Triggering regeneration and tackling apoptosis: a combinatorial approach to treating congenital muscular dystrophy type 1A

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Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is an autosomal recessive disorder caused by mutations in the laminin-α2 gene (OMIM: 607855). Currently, no treatment other than palliative care exists for this disease. In our previous work, genetic interventions in the Lama2Dy-w mouse model for MDC1A demonstrated that limited regeneration and uncontrolled apoptosis are important drivers of this disease. However, targeting one of these disease drivers without addressing the other results in only partial rescue of the phenotype. The present study was designed to determine whether utilizing a combinatorial treatment approach can lead to a more profound amelioration of the disease pathology. To accomplish this task, we generated Bax-null Lama2Dy-w mice that overexpressed muscle-specific IGF-1 (Lama2Dy-wBax2/21+IGF-1tg). Further to test the translational potential of IGF-1 administration in combination with Bax inhibition, we treated Lama2Dy-wBax2/2 mice postnatally with systemic recombinant human IGF-1 (IPLEX™). These two combinatorial treatments lead to similar, promising outcomes. In addition to increased body and muscle weights, both transgenic overexpression and systemic administration of IGF-1 combined with Bax-inhibition resulted in improved muscle phenotype and locomotory function that were nearly indistinguishable from wild-type mice. These results provide a fundamental proof of concept that justifies the use of a combination therapy as an effective treatment for MDC1A and highlights a compelling argument toward shifting the paradigm in treating multifaceted neuromuscular diseases.

INTRODUCTION

Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is the second-most common form of congenital muscular dystrophy. Patients with this disease have poor muscle tone at birth, extremely compromised neuromuscular function and rarely achieve independent ambulation (1). Most patients with MDC1A succumb to a premature death due to either respiratory complications or failure to thrive (1–3). Although significant strides have been made toward understanding the molecular and biochemical mechanisms underlying MDC1A, there remains no effective therapy in place to combat this lethal disease.

Unlike other dystrophies, which result from defects in membrane proteins, MDC1A is caused by mutations in the LAMA2 gene that encodes for the alpha 2 chain of the laminin-211 protein complex, an essential component of the extracellular matrix (ECM). ECM proteins are known to play a critical role in preserving the integrity of the cellular niche and are required for proper cell function (4,5). Because of its multiple binding partners in the ECM and at the plasma membrane, defects in laminin-211 result in a major disruption of structural stability and signal transduction.

Of the many animal models for MDC1A currently available, the Lama2Dy-w is a mouse model whose pathology most closely resembles that of the human disease (6–8). Like humans, mice homozygous for the mutant allele show accelerated muscle degeneration with limited or no regenerative capacity and have a severely shortened lifespan. These mice also show an increase in apoptosis, fibrosis and severe inflammation (9–13). Proof-of-concept studies designed to correct the primary defect of laminin-α2 deficiency by gene or protein replacement therapies greatly ameliorated pathology in MDC1A mouse models (14–16). However, the clinical application of these therapies is still several years away (17). In an effort to improve the length and quality of life for patients with MDC1A and other muscular dystrophies, a more immediate approach is to treat some of the secondary pathophysiologies (10,11,17–19).

Previously, we demonstrated that blocking apoptosis by the inactivation of Bax, a proapoptotic member of the Bcl2 protein
family (20), resulted in an increase in the lifespan of Lama2Dy-w mice (21). Additionally, we also found that the overexpression of insulin-like growth factor-1 (mIGF-1) under a muscle-specific promoter significantly improved the lifespan and the overall growth of Lama2Dy-w mice by improving muscle regeneration (22). While these strategies led to a measurable alleviation of pathology, none resulted in complete recovery, stressing that a single therapy may not be sufficient to treat MDClA. An emerging idea in the study of multifaceted diseases such as atherosclerosis, hepatic fibrosis, pulmonary emphysema and chronic obstructive pulmonary disease is that different pathological processes are intimately connected and can even synergize to manifest the disease pathology (23–25). Thus, the complex pathology seen in MDClA may also be the result of dysregulation of multiple cellular functions and processes. Strategies that simultaneously target several of these mechanisms might lead to a greater amelioration of pathology than a single mode therapy. Although the idea of a combinatorial treatment strategy seems intuitive for the treatment of complex diseases, very few studies have utilized the power of combination therapy in the context of muscular dystrophy.

Here, we describe a novel combinatorial treatment strategy that enhances muscle regeneration and inhibits apoptosis. We studied the outcome of combining these single mode treatment models by generating Lama2Dy-wBax+/−/IGF-1tg mice (Bax-null Lama2Dy-w mice overexpressing mIGF-1). Further, to verify the therapeutic potential of increased systemic IGF-1 in combination with Bax inhibition, we administered recombinant human IGF-1 (IPLEX™, Insmed Inc.) in Lama2Dy-wBax+/− animals. Our results show that combination therapy leads to a marked improvement in the muscle pathology of Lama2Dy-w mice. The most impressive outcome was the improved activity and mobility of the Lama2Dy-wBax−/−/IGF-1tg mice, most likely due to a significant resolution of inflammation and fibrosis. Our study is a proof of concept that demonstrates the addition of IGF-1, introduced transgenically or administered systemically, in combination with Bax inhibition leads to a more remarkable improvement in phenotype than we have seen with any single mode therapy.

RESULTS
Combination therapy results in improved body weight and muscle function

In all treated and wild-type (WT) mice, there is a growth spurt between weeks 3 and 5, which is absent in the Lama2Dy-w mice, who maintain the smallest body weights. Our results show that the combined therapy exhibited the greatest increase in body weight of any treated group. The Lama2Dy-wBax−/−/IGF-1tg group had significantly higher body weights at week 8 than the Lama2Dy-wBax−/− (P < 0.05) and Lama2Dy-w+/−/IGF-1tg groups (P < 0.0001; Fig. 1A). The tibialis anterior (TA), gastrocnemius/soleus (GS) and quadriceps (QD) muscles from all treated groups were significantly larger than muscles from all treated groups were significantly larger than TA, gastrocnemius/soleus (GS) and quadriceps (QD) IGF-1tg groups (22). While these strategies led to a measurable alleviation of pathology, none resulted in complete recovery, stressing that a single therapy may not be sufficient to treat MDClA. An emerging idea in the study of multifaceted diseases such as atherosclerosis, hepatic fibrosis, pulmonary emphysema and chronic obstructive pulmonary disease is that different pathological processes are intimately connected and can even synergize to manifest the disease pathology (23–25). Thus, the complex pathology seen in MDClA may also be the result of dysregulation of multiple cellular functions and processes. Strategies that simultaneously target several of these mechanisms might lead to a greater amelioration of pathology than a single mode therapy. Although the idea of a combinatorial treatment strategy seems intuitive for the treatment of complex diseases, very few studies have utilized the power of combination therapy in the context of muscular dystrophy.

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This observed increase in body and muscle mass correlates with improved activity. We used standup and tail suspension tests as functional measures of overall activity and hind limb muscle strength (22,26). Normal exploratory behavior of WT mice involves frequent standing up on their hind legs, whereas Lama2Dy-w mice are visibly less active and rarely exhibit this behavior due to weaker muscles (20,22). Lama2Dy-wBax+/−/IGF-1tg mice showed a significant improvement compared with Lama2Dy-wBax−/−, Lama2Dy-w+IGF-1tg and Lama2Dy-w mice (44 ± 5.76, 32 ± 2.83, 17.25 ± 5.47 and 4.27 ± 1.62 standups/5 min, respectively; P < 0.0001; Fig. 1C). In tail suspension tests, WT mice are capable of holding their legs in an extended position for longer than 10 s, while Lama2Dy-w mice quickly retract their legs to an adducted position (2.67 ± 0.99 s) due to muscle weakness. In this study, mice on the combined therapy had the longest time-to-leg retraction (9.68 ± 0.40 s) compared with all other laminin-α2-deficient groups (P < 0.0001; Fig. 1D).

Laminin-211 is also found in the basement membranes of Schwann cells. Loss of laminin-α2 has been associated with peripheral neuropathy characterized by demyelinated axons and reduced conduction velocity (7,27,28). In an initial qualitative assessment, we looked at cross-sections of sciatic nerves stained with Luxol Fast Blue to evaluate whether these different interventions prevent demyelination (Fig. 2). We demonstrate here that the global inhibition of Bax greatly improves the myelination patterns in Lama2Dy-w mice. Interestingly, the muscle-specific overexpression of IGF-1 alone also improves the myelination of axons (although to a lesser degree compared with Bax inhibition), suggesting the possibility of retrograde signaling. The most complete recovery, however, is seen with the combined treatment, which is nearly indistinguishable from the WT (Fig. 2).

While the majority of Lama2Dy-w mice died within 40 days after birth (21,29), no deaths occurred in the Lama2Dy-wBax+/−/IGF-1tg group up to the time animals were sacrificed for tissue analysis at 8 weeks of age. While it is warranted, a longer-term survival study was beyond the scope of this study due to IACUC restrictions and the difficult nature of generating these transgenic mice.

Combination therapy improves muscle pathology

Histological analyses using hematoxylin and eosin (H&E) staining of TA muscles showed that combined therapy more completely restored muscle phenotype relative to either treatment alone. Lama2Dy-w muscles display an increased proportion of small myofibers with large interstitial spaces filled with infiltrating cells (Fig. 3A). While we see many large fibers and only a few centrally nucleated ones (a hallmark of regeneration) in the Lama2Dy-wBax−/− muscles, Lama2Dy-w+IGF-1tg muscles primarily exhibit a large increase in centrally nucleated myofibers with a few hypertrophied ones (Fig. 3B and C). Lama2Dy-wBax+/−/IGF-1tg muscles, however, show an increase in the number of both hypertrophied and regenerating myofibers compared with the inhibition of Bax alone. Additionally, the muscle architecture of combination treated animals most
closely resembles the WT with very little interstitial space and few infiltrating cells (Fig. 3D and E).

The total cross-sectional area of muscles in the combinatorial treatment group was significantly increased compared with that of Lama2Dy-w (P, 0.001) and Lama2Dy-w+IGF-1tg (P, 0.05), but not the Lama2Dy-wBax−/− group (Fig. 3F). To quantify the myofiber size distribution, the minimum Feret diameter was measured. The minimum Feret diameter is the shortest minimum distance between parallel lines tangent to the perimeter of an object. This parameter is considered a more reliable measurement of muscle fiber size than area (30). The minimum Feret distribution shows that Lama2Dy-w muscles have many small fibers with a peak range of 5–10 μm. This fiber size distribution and peak is shifted toward the WT in all treated groups. Bax inhibition alone and the combined therapy show the greatest shift and have a similar minimum Feret distribution (peak of 35–45 μm). Of the two groups, however, the combination therapy resulted in a wider fiber distribution with a greater number of hypertrophied fibers (Fig. 3G).

In order to assess the extent of muscle injury, we measured changes in levels of serum creatine kinase (CK), a known biomarker for muscle damage that has been shown to be elevated in laminin-α2-deficient muscle (31,32). CK levels in the combined treatment group were significantly lower than that of both Lama2Dy-w (n = 4) and Lama2Dy-w+IGF-1tg (n = 5) muscles. However, only the QD of Lama2Dy-wBax−/−+IGF-1tg mice were significantly larger than those of Lama2Dy-wBax−/− (n = 4). (C and D) Lama2Dy-wBax−/−+IGF-1tg mice show further improvements in muscle strength over the single mode therapies, as measured by their ability to stand on their hind limbs and time-to-leg retraction in tail suspension tests (n > 5 for all groups). ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.0001; ∗∗∗∗P < 0.0001, dP < 0.01 between all marked groups.

Figure 1. IGF-1 overexpression in combination with Bax inhibition leads to the greatest increase in body weight and maintenance of muscle strength over time. (A) Lama2Dy-wBax−/−+IGF-1tg mice have higher body weights than all other laminin-deficient groups beginning at postnatal week 2 and continuing throughout development. At week 8, Lama2Dy-wBax−/−+IGF-1tg mice (n = 5) were significantly larger in body weight compared with Lama2Dy-w (n = 14), Lama2Dy-wBax−/− (n = 5) and Lama2Dy-w+IGF-1tg mice (n = 10) (P < 0.05 and P < 0.0001, respectively). (B) Weights of TA, GS complex and QD were significantly increased with the combined treatment (n = 4) compared with Lama2Dy-w (n = 4) and Lama2Dy-w+IGF-1tg (n = 5) muscles. However, only the QD of Lama2Dy-wBax−/−+IGF-1tg mice were significantly larger than those of Lama2Dy-wBax−/− (n = 4). (C and D) Lama2Dy-wBax−/−+IGF-1tg mice show further improvements in muscle strength over the single mode therapies, as measured by their ability to stand on their hind limbs and time-to-leg retraction in tail suspension tests (n > 5 for all groups). ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.0001; ∗∗∗∗P < 0.0001, dP < 0.01 between all marked groups.
macrophages. We found that untreated muscle had the highest instance of apoptotic cells (8.3 ± 2.69 TUNEL+/mm²), while all treatments resulted in a significant reduction in TUNEL+/mm² (P < 0.01), with the greatest decrease seen with the combined treatment (0.63 ± 0.12 TUNEL+/mm²; Fig. 5).

**Inflammation and fibrosis are reduced in response to the combination therapy**

Inflammation and fibrosis are recognized as major drivers of disease pathology in many dystrophies, including MDC1A. We performed immunohistochemistry to help elucidate the extent of inflammation using CD11b antibody, a marker for infiltrating macrophages. We found that untreated Lama2ΔBw−/− mice have an extremely elevated number of infiltrating cells. While there was a slight decrease in CD11b-positive cells with the single mode therapies compared with no treatment, the combination approach showed the least number of infiltrating inflammatory cells indicating a less inflamed environment (Fig. 6A–D). These results are supported by a significant decrease in CD11b mRNA expression levels with the combination treatment compared with Lama2ΔBw−/− muscle (P < 0.01; Fig. 6E).

We have also found that Lama2ΔBw−/− muscles have markedly increased expression levels of the key proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin (IL)-1α. In response to the combined treatment, we saw a decreased expression of both genes to a greater extent than was observed with either treatment alone (P < 0.01 for TNF-α and IL-1α; Fig. 6F). Since nuclear factor κ B (NF-κB) activation is known to regulate the expression of these cytokines, we documented the activity of this pathway by looking at the phosphorylation of the NF-κB subunit p65. We have found phospho-NF-κB p65 to be greatly increased in Lama2ΔBw−/− muscles. Elimination of Bax resulted in a drastic reduction in phospho-NF-κB p65 levels, while the overexpression of mIGF-1 had no additive effect in combination with Bax inhibition. In fact, consistent with the histologically observed inflammatory phenotype, IGF-1 overexpression as a standalone treatment actually led to an increase in the expression levels of phospho-NF-κB p65 (Fig. 6G).

In addition to elevated inflammation, laminin-α2-deficient muscles exhibit extensive fibrosis. In order to measure the fibrotic index, TA muscle sections from the various treated groups were stained with Picrosirius Red to delineate the amount of collagen present. While neither Bax inhibition nor IGF-1 overexpression alone reduced the amount of fibrosis in muscle, Lama2ΔBw−/−Bax−/−/+IGF-1tg TA muscles showed a significant decrease in the fibrotic tissue (Fig. 7A–E). We measured interstitial space as a percent of the cross-sectional area (%IS) to quantify fibrotic regions. Among the three treatment groups, we found the lowest %IS in muscles of the combined therapy group (P < 0.001; 12.1 ± 3.48%) when compared with the individual treatment groups Lama2ΔBw−/−Bax−/−/+IGF-1tg (21.51 ± 2.00%; P < 0.05) or Lama2ΔBw−/−/+IGF-1tg groups (28.36 ± 4.46%, P < 0.001; Fig. 7F).

Because tumor growth factor β (TGF-β) is a key regulator of fibrosis, we looked at gene expression levels of TGF-β and its downstream targets COL1a (collagen 1a) and FN1 (fibronectin). While there was a slight reduction in the expression of these genes with single mode therapies, the only significant decrease was found in Lama2ΔBw−/−Bax−/−/+IGF-1tg muscles (TGF-β, P < 0.0001; COL1a, P < 0.01; FN1, P < 0.001; Fig. 7G). Moreover, the combined treatment also showed the only significant reduction in levels of phospho-SMAD2/3 protein, an indicator of activated TGF-β signaling (Fig. 7H).

**IPLEX™ treatment shows translational potential for combination therapy**

To test the therapeutic potential of systemically administered IGF-1 compared with muscle-specific overexpression, we treated Lama2ΔBw−/−Bax−/− animals with IPLEX™ (Insmed Inc.) for 7 weeks. Our results show that postnatal administration of IGF-1, like the transgenic therapy, shows a substantial increase in body weights compared with vehicle control (or untreated) animals (two-way ANOVA, P < 0.0001; Fig. 8A). In addition, standup and tail suspension tests displayed significant improvements in activity and hind limb muscle strength of Lama2ΔBw−/− IPLEX™ mice compared with Lama2ΔBw−/−Bax−/− PBS mice (standup test: one-way ANOVA, P < 0.001; tail suspension test: one-way ANOVA, P < 0.01; Fig. 8B and C).

As seen in the transgenic mice, H&E staining of Lama2ΔBw−/−IPLEX™ treated animals showed improved muscle phenotype compared with the vehicle control group (Fig. 8D). Minimum Feret diameter measurements indicate a similar fiber size distribution in the treated and control groups.
**Figure 3.** *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup> + IGF-1tg mice demonstrate the greatest improvement in muscle phenotype compared with single mode therapies. (A–E) Hematoxylin and eosin (H&E) staining of TA muscle cross-sections shows remarkable improvement in muscle phenotype with the combination therapy relative to single treatments. Bar = 50 μm. (F) Bax inhibition alone (n = 4) or in combination with IGF-1 overexpression (n = 4) resulted in the greatest increase in the cross-sectional area of TA muscles compared with *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>* mice (n = 4). IGF-1 overexpressing animals (n = 4) show a more modest, but still significant, increase. (G) Minimum Feret analysis shows that Bax-null (n = 3; yellow) and combination treated animals (n = 6; blue) have the greatest shift in fiber size distribution toward the WT (n = 4; black). Of the two groups, the *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup> + IGF-1tg mice exhibit a more uniform distribution with more hypertrophied fibers. *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>* (n = 3; red), *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup> + IGF-1tg (n = 4; green). *P < 0.05; **P < 0.01; ***P < 0.001.

(Fig. 8E), however *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup>-IPLEX<sup>TM</sup> muscle fibers are more tightly packed with little interstitial space (Fig. 8D). Additionally, like our transgenic approach, *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup>-IPLEX<sup>TM</sup> also exhibits a slightly larger number of hypertrophied fibers compared with the vehicle treated groups. Picrosirius Red staining also shows reduced fibrosis in TA muscles of *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup>-IPLEX<sup>TM</sup> mice (Fig. 8F). Each of these improvements in the pathology of IPLEX<sup>TM</sup>-treated *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup> mice are similar to those seen in *Lama2*<sup>Δ<sub>dy</sub>-<sup>W</sup>*<sup>−/−</sup> + IGF-1tg mice.

**DISCUSSION**

Over the last few years, it has been found that the complex pathology seen in MDC1A results from the dysregulation of many cellular mechanisms. Following this line of thought, it stands...
to reason that simultaneously targeting more than one mechanism may lead to an improved outcome. However, the efficacy of a combinatorial treatment in this disease, to our knowledge, has only been tested in only one other study. Our previous research showed that targeting uncontrolled apoptosis or limited regenerative capacity results in only partial amelioration of the disease. This study is a proof of concept that demonstrates simultaneous targeting of these two disease drivers leads to a near complete amelioration of disease pathology.

While the benefits of IGF-1 overexpression include overall muscle growth by improving regeneration (22), IGF-1 signaling also has been shown to negatively affect inflammatory and fibrotic pathways (33–36). Due to the continuation of the noxious environment, it is possible that the pro-mygogenic effects of IGF-1 overexpression are under-utilized in Lama2-Dy-w muscle. If it is indeed this inflammatory environment that plays a role in negating the potential benefits of IGF-1 signaling, it would be of interest to combine IGF-1 overexpression with an anti-inflammatory therapy. This study also shows the inhibition of Bax yielded a more robust rescue of the Lama2-Dy-w phenotype compared with IGF-1 overexpression. Therefore, one can hypothesize that the IGF-1/Akt signaling axis plays a secondary role to Bax-mediated apoptosis in driving the muscle pathology. However, Bax inhibition is also limited in that it does not fully address chronic inflammation or fibrosis and shows very little improvement in the regenerative index. These findings highlight the limitations of targeting only one of these pathways in treating MDC1A.

By combining the two therapies, we found that in addition to improved postnatal growth, Lama2-Dy-w Bax−/− + IGF-1tg mice showed impressive locomotory capacities and remarkable improvement in muscle pathology. The most striking result of the combinatorial approach is the reduction in inflammation and fibrosis. Consistent with histological findings, real time polymerase chain reaction (RT–PCR) analysis revealed decreased expression of several proinflammatory and fibrotic genes. We found that the expression of TNF-α, IL-1α, CD11b, FN1 and COL1a are all significantly down-regulated in the Lama2-Dy-w Bax−/− + IGF-1tg mice compared with either of the single mode therapies. Following these results, we then demonstrated that levels of both phospho-NF-κB p65 and phospho-SMAD/3, major mediators of NF-κB and TGF-β signaling, are also reduced in the combinatorial treatment. The activation of NF-κB has been shown to contribute to fibrogenesis (37,38) and, therefore, it is possible that the attenuation of this signaling pathway leads to subsequent resolution of fibrosis. However, it is interesting to note that while Bax inhibition alone did result in a significant reduction in NF-κB signaling, only the combination therapy caused a significant change in TGF-β signaling. These results suggest that apoptotic and regeneration axes are likely upstream players in triggering inflammatory and fibrotic responses. While it remains to be elucidated whether these decreases in inflammation and fibrosis are causative or correlative, the drastic improvements in the health of dual treated animals indicate the importance of their attenuation on overall muscle health.

Since demyelinating neuropathy is another important aspect of MDC1A (15,39–41), a therapeutic approach that addresses both the muscle and nerve pathology is appealing in the context of MDC1A. Of the many therapeutic interventions studied so far, only few have alleviated neuropathy (15,28). In laminin-α2-deficient mice, we observe large patches of demyelinated axons in the sciatic nerve. With the single mode therapies, we see some alleviation of this loss of myelination, but large patches of the nerve are still bare. Remarkably, this study shows that mice receiving the combination treatment not only exhibited greater levels of activity than all other treatment
groups, but also had comparable sciatic nerve myelination patterns to those of the WT. Further, the Lama2Dy-wBax\(^{2-/2}\)+IGF-1tg is the only treated group in which we have never observed paralysis. Although it was beyond the scope of this study, these promising results warrant a more detailed study to elucidate whether combination treatment directly targets the nerve pathology or indirectly alleviates neuropathy as a consequence of retrograde signaling from a healthier muscle phenotype.
The greatest reduction in fibrosis was seen in \textit{Lama2}^{2+}/2 + IGF-1tg mice. (A–E) Picrosirius Red staining (red, collagen; yellow, cytoplasm) of \textit{Lama2}^{2+}/2 + IGF-1tg TA muscle cross-sections show a reduction in fibrotic tissue that is not addressed by either treatment alone. Bar = 50 μm. (F) Percent interstitial space was significantly decreased in the \textit{Lama2}^{2+}/2 + IGF-1tg group \((n = 5)\) compared with all other laminin-α2-deficient groups \((n = 3\) within each of those groups). (G) RT–PCR of hind limb muscles in 8-week-old mice shows that \textit{COL1a} and \textit{FN1} are significantly reduced only in the \textit{Lama2}^{2+}/2 + IGF-1tg group. Tumor growth factor β (TGF-β) gene expression was reduced in all treated groups, with the greatest reduction in the combined therapy (asterisks represent significance between \textit{Lama2}^{2+}/2 and \textit{Lama2}^{2+}/2 + IGF-1tg groups when not specified by a bar). (H) pSMAD2/3 levels were determined by western blot analysis; the top panel shows a representative western blot. Elevated expression levels of pSMAD2/3 seen in laminin-α2-deficient muscle were only decreased in response to combination therapy. Total SMAD2/3 did not change (data not shown). Lane 1, \textit{Lama2}^{2+}/2; lane 2, \textit{Lama2}^{2+}/2 + IGF-1tg; lane 3, \textit{Lama2}^{2+}/2 + IGF-1tg; lane 4, \textit{Lama2}^{2+}/2 + IGF-1tg; lane 5, WT; top band = pSMAD2/3; bottom band = α-tubulin. *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\)
As a proof of concept for the therapeutic potential of IGF-1, we systematically administered IPLEX™ to *Lama2*^{Dv-w*}Bax^{-/-} animals. IPLEX™ treatment indeed showed significant improvements in the disease outcome, comparable with what was seen in the transgenic *Lama2*^{Dv-w*}Bax^{-/-} + IGF-Itg animals. As in the transgenic combination study, we saw remarkable improvements in body growth, muscle function and muscle pathology. It is important to note that our results demonstrate that a systemic delivery of IGF-1 after birth still leads to a substantial amelioration of the disease pathology when combined with Bax inhibition. This is important because many children are often not diagnosed and do not start treatment until well after birth. In a clinical trial for myotonic dystrophy, IPLEX™ led to increased lean muscle mass; however, it did not prove efficacious in terms of muscle strength (42). Since gaining muscle mass is a major challenge for patients with MDC1A, the increases seen in response to IPLEX™ treatment could have great implications in treating this disease. These encouraging results warrant a future study testing the efficacy of combining IPLEX™ with an anti-apoptotic drug (doxycycline or omigapil); however, this was beyond the scope of this study.

**CONCLUSION**

Within the last decade, tremendous progress has been made in identifying therapeutic targets to treat MDC1A. We show for...
the first time that targeting more than one downstream pathome-
chanism of MDC1A potentiates a more profound recovery from this
disease. This is a proof-of-concept study that supports the
use of dual therapies in MDC1A. Meinen et al. (32) have also
shown that anti-apoptotic therapy combined with mini-agrin
gene therapy results in improved muscle function and pheno-
type. While the successful application of gene-based therapy is
not currently available to patients, both IGF-1 (IPLEX™/Incre-
lex) and anti-apoptotic (omigapil or doxycycline) therapies have
been clinically tested in the context of other diseases and there-
fore have more immediate pharmacological promises for
MDC1A patients. Together, these results support our hypothesis
that a combinatorial approach has synergistic benefits to treat
laminin-α2 deficiency and could have the potential to treat
MDC1A patients.

**METHODS**

**Animal breeding and care**

All animals were housed at the Laboratory Animal Care Facility,
Boston University on a 12:12 h light–dark cycle. Food and water
were provided *ad libitum*. All procedures were performed in ac-
cordance with the IACUC guidelines at Boston University. Het-
erozygous B6.129 Lama2<sup>Bax<sup>-/-+mIGF-1<sup>Tg</sup></sup></sub> mice carrying a mutation in the
*LAMA2* gene and MLC/mIGF-1 mice were kindly provided by
Dr Eva Engvall (Burnham Institute, La Jolla, CA, USA) and
Dr Elizabeth Barton (University of Pennsylvania), respectively
(43). Heterozygous C57BL/6-<sup>Bax<sup>-/-</sup></sub> mice with or without the mIGF-1 transgene
in the *Bax* gene were obtained from the Jackson Laboratory
(44). Five groups of 8-week-old animals were generated through mul-
tiple rounds of breeding: *Lama2<sup>Bax<sup>-/-</sup></sub>, *Lama2<sup>Bax<sup>-/-</sup></sub>* mice overexpressing the mIGF-1 transgene (*Lama2<sup>Bax<sup>-/-</sup>+mIGF-1<sup>Tg</sup></sub>* and
*Lama2<sup>Bax<sup>-/-</sup></sub>*-null mice with or without the mIGF-1 transgene
(*Lama2<sup>Bax<sup>-/-</sup>+mIGF-1<sup>Tg</sup></sub>* and *Lama2<sup>Bax<sup>-/-</sup></sub>*), respectively) and WT mice. Mice were genotyped as described previ-
ously (21,43). All interventions and treatments refer to
transgenic modifications, with the exception of the IPLEX™
therapy.

**Systemic administration of IPLEX™**

*Lama2<sup>Bax<sup>-/-</sup></sub>* mice were treated with IPLEX™ (recombin-
ant human IGF-1 and its binding protein IGFBP-3, rhIGF-1/
rhIGFBP-3). They received intra-peritoneal injection at
15 mg/kg/day from postnatal weeks 1 through 8, and the
control group received isovolumetric phosphate-buffered saline
(PBS).

**Functional capability**

Two simple non-invasive tests were performed to assess muscle
function at week 6. A standup test was conducted to evaluate the
exploratory behavior of mice by counting the number of times
the animal stood up on their hind limbs during a 5 min interval.
As a measure of hind limb muscle strength, the average
time-to-leg retraction was calculated from three 10 s tail suspen-
sion tests per animal. A resting period of 1 min was allowed
between each trial.

**Tissue collection**

Animals were euthanized by an overdose of isoﬂurane. Tissues
were excised, weighed and snap frozen in liquid nitrogen for
RNA and protein extraction. TA muscles for histology were em-
bedded in Tissue-Tek OCT Compound (Sakura Finetek USA,
Inc., Torrance, CA, USA) and frozen in isopentane chilled in
liquid nitrogen (Sigma-Aldrich, St Louis, MO, USA). Serial sec-
tions (7 μm) were prepared using the Leica CM 1850 cryostat
(Leica Microsystems, Inc.).

**Histology**

For histological analysis mid-belly cross-sections of muscles
were stained with H&E and Picrosirius Red using protocols
that were described previously (22). Morphometric analyses
were performed using NIS-Elements Basic Research 3.0 soft-
ware. Myofiber size, percent centrally nucleated fibers, relative
fibrotic area and cross-sectional area were measured.

**Sciatic nerve histology**

Sciatic nerves of 8-week-old mice were also embedded in
Tissue-Tek OCT Compound (Sakura Finetek USA, Inc.) and
frozen in isopentane (Sigma-Aldrich) chilled on liquid nitrogen.
Cryosectioning and Luxol Fast Blue staining were outsourced to
Mass Histology Service, Inc. (Worcester, MA, USA).

**CK assay**

Serum collected from mice was used to measure CK levels. Non-
hemolyzed serum samples collected from 8-week-old mice were
assayed for CK levels using the Creatine Kinase Enzyme Assay
Kit (Cat. No. C7909-98, US Biological Marblehead, MA, USA)
following the manufacturer’s instructions.

**Immunohistochemistry**

Frozen tissue sections were fixed in 2% paraformaldehyde, blocked
for 60 min with 2% bovine serum albumin, 2% goat serum, 0.1%
Triton X-100 in 1 x PBS and incubated with anti-CD11b (1:200)
(BD Biosciences, San Jose, CA, USA) for 60 min in the dark.
Nuclei were stained with 0.1 μg/ml 4′,6-diamidino 2-phenylindole
(DAPI) for 5 min. After washing with PBS, sections were mounted
with Vectashield (Vector Laboratories).

**TUNEL assay**

Frozen muscle sections were fixed with 1% paraformaldehyde
and immunostained for the visualization of apoptotic nuclei by
the TUNEL assay, using ApopTag Plus Fluorescein *In Situ*
Apoptosis Detection Kit (Chemicon International-Millipore,
Billerica, MA, USA; Cat. No. S7111). These sections were sub-
sequently stained with mouse anti-dystrophin (1:200; BD Bios-
ciences) for 1 h. Goat anti-mouse Alexa Fluor 568 (Invitrogen,
Carlsbad, CA, USA) was used as the secondary antibody.
Gene expression

RNA was extracted from 25 mg of hind limb muscle per biological sample using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions. Isolated total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA). Analysis of gene expression was performed by TaqMan assays (Applied Biosystems) on ABI 7300 Real Time PCR system. Glyceraldehyde 3-phospho dehydrogenase served as the endogenous control and gene expression was calculated by using the ΔΔCt method.

Western blot

Five biological samples per animal group were pooled for protein extraction. Muscle lysates were prepared by homogenizing 25 mg of muscle in radio immuno precipitation assay buffer containing complete mini protease inhibitor cocktail (Cat No. 04 693 195 001; Roche Diagnostic Gmb Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail (Cat No. 04 906 837 001; Roche Diagnostic Gmb Mannheim). Protein concentration was estimated by Bio-Rad DC assay (Cat No. 500-0114-6, Bio-Rad Laboratories, Hercules, CA, USA). 35 mg of protein were resolved on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane by semi-dry electrophoretic transfer (Trans-Blot SD Bio-Rad Laboratories). Membranes were blocked with odyssey blocking buffer (Cat No. 927-40000 LI-COR-Biosciences, Nebraska, USA) and probed with 1:1000 anti phospho-SMAD2/3 and anti phospho-p65 rabbit antibodies at 4°C overnight. The blots were washed and scanned for analysis using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistics

Unless otherwise specified, one- and two-way ANOVA, followed by Newman–Keuls post hoc analysis, were done for all mentioned statistical analyses using GraphPad Prism 5 software. Data are presented as the mean ± standard deviation.

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Conflict of Interest statement. None declared.

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