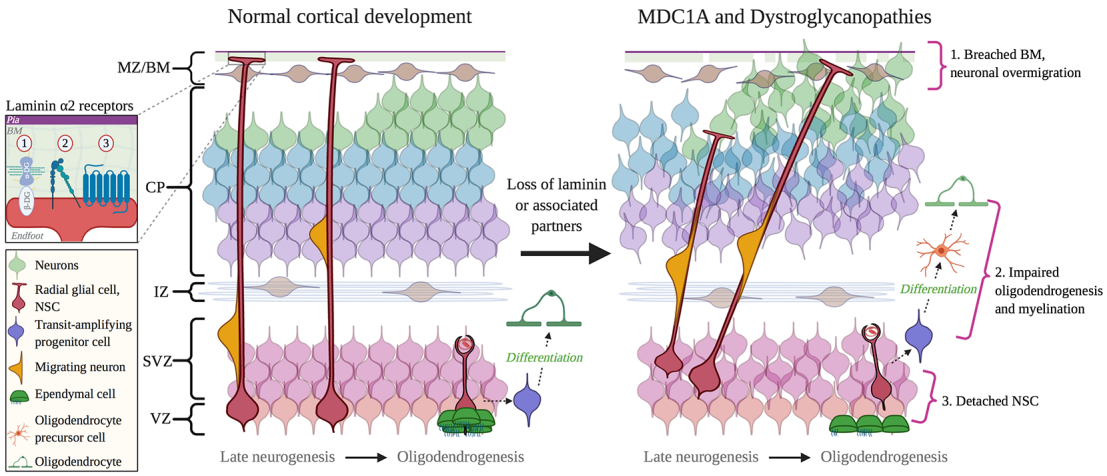

FIGURE 4 | Cortical dysplasia and NSC dysfunction in Congenital Muscular Dystrophies (CMD). *Left*: normal cortical development. *Right*: abnormal cortical development in CMD. 1. Breached BM caused by mutations in DAG1 and GPR56 genes, or abnormal glycosylation by fukutin, FKRP, and LARGE1. 2. Impaired oligodendrogenesis due to loss of LAMA2 and DAG1 expression or function. 3. The detachment of NSCs due to the loss of a functional interaction between laminin α2 and β1 integrins. Inset: receptors mediate interactions between radial glial cell endfeet and with laminin α2: 1, Dystroglycan; 2, Integrin; 3, GPR56 and (possibly) GPR126. Abbreviations: BM, basement membrane; CP, cortical plate; IZ, intermediate zone; MZ/BM, Marginal Zone/Basement Membrane; NSC, neural stem cell; SVZ, subventricular zone; VZ, ventricular zone.

dysgenesis, and ocular abnormalities (e.g., myopia and retinal degeneration; Tsutsumi et al., 1989; Angelini, 2016).

The LARGE1 gene also encodes for a glycosyltransferase that is critical for the glycosylation of α-dystroglycan (Jimenez-Mallebrera et al., 2005). Mutations in LARGE1 result in the hypoglycosylation of α-dystroglycan, subsequently impairing α-dystroglycan binding to Lm-211 (Longman et al., 2003). Mutations in LARGE1 cause Congenital Muscular Dystrophy Type 1D (MDC1D), which can present with cognitive deficits that are accompanied by structural brain abnormalities including abnormal white matter and neuronal migration defects that manifest as "cobblestone" ectopias (Longman et al., 2003; Montanaro and Carbonetto, 2003). Mutations in LARGE1 can also result in WWS, which can also be caused by mutations in FKRP and, most notably, POMT1. In addition to being a more severe muscular dystrophy, WWS is characterized by cobblestone lissencephaly and ocular malformations, which include optic nerve hypoplasia and retinal malformation (Dobyns et al., 1989). A final example is Muscle Eye Brain disease (MEB), caused by a mutation in the POMGnT1 gene, which is also responsible for correct glycosylation of α-dystroglycan (Yis et al., 2014). MEB presents with similar symptoms as other CMDs (i.e., hypotonia and muscle weakness), but unlike classical MCD1A, MEB presents with severe ophthalmological findings such as severe myopia (>10 diopters), glaucoma, retinal malformation and uncontrolled eye movements (Santavuori et al., 1989; Haltia et al., 1997; Yis et al., 2014). Similar to MDC1A, however, MEB is usually accompanied by epilepsy (Santavuori et al., 1989).

Not surprisingly, given the central role of the laminin-DGC connection in skeletal muscle, many dystroglycanopathies share features with MDC1A. The neurological features of these diseases also have many similarities with MDC1A. Despite this, there are differences, particularly in the degree of severity in both neurological problems and CNS structural deficits. The basis of these differences remains unknown, but the fact that α2-containing laminins have other roles besides interacting with dystroglycan, and dystroglycan has multiple LG-containing ECM protein partners in the brain (e.g., neurexin), likely contributes. Overall, the precise network and interplay of laminin- and dystroglycan-interactions in the brain is incomplete and will require a concerted effort to understand, for example, efforts to interrupt the expression of both proteins in a temporal and cell-specific fashion during neurodevelopment, as well as the to express LAMA2 with domain-specific mutations designed to abolish particular binding interactions.

GPR126-Related Cortical Dysplasia

An additional receptor for α2-containing laminins is the adhesion G protein-coupled receptor, GPR126. Upon ECM binding, adhesion G protein-coupled receptors (GPCRs) undergo autoproteolysis that results in two cleaved products: an N-terminal fragment, which contains the GAIN domain responsible for autoproteolysis, and a seven-transmembrane-containing C-terminal fragment (Langenhan et al., 2013). GPR126 is required in Schwann cells, the myelinating cells of the PNS, where it acts by increasing cAMP levels to activate protein kinase A, which initiates the upregulation of transcription factors required for Schwann cell myelination (Glenn and Talbot, 2013). Lm-211 interacts with the GAIN domain in the N-terminal GPR126 fragment to either promote myelination or suppress myelination, as well as favor radial sorting of axons in the PNS (Petersen et al., 2015). Although it remains unknown if GPR126 interacts with α2-containing laminins in the CNS, the clinical phenotype of patients with mutations in GPR126 indicates that GPR126 likely interacts with ECM

proteins in the brain. For example, a recent study described two patients with GPR126 mutations with intellectual disabilities (Hosseini et al., 2019). The first patient had normal motor development until 13 months of age when they developed a generalized seizure. At 16 years old, the patient had a low IQ estimated between 20–25, and MRI revealed cerebellar hypoplasia (Hosseini et al., 2019). The second patient developed refractory epilepsy by 12 months of age and again had a low IQ, estimated between 20–25 (Hosseini et al., 2019). Both patients had mutations in the seven-transmembrane-containing C-terminal fragment, which is responsible for increasing cAMP to induce Schwann cell myelination (Langenhan et al., 2013), but interestingly, these patients had CNS symptoms, rather than peripheral neuropathy. Given the overlap in neurological deficits between MDC1A patients and patients with GPR126 mutations, it seems likely that failed interactions between GPR126 and α2-containing laminins underlie at least some of the CNS features of patients with GPR126 mutations.

GPR56- Related Cortical Dysplasia

Mutations in GPR56, another adhesion GPCR for α2-containing laminins, also cause neurological symptoms. Like all GPCRs, GPR56 contains a seven-transmembrane-containing domain and has a long N-terminus extension similar to GPR126. Interestingly, GPR56 contains a mucin-rich domain similar to that found in dystroglycan (Piao et al., 2004). *In situ* hybridization experiments in mice demonstrated that GPR56 localizes to the VZ-SVZ during embryonic development but minimally in other areas of the cortex, which suggests GPR56 involvement in neurogenesis (Piao et al., 2004). Mutations in the gene encoding GPR56 result in bilateral frontoparietal polymicrogyria, which is characterized by abnormal cortical lamination and gyral organization (Piao et al., 2004; Bahi-Buisson et al., 2010). GPR56 is localized in radial glial endfeet, and in GPR56$^{-/-}$ mice there is rupture of the pial BM, which results in neuronal over migration (Li et al., 2008). Desai and Udani (2015) reported four cases of patients with a mutation in GPR56 with diffuse bilateral polymicrogyria in the frontoparietal lobes as well as ocular findings such as strabismus. MRI furthermore revealed diffuse white matter abnormalities in two out of four patients and frontal periventricular white matter changes in the other two patients. In a previous study of 30 patients with bilateral frontoparietal polymicrogyria, 14 were found to have mutations in GPR56 (Bahi-Buisson et al., 2010). All 14 patients had a severe cognitive delay, 7 out of 14 had eye movement abnormalities, and 12 out of 14 had epilepsy (Bahi-Buisson et al., 2010). Given that MDC1A neurological symptoms overlap with those in patients with GPR56 mutations, combined with the knowledge that GPR56 and α2-containing laminins both are required for the correct regulation of NSCs during brain development in mice, it appears very likely that GPR56 and α2-containing laminins interact during human CNS neurodevelopment.

CONCLUSION

While the degree of brain involvement is highly variable in different MDC1A patients, an emerging theme is that the severity of dystrophic symptoms and neurological symptoms can be uncoupled and do not always follow a strict pattern depending on LAMA2 mutation type. However complete loss of LAMA2 expression is correlated with more severe CNS involvement. Together these themes suggest a large degree of variability in how different patterns of gene expression and function, both from patient to patient and tissue to tissue, can alter the trajectory of this complex disease. Lastly, it is increasingly clear that α2-containing laminins have diverse roles in the developing brain, both during embryonic development and during postnatal development, from ensuring correct BBB function, to the organization of the developing cortical plate, to the proper development of oligodendroglia. The precise receptor interactions as well as the precise chemical and mechanical signaling properties of α2-containing laminins in the brain are yet to be discovered but will be critical in the development and feasible timing of future avenues of intervention in neurological aspects of MDC1A as well as other CMDs with brain involvement.

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AJA and HC wrote the manuscript. AJA created the figures.

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frontiers in Molecular Neuroscience

REVIEW
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LAMA2-Related Dystrophies: Clinical Phenotypes, Disease Biomarkers, and Clinical Trial Readiness

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Mutations in the *LAMA2* gene affect the production of the α2 subunit of laminin-211 (= merosin) and result in either partial or complete laminin-211 deficiency. Complete merosin deficiency is typically associated with a more severe congenital muscular dystrophy (CMD), clinically manifested by hypotonia and weakness at birth, the development of contractures of large joints, and progressive respiratory involvement. Muscle atrophy and severe weakness typically prevent independent ambulation. Partial merosin deficiency is mostly manifested by later onset limb-girdle weakness and joint contractures so that independent ambulation is typically achieved. Collectively, complete and partial merosin deficiency is referred to as LAMA2-related dystrophies (LAMA2-RDs) and represents one of the most common forms of congenital muscular dystrophies worldwide. LAMA2-RDs are classically characterized by both central and peripheral nervous system involvement with abnormal appearing white matter (WM) on brain MRI and dystrophic appearing muscle on muscle biopsy as well as creatine kinase (CK) levels commonly elevated to >1,000 IU/L. Next-generation sequencing (NGS) has greatly improved diagnostic abilities for LAMA2-RD, and the majority of patients with merosin deficiency carry recessive pathogenic variants in the *LAMA2* gene. The existence of multiple animal models for LAMA2-RDs has helped to advance our understanding of laminin-211 and has been instrumental in preclinical research progress and translation to clinical trials. The first clinical trial for the LAMA2-RDs was a phase 1 pharmacokinetic and safety study of the anti-apoptotic compound omigapil, based on preclinical studies performed in the dy^{W}/dy^{W} and dy^{2J}/dy^{2J} mouse models. This phase 1 study enabled the collection of pulmonary and motor outcome measures and also provided the opportunity for investigating exploratory outcome measures including muscle ultrasound, muscle MRI and serum, and urine biomarker collection. Natural history studies, including

a five-year prospective natural history and comparative outcome measures study in patients with LAMA2-RD, have helped to better delineate the natural history and identify viable outcome measures. Plans for further clinical trials for LAMA2-RDs are presently in progress, highlighting the necessity of identifying adequate, disease-relevant biomarkers, capable of reflecting potential therapeutic changes, in addition to refining the clinical outcome measures and time-to-event trajectory analysis of affected patients.

Keywords: LAMA2, phenotype, biomarkers, clinical trial, natural history

INTRODUCTION

The laminin α2–related muscular dystrophies (LAMA2-RDs) are a subtype of congenital muscular dystrophy (CMD) caused by recessive variants in the *LAMA2* gene [6q22–q23; OMIM*156225] (Helbling-Leclerc et al., 1995; Zhang et al., 1996). *LAMA2* encodes for the alpha-2 subunit of the heterotrimeric laminin-2 protein (made up of α2, β1, and γ1 subunits) with the α2 subunit called laminin-211 or merosin serving as a tissue-specific component of the extracellular matrix with a key role in myotubes stability and apoptosis (Vachon et al., 1996). The clinical spectrum of LAMA-RDs is wide, ranging from a severe, early-onset, and progressive presentation to a milder, later-onset form. To date, there are no effective treatments for LAMA2-RDs.

In this review article, we present a detailed overview of the most relevant clinical aspects of LAMA2-RDs and provide an update on translational developments, in particular natural history studies and available disease-related biomarkers.

LAMA2-RD: CLINICAL ASPECTS

LAMA2-RDs are classically divided into two main phenotypic categories: a more common severe, early-onset form, presenting with features of CMD, also known as Merosin Deficient Congenital Muscular Dystrophy type 1A (MDC1A), and a much less common, milder, later-onset form often presenting with a phenotype suggestive of limb-girdle muscular dystrophy (LGMD) with prominent joint contractures. Severe LAMA2-RD is one of the most common forms of CMD, accounting for ∼1/3 of patients with a diagnosis of CMD (Allamand and Guicheney, 2002; Muntoni and Voit, 2004; Sframeli et al., 2017), with an estimated prevalence in UK and Italy of 0.6–0.7/100,000 (Mostacciuolo et al., 1996; Norwood et al., 2009). The prevalence of the milder LAMA2-RD form is not fully known. The UK and Danish studies showed *LAMA2* variants in about 2–3% of patients with mild muscular dystrophies or LGMD (Løkken et al., 2015; Sframeli et al., 2017). As a general rule, patients with the severe CMD-like phenotype have a (virtually) complete absence of merosin as detected by immunohistochemical staining on muscle or skin biopsy. Conversely, patients with milder clinical presentations have some residual (or partial) merosin expression (Naom et al., 1998; Pegoraro et al., 1998; Topaloğlu et al., 1998; Tezak et al., 2003; Oliveira et al., 2008; Geranmayeh et al., 2010; Gavassini et al., 2011; Xiong et al., 2015). However, exceptions do exist and extreme intrafamilial variability is reported, suggesting that disease modifiers play a role in defining phenotypes and severity (Prandini et al., 2004; Geranmayeh et al., 2010).

Severe LAMA2-RD

The classic, severe LAMA2-RD presentation is a relatively homogenous CMD phenotype and is most typically associated with complete merosin deficiency on muscle biopsy immunohistochemical studies. Clinical hallmarks are early-onset severe hypotonia, axial weakness, inability to achieve independent ambulation, and elevated creatine kinase (CK) levels, commonly >1,000 IU/L (**Figure 1**). Progressive joint contractures, respiratory insufficiency, and scoliosis are observed in almost all patients. About 2/3 of patients are symptomatic at birth, with a further 1/3 with symptoms recognized by age 6 months of age (Geranmayeh et al., 2010; Xiong et al., 2015). Presenting symptoms include hypotonia, a weak cry, and reduced spontaneous movements. Respiratory problems, feeding difficulties, and mild distal contractures can also be present at birth, but severe arthrogryposis is not usually observed. At the onset, weakness primarily affects axial muscles, with severe head lag and predominant upper more than lower limb involvement. The great majority of patients present with motor developmental delay. Not infrequently, a sharp decline in motor function during the first weeks of life followed by a degree of improvement and partial attainment of motor milestones is subsequently observed during the first year/s of life (personal observation). While the majority of patients eventually attain trunk control, antigravity strength is typically not achieved in neck flexion, trunk flexion, and the deltoid muscles. Some patients achieve the ability to stand with support for variable periods; however, independent ambulation is only achieved exceptionally. A review of 33 LAMA2-RD patients documented independent ambulation in two patients with complete merosin deficiency, namely at 3.6 and 4 years of age (Geranmayeh et al., 2010). It is noteworthy that none of these two children had feeding or respiratory complications at the time of ambulation. A more recent review of a cohort of LAMA2-RD patients seen at the Dubowitz Neuromuscular Centre identified 6/42 patients (14%) who were ambulant with variable support and variable lengths of time (Zambon AA, Muntoni F and Sarkozy A, personal observation). Similar prevalences emerge from further published cohorts, overall indicating that ambulation during childhood (mostly with support and for limited periods only) is possible in up to about 10% of LAMA2-RD patients with complete merosin deficiency (Jones et al., 2001). Of note, the presence of residual merosin expression was demonstrated in biopsies of a number

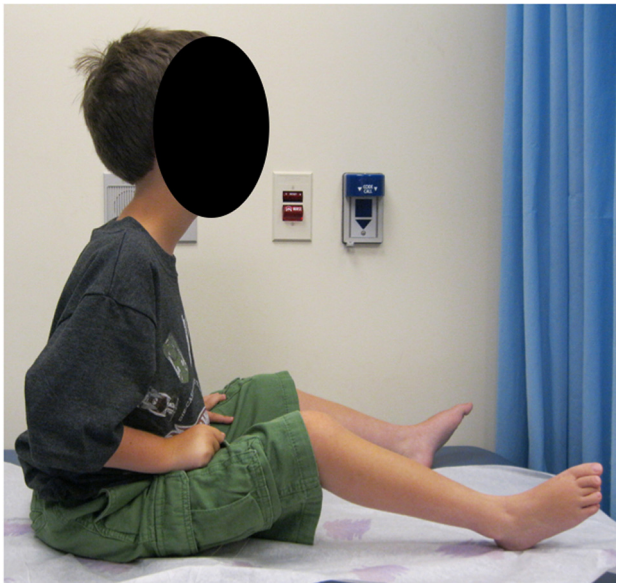

FIGURE 1 | Clinical phenotype. A patient with complete merosin deficiency, with evidence of hip, knee, and ankle contractures with lordotic posture which is typical of LAMA2-RD patients from an early age (written informed consent for the publication of the clinical image was obtained from a parent of the patient).

of these patients, and thus it is possible that in some of these patients with a relatively milder phenotype a low level of merosin expression provided a partial benefit.

Facial weakness, typically with drooling and elongated face, macroglossia, and the protruding tongue is common in patients with severe LAMA2-RD. A progressive limitation of extraocular movements, in particular of upward gaze, is noted as early as at 2 years of age, with clear ophthalmoparesis in the horizontal and upwards direction becoming more evident by the end of the first decade. Interestingly, a deficit of downwards movements or intrinsic muscles as well as ptosis are not observed (Philpot and Muntoni, 1999).

Progressive, restrictive pulmonary insufficiency due to weakness of intercostal and accessory muscles is the most common cause of morbidity and mortality in LAMA2-RD. Inefficient cough is usually present from the first months of life. Patients will often need the use of cough assistance during respiratory infections, with ∼1/3 of patients requiring non-invasive ventilatory (NIV) support in early childhood. Patients started on NIV in the first year of life can sometimes be subsequently weaned-off (Geranmayeh et al., 2010). In a previously published Dubowitz Neuromuscular Centre series of patients, NIV was needed in 4/18 patients <5 years and 8/9 patients aged >10 years with complete merosin deficiency (Geranmayeh et al., 2010). The median age at NIV initiation reported in this cohort was approximately 13 years. Of note, while diaphragm excursion is well-preserved, on dynamic MRI imaging LAMA2-RD patients demonstrate reduced chest wall expansion (Foley AR and Bönnemann CG, personal observation). While invasive ventilation might become necessary for short periods and typically during times of respiratory infections, long-term use of invasive ventilation or use of ventilation via a tracheostomy is rare in pediatric LAMA2-RD patients.

Feeding is variably impaired in its oral, pharyngeal, laryngeal, and/or esophageal phases (Philpot et al., 1999a). Swallowing difficulties can lead to aspiration, recurrent chest infections, and failure to thrive. Macroglossia and facial weakness might further contribute to the defective oral phase in LAMA2-RD patients. Prolonged mealtimes can be distress for families and children. While gastrostomy tube placement is a common and effective procedure for weight and infections' control, safe oral feeding has been observed post-gastrostomy insertion in ∼60% of severe LAMA2-RD patients in a Dubowitz Neuromuscular Centre cohort.

Severe, progressive proximal and distal joints' contractures, in upper and lower limbs, can be present from as early as birth (Prandini et al., 2004), with a considerable detrimental effect on motor function. Progressive scoliosis is common after 6 years of age, leading to a surgical correction in most patients. Severe lordosis, spine, and neck rigidity is also observed. Geranmayeh et al. (2010) reported scoliosis in 14/33 patients with complete merosin efficiency. Interestingly, only two of these 14 patients achieved some form of ambulation, and all but one had decreased or insufficient respiratory function, suggesting a positive correlation between the severity of motor, respiratory, and spinal involvement.

In pediatric patients, independent from the overall clinical severity, cardiac involvement is not often significant (Muntoni, 2003). However, several reports highlight the considerable frequency of subclinical cardiac involvement, in particular, right bundle branch block and left ventricular dysfunction (Spyrou et al., 1998; Finsterer et al., 2010), with rare reports of heart failure at various ages, and thus regular cardiac monitoring with cardiac rhythm assessment by Holter monitoring and cardiac imaging by echocardiogram is recommended in all LAMA2-RD patients.

Central nervous system (CNS) and peripheral nervous system involvement is frequent in LAMA2-RD. Characteristic brain white matter (WM) hypointensity on T1 magnetic resonance imaging (MRI), and increased T2 signal in the periventricular and subcortical WM, are invariably observed in most patients older than 6 months (Farina et al., 1998; Philpot et al., 1999b; Leite et al., 2005), independently from clinically evident CNS involvement (**Figures 2A–D**). Cerebral atrophy and neuronal migration defects (such as focal cortical dysplasia, polymicrogyria, or cortical anomalies typically affecting the occipital regions) can be observed in ∼10% to ∼40% of patients from various series. Seizures, usually responsive to antiepileptic medications in the absence of an underlying cortical anomaly, are observed in up to ∼30% of patients (Jones et al., 2001; Bönnemann et al., 2014). Seizures can be simple or complex partial episodes, occasionally spreading to secondary generalized tonic-clonic seizures (Prandini et al., 2004). The most consistent hypothesis regarding the etiology of these seizures is abnormal neuronal firing due to deficient neuronal migration in specific areas of the cortex (Ahmed et al., 2019), which

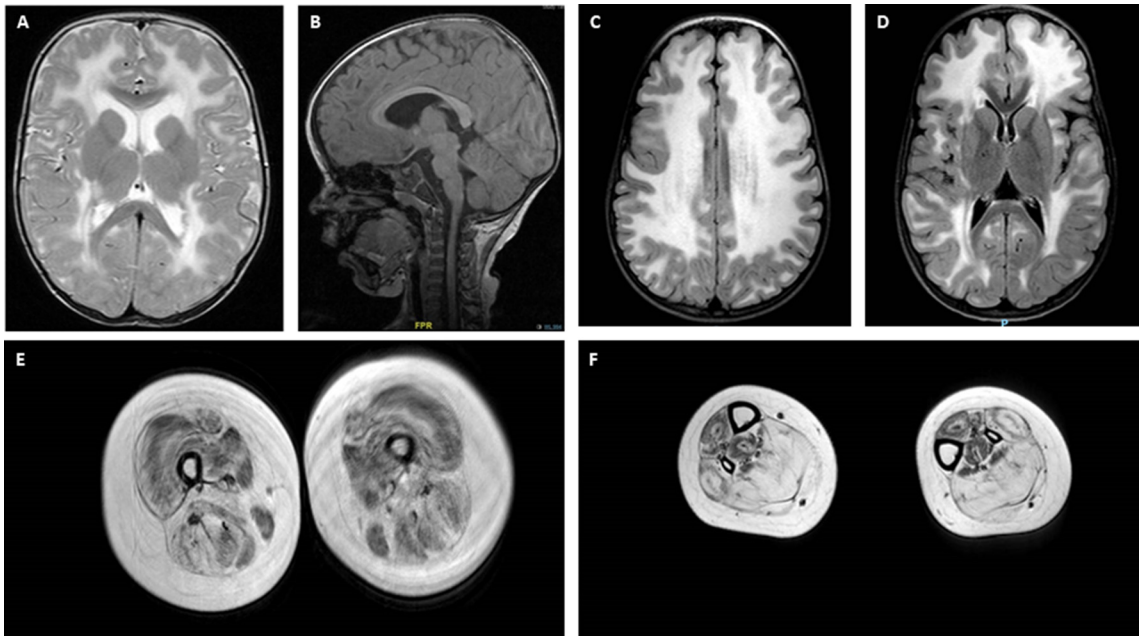

FIGURE 2 | Brain and muscle imaging findings in LAMA2-RD. **(A–D)** Typical brain MRI findings in LAMA2-RD with abnormal appearance of myelin with sparing of the U fibers seen on T2 axial images **(A,C,D)**. T1 sagittal image **(B)** demonstrates evidence of occipital polymicrogyria, which has been observed in some LAMA2-RD patients and may predispose to occipital lobe seizures. Muscle MRI of the upper leg **(E)** and lower leg **(F)** in a 9-year-old patient with complete merosin deficiency with evidence of abnormal signaling in most muscles with relative sparing of the sartorius and gracilis muscles **(E)**. In the lower leg, the muscles of the anterior compartment seem relatively spared compared to muscles of the posterior compartment, namely the soleus and gastrocnemius muscles, which demonstrate the apparent replacement of muscle with fibrofatty tissue.

may be present even when not appreciated on neuroimaging. Mild-to-moderate cognitive disability is reported in a small proportion of LAMA2-RD patients (Messina et al., 2010) and one series was associated with additional structural occipital cortex abnormalities (Mercuri et al., 1999). Mild, sensorimotor demyelinating neuropathy is commonly observed, but its contribution to muscle weakness is considered to be minimal in the human, while it plays a substantial role in mouse models of lama2 deficiency (Shorer et al., 1995; Previtali and Zambon, 2020).

Long term survival has not been well documented in LAMA2-RD. A life expectancy up to the third decade is usually observed, but death as early as the first decade has been described in some patients mainly due to complications of respiratory insufficiency (Philpot et al., 1995; Xiong et al., 2015).

Mild LAMA2-RD

Patients with partial merosin deficiency present with a variable, milder clinical form of LAMA2-RD. Phenotypes range from CMD-like to milder and later presentations including late-onset LGMD-like phenotypes (Naom et al., 1998; Pegoraro et al., 1998; Topaloğlu et al., 1998; Di Blasi et al., 2000; Tezak et al., 2003; Gavassini et al., 2011; Løkken et al., 2015; Nelson et al., 2015; Harris et al., 2017). Partial LAMA2-RD with LGMD presentation is now also classified as LGMDR23 (Straub et al., 2018). The majority of patients with mild LAMA2-RD present their first symptoms during childhood, often with delayed motor milestones and raised CK values. However, a significant proportion of patients achieve and maintain independent ambulation. For example, one large series reported that >60% of patients with residual merosin gained independent or supported ambulation for variable lengths of time (Geranmayeh et al., 2010).

In partial merosin deficiency scoliosis is generally less frequent and progressive than in patients with complete merosin deficiency. Indeed, in a series with 13 patients with partial merosin deficiency, one had scoliosis (Geranmayeh et al., 2010). This patient, aged 15 years at the last assessment, was ambulant with support and presented severe contractures. Conversely, all but one of the 13 patients with partial merosin deficiency (who was aged 1.3 years at last assessment) showed variable degrees of joint contractures, suggesting mild LAMA2-RD has relevant contractual features. In keeping with this finding, recent reports provided evidence that residual merosin deficiency can also clinically manifest as a prominent contractual presentation resembling Emery Dreifuss MD (Nelson et al., 2015).

Patients with partial merosin deficiency are statistically less likely to require ventilatory support or enteral feeding during their lifetime with NIV needed in 1/13 patients with partial merosin deficiency vs. 13/33 patients with complete merosin deficiency (*P*-value 0.0354; Geranmayeh et al., 2010). Similarly, only one of seven patients with partial deficiency aged less than 5 years needed enteral feeding vs. 9/19 of patients with complete merosin deficiency.

Cardiac monitoring in patients with partial merosin deficiency has frequently detected subclinical, primary dilated cardiomyopathy as well as rhythm and conduction disturbances that could be potentially life-threatening, in particular after the third decade of life (Carboni et al., 2011; Marques et al., 2014; Nelson et al., 2015; Harris et al., 2017).

CNS involvement, with epilepsy, typical WM changes, and cortical alterations are not uncommon, and peripheral sensorimotor demyelinating neuropathy is also observed (Geranmayeh et al., 2010; Chan et al., 2014; Harris et al., 2017; Kim et al., 2017; Kubota et al., 2018).

Genotype-Phenotype Correlations

Prediction of clinical severity is based not only on knowledge of the residual amount of merosin but also on location and mutational mechanism of the *LAMA2* variants (Ge et al., 2018; Oliveira et al., 2018). As of December 2017, the *LAMA2* gene variant database[1] listed 309 disease-associated variants (de Oliveira et al., 2014). Overall, homozygous or biallelic loss-of-function mutations (including larger deletions/duplications) in *LAMA2* preferentially lead to severe phenotypes and complete merosin deficiency on muscle biopsy. Conversely, missense variants, in particular those occurring in the N-terminal region with preserved C-terminal expression, are associated with residual merosin expression and milder clinical presentations (Naom et al., 1998; Oliveira et al., 2008, 2014, 2018; Ding et al., 2016). Among these, the *LAMA2* variant c.2461A>C (p.Thr821Pro; in homozygosity or compound heterozygosity with loss-of-function variants) has now been described in several patients with milder EDMD or LGMD-like clinical presentations (Marques et al., 2014; Nelson et al., 2015), as well as in patients with mild, atypical forms of LAMA2-RD with predominant CNS involvement (Marques et al., 2014; Nelson et al., 2015; Oliveira et al., 2018). Conversely, in-frame deletions involving the G-domain can still result in a severe LAMA2-RD presentation, often with residual merosin on muscle biopsy, highlighting the importance of using antibodies directed towards different epitopes of the protein for the immunodiagnosis. A nonsense variant c.4645C>T; p.(Arg1549Ter) has now been associated with milder phenotype both in homozygosity and compound heterozygosity, possibly due to alternative in-frame splicing of exon 32 (Di Blasi et al., 2000, 2001; Geranmayeh et al., 2010). However, we identified the same truncating variant in patients with severe LAMA2-RD, suggesting that genotype-phenotype correlations are still challenging (personal observation). Intrafamilial clinical variability is often reported, one of the most extreme examples being an Italian sibship with two sisters carrying a homozygous loss-of-function *LAMA2* variant, presenting with severe and mild LAMA2-RD, respectively (Prandini et al., 2004).

Diagnosis

The identification of two pathogenic variants in the *LAMA2* gene is the diagnostic gold standard for LAMA2-RD. However, diagnosis can be strongly aided by a combination of clinical features and results of investigations. Elevated serum CK (commonly >1,000 IU/L) is nearly a constant finding in all LAMA2-RD patients, though normal CK has been rarely reported (Sframeli et al., 2017). Brain, and increasingly also body, MRI may provide supportive diagnostic evidence in atypical patients and may support the pathogenicity of unclear *LAMA2* variants. For complete merosin deficiency patients, the role of muscle MRI may be less helpful than brain MRI, however, since muscles can demonstrate abnormal signaling on MRI from an early age (**Figures 2E,F**). Interestingly, muscle MRI features in the lower limbs of LAMA2-RD patients with milder phenotypes are similar to what has been reported in patients with COL6-related dystrophies, with the typical inside-out pattern of fatty replacement (Nelson et al., 2015; Harris et al., 2017).

Muscle biopsies of LAMA2-RD patients show dystrophic changes that can range from mild to severe (**Figures 3A,E**). Deficiency of laminin-211 can be demonstrated in sections using specific antibodies such as Alexis 4H8 recognizing the N terminal 300 kDa fragment of the protein. The deficiency ranges from partial in milder cases (**Figures 3F,G**) to complete in severe cases (**Figure 3B**) and is also observed in the intramuscular motor nerves (**Figure 3B** stars, **3G**, arrows). Absence of laminin alpha 2 at the epidermal and adnexal basement membranes and intradermal sensory nerves can be demonstrated in skin biopsies, particularly in cases of complete laminin alpha 2 deficiency (Sewry et al., 1996, 1997). Thus, a skin biopsy can be offered as a less invasive alternative diagnostic tool in cases where a muscle biopsy is not feasible.

Next-generation sequencing (NGS) is now able to identify pathogenic *LAMA2* variants in close to 100% of patients with suspected LAMA2-RD (Oliveira et al., 2018). Of note, a considerable number of patients have been shown to carry variable-sized deletions/duplications in the *LAMA2* gene and thus, NGS with copy number variation testing is recommended in case two pathogenic variants are not found on NGS alone (Ge et al., 2018). Furthermore, deep intronic variants, synonymous variants leading to abnormal splicing, or copy neutral variations (such as inversions) can be identified by whole genome or RNA sequencing in patients that remain undiagnosed after whole gene sequencing (Gonorazky et al., 2019). Molecular preimplantation and prenatal diagnosis could be offered to families where two clear pathogenic changes are found in the previously affected child. As laminin-211 is expressed in trophoblasts from the 9th week of gestation, analysis of laminin-211 on fetal cells could be offered for families were two pathogenic variants were not identified (Vainzof et al., 2005; **Figures 3I–M**). However, interpretation of partial deficiencies is challenging and thus this analysis is only recommended in cases where complete merosin deficiency was observed in the index patient. At present, neonatal screening is not available for LAMA2-RD.

Management

Clinical management of LAMA2-RD patients is focused on the prevention and treatment of complications. Early interventions with physical, occupational, and speech therapy should be arranged to optimize motor and cognitive development. Regular physical activity, including stretching of joints and providing

[1]https://databases.lovd.nl/shared/genes/LAMA2

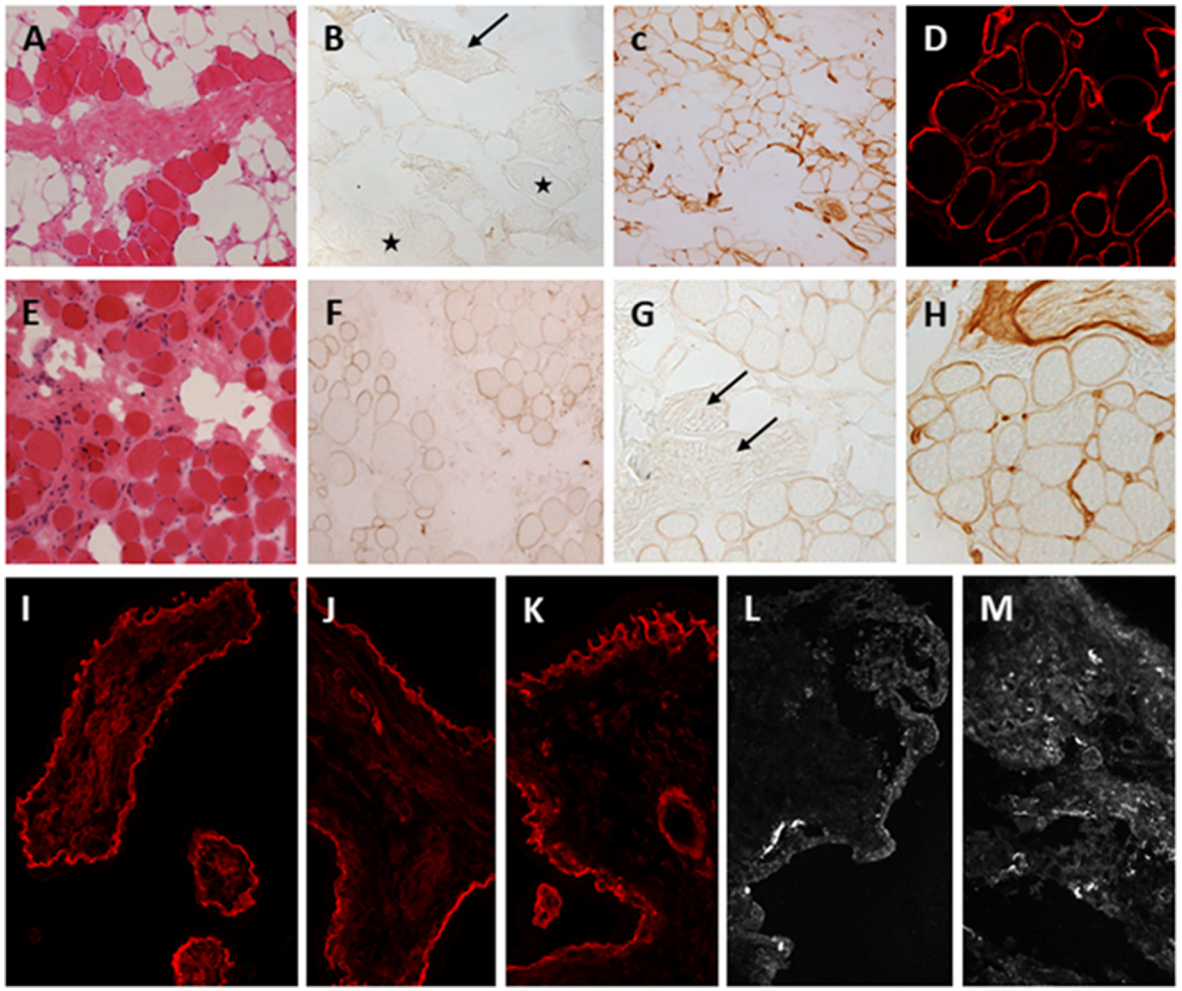

FIGURE 3 | Laminin-211 deficiency in diagnostic quadriceps muscle biopsies and chorionic villus biopsies. Complete laminin-211 deficiency in quadriceps muscle biopsy **(A–D)**. Hematoxylin and eosin **(H & E)** stained section **(A)** shows marked dystrophic changes. Immunolabeling with the 300 kDa laminin-211 antibody **(B)** shows complete absence at the myofiber basal lamina (**B**, stars) and on an intramuscular motor nerve (**B**, arrow). There is widespread secondary upregulation of laminin alpha 5 **(C)**, whereas labeling with the IIH6 antibody against the glycosylated alpha-dystroglycan epitope appears normal. Partial laminin-211 deficiency in quadriceps muscle biopsy **(E–H)**. H&E stained section **(E)** shows moderate dystrophic changes. Immunolabeling with the 300 kDa laminin-211 antibody shows moderate, patchy reduction at the myofiber basal lamina **(F)** and complete absence on an intramuscular motor nerve (**G**, arrows). There is widespread secondary upregulation of laminin alpha 5 **(H)**. Prenatal testing for laminin-211 deficiency in chorionic villus biopsies **(I–M)**. Labeling of a positive control sample (fetus unaffected by LAMA2-RD) **(I)** with the 300 kDa laminin-211 antibody shows distinct membranous labeling at the trophoblastic basement membrane of chorionic villi. Example of a test case showing normal labeling **(J,K)** comparable to the positive control, and another case showing complete absence of laminin-211 at the trophoblastic basal lamina **(L,M)** of first-trimester chorionic villi. In this case, the complete absence of laminin-211 expression suggests that the fetus is affected by LAMA2-RD. Normal labeling for laminin-211 in chorionic villi does not exclude carrier status for LAMA2-RD.

orthosis for upper and lower limbs should be started from the time that a diagnosis of LAMA2-RD is suspected. Ongoing scoliosis monitoring and surgical intervention as needed should be offered to patients. Referral to respiratory care teams should be prompt, given the known natural history of respiratory insufficiency which can manifest with recurrent chest infections from the very first years of life in patients with complete merosin deficiency. Chest physiotherapy and the use of the Cough Assist machine can help in reducing hospitalizations and avoid potentially life-threatening episodes. Pulmonary surveillance includes pulmonary function tests, pulse oximetry, and sleep studies. For patients with complete merosin deficiency, the use of nocturnal NIV becomes necessary during the first decade of life. Weight monitoring from early infancy should focus on addressing feeding difficulties to minimize failure to thrive. As silent aspiration is possible in complete merosin deficiency patients, baseline swallowing evaluation should be obtained regardless of symptoms. Many patients will need the placement of a gastrostomy tube to help in supplementing caloric input and avoiding frequent chest infections in those patients with silent aspiration. Cardiac monitoring in the first two decades of life should focus on assessing silent cardiomyopathies or conductions defects, that might require medical treatment or implantable

devices. Epilepsy should be monitored for and usually can be controlled with first-line antiepileptic drugs. While potential therapeutic interventions are in development for the LAMA2-RDs, disease-modifying medications are not available at present. It remains essential, however, that clinical management is optimized for both decreasing morbidity and improving clinical trial readiness.

BIOMARKERS AND CLINICAL TRIAL NEEDS IN LAMA2-RD

Research in the LAMA2-RDs has benefited from the availability of multiple mouse models which, to varying degrees, recapitulate the human clinical phenotype of skeletal muscle weakness, respiratory insufficiency, and neuropathy. In particular, there are five separate mouse models of laminin-211 deficiency: *dy/dy*, dy^{2J}/dy^{2J} and dy^{nmf417}/dy^{nmf417} (spontaneous mutants); and dy^{W}/dy^{W} and dy^{3k}/dy^{3k} (generated mutants). The recognition of the involvement of apoptotic pathways as a potential disease mechanism in the LAMA2-RDs was demonstrated by studies performed in the dy^{W}/dy^{W} mouse model in which the transgenic overexpression of *Bcl-2* (an apoptosis inhibitor) or inactivation of proapoptotic *Bax* resulted in prolonged survival (Girgenrath et al., 2004; Dominov et al., 2005). Preclinical studies were performed in the dy^{W}/dy^{W} and dy^{2J}/dy^{2J} mouse models of an antiapoptotic compound [N-(dibenz(b,f)oxepin-10-ylmethyl)-N-methyl-N-prop-2-ynylamine maleate] also known as TCH346 and omigapil, which binds to GAPDH thus inhibiting the Siah1-mediated nuclear translocation of GAPDH and the subsequent activation of the apoptotic pathway (Hara et al., 2005). Studies in the dy^{W}/dy^{W} mouse demonstrated inhibition of GAPDH-Siah1-mediated apoptosis in muscle and improved locomotor activity, and studies in the dy^{2J}/dy^{2J} mouse demonstrated decreased fibrosis in the skeletal muscles and the diaphragm muscle along with the improved respiratory rate. These findings formed the basis of the development of a phase 1 pharmacokinetic and safety study of omigapil in the LAMA2-RDs, the first clinical trial for this patient population (Erb et al., 2009; Yu et al., 2013). This study was an open-label, sequential group, ascending oral dose, cohort study with patients with either LAMA2-RD or COL6-related dystrophy (COL6-RD) who were stratified by disease type and weight and randomly assigned to one of three pre-specified dose cohorts. Omigapil was administered at a dose of 0.02–0.08 mg/kg/day for 12 weeks duration, and slightly greater than dose-proportional increases in systemic exposure to omigapil were seen. Overall, omigapil was found to be safe and well-tolerated, and the dose selected to achieve exposure within the pre-established target of the AUC0-24h range was found to be 0.06 mg/kg/day (Clinicaltrials.gov Identifier NCT01805024). Given the short duration of the study, no consistent changes were seen in disease-relevant clinical assessments; however, the study enabled the collection of pulmonary and motor outcome measures and provided an opportunity for the collection and investigation of exploratory outcome measures, including muscle ultrasound, muscle MRI and serum and urine biomarker collection.

Data for additional therapeutic approaches for LAMA2-RDs have emerged including from preclinical studies of transgenic expression of mini-agrin (mag) together with laminin-alpha1 LN-domain nidogen-1(alphaLNNd) in the dy^{W}/dy^{W} mouse model which have demonstrated the restoration of basement membrane stability and the survival of this mouse model to over 2 years (5 times the typical survival). Given that the cDNA for mag and alphaLNNd can each fit into AAV vectors, and AAV vectors are utilized for transgene delivery for gene therapy studies for other muscle diseases presently in progress (NCT03362502, NCT03375164, NCT03652259, NCT03199469), this approach carries high promise for the potential of translation into clinical trials (Reinhard et al., 2017). Work demonstrating that transgenic overexpression of laminin α1 in the dy^{3k}/dy^{3k} mouse model of laminin α1 deficiency improved muscle histological appearance, health, and longevity of the mice (Gawlik et al., 2004; Gawlik and Durbeej, 2010) and that transgenic overexpression of laminin α1 in the dy^{2J}/dy^{2J} mouse model decreased disease severity (Gawlik et al., 2018) led to the subsequent development of a therapeutic approach of using the compensatory upregulation of endogenous Lama1 via AAV9 delivered, catalytically inactivated Cas9 (dCas9) linked VP64 transactivators and multiple guide RNAs in the dy^{2J}/dy^{2J} mouse model (Kemaladewi et al., 2019). Another therapeutic approach developed based on findings from the transgenic overexpression of laminin α1 (Gawlik et al., 2004; Gawlik and Durbeej, 2010; Gawlik et al., 2018) is a protein substitution therapy approach, which has been studied in the dy^{W}/dy^{W} mouse model via the systemic delivery of the embryonically present laminin-111 protein, which has demonstrated stabilization of the basement membrane (Rooney et al., 2012). Protein therapy with laminin-111 was also studied in a $lama2^{-/-}$ zebrafish model of LAMA2-RD and was reported to prevent contraction-induced damage to myofibers and promote reattachment of myofibers to the extracellular matrix (Hall et al., 2019).

Natural History Studies

Collectively, these promising therapeutic avenues currently in development have galvanized efforts to better define the natural history of LAMA2-RDs and to identify viable clinical outcome measures to improve clinical trial readiness for individuals with LAMA2-RD. Identifying outcome measures that correlate with muscle function and are sensitive to change over time is an essential component of clinical trial readiness. One such effort to identify disease-specific outcome measures was a prospective study performed at the National Institutes of Health which measured longitudinal changes in LAMA2-RD patients (n = 24) and COL6-RD patients (n = 23) ages 4–22 years using the Motor Function Measure 32 (MFM32), myometry (of knee flexors and extensors and elbow flexors and extensors) and goniometry (of knee and elbow extension), pulmonary function tests and quality of life measures with five annual assessments. This study found that the MFM32 was sensitive to change in individuals with LAMA2-RD or COL6-RD in ambulatory and non-ambulatory children and adults. In particular, for non-ambulatory patients with LAMA2-RD, the rate of decline

in total MFM32 score was −2.16 points/year ($p < 0.01$). In terms of longitudinal myometry measurements in LAMA2-RD patients, knee flexion strength in non-ambulatory patients declined by 2.47% per year ($p < 0.01$). Longitudinal goniometry measurements were statistically significant in non-ambulatory LAMA2-RD patients in left elbow extension (−4.11° per year; $p < 0.01$). The annual rate of change in forced vital capacity (FVC) was not found to be significant in LAMA2-RD patients in this longitudinal study; however, one potential reason could be that the non-ambulatory patients with LAMA2-RD in this study may have already reached a nadir in their respiratory insufficiency, resulting in a lack of further decline during this study which took place over 4 years (Jain et al., 2019). It is notable that in a separate, retrospective study of pulmonary function in 65 patients with LAMA2-RD, the annual rate of decline of FVC in non-ambulatory LAMA2-RD patients was found to be 1.73% per year ($p < 0.01$; Collins et al, personal observation). Currently, efforts are underway to perform a prospective natural history study focused on LAMA2-RD patients less than 5 years of age. This study is being coordinated among several specialist neuromuscular centers internationally to maximize the cohort size and thus increase the quantity of data collected. Given the paucity of early natural history data in LAMA2-RD and the goal of promising preclinical research efforts to translate into treatments aimed at treating patients with LAMA2-RD from a young age, this early natural history study is timely.

Disease Biomarkers

Adequate, disease-relevant biomarkers, which could be capable of reflecting potential therapeutic changes in individuals with LAMA2-RD are also imminently needed. Such biomarkers would ideally provide a measure of disease and target engagement in patients participating in clinical trials without necessitating sequential muscle biopsies (Szigyarto and Spitali, 2018). In theory, developing comprehensive omics platforms may provide the best chance of identifying disease-relevant biomarkers. Investigations into proteomic evaluations for monitoring disease progression and predicting clinical course have been performed using samples from individuals with Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). These proteomic evaluations were used as a non-invasive way of monitoring disease progression and predicting future clinical courses (Spitali et al., 2018).

In a study analyzing the proteomic signature of the vastus lateralis muscle in four patients with genetically confirmed LAMA2-RD, 86 proteins were identified of which 35 were increased and 51 decreased [using liquid chromatography with tandem mass spectrometry (LC-MS/MS)] (Kölbel et al., 2019). Using the following bioinformatics programs: the Database for Annotation, Visualization, and Integrated Discovery (DAVID[2]), Kyoto Encyclopedia of Genes and Genomes (KEGG[3]), Reactome[4] and Proteomap[5] pathway analyses were performed. This data was then analyzed along with proteomic data from diaphragm and gastrocnemius muscles of the dy^{3k}/dy^{3k} mouse model and identified nine common proteins that appear to be vulnerable, namely decreased proteins related to mitochondrial function in both (de Oliveira et al., 2014). Overall, this study offered preliminary data, for which more comprehensive studies are needed to more definitively identify tissue biomarkers of LAMA2-RD, including at particular stages of disease (Kölbel et al., 2019).

To be able to identify relevant biomarkers from thousands of candidates, networks and consortia with a data-sharing agreement and a common goal in mind need to be established. One such effort has resulted in the creation of Muscle Gene Sets (MGS[6]), which is also accessible via Enrichr, MsigDB/GSEA, and WebGestalt has provided a tool for functional genomics in neuromuscular conditions (Malatras et al., 2019). While not yet extended to proteomic data, this tool offers the ability to study the behavior of genes lists across more than 1,100 comparisons of muscle conditions. Extending such network and consortium efforts to tissue biobanks would provide the opportunity for using platform-type approaches for analyzing proteomic data, thus leveraging the collective efforts of various muscle research groups resulting in increased protein-protein interaction/interactome level data.

Beyond the prospect of identifying specific serum and protein biomarkers for LAMA2-RDs remain the prospect of muscle imaging modalities to serve as disease biomarkers, as well. Efforts towards performing quantitative magnetic resonance imaging (qMRI) to quantify fat replacement in muscular dystrophies include methods of chemical shift imaging (Dixon or IDEAL) or spectroscopy (Burakiewicz et al., 2017). In particular, qMRI of fat replacement has been found to have higher sensitivity than clinical assessments for capturing the progression of neuromuscular disease, including in DMD using two-point Dixon (Bonati et al., 2015) and transverse relaxation time constant (MRI-T2; Willcocks et al., 2016), LGMD type 2I using two-point and three-point Dixon (Willis et al., 2013), oculopharyngeal muscular dystrophy using two-point Dixon (Fischmann et al., 2012), and inclusion body myositis and Charcot-Marie-Tooth disease 1A using three-point Dixon (Morrow et al., 2016). While not evaluated in comparison to motor outcome measure assessments, muscle MRI has been performed in LAMA2-RD patients with preliminary quantitative MRI-T2 and MRS fat fraction evaluations performed (Walter et al, personal observation). If the challenges which joint contractures and dependency on non-invasive ventilation pose to comfortable positioning and stable ventilation during the MRI could be overcome, further use of qMRI in the LAMA2-related population could be evaluated and, such as in other muscular dystrophies, may prove to be highly sensitive to disease progression.

The identification of biomarkers sensitive and specific enough for measuring clinical benefit could enable a smoother road for

[2]www.david.ncifcrf.gov
[3]www.genome.jp/kegg/pathway.html
[4]www.reactome.org
[5]www.proteomaps.net
[6]http://www.sys-myo.com/muscle_gene_sets/

demonstrating potential efficacy or lack of efficacy of particular interventions, thus improving the efficiency of the journey of translational research along the so-called "bench-to-bedside" pipeline for bringing promising therapeutics to patients. It is important to note that in LAMA2-RD caused by biallelic loss-of-function mutations and resulting in a "null" status for the LAMA2 protein/complete merosin deficiency, the onset of the disease is before birth. Thus, clinical and pathological evidence of the disease is already established at birth, at which time severe muscle weakness and muscle biopsy evidence of degeneration and inflammation are seen (Pegoraro et al., 1996). It is therefore essential that therapeutic efforts for the LAMA2-RDs be focused on targeting patients at a very early age and be robust enough in their potential therapeutic effects to demonstrate target engagement and clinical improvements in patients with established disease symptoms at birth. Clinical trial readiness for individuals with LAMA2-RD will depend on combining biobank efforts in a joint quest of identifying disease-relevant biomarkers which are capable of capturing potential changes due to therapeutic interventions. The promising therapeutic approaches in preclinical development for LAMA2-RD all share the overall goal of resulting in meaningful clinical improvements for individuals affected by this CMD subtype. To this end, clinical trial endpoints- as measured via outcome measures- need to be capable of capturing biological and physical improvements which are meaningful to patients as well as recognized as significant from the perspective of regulatory agencies, who will ultimately determine which therapeutics are approved and thus made available for use by the entire patient population.

ETHICS STATEMENT

Written informed consent was obtained from the parent of the patient for the publication of the clinical image included in this article.

AUTHOR CONTRIBUTIONS

AS and AF equally contributed to the conception and design of the article, acquisition of data, drafting and critical revision of the final manuscript. AZ contributed to the literature review and drafting of the manuscript. CB and FM contributed to the conception and design of the article, critical revision of the manuscript, review, and approval of the final version.

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