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Role of dystrophin deficiency in fibrosis-related gene expression during myogenesis

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Abstract

Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by progressive muscle degeneration and fibrosis, primarily driven by excessive extracellular matrix (ECM) deposition. TGF-β cytokines and their associated latent TGF-β binding proteins (LTBPs) are key regulators of ECM composition and remodeling. While the roles of these molecules in skeletal muscle fibrosis are well established, the contributions of myoblasts to their production and the effects of dystrophin deficiency on their expression remain unclear. This study aimed to investigate the impact of dystrophin deficiency on myogenesis and the expression dynamics of fibrosis-related genes using wild-type (WT) and dystrophin-deficient (mdx) H2K myoblasts. Myogenesis was investigated through three key stages: proliferation, fusion, and terminal differentiation, by assessing both morphological changes and the expression levels of *Tgfb* and *Ltbp* genes at each stage. Dystrophin deficiency did not impair the morphological progression or timing of myogenesis, as mdx cells displayed comparable differentiation patterns to WT cells in terms of myotube formation, length, and alignment. However, dystrophin deficiency significantly altered the expression profiles of *Tgfb* and *Ltbp* genes. Notably, *Tgfb3* expression was consistently elevated in mdx cells across all stages, reaching a 4.26-fold increase during proliferation and 2.0-fold during terminal differentiation. In contrast, the expression of *Ltbp1*, *Ltbp3*, and *Ltbp4* was initially reduced in mdx cells but increased significantly in terminally differentiated myotubes. These findings reveal that dystrophin deficiency does not disrupt myogenesis morphologically or temporally but significantly impacts the expression of key fibrosis-related genes. This suggests that altered *Tgfb* and *Ltbp* expression dynamics may contribute to ECM accumulation and fibrosis in DMD, providing potential targets for therapeutic interventions.

Keywords: Duchenne muscular dystrophy, skeletal muscle fibrosis, TGF-β signaling, gene expression dynamics

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder caused by mutations in the *DMD* gene, which encodes dystrophin, a protein essential for muscle fiber integrity. These mutations prevent the production of functional dystrophin, leading to progressive muscle weakness. The incidence of DMD is approximately 1 in 5,000 live male births [1]. Symptoms typically manifest in early childhood, with delays in motor skills, frequent falls, and difficulty walking. As the disease progresses, affected children lose the ability to run and jump, and by the ages of 10–12, they typically become wheelchair-dependent. Mortality in individuals with DMD is primarily due to respiratory failure caused by weakening of respiratory muscles and heart failure resulting from progressive cardiomyopathy, with life expectancy rarely extending beyond the early 30s [2,3].

Dystrophin, located on the inner surface of the sarcolemma, forms a critical structural link between the actin cytoskeleton and the extracellular matrix (ECM), enabling muscle fibers to endure mechanical stress and contraction. In the absence of dystrophin, increased sarcolemmal permeability allows calcium influx into the sarcoplasm, leading to muscle fiber necrosis and degeneration. This necrosis triggers an inflammatory response that promotes fibrosis and adipose tissue accumulation within the muscle [4,5]. Transforming growth factor-beta (TGF-β) plays a central role in fibrosis by activating fibroblasts, stimulating ECM protein production, and supporting pro-fibrotic pathways. Additionally, fibrotic ECM accumulation in DMD patients disrupts satellite cell activation and differentiation, further impairing muscle repair and regeneration. Excessive fibrotic tissue also hinders the bioavailability of growth factors within the ECM, compromising

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the structural integrity and nourishment of muscle fibers and contributing to progressive muscle mass loss [6-9].

TGF-β is a multifunctional cytokine family involved in cellular processes such as growth, differentiation, and apoptosis, comprising three main isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β is synthesized as an inactive precursor molecule (latent TGF-β) that requires proteolytic processing for activation. Latent TGF-β binding proteins (LTBPs) regulate its bioavailability by directing it to the ECM, controlling its storage and activation [10-12]. TGF-β promotes fibrosis in muscle tissue by increasing the synthesis of ECM proteins, such as collagen and fibronectin [13,14]. It also induces the differentiation of fibroblasts into myofibroblasts, resulting in excessive ECM production and fibrosis progression [15,16]. Additionally, TGF-β inhibits the activity of ECM-degrading proteins such as matrix metalloproteinases (MMPs) while increasing the production of their inhibitors (TIMPs), thereby exacerbating ECM accumulation [17-19]. Although the contribution of TGF-β to the development of fibrotic processes in skeletal muscle is well-known, the role of myoblasts and muscle fibers in producing TGF-β and contributing to its ECM availability remains poorly understood. Therefore, this study investigates the expression of *Tgf-β* and *Ltbp* members in a conditionally immortal primary-like H2K myoblast cell line at different stages, including proliferation, fusion, and the maturated myotube stage. To further elucidate the impact of dystrophin deficiency on *Tgf-β* and *Ltbp* member expression, a dystrophin-deficient mutant H2K cell line was also utilized.

Material and Methods

Cell Culture Conditions

Immortalized mouse H2K myogenic cell lines were used for gene expression analyses: the H2K-mdx23 clone 1A5, which harbors a nonsense point mutation in exon 23 of the *Dmd* gene resulting in a premature stop codon in the *Dmd* mRNA, and the H2K wild-type clone, which lacks any mutations in the *Dmd* gene [20,21]. Since the experiments were conducted using cell lines, ethical committee approval is not required for this study. Before seeding, the plates were coated with Matrigel (100 µg/mL; Corning) to promote cell attachment. To ensure proper adherence of Matrigel to the plate surfaces, the plates were incubated at room temperature for 1 hour. Myoblast proliferation was carried out in growth medium (GM) comprising high-glucose DMEM (Dulbecco's Modified Eagle Medium; Invitrogen, Gibco), supplemented with 20% FBS (Fetal Bovine Serum; Biochrom), 0.5% chick embryo extract (CEE; US Biological), and 20 U/mL interferon (IFN)-γ (Roche). Cells were maintained in a humidified incubator at 33°C with 10% CO_2. For differentiation, once cells reached 80%–90% confluency, GM was replaced with differentiation medium (DM) containing high-glucose DMEM (Gibco) and 5% horse serum (HS; Biochrom). The cells were incubated at 37°C with 5% CO_2 for 4 days to allow differentiation. Phase-contrast brightfield microscopy was used to visualize the cells, and images were captured at 100× magnification.

RNA Extraction and Gene Expression Analysis

RNA extraction was performed from cells at three distinct stages: during proliferation, at the early stages of fusion, and from terminally differentiated cells. For the proliferation stage, myoblasts were harvested when they reached 50–60% confluency in GM. To obtain samples from the early fusion stage, cells were collected 24 hours after the induction of differentiation with DM. At this stage, myoblasts were closely aligned, primitive myotubes had begun to form, and myotube maturation had not yet occurred. For mature, terminally differentiated multinucleated myotubes, RNA was isolated from cells on the fourth day of differentiation.

Total RNA was extracted using the TRIzol reagent (Invitrogen) following the manufacturer's guidelines. The quality and integrity of the RNA samples were assessed through denaturing agarose gel electrophoresis and UV absorbance measurements. RNA concentrations were determined using a NanoDrop 3000 spectrophotometer prior to cDNA synthesis. For cDNA synthesis, 500 ng of total RNA was used in a 20-µL reaction mixture containing oligo (dT) primers and SuperScript III Reverse Transcriptase (Invitrogen), prepared according to the manufacturer's instructions. The synthesized cDNA was subsequently used for quantitative real-time PCR (qPCR) analysis. qPCR was conducted using the SYBR Green method, with equal amounts of cDNA from each sample. Reactions were performed in technical triplicates to reduce PCR variability. Each 25-µL reaction included JumpStart SYBR Green Mix (Sigma-Aldrich) and 1.5 µL of cDNA, with an annealing temperature set at 60°C. The relative expression levels of target genes were calculated using the ΔΔCt method and normalized to *Gapdh* as the reference gene. Primers were specifically designed to amplify coding sequences while excluding intronic regions. Primer sequences are available upon request.

Results

The differentiation of myoblasts into mature muscle fibers observed during skeletal muscle regeneration was mimicked in vitro using conditionally immortal primary-like H2K myoblast cell lines. Wild-type (WT) and dystrophin-deficient (mdx) H2K cells were morphologically compared at three different stages: the proliferation stage (50–60% confluence), the fusion stage (the first day of differentiation), and the maturated myotube stage (the fourth day of differentiation). It was observed that dystrophin deficiency did not hinder the myogenesis performance of mdx cells, and they exhibited a differentiation pattern similar to that of WT myoblasts. Moreover, mdx myoblasts began fusion at the same time as WT myoblasts and did not exhibit any delay in maturated myotube formation. Additionally, mdx myotubes displayed similar maturation characteristics to WT myotubes in terms of length, diameter, and alignment with one another (Figure 1).

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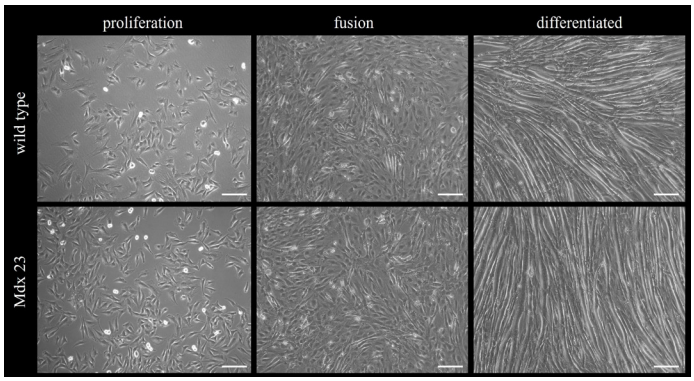

Figure 1. Morphological appearance of dystrophin-deficient and dystrophin-expressing H2K myoblast cell lines during the differentiation process in culture; Representative images illustrate the morphological changes of the cells from the proliferation stage to the terminally differentiated stage; The scale bars are 100 µm

To assess whether the expression of *Tgfb1, Tgfb2, Tgfb3*, and their associated ECM regulators *Ltbp1, Ltbp3,* and *Ltbp4* is influenced by dystrophin deficiency, mRNA levels were analyzed comparatively in WT and mdx H2K cells during proliferation, fusion, and maturated myotube stages. During proliferation, mdx cells exhibited significantly higher expression levels of *Tgfb2* and *Tgfb3* (1.6-fold and 4.26-fold, respectively) compared to WT, while the expression of *Tgfb1, Ltbp1, Ltbp3,* and *Ltbp4* was lower by 52%, 36%, 39%, and 27%, respectively (Figure 2A). At the fusion stage, only *Tgfb3* expression was elevated (1.58-fold) in mdx cells, while *Tgfb1, Tgfb2, Ltbp1,* and *Ltbp4* were reduced by 36%, 20%, 17%, and 32%, respectively. Notably, Ltbp3 expression remained unchanged between WT and mdx cells at this stage (Figure 2B). In maturated mdx myotubes, all genes showed higher expression levels compared to WT. The greatest increase was observed in *Tgfb3* (2.0-fold), followed by *Tgfb1* (1.47-fold), *Tgfb2* (1.39-fold), *Ltbp1* (1.19-fold), *Ltbp3* (1.68-fold), and *Ltbp4* (1.78-fold) (Figure 2C).

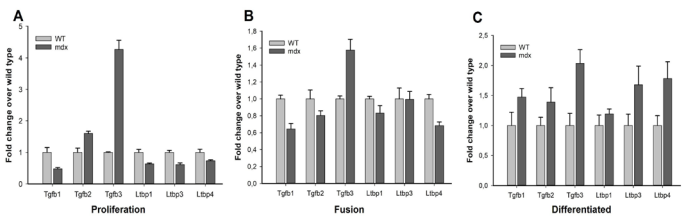

Figure 2. Expression profiles of Tgfb and Ltbp genes in H2K cell lines at the proliferation, fusion, and terminally differentiated stages; Each gene expression in mdx cells is normalized to the corresponding gene expression in WT cells; The data represent the mean±SD

Additionally, the effects of dystrophin deficiency on the progression of gene expression from the initial proliferation stage to the terminally differentiated maturated myotube stage were analyzed. In mdx cells, *Tgfb1* expression increased during the fusion stage and remained stable in the myotube stage, whereas in WT cells, *Tgfb1* expression was unchanged during fusion but decreased significantly at the terminal differentiation stage (Figure 3A). *Tgfb2* expression in mdx cells markedly increased at the fusion stage but decreased significantly in maturated myotubes; in contrast, WT cells showed a consistent decrease in *Tgfb2* expression throughout differentiation (Figure 3B). *Tgfb3* expression in mdx myoblasts increased during the fusion stage but returned to proliferation levels in the myotube stage, while in WT cells, *Tgfb3* expression steadily decreased throughout differentiation (Figure 3C). Both *Ltbp1* and *Ltbp4* expression decreased throughout differentiation in both cell types, with the reduction in *Ltbp1* expression being more pronounced in WT cells (Figure 3D and 3F). In mdx cells, *Ltbp3* expression significantly increased during the fusion stage but partially reverted to proliferation levels in the myotube stage, whereas WT cells exhibited a gradual decrease in *Ltbp3* expression during differentiation (Figure 3E).

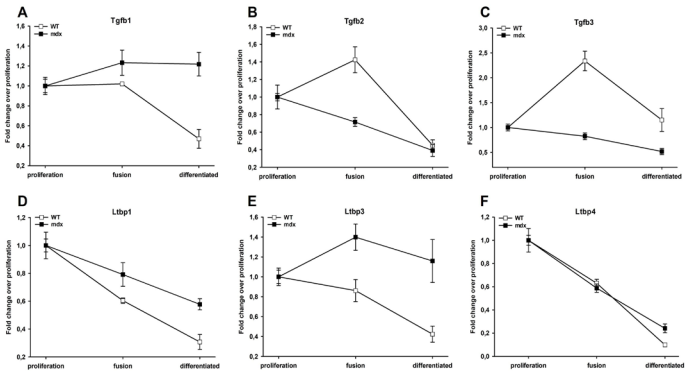

Figure 3. Expression profiles of Tgfb and Ltbp genes during the progression from the proliferation stage to the terminal differentiation stage in H2K cell lines; Gene expression levels at the fusion and terminal differentiation stages were normalized to their respective levels at the proliferation stage; The data represent the mean±SD

Discussion

In patients with DMD, the development of fibrosis due to excessive accumulation of ECM components within skeletal muscle tissue severely limits the regenerative capacity of the muscle and ultimately leads to muscle tissue loss over time [22-24]. Cytokines of the TGF-β family are the primary regulators of ECM composition, remodeling, and maintenance, as they control the timing and frequency of ECM component production and degradation [25,26]. In DMD patients, the continuous inflammatory response triggered by lifelong muscle fiber necrosis abnormally affects TGF-β signaling, resulting in excessive ECM deposition between muscle fibers [6,27]. This persistent disruption of the ECM environment not only impairs satellite cell function but also contributes to the progressive stiffening of muscle tissue [9]. Another crucial group of molecules responsible for the increased fibrotic content are the LTBP family members, which regulate the availability and activation of TGF-β cytokines in the ECM. Each member of the LTBP family interacts differently with TGF-β isoforms, leading to diversity in the regulation of TGF-β signaling [28]. Recent studies have also highlighted the role of LTBP polymorphisms in modulating the severity of fibrosis and functional decline in DMD patients [12]. Notably, LTBP4 plays a critical role in maintaining TGF-β in its inactive form within muscle tissue [29]. Understanding the dynamic interplay

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between LTBPs and TGF-β isoforms in dystrophic muscle may offer novel therapeutic targets for limiting fibrosis.

Several therapeutic approaches targeting TGF-β signaling have been proposed to mitigate skeletal muscle fibrosis. In an mdx mouse model, suramin, a TGF-β1 receptor antagonist, was shown to reduce muscle fibrosis [30]. Similarly, decorin, a TGF-β inhibitor, decreased collagen I mRNA expression in diaphragm muscle and inhibited the fibrotic differentiation of myogenic cells in mdx mice [31]. Additionally, blocking the angiotensin II type 1 receptor in mdx mice improved muscle regeneration affected by TGF-β and reduced fibrotic tissue, as shown with drugs like losartan [32,33]. Another study demonstrated that halofuginone, an inhibitor of TGF-β signaling, prevented fibrosis by blocking TGF-β-induced SMAD3 activation [34]. Furthermore, LTBP4 antibodies were shown to reduce muscle damage and fibrosis while improving muscle function by inhibiting TGF-β release and activation [35]. In a study using cultured myotubes derived from DMD patients, SETDB1 silencing was found to suppress the expression of TGF-β target genes and mitigate the fibrotic response of myoblasts [36].

While the role of TGF-β cytokines and LTBP proteins in the development of skeletal muscle fibrosis is well-established, there is limited knowledge regarding the contribution of myoblasts and muscle fibers to the production of these regulators, as well as the potential effects of dystrophin deficiency on their gene expression [27,29]. Therefore, in this study, the myogenesis process of DMD skeletal muscle was mimicked using dystrophin-deficient H2K myoblasts, with a focus on examining morphological changes and gene expression profiles of the related genes during the proliferation, fusion, and terminal differentiation stages. The findings demonstrate that dystrophin deficiency does not negatively impact the morphological changes occurring during the myogenesis process. Additionally, it does not cause any temporal delay in the completion of myogenesis. Although the fact that DMD patients are born with fully developed muscles, similar to healthy individuals, serves as evidence that dystrophin deficiency does not impair myogenesis, this study is valuable in demonstrating that the completion time of myogenesis is also unaffected by dystrophin deficiency [1,3,4]. Nevertheless, the findings also indicate that, while dystrophin deficiency does not impair the morphological or temporal aspects of myogenesis, it significantly alters the expression of *Tgfb* and *Ltbp* genes. These results suggest that dystrophin deficiency impacts the expression dynamics of genes associated with fibrosis during the three key stages of myogenesis: proliferation, fusion, and terminal differentiation. This highlights the potential contribution of altered *Tgfb* and *Ltbp* gene expression to the fibrotic processes observed in DMD skeletal muscle [7,8].

Conclusion

This study provides critical insights into the effects of dystrophin deficiency on skeletal muscle myogenesis and fibrosis-related gene expression. Using a dystrophin-deficient H2K myoblast model, we demonstrated that dystrophin deficiency does not impair morphological progression or the timely completion of myogenesis during the proliferation, fusion, and terminal differentiation stages. However, significant alterations in the expression of *Tgfb* and *Ltbp* genes were observed, suggesting that dystrophin deficiency influences the regulatory pathways linked to ECM remodeling and fibrosis. These findings underscore the potential role of myoblasts and myotubes in contributing to fibrosis through altered gene expression, even in the absence of direct effects on myogenesis. Understanding these gene expression dynamics could aid in identifying novel therapeutic targets to mitigate fibrosis in DMD patients. Ultimately, this study bridges a critical gap in our knowledge by linking dystrophin deficiency to molecular mechanisms that may drive fibrosis during muscle regeneration.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Not applicable, as the study did not involve patients, volunteers or animals.

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