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Research Report

# Integrin dysregulation as a possible driver of matrix remodeling in Laminin-deficient congenital muscular dystrophy (MDC1A)

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**Abstract**.
**Background**: Merosin-deficient congenital muscular dystrophy (MDC1A) is caused by a loss of Laminin-α2. Secondary manifestations include failed regeneration, inflammation, and fibrosis; however, specific pathomechanisms remain unknown.
**Objectives**: Using the LAMA2$^{DyW}$ (DyW) mouse model of MDC1A, we sought to determine if Integrin-αV and -α5, known drivers of pathology in other diseases, are dysregulated in dystrophic muscle. Additionally, we investigated whether Losartan, a drug previously shown to be antifibrotic in dystrophic scenarios, rescues integrin overexpression in DyW mice.
**Methods**: qRT-PCR, ELISA, and immunohistochemistry were utilized to characterize integrin and matricellular protein dysregulation in hind limb muscles from WT and untreated/ Losartan-treated DyW mice.
**Results**: Integrin-αV and –α5 are significantly upregulated on both gene and protein level in DyW muscle- Losartan treatment attenuates this dysregulation. Immunohistochemistry showed that Integrin-αV is expressed on both infiltrating cells as well as on muscle cells- Losartan attenuates expression in both compartments. In addition, transcriptional overexpression of common matricellular and beta binding partners is rescued close to WT levels with Losartan. Lastly, latent and active TGF-β are upregulated in the serum of DyW mice, but only active TGF-β levels are attenuated by Losartan treatment.
**Conclusions**: Our results suggest that overexpression of Integrin-αV and –α5 are likely contributing to secondary pathologies in MDC1A. We also believe that downregulation of Integrin-αV could be partially responsible for Losartan's antifibrotic effect and therefore could serve as a novel therapeutic target in MDC1A and other degenerative fibrotic diseases.

Key words: MDC1A, Congenital muscular dystrophy, ECM, Integrin, TGF-β

## INTRODUCTION

Congenital Muscular Dystrophy type 1A (MDC1A) is a devastating neuromuscular disease caused by defects in the alpha 2 chain of the muscle-specific extracellular matrix (ECM) protein Laminin-211. Absence of a functional copy of this protein results in structural instability as well as severe signaling dysregulation across the sarcolemma. MDC1A patients present with muscle weakness and hypotonia either at or soon after birth, rarely achieve independent ambulation, and often die prematurely from respiratory complications or failure to thrive in their teen years [1–4]. While great progress has been made in the understanding of this disease, many of the pathomechanisms driving this disease still remain unknown.

Using the DyW mouse, we have shown that Laminin-deficient muscles have massive dysregulation of the matricellular genes Osteopontin, Fibronectin, and Periostin. We have also demonstrated increases in TGF-β and NF-κB signaling via Smad2/3 and p65 activation, respectively, beginning from a very young age [5]. Past research has shown that the production and activation of each of these extracellular and signaling proteins converges on Integrin -αV and -α5 [6–8]. Integrins comprise a family of alpha- and

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beta-containing heterodimeric membrane spanning proteins where 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 matricellular proteins and participate in a wide array of cellular functions including migration, signal transduction and cell stability [9, 10]. In particular, dysregulation of these two integrins, as well as their beta dimer partners -β1, -β3, -β5, -β6, and -β8 have been shown to contribute to the progression of many diseases including cancer, heart disease, and the fibrosis of various organs [8, 11–14]. In this study we report that both Integrin -αV and -α5, along with previously mentioned beta dimer partners, are overexpressed in Laminin-deficient muscle. Thus, it is possible that they are playing a critical role in driving the secondary pathologies associated with MDC1A. While we found that these integrins are dysregulated in Laminin-deficiency, this paper mainly focuses on Integrin-αV and its proposed role in TGF-β mediated fibrosis.

Angiotensin II Type 1 Receptor Blockers, like Losartan, have been shown to cause a robust recovery in fibrotic phenotype via attenuation of dysregulated TGF-β signaling although the specific mechanism by which this occurs remains to be elucidated [15, 16]. Interestingly, Integrin-αV-containing dimers have been shown to play a critical role in the activation of TGF-β via its release from the Large Latent Complex (LLC). Specifically, the Latency Associated Peptide (LAP) of the LLC contains an Arginine-Glycine-Aspartate (RGD) domain that binds to the RGD binding motif on Integrin-αV causing a conformational change that leads to activation of TGF-β by either mechanical or proteolytic release from the LLC. This mechanism plays such an important role in TGF-β activation that Integrin-αV KO mice have a phenotype similar to TGF-β KO models [17, 18]. In this study, we present data that suggests the decrease in TGF-β-mediated fibrosis in dystrophic skeletal muscle in response to Losartan could be potentially linked to downregulation of Integrin-αV.

To our knowledge, the role of Integrin-αV has never been explored in the context of degenerative muscle disease even though it can have a major impact on extracellular remodeling, fibrosis, and inflammation. Because all of these pathologies are major hallmarks of dystrophies including Laminin-deficiency, the findings presented in this paper open up new avenues for investigation into Integrin-αV as a novel therapeutic target in MDC1A and other fibrotic, degenerative muscle diseases.

## RESULTS

### *Losartan leads to decreased gene and protein levels of Integrin-αV and -α5 in Laminin-deficiency*

Using qRT-PCR, we show that Laminin-deficient mice have an increase in the transcription of Integrin-αV and -α5 at 7 weeks of age ($p < 0.05$, $n = 6$ one-way ANOVA) (Fig. 1A, B). Treatment with Losartan rescued Integrin-αV and -α5 gene expression almost completely back to WT levels. These results were also substantiated on the protein level using ELISA (specificity of the antibody used and its sensitivity to quantitative change was also confirmed by western blot, see supplemental figure 1). DyW mice had significantly higher levels of Integrin-αV and -α5 at 7 weeks of age ($p < 0.05$, $n = 6$ one-way ANOVA) compared to wild type, which was reverted almost completely back to WT levels in the case of Integrin-αV and close to, but still significantly greater than, WT levels for -α5 following treatment with Losartan (Fig. 1C, D).

### *Immunohistochemistry reveals that Integrin-αV is expressed by infiltrating cells as well as muscle cells*

Immunostaining with CD-51 (Integrin-αV) antibodies shows a large increase in Integrin-αV expression in 7-week DyW mice as compared to age-matched WT mice (Fig. 2). While much of the Integrin-αV expression in DyW muscle can be seen in the interstitial space, costaining with Desmin reveals that the muscle fibers themselves are also expressing Integrin-αV by seven weeks. This was verified by isolating single fibers from both WT and DyW hind limb muscle. Figure 3 shows distinct Integrin-αV staining at the sarcolemma of DyW myofibers (Fig. 3A) that is completely absent on WT fibers (Fig. 3B). Upon Losartan treatment, there is a marked decrease in Integrin-αV staining on muscle fibers as well as in the interstitial space (Fig. 2). Treatment with Losartan has been previously shown to reduce the number of macrophages in triceps and diaphragm of DyW mice [15]. Using CD11b gene expression (Fig. 4B) as well as Mac-1 staining (Fig. 4A) we see a similar decrease in DyW hind limb muscle. While interstitial Integrin-αV expression decreases in the treated muscles, this decrease is a function of a reduced presence of infiltrating macrophages (Fig. 4A).

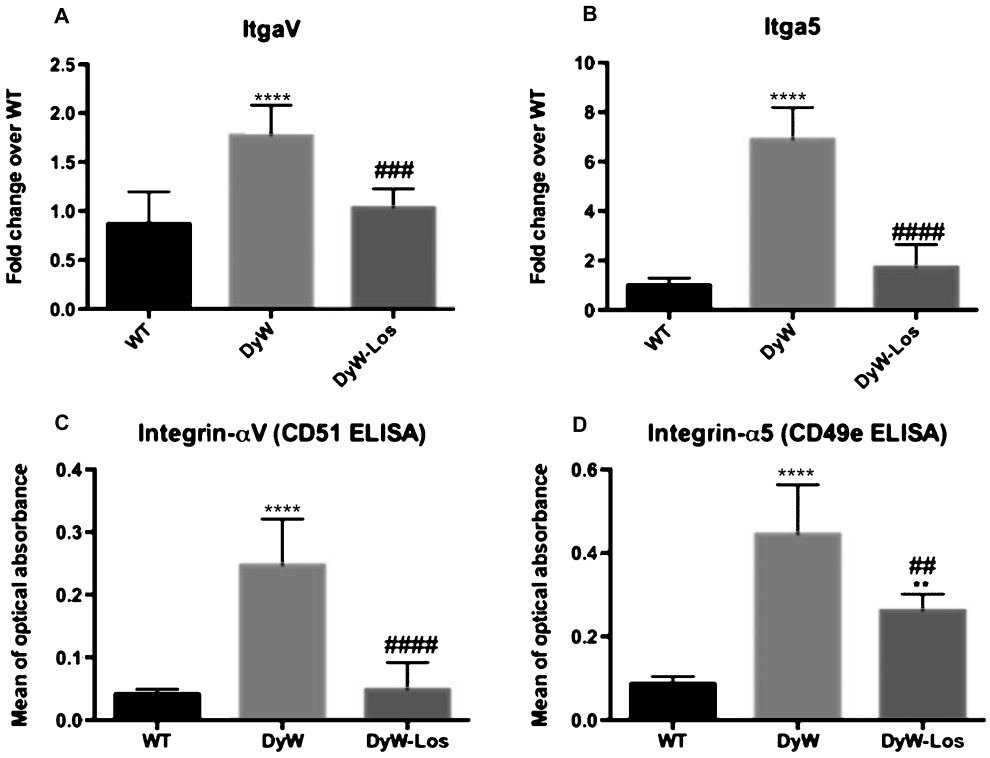


Fig. 1. **Gene and protein expression of Integrin-αV and -α5 are upregulated in DyW mice and are rescued by Losartan.** (A, B) Gene expression measured by qRT-PCR of Integrin-αV and -α5 show upregulation in 7 week DyW mice compared to wild type ($p < 0.05$, $n = 6$, one-way ANOVA) that is rescued by Losartan. (C, D) Protein expression quantified by ELISA show significantly greater expression of Integrin-αV and -α5 in DyW mice ($p < 0.05$, $n = 6$, one-way ANOVA) that is rescued by Losartan ($p < 0.05$, $n = 6$, one-way ANOVA) but is still greater than the wild type in the case of Integrin-α5 ($p < 0.05$, $n = 6$, one-way ANOVA). (* is used to denote significance between WT and DyW; # is used to denote significance between DyW and DyW Losartan-treated; *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$, this also applies to the other symbols).

*Overexpression of beta dimer partners of Integrin-αV and -α5 is rescued following Losartan treatment*

Gene expression of several beta integrins that preferentially partner with Integrin-αV and -α5 was also measured using qRT-PCR. We found that at 7 weeks of age, DyW mice have significantly higher expression of Integrin-β1, -β3, -β5, -β6, and -β8 compared to age-matched WT littermates (Fig. 5A–E) ($p < 0.05$, $n = 6$, one-way ANOVA). Similar to -αV and -α5, treatment with Losartan returned expression close to WT levels ($p < 0.05$, $n = 6$, one-way ANOVA).

*Treatment with Losartan leads to decreased expression of Integrin-αV matricellular binding partners*

We have previously reported dysregulation of matricelluar proteins that are known to bind and activate Integrin-αV [5]. At seven weeks post-natal, DyW animals show increased transcription of Fibronectin, Osteopontin, and Periostin ($p < 0.001$, $n = 6$, one-way ANOVA) (Fig. 6A–C). Treatment with Losartan led to a significant decrease in all three genes close to WT levels. These results confirm past publications that have shown Losartan treatment leads to a decrease in Periostin on the protein level, as well as an overall improved fibrotic phenotype [15, 16].

*Serum levels of TIMP1 are also rescued in response to Losartan treatment*

TIMP1 is known to promote ECM stability in fibrotic diseases [19, 20]. Since Integrin-αV has been shown to modulate its expression [21], we looked at serum levels of TIMP1 which we have previously shown to be upregulated in DyW muscle [5]. Protein levels quantified by ELISA show that TIMP1 is overexpressed in the serum of 7 week DyW mice compared to age-matched WT littermates (Fig. 7) ($p < 0.05$, $n = 5$, one-way ANOVA). In response to Losartan treatment,

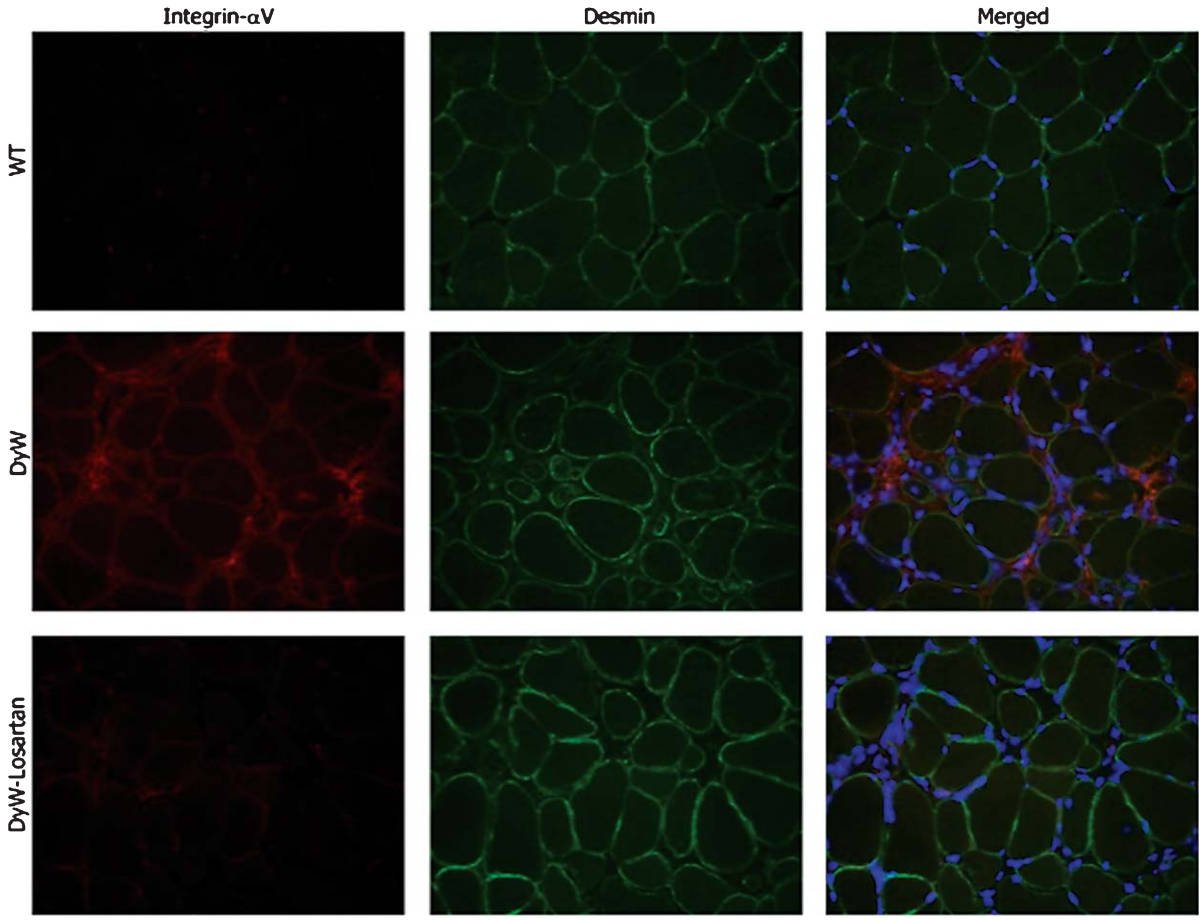


Fig. 2. **Immunohistochemistry shows overexpression of Integrin-αV in DyW mice that is rescued by Losartan**. Immunostaining with CD51 and Desmin antibodies show overexpression of Integrin-αV in 7 week DyW mice compared to WT which can be seen located on both infiltrating cells in the interstitium as well as on muscle fibers themselves. In response to Losartan treatment, DyW expression returns close to WT levels in both compartments.

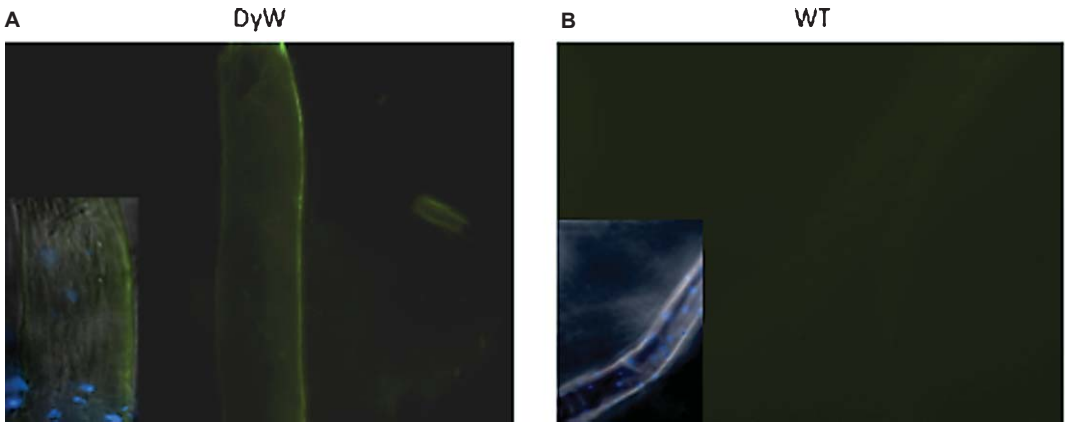


Fig. 3. **Single fiber immunohistochemistry shows expression of Integrin-αV on the sarcolemma of DyW myofibers.** (A, B) Staining of single DyW and WT myofibers with anti-CD51 antibodies shows expression of Integrin-αV on the sarcolemma of DyW mice that is not seen on WT fibers thus demonstrating a novel shift in adult muscle phenotype. Insets at bottom left are of the merged channels (FITC, DAPI and bright field). Note: DyW inset is at 40x magnification while WT inset is 20x.

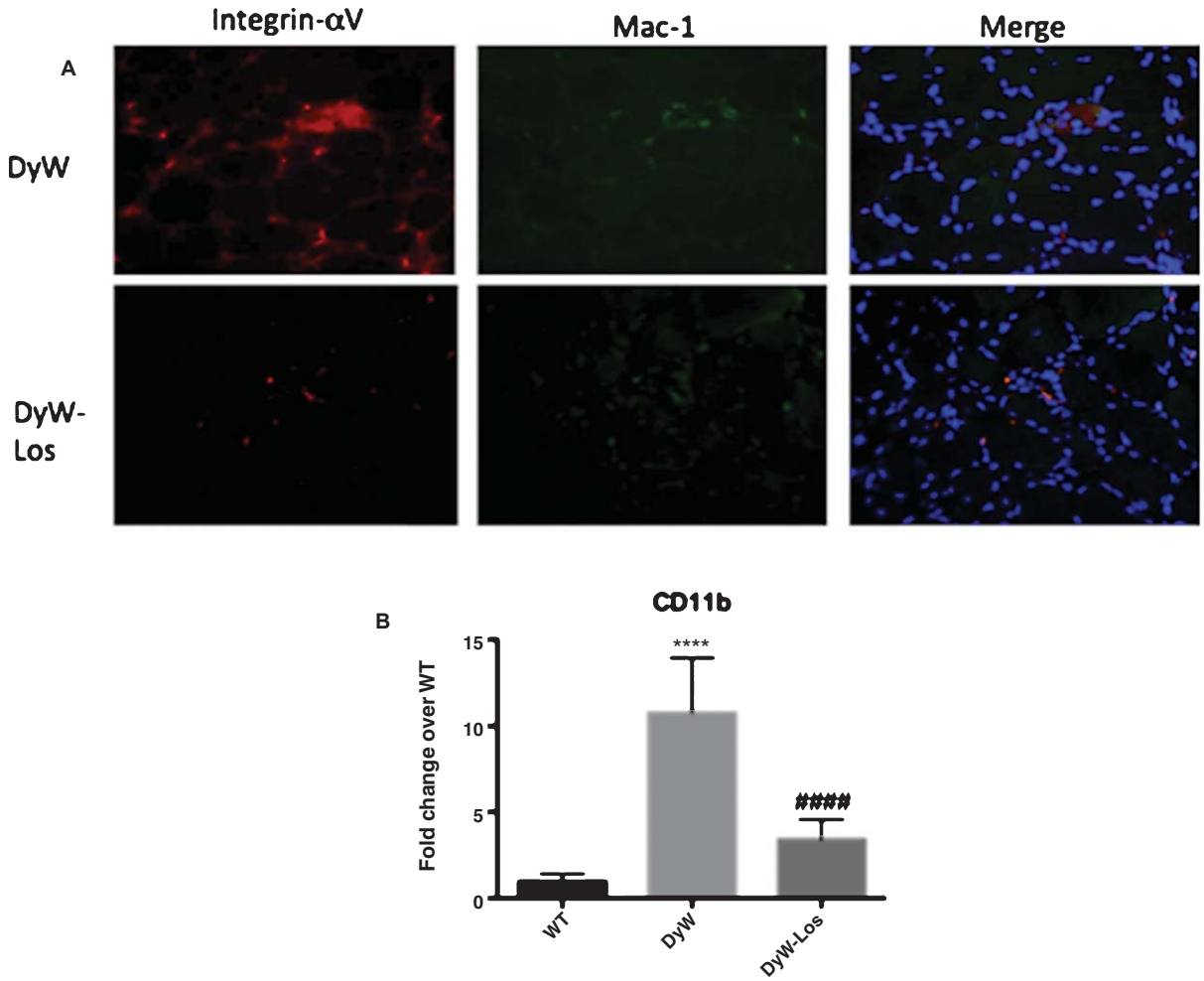


Fig. 4. **Immunohistochemistry shows overexpression of Integrin-αV in interstitial space is due to infiltrating macrophages which are reduced in response to Losartan.** (A) Costaining of CD51 and CD11b show that much of the interstitial expression of Integrin-αV is due to the presence of infiltrating macrophages. Following treatment with Losartan, interstitial expression of Integrin-αV is decreased as a result of a reduced number of infiltrating macrophages. (B) Gene expression of CD11b quantified by qRT-PCR shows significant upregulation in DyW mice that is decreased close to WT levels in response to Losartan treatment.

TIMP1 expression returned to WT levels ($p < 0.05$, $n = 6$, one-way ANOVA).

*Overexpression of active, but not latent TGF-β, is attenuated by treatment with Losartan*

Integrin-αV is known to activate TGF-β by releasing it from the latent complex [18, 22]. To test whether the decrease in TGF-β signaling and fibrosis in Losartan treated muscle is possibly a function of a lower availability of active TGF-β, we compared levels of both active and latent TGF-β in serum samples of treated and untreated DyW mice as well as WT. Using ELISA, we found that at 7 weeks of age, DyW mice have significantly increased levels of both active and latent TGF-β compared to their age-matched WT littermates ($p < 0.05$, $n = 6$, one-way ANOVA) (Fig. 8A, B). While latent TGF-β remained unchanged in response to Losartan, active TGF-β was significantly reduced back to WT levels ($p < 0.05$, $n = 6$, one-way ANOVA).

## DISCUSSION

As previously mentioned, Laminin-deficient muscles have massive dysregulation of matricellular proteins, as well as increases in TGF-β and NF-κB signaling beginning from a very young age [5]. This paper highlights that not only are these proteins dysregulated, but so too are their receptors, specifically Integrin-αV and -α5. Upon activation by binding to the

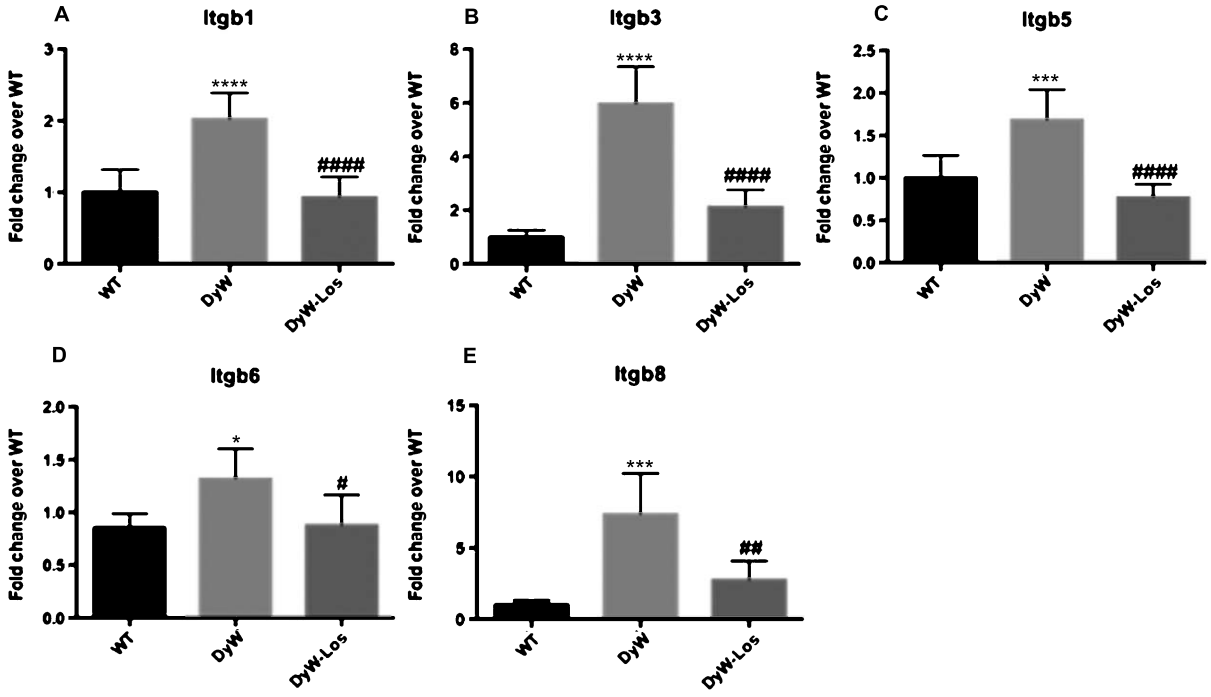


Fig. 5. **Upregulation of common beta binding partners of Integrin-αV and -α5 is rescued following treatment with Losartan.** (A-E) Gene expression measured by qRT-PCR shows upregulation of beta dimer partners Integrin-β1, -β3, -β5, -β5, and -β8 ($p < 0.05$, $n = 6$, one-way ANOVA). Following treatment with Losartan, expression of these integrins returns close to WT levels ($p < 0.05$, $n = 6$, one-way ANOVA). (* is used to denote significance between WT and DyW; # is used to denote significance between DyW and DyW Losartan-treated; *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$, this also applies to the other symbols).

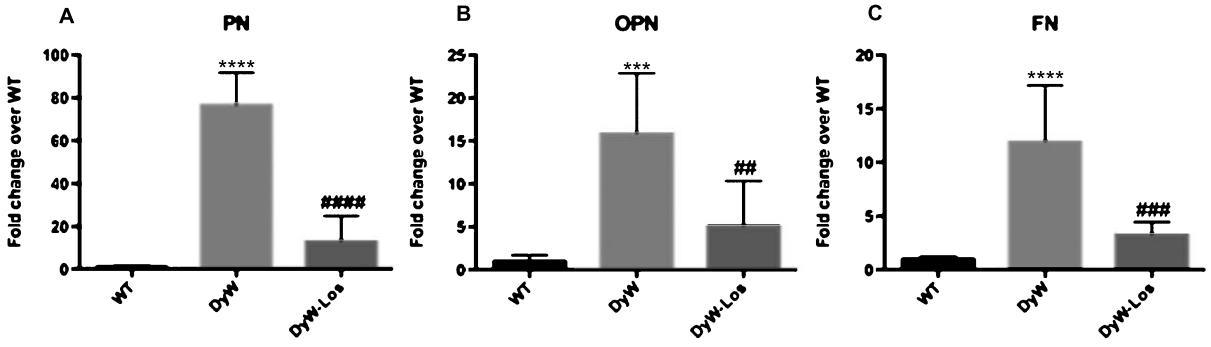


Fig. 6. **The gene expression of common Integrin-αV and -α5 matricellular binding partners is elevated in DyW mice and rescued in response to Losartan treatment.** (A-C) Using qRT-PCR we found that the gene expression of Osteopontin, Periostin, and Fibronectin (common binding partners of Integrins-αV and -α5 in the ECM) are elevated in the muscles of 7 week old DyW mice ($p < 0.05$, $n = 6$, one-way ANOVA), and that this expression is almost completely rescued in response to Losartan treatment. (* is used to denote significance between WT and DyW; # is used to denote significance between DyW and DyW Losartan-treated; *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$, this also applies to the other symbols).

alpha subunit, these integrins facilitate either immediate changes to the ECM or intracellular signaling through associated kinases and adaptor proteins via the beta subunit. Integrin-αV activation occurs primarily upon binding matricellular proteins, such as Osteopontin and Periostin, and has been shown to cause the downstream activation of NF-κB in macrophages, as well as serving as a critical component of the mechanical and proteolytic release of TGF-β from its latent complex in the ECM [23–27]. Integrin-α5, a common binding partner for Fibronectin, has also been shown to induce NF-κB activation, as well as promote macrophage migration and adhesion [28, 29]. As DyW muscles are known to have large numbers

of infiltrating cell types that are known to express -αV and -α5, we were not surprised to find that expression of both of these integrins are elevated in DyW mice on the gene and protein levels. What was interesting, however, is that Integrin-αV appears to be expressed not only on infiltrating cells in the interstitium (mainly macrophages) but also on the surface of adult muscle fibers in DyW muscle. Because adult muscle fibers do not normally express Integrin-αV ([30] and our own data presented here), these results demonstrate a novel shift in adult muscle cell phenotype resulting from a loss of laminin. While further experimentation needs to be done in order to determine if integrin upregulation is a direct consequence of a Laminin-deficiency, it is clear that the overexpression of Integrins-αV and -α5, coupled with the overexpression of matricellular proteins, could be acting as a double-edged sword toward exacerbating inflammatory and fibrotic pathology.

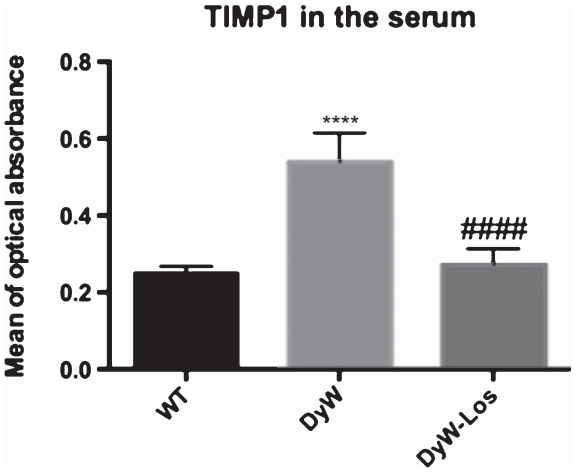


Fig. 7. **Serum levels of TIMP1 are rescued in response to Losartan treatment.** Protein levels quantified by ELISA assays show that TIMP1 in the serum of 7 week DyW mice is significantly upregulated compared to age-matched WT litter mates ($p < 0.05$, $n = 5$, one-way ANOVA). In response to Losartan treatment, TIMP1 levels return close to WT levels. (* is used to denote significance between WT and DyW; # is used to denote significance between DyW and DyW Losartan-treated; *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$, this also applies to the other symbols).

We also found that many beta dimer partners known to act as pathological drivers with Integrin-αV and -α5 are transcriptionally overexpressed in DyW mice. Specifically, we determined that Integrin-β1, -β3, -β5, -β6, and -β8 are all upregulated in 7 week old DyW mice. It has been shown that Integrin-αVβ1,-αVβ3, -αVβ5, -αVβ6, and -αVβ8 dimers can all bind the LAP and release TGF-β from the LLC [27, 31–33]. In addition, Integrin-β6 expression has been shown to be upregulated in kidney, liver, and lung fibrosis, and that mice lacking this integrin are protected from bleomycin-induced lung fibrosis [13, 27, 34]. Further, Integrin-β1 has been shown to be a driver of hepatic fibrosis through stimulation of ECM protein synthesis and protection from degradation [26].

Angiotensin II Type I Receptor (AT1) antagonists, such as Losartan and its derivatives, have been proven to be potent antifibrotic agents. Specifically in Laminin-deficiency, Elbaz *et. al* have shown that Losartan treatment leads to decreased smad phosphorylation, MAPK activation, and improved muscle function in $Dy^{2J}$ mice. Further, Meinen *et. al* showed similar functional results in the DyW model using the

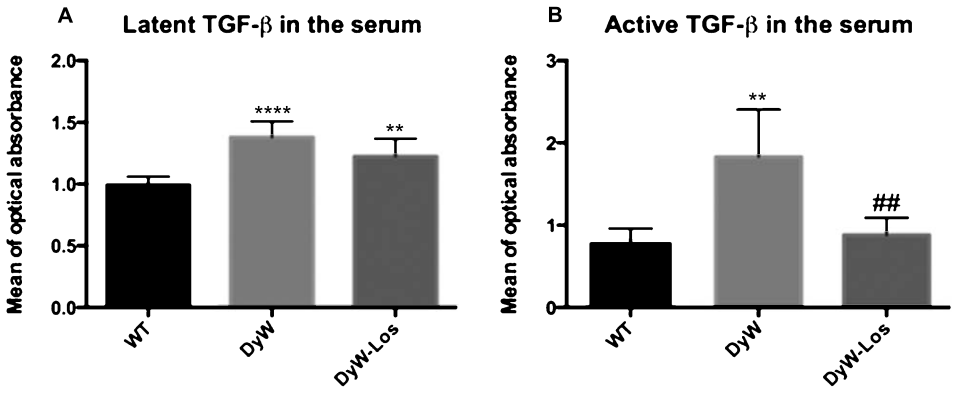


Fig. 8. **Active, but not latent TGF-β levels are attenuated by treatment with Losartan.** ELISA analysis of TGF-β levels in the serum of DyW animals shows elevated levels of both latent ($P < 0.001$, $n = 6$, one-way ANOVA) and active TGF-β ($p < 0.05$, $n = 6$, one-way ANOVA). We also found that while treatment with Losartan led to no reduction in levels of latent TGF-β, activated levels of the cytokine were reduced back to WT expression levels. (* is used to denote significance between WT and DyW; # is used to denote significance between DyW and DyW Losartan-treated; *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$, this also applies to the other symbols).

Losartan derivative L-158809, as well as demonstrated that Angiotensin II Receptor Blockers (ARBs) are capable of preventing fibrosis and inflammatory cell infiltration. While it is known that Losartan is able to inhibit fibrosis by attenuation of TGF-β signaling, the specific mechanism by which AT1 inhibition affects fibrotic and inflammatory signaling remains largely unknown. Here, we show that treatment with Losartan significantly rescues the gene and protein expression of -α5 and -αV integrins in DyW mice. Based on these results, and the known role of Integrin-αV in the activation of TGF-β, we propose that integrin signaling, specifically by Integrin-αV, could be a nodal point for TGF-β-mediated fibrosis, and its downregulation serves to drive the reduction in fibrosis caused by Losartan.

The correlation between the Renin-Angiotensin system and integrins was demonstrated in rat cardiac neonatal fibroblasts [35]. Kawano *et. al* showed that Angiotensin II promotes increased expression of Integrin-αV, -β1, -β3, -β5 and that treatment with an Angiotensin II Type 1 Receptor blocker, like Losartan, completely blocked this increased expression. Indeed, we found that Losartan was able to rescue the dysregulation of Integrin-αV, -α5, -β1, and -β3 towards wild type levels. Further, it has also been noted in cardiac neonatal fibroblasts that AngII caused increased expression of matricellular proteins Osteopontin and Fibronectin, which was blocked by treatment with Losartan. We found similar results in DyW skeletal muscle, which showed a rescue in the gene expression of these matricellular proteins in response to AT1 inhibition. Further, we show here TIMP1 that is significantly upregulated in the serum of DyW mice at 7 weeks of age is reduced to WT levels in response to Losartan treatment. Interestingly, it has been shown that Integrin-αV antagonists can cause significant downregulation of TIMP1 [21].

Finally, in response to Losartan, we see that activated TGF-β is significantly reduced while latent TGF-β remains unchanged. This finding is of significance because as previously mentioned, Integrin-αV containing dimers facilitate the release of TGF-β from the LLC [32]. The finding that only activated TGF-β is reduced in response to Losartan while latent TGF-β remains unchanged further supports the notion that Integrin-αV could be a key player in mediating fibrotic pathology in MDC1A via TGF-β activation.

Overall, our results suggest that the overexpression of Integrin-αV and -α5 are likely contributing to fibrotic pathology in MDC1A. Although correlative, our results establish a possible link between Integrin-αV and TGF-β activation in dystrophic skeletal muscle. On a different note, given the chronic nature of this disease in very young kids, it is important to identify noninvasive biomarkers. If, in fact, these results are paralleled in humans, we believe that tracking levels of activated TGF-β as well as TIMP1 in the serum of patients could serve as viable biomarkers for longitudinal treatment/survival studies. While future studies are needed to further elucidate the underlying cause of integrin overexpression and its role in driving fibrotic and inflammatory pathologies, we nevertheless believe these results point to Integrin-αV as a novel target for therapy in MDC1A as well as other degenerative fibrotic muscle diseases.

## MATERIALS AND METHODS

### *Animal breeding and care*

All animals were housed at the Animal Care Facility – Charles River Campus (LACF-CRC) of Boston University on a 12 : 12-hr light-dark cycle. Food and water were provided *ad libitum*. We performed all procedures in accordance to the protocol approved by the IACUC of Boston University. Heterozygous B6.129 *Lama2dy-W/+*mice, carrying a targeted mutation in the *Lama2* gene, were kindly provided by Dr. Eva Engvall (Burnham Institute, La Jolla, CA, USA). Losartan treatment was started at two weeks post-natal and continued until collection at 7 weeks of age. Losartan was provided in the drinking water *ad libitum* (600 mg/L, Cozaar by Merck pharmaceuticals).

### *Muscle tissue and serum collection*

Mice were first briefly bled from the submandibular vein to obtain blood samples and left to clot for 15 minutes at room temperature then spun at 2,000 g for 8 minutes to separate serum. Animals were then euthanized with isoflurane (Webster Veterinary, Devens, MA, USA) before isolating the tibialis anterior (TA), gastrocnemius/soleus complex (GS), and quadriceps muscles (QD). Tissues were weighed and snap frozen in liquid nitrogen for RNA and protein extraction. TA muscles used for histology were embedded in Tissue-Tek OCT Compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in isopentane (Sigma-Aldrich, St. Louis, MO, USA) chilled in liquid nitrogen. Serial transverse sections (7 μm), were prepared using the Leica CM 1850 cryostat (Leica Microsystems, Inc.) and stored at –80°C.

*Gene expression*

RNA from 25 mg liquid nitrogen snap frozen pooled hind limb muscles was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 1μg 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, Foster City, CA, USA) on ABI 7300 Real Time PCR system. 18 s ribosomal subunit RNA served as the endogenous control and gene expression was calculated by using the ΔΔCt method.

*Integrin, TIMP1 and TGF-β ELISA*

Amount of Integrin-αV and –α5, as well as free and latent TGF-β and TIMP1 were determined using ELISA kits (CD51:Biolegend, San Diego, CA; CD49e:Biolegend, San Diego, CA; LEGEND MAXTM TGF-β kits:Biolegend, San Diego, CA, TIMP1:Biolegend, San Diego CA) following manufacturer's instructions. Using lysis buffer containing protease and phosphatase inhibitor cocktails, protein was isolated from hind limb and serum samples of WT, DyW, and Losartan-treated mice. The lysates were incubated with the capture antibody for 3 hours at room temperature followed by 5 washes with the wash buffer. The wells were washed 5 times for 3 minutes each and then incubated with detection antibodies for 2 hours at room temperature. The wells were then washed as described above and then incubated with Avidin-HRP conjugates. The washing step was repeated again as above and incubated with the substrate for 15 minutes. The reactions were stopped subsequently and the absorbance was recorded at 450 and 570 nm.

*Isolation of single myofibers*

Animals were euthanized by overdose of isoflurane. Fur was wetted with 70% ethanol and alcohol was allowed to evaporate. Extensor Digitorum Longus (EDL) muscles from both hind limbs were removed quickly and incubated in 2 mL DMEM containing 0.2 % Collagenase type I (Worthington Biomedical, Lakewood NJ, USA). Digestion time of 30 minutes for WT and 45 minutes for DyW muscle was found optimal to release single myofibers from these animals respectively. Muscle was triturated gently with a wide bore glass pipette to release single myofibers. Single fibers were washed twice with pre-warmed DMEM and transferred to matrigel-coated culture dish containing growth medium (DMEM with 20% FCS, 1% chick embryo extract and 1x penicillin streptomycin). They were fed with fresh pre-warmed growth medium every 24 hours.

*Immunohistochemistry*

Frozen tissue sections were incubated in Acetone for 20 minutes then left to air dry for 15 minutes. Protocol for Mouse on Mouse (M.O.M.) serial immunostaining for frozen sections provided by Vector Labs (Burlingame, CA) was followed using anti-CD51 (Biolegend, San Diego, CA) anti-Desmin antibodies (Sigma Aldrich, St. Louis, MO). Sections were mounted with Vectashield with DAPI (Vector Labs, Burlingame, CA) and imaged with a Nikon DSFi1 camera head attached to a Nikon ECLIPSE 50i light microscope system. These images were analyzed using NIS-Elements Basic Research 3.0 software. Single myofibers were fixed in 4% PFA and stained using the same protocol as frozen muscle sections with anti-CD51.

*Integrin-αV western blot*

Four biological samples per animal group were pooled for protein extraction. Muscle lysates were prepared by homogenizing 25 mg of muscle in radioimmunoprecipitation assay buffer containing a Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) and a PhosSTOP Phosphatase Inhibitor Cocktail tablet (Roche Diagnostics GmbH). Protein concentration was estimated by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), and 35 μg of protein were resolved on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane by semidry electrophoretic transfer (Trans-Blot SD; Bio-Rad Laboratories). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and probed with primary antibodies for 1 : 2,000 anti-CD51 (BioLegend, San Diego, CA) and 1 : 5,000 anti-α-tubulin rabbit primary antibodies (Sigma-Aldrich) at 4°C overnight. The blot was washed three times for 5 minutes each time with Tris-buffered saline containing 0.1% Tween 20 and subsequently stained with 1 : 2,000 goat anti-rabbit IRDye 800CW IgG2b and IRDye 680LT IgG2b antibodies (LI-COR Biosciences). The blots were then washed and scanned for analysis using an Odyssey infrared imaging system (LI-COR Biosciences).

*Statistics*

One-way ANOVA tests were performed using GraphPad Prism 6 software. Data are presented as mean ± standard deviation.

## ACKNOWLEDGMENTS

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## COMPETING INTERESTS

The authors declare no competing interests.

## ABBREVIATIONS LIST

Transforming Growth Factor-β (TGF-β), Nuclear Factor-κB (NF-κB), quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Mitogen-Activated Protein Kinase (MAPK), Large Latent Complex (LLC), Latency Associated Peptide (LAP), Tissue Inhibitor of Matrix Metalloprotease 1 (TIMP1).

## AUTHOR CONTRIBUTIONS

TM and AA conducted gene expression and immunohistochemical experiments and wrote the body of the manuscript. AK performed the ELISA assays and contributed to all other aspects of the experimental procedures. YR performed gene expression experiments and manuscript revisions. SG Conceptualized and designed the study and helped in drafting the manuscript. All authors read and approved the final manuscript.

## REFERENCES

[1] Gawlik, K. I., Durbeej, M. Skeletal muscle laminin and MDC1A: Pathogenesis and treatment strategies. Skeletal muscle. 2011; 1(1): 9.

[2] Collins, J., Bonnemann, C. G. Congenital muscular dystrophies: Toward molecular therapeutic interventions. Curr Neurol Neurosci Rep. 2010; 10(2): 83-91.

[3] Philpot, J., Bagnall, A., King, C., Dubowitz, V., Muntoni, F. Feeding problems in merosin deficient congenital muscular dystrophy. Archives of disease in childhood. 1999; 80(6): 542-547.

[4] Muntoni, F., Bertini, E., Bonnemann, C., Brockington, M., Brown, S., Bushby, K., et al. 98th ENMC International Workshop on Congenital Muscular Dystrophy (CMD), 7th Workshop of the International Consortium on CMD, 2nd Workshop of the MYO CLUSTER project GENRE. 26–28th October, 2001, Naarden, The Netherlands. Neuromuscular disorders: NMD. 2002; 12(9): 889-896.

[5] Mehuron, T., Kumar, A., Duarte, L., Yamauchi, J., Accorsi, A., Girgenrath, M. Dysregulation of matricellular proteins is an early signature of pathology in laminin-deficient muscular dystrophy. Skelet Muscle. 2014; 4:14.

[6] Lygoe, K. A., Norman, J. T., Marshall, J. F., Lewis, M. P. AlphaV integrins play an important role in myofibroblast differentiation. Wound Repair Regen. 2004; 12(4): 461-470.

[7] Ruoslahti, E., Engvall, E. Integrins and vascular extracellular matrix assembly. J Clin Invest. 1997; 100(11 Suppl): S53-S56.

[8] Sheppard, D. Roles of alphav integrins in vascular biology and pulmonary pathology. Curr Opin Cell Biol. 2004; 16(5): 552-557.

[9] Busk, M., Pytela, R., Sheppard, D. Characterization of the integrin alpha v beta 6 as a fibronectin-binding protein. J Biol Chem. 1992; 267(9): 5790-5796.

[10] DeSimone, D. W., Stepp, M. A., Patel, R. S., Hynes, R. O. The integrin family of cell surface receptors. Biochem Soc Trans. 1987; 15(5): 789-791.

[11] Desgrosellier, J. S., Cheresh, D. A. Integrins in cancer: Biological implications and therapeutic opportunities. Nat Rev Cancer. 2010; 10(1): 9-22.

[12] Lal, H., Verma, S. K., Smith, M., Guleria, R. S., Lu, G., Foster, D. M., et al. Stretch-induced MAP kinase activation in cardiac myocytes: Differential regulation through beta1-integrin and focal adhesion kinase. J Mol Cell Cardiol. 2007; 43(2): 137-147.

[13] Hahm, K., Lukashev, M. E., Luo, Y., Yang, W. J., Dolinski, B. M., Weinreb, P. H., et al. Alphav beta6 integrin regulates renal fibrosis and inflammation in Alport mouse. Am J Pathol. 2007; 170(1): 110-125.

[14] Popov, Y., Patsenker, E., Stickel, F., Zaks, J., Bhaskar, K. R., Niedobitek, G., et al. Integrin alphavbeta6 is a marker of the progression of biliary and portal liver fibrosis and a novel target for antifibrotic therapies. J Hepatol. 2008; 48(3): 453-464.

[15] Meinen, S., Lin, S., Ruegg, M. A. Angiotensin II type 1 receptor antagonists alleviate muscle pathology in the mouse model for laminin-alpha2-deficient congenital muscular dystrophy (MDC1A). Skeletal muscle. 2012; 2(1): 18.

[16] Elbaz, M., Yanay, N., Aga-Mizrachi, S., Brunschwig, Z., Kassis, I., Ettinger, K., et al. Losartan, a therapeutic candidate in congenital muscular dystrophy: Studies in the dy(2J) /dy(2J) mouse. Ann Neurol. 2012; 71(5): 699-708.

[17] Goodwin, A., Jenkins, G. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. Biochem Soc Trans. 2009; 37(Pt 4): 849-854.

[18] Henderson, N. C., Sheppard, D. Integrin-mediated regulation of TGFbeta in fibrosis. Biochim Biophys Acta. 2013; 1832(7): 891-896.

[19] Takahara, T., Furui, K., Yata, Y., Jin, B., Zhang, L. P., Nambu, S., et al. Dual expression of matrix metalloproteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers. Hepatology. 1997; 26(6): 1521-1529.

[20] Milani, S., Herbst, H., Schuppan, D., Grappone, C., Pellegrini, G., Pinzani, M., et al. Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol. 1994; 144(3): 528-537.

[21] Zhou, X., Murphy, F. R., Gehdu, N., Zhang, J., Iredale, J. P., Benyon, R. C. Engagement of alphavbeta3 integrin regulates proliferation and apoptosis of hepatic stellate cells. J Biol Chem. 2004; 279(23): 23996-24006.

[22] Dallas, S. L., Rosser, J. L., Mundy, G. R., Bonewald, L. F. Proteolysis of latent transforming growth factor-beta (TGF-beta)-binding protein-1 by osteoclasts. A cellular mechanism for release of TGF-beta from bone matrix. J Biol Chem. 2002; 277(24): 21352-21360.

[23] Liaw, Y. S., Yu, C. J., Shun, C. T., Lee, Y. C., Kuo, S. H., Luh, K. T., et al. Expression of integrins in human cultured mesothelial cells: The roles in cell-to-extracellular matrix adhesion and inhibition by RGD-containing peptide. Respiratory medicine. 2001; 95(3): 221-226.

[24] Li, G., Jin, R., Norris, R. A., Zhang, L., Yu, S., Wu, F., et al. Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and alphavbeta5 and focal adhesion kinase (FAK) pathway. Atherosclerosis. 2010; 208(2): 358-365.

[25] Scatena, M., Almeida, M., Chaisson, M. L., Fausto, N., Nicosia, R. F., Giachelli, C. M. NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. J Cell Biol. 1998; 141(4): 1083-1093.

[26] Hinz, B. It has to be the alphav: Myofibroblast integrins activate latent TGF-beta1. Nat Med. 2013; 19(12): 1567-1568.

[27] Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis. Cell. 1999; 96(3): 319-328.

[28] Klein, S., de Fougerolles, A. R., Blaikie, P., Khan, L., Pepe, A., Green, C. D., et al. Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. Mol Cell Biol. 2002; 22(16): 5912-5922.

[29] Akimov, S. S., Belkin, A. M. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. Blood. 2001; 98(5): 1567-1576.

[30] Hirsch, E., Gullberg, D., Balzac, F., Altruda, F., Silengo, L., Tarone, G. Alpha v integrin subunit is predominantly located in nervous tissue and skeletal muscle during mouse development. Dev Dyn. 1994; 201(2): 108-120.

[31] Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J. L., Munger, J. S., Kawakatsu, H., et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. J Cell Biol. 2002; 157(3): 493-507.

[32] Wipff, P. J., Hinz, B. Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. Eur J Cell Biol. 2008; 87(8-9): 601-615.

[33] Munger, J. S., Harpel, J. G., Giancotti, F. G., Rifkin, D. B. Interactions between growth factors and integrins: Latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. Mol Biol Cell. 1998; 9(9): 2627-2638.

[34] Wang, B., Dolinski, B. M., Kikuchi, N., Leone, D. R., Peters, M. G., Weinreb, P. H., et al. Role of alphavbeta6 integrin in acute biliary fibrosis. Hepatology. 2007; 46(5): 1404-1412.

[35] Kawano, H., Cody, R. J., Graf, K., Goetze, S., Kawano, Y., Schnee, J., et al. Angiotensin II enhances integrin and alpha-actinin expression in adult rat cardiac fibroblasts. Hypertension. 2000; 35(1 Pt 2): 273-279.