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miR-24 and miR-122 Negatively Regulate the Transforming Growth Factor-β/Smad Signaling Pathway in Skeletal Muscle Fibrosis

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Fibrosis is common after skeletal muscle injury, undermining tissue regeneration and function. The mechanism underlying skeletal muscle fibrosis remains unveiled. Transforming growth factor-β/Smad signaling pathway is supposed to play a pivotal role. However, how microRNAs interact with transforming growth factor-β/Smad-related muscle fibrosis remains unclear. We showed that microRNA (miR)-24-3p and miR-122-5p declined in skeletal muscle fibrosis, which was a consequence of transforming growth factor-β. Upregulating Smad4 suppressed two microRNAs, whereas inhibiting Smad4 elevated microRNAs. Luciferase reporter assay and chromatin immunoprecipitation confirmed that Smad4 directly inhibited two microRNAs. On the other hand, overexpression of these two miRs retarded fibrotic process. We further identified that Smad2 was a direct target of miR-24-3p, whereas miR-122-5p targeted transforming growth factor-β receptor-II. Both targets were important participants in transforming growth factor-β/Smad signaling. Taken together, a positive feedback loop in transforming growth factor-β/Smad4 signaling pathway in skeletal muscle fibrosis was identified. Transforming growth factor-β/Smad axis could be downregulated by microRNAs. This effect, however, was suppressed by Smad4, the downstream of transforming growth factor-β.

INTRODUCTION

Skeletal muscle injury is a common disorder with prevalence up to 50% in all sports-related injuries.[1] It can occur in various circumstances, including direct mechanical injury or indirect destruction related to blood supply and neurological dysfunctions.[2–5] Though the healing of skeletal muscle experiences degeneration, regeneration, and remodeling phase, the injured tissue cannot reach a total recovery, weakening its physiological properties.[6] In order to improve the quality of healed skeletal muscle, methods aiming at promoting skeletal muscle regeneration are developed. By using various growth factors, including hepatocyte growth factor, fibroblast growth factors, and insulin-like growth factor, properties of newly formed skeletal muscle tissue can be similar to that of native muscle.[7–10] Regardless of the regenerating potential of skeletal muscle, however, scar formation is unavoidable.[11] Scar, or fibrosis, is the abnormal accumulation of extracellular matrix (ECM). Severe fibrotic tissue leads to chronic healing problems with tissue/organ dysfunction.[12–14] In skeletal muscle, fibrosis hinders regeneration, undermines muscle function, and alters the tissue environment, resulting in susceptibility to re-injury,[11] hence undermining the performance of skeletal muscle. Intervention against the fibrotic progress is of vital importance.

Myofibroblast, the initiator of fibrotic process, is acknowledged to be derived from different cell lines, including inflammatory cells, fibro/adipogenic progenitors, and fibroblasts.[15] Transforming growth factor-β (TGF-β),[16] connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF) families have been identified to play a role in this pathology, among which TGF-β, a strong profibrotic cytokine, can stimulate the synthesis of ECM, and then result in skeletal muscle fibrosis. As the canonical TGF-β pathway, TGF-β/Smad pathway plays a pivotal role in mediating the fibrotic process,[17] and modalities against TGF-β-related pathways have been proven to potentially alleviate the pathology.[18]

However, the cross-talking between multiple signaling pathways makes it difficult to delineate the key factor accounting for fibrosis, making it necessary to look for novel regulations. In recent years, microRNA (miR) has attracted large attention and been proven to participate in various physiological activities by interactions with the 3′ UTRs of mRNA and resulting in mRNA degeneration.[19] As a set of small endogenous non-coding RNA, increasing evidences suggest that miRs are critically involved in the fibrotic process in many tissues, including lung, heart, kidney, and skeletal muscle.[20–23]

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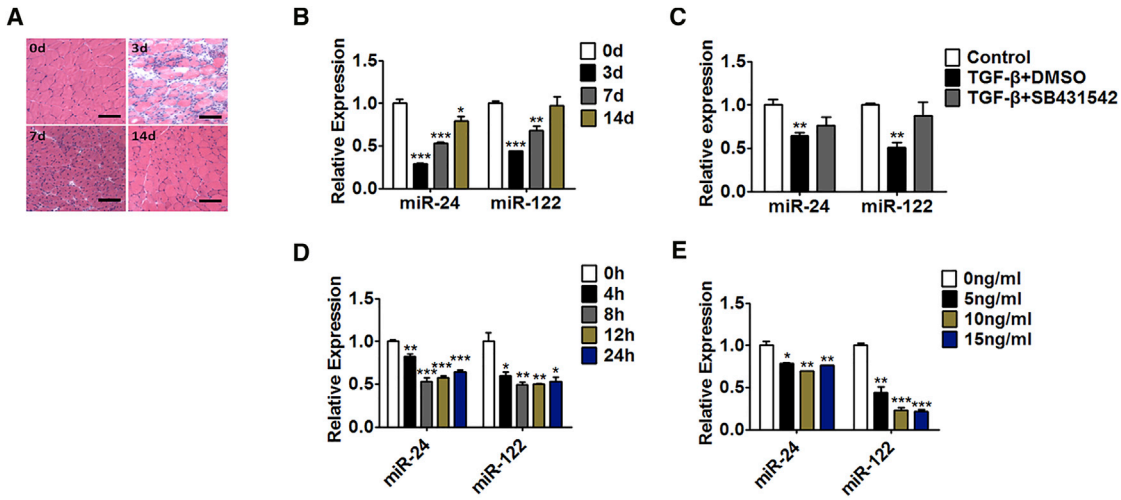

Figure 1. A Downregulation of miR-24 and miR-122 Is Noticed in Injured Skeletal Muscle and Is Related to TGF-β

(A) H&E staining of tibialis anterior (TA) muscle of mice at the indicated time points following acute contusion. (B) Real-time qPCR analysis of the time course expression of miR-24 and miR-122 in TA muscle of mice following acute contusion is shown. (C) Real-time qPCR analysis of the *in vitro* expression of miR-24 and miR-122 under normal conditions, under TGF-β stimulation, and under TGF-β stimulation with TGF-β signaling inhibitor SB431542 is shown. (D) After culturing for 0, 4, 8, 12, and 24 hr with TGF-β stimulation (10 ng/mL), the level of miR-24 and miR-122 in C2C12 cells was quantified by using real-time qPCR. (E) After culturing for 24 hr with TGF-β stimulation 12 hr of different concentration (0, 5, 10, and 15 ng/mL), the level of miR-24 and miR-122 in C2C12 cells was quantified by real-time qPCR. Data are expressed as mean ± SD; compared with control, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The scale bar represents 100 μm.

Nevertheless, how the level of miRs changed and the function of miRs in skeletal muscle fibrosis are not fully understood. Researchers have found that TGF-β signaling pathway can influence the expression and maturity of miRs, which conversely regulate the activity of the pathway.[24,25] Therefore, we hypothesize that, in skeletal muscle fibrosis, the upregulated TGF-β/Smad4 signaling pathway may alter the expression of miRs, which correspondingly have an impact on the activity of the same signaling pathway.

In the current study, a mice skeletal muscle fibrosis model was created as described previously.[26] By reviewing published studies and preliminary experiments, mmu-miR-24-1-3p (miR-24) and mmu-miR-122-5p (miR-122) were identified to be significantly downregulated in fibrotic process, which was confirmed to be the transcriptional inhibition of Smad4. Both miRs are proven to be anti-fibrotic by directly targeting Smad2 and TGF-β receptor-II (Tgfbr2), which are both located in TGF-β/Smad4 pathway. Restoration of two miRs lowered the activity of this pathway and inhibited the formation of fibrosis. These findings indicated that miR-24 and miR-122 are inhibitors of scar formation in skeletal muscle by directly suppressing pro-fibrotic pathways and suggested that both miRs might show potential in the treatment for skeletal muscle fibrosis.

RESULTS

A Downregulation of miR-24 and miR-122 Was Noticed in Injured Skeletal Muscle and Was Related to TGF-β

Acute contusion was usually used to harm skeletal muscle and cause subsequent fibrosis.[27,28] We found that the injured skeletal muscle was most disordered at 3–7 days post-injury (Figure 1A). Correspondingly, the levels of miR-24 and miR-122 were downregulated mostly at 3 days, followed by 7 days after contusion (Figure 1B). Previously, we have proven that, in the same skeletal muscle injury model, fibrosis was remarkable at 7 days after acute contusion;[29] therefore, we questioned whether there was a relationship between miR-24 and miR-122 and pro-fibrotic factors, especially TGF-β. C2C12 cells were treated with TGF-β or TGF-β plus SB431542, the TGF-β receptor-1 (Tgfbr1) inhibitor,[30] and the levels of both miRs were detected. As expected, the levels of miRs declined significantly under TGF-β stimuli, but this effect was antagonized by SB431542 (Figure 1C). To strengthen our hypothesis, cells were exposed to TGF-β in a time- and dose-dependent manner. We found that miR-24 and miR-122 decreased early after exposure (4 hr) and maintained to 24 hr (Figure 1D), and the inhibitory effect was significant no matter whether the concentration was high or low (Figure 1E). To investigate whether this decrease was a general effect resulting from exogenous stimulus or not, the level of miR-345, a previously reported miR free from TGF-β regulation,[31] was measured. Not surprisingly, the expression of miR-345 was not significantly influenced by TGF-β (Figure S1). These findings suggested that the expression levels of miR-24 and miR-122 were specifically downregulated by TGF-β in skeletal muscle.

Smad4 Is a Transcription Factor of miR-24 and miR-122

As mentioned above, Smad4 acts as a key transcription factor in canonical TGF-β pathway; therefore, we wondered whether there were any interactions between miRs and Smad4. The upregulation of Smad4 resulted in a significant decrease of the levels of both miRs (Figure 2A) and an upregulation of TGF-β (Figure 2B) *in vitro*. Because Smad4 is usually transported into nuclei in

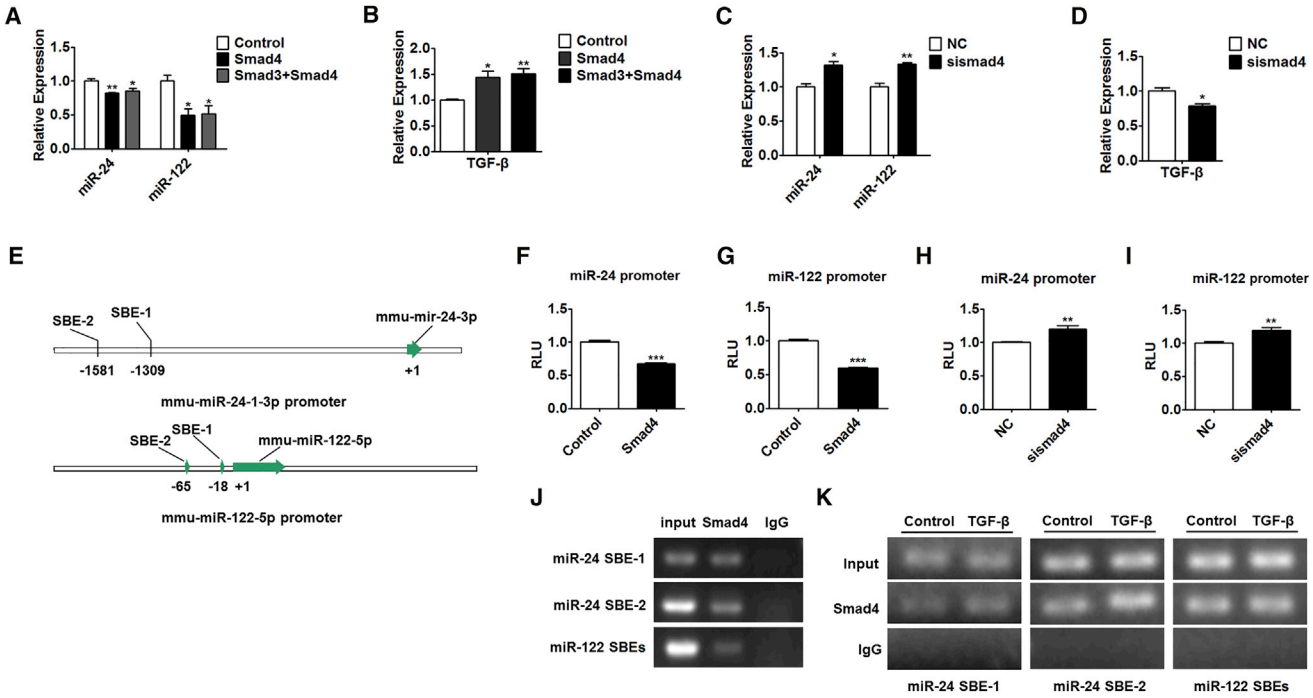

Figure 2. Smad4 Is a Transcription Factor of miR-24 and miR-122

(A and B) The influence of Smad4 or Smad3 plus Smad4 co-transfection on the expression of miR-24 and miR-122 (A) as well as TGF-β (B) *in vitro*. (C and D) The influence of smad4 interference on the expression of miR-24 and miR-122 (C) as well as TGF-β (D) *in vitro* is shown. NC, negative control. (E) Predicted Smad binding element (SBE) location in the promoter area of miR-24 and miR-122, respectively, is shown. (F and H) The transcriptional activity of promoter area in miR-24 when Smad4 was knocked down or upregulated. (G and I) The transcriptional activity of promoter area in miR-122 when Smad4 was knocked down or upregulated. (J) Chromatin immunoprecipitation (ChIP) showed that Smad4 directly bound with the two SBEs of miR-24 and the SBE of miR-122. (K) ChIP assay showed that TGF-β stimulation could enhance the binding of Smad4 with SBEs of miR-24 and miR-122. Data are expressed as mean ± SD; compared with control, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

combination with other Smads, especially Smad3, we therefore transfected Smad3 together with Smad4. Similar to the transfection of Smad4, co-transfection of Smad3 and Smad4 resulted in a downregulation of miRs (Figure 2A) and upregulation of TGF-β (Figure 2B). On the contrary, when Smad4 was silenced, the expressions of miRs and TGF-β changed inversely (Figures 2C and 2D), implying that Smad impeded the expression of miRs.

It is well acknowledged that Smad regulates the transcriptional activity of target genes via binding the Smad binding element (SBE),[32] a sequence of CAGAC;[33] therefore, SBEs in the promoter of miR-24 and miR-122 were searched within a length of 2,000 bp. For miR-24, there are two SBEs at 1,581 bp and 1,309 bp upstream of transcriptional start site. For miR-122, the two SBEs are located at 65 bp and 18 bp upstream (Figure 2E). Luciferase assay reported that the activities of both miR-24 and miR-122 promoters were prohibited when Smad4 level was upregulated (Figures 2F and 2G). Correspondingly, by using small interfering RNA (siRNA) to abrogate Smad4 expression, the activity of both miRs inversely changed (Figures 2H and 2I). The results of chromatin immunoprecipitation (ChIP) further suggested that Smad4 could directly bind to the SBEs of miR-24 and SBEs of miR-122 (Figure 2J), and this effect was inspired in the presence of TGF-β (Figure 2K). It should be noted that the distance between two SBEs of miR-122 was too close to be separated from each other, and the activity was measured as a whole. Together, these observations indicated that Smad4 directly inhibited the expression of miR-24 and miR-122, which was exacerbated by TGF-β stimulation.

miR-24 and miR-122 Suppressed Fibrotic Process

In the fibrotic tissue, type I collagen was the most deposited component in ECM, and its alpha1 chain (Col1a1) is commonly used to indicate the level of collagen accumulation.[34] Myofibroblast expressing α-smooth muscle actin (α-SMA)[26] and vimentin (VIM)[18] is the major cell source accounting for fibrotic process. The level of these two markers provides evidence for fibrosis formation. In the current experiments, we found that, in either physiological condition or stimulated with TGF-β *in vitro*, the mRNA level (Figure 3A) and protein levels (Figures 3B–3D; quantification of western blot was shown in Figure S2) of Col1a1, α-SMA, and VIM were downregulated in samples transfected with miR-24 or miR-122, implying an inhibition of TGF-β-induced fibrotic process. Similarly, injection of miR agomir via tail vein decreased the levels of these ECM genes (Figures 3E–3H; quantification of western blots was shown in Figure S2) *in vivo*. Immunohistochemistry (IHC) tests further confirmed these data. Compared with normal tissue, excessive Col1a1, α-SMA, and VIM were noticed in the injured tissue with negative control oligo

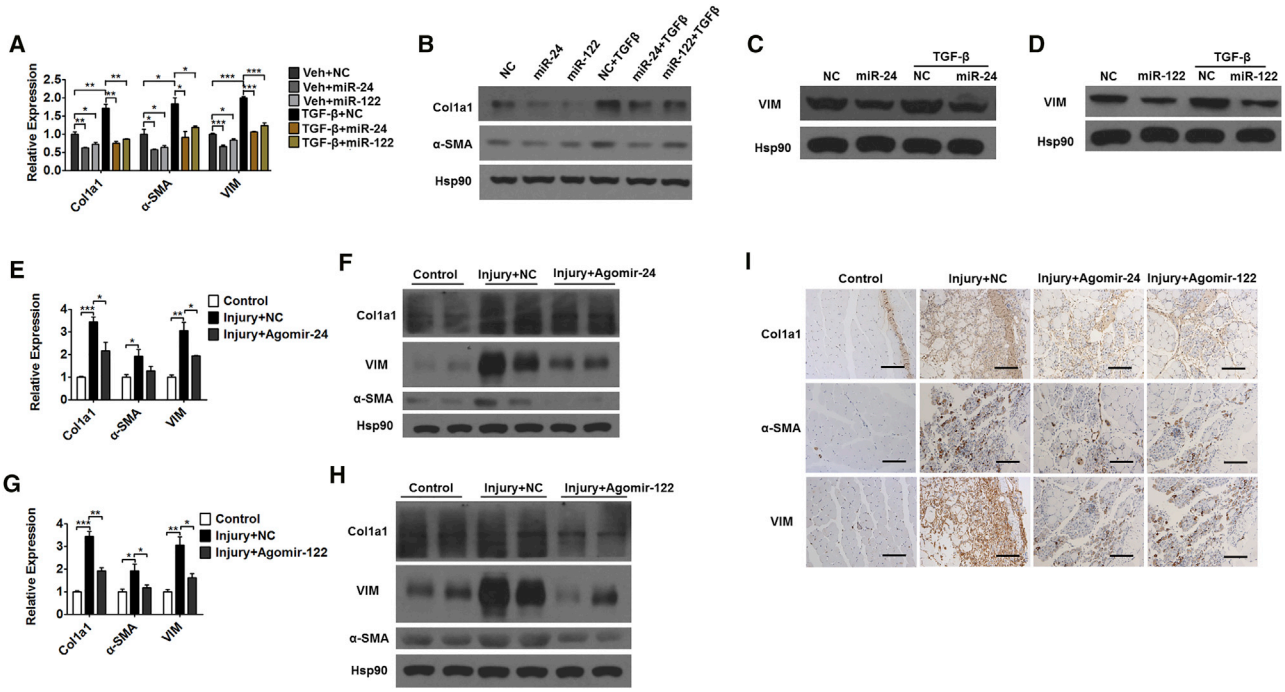

Figure 3. miR-24 and miR-122 Suppressed Fibrotic Process

(A) Real-time qPCR showed that, in either normal condition or with stimulation of TGF-β, Col1a1, α-SMA, and VIM decreased significantly in C2C12 cells transfected with either miR-24 or miR-122 compared with control. (B–D) Western blot showed that, in either normal condition or with stimulation of TGF-β, Col1a1, α-SMA (B), and VIM (C and D) expression were inhibited in C2C12 cells transfected with either miR-24 or miR-122 compared with control. (E–H) At three days after contusion, agomir-24 or agomir-122 was administered via tail vein injection. The mRNA (E and G) and protein (F and H) level of Col1a1, α-SMA, and VIM were detected at seven days after contusion. (I) The positive area of Col1a1, α-SMA, and VIM were detected in normal mice, contusion mice with control agomir intervention, and contusion mice with agomir-24 or agomir-122 injection, as shown as immunohistochemical staining. Data are expressed as mean ± SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The scale bar represents 100 μm.

supplement, indicating an accumulation of collagen with activation of myofibroblast. However, the ECM-positive area reduced after miR-24 or miR-122 intervention (Figure 3I). Therefore, these results demonstrated that miR-24 and miR-122 downregulated fibrosis formation in injured skeletal muscle.

The Anti-fibrotic Property of miR-24 and miR-122 Is Based on Their Target in TGF-β/Smad Signaling Pathway

Given the anti-fibrotic role of miRs, we tried to identify potential target genes, especially those located in TGF-β/Smad pathway. Bioinformatic analysis showed that Smad2 might be a target of miR-24, whereas miR-122 might target Tgfbr2. The potential binding sites in the mRNA in both Smad2 and Tgfbr2 were the same among mouse, rat, and human, implicating a conservation (Figures 4A and 4H). Luciferase assay showed that miR-24 could bind with the 3′ UTR of Smad2, whereas miR-122 could bind with the coding sequence (CDS) of Tgfbr2 (Figures 4B and 4I). No matter whether cells were stimulated by TGF-β or not, the expression of target genes was inhibited by miR-24 and miR-122, respectively (Figures 4C, 4D, 4J, and 4K; quantification of western blots was shown in Figure S3), *in vitro*.

It is well acknowledged that Smad2 and Smad3 maintained pro-fibrotic potential when they were phosphorylated into p-Smad2 and p-Smad3 by TGF-β receptors;[35] therefore, the level of p-Smad2, Smad3, as well as p-Smad3 were determined. Downregulation of miRs ascended the protein levels of p-Smad2, Smad3, and p-Smad3 (Figures 4D and 4K; quantification of western blots were shown in Figure S3), whereas upregulation of miRs suppressed the level of p-Smad2, Smad3, and p-Smad3 (Figures 4E and 4L; quantification of western blots were shown in Figure S3). These observations suggested that the miR-24 and miR-122 impeded the activity of TGF-β/Smad signaling pathway *in vitro*.

Similar to the results *in vitro*, the expression of Smad2 and Tgfbr2 were upregulated in the injured muscle with control oligo interference compared with normal muscle, but the injection of miR agomir downregulated the level of target genes (Figures 4F and 4M; quantification of western blots were shown in Figure S3). The transcription levels of key factors in the TGF-β/Smad signaling pathway, i.e., TGF-β, Smad3, and Smad4, were also detected. Compared to normal tissue, the levels of TGF-β, Smad3, and Smad4 were significantly higher in injured muscle with control agomir injection, but this trend was offset, at least partially, by miR-24 and miR-122 agomir injection, respectively (Figures 4G and 4N). Taken together, our data supported that the anti-fibrotic effect of miR-24 and miR-122 was a consequence of an obstruction with TGF-β/Smad signaling pathway.

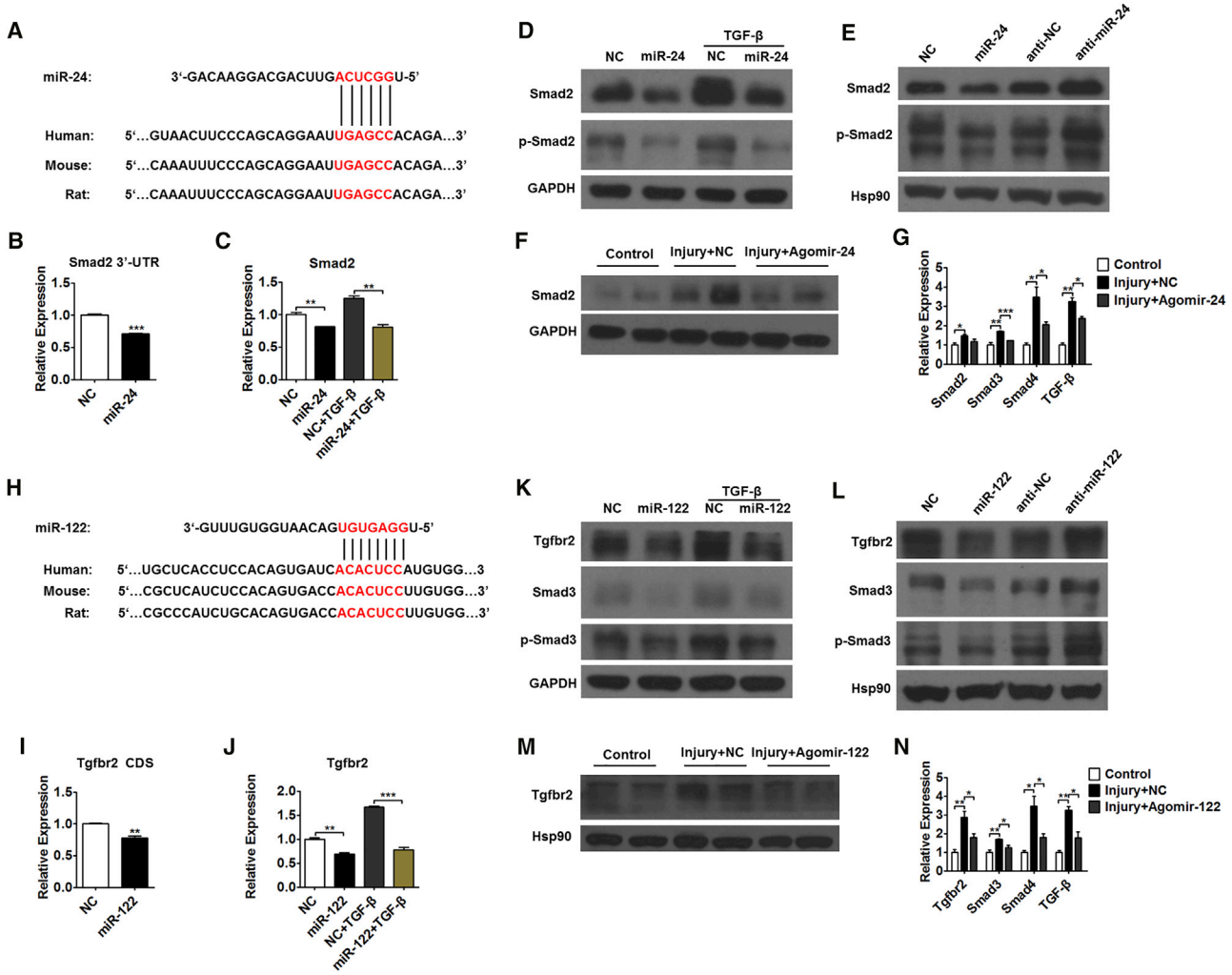

Figure 4. The Anti-fibrotic Property of miR-24 and miR-122 Is Based on Their Target in the TGF-β/Smad Signaling Pathway

(A and H) Bioinformatic analysis showed the predicted binding site of (A) miR-24 and (H) miR-122 with target genes in mouse, rat, and human. (B and I) After (B) miR-24 and (I) miR-122 transfection, the change in translational activity of target genes as indicated by luciferase reporter assay in HEK293T cell is shown. (C and J) In either normal condition or with TGF-β stimulation, the relative mRNA level of target genes Smad2 or Tgfbr2 in C2C12 cells in miR-24 (C) or miR-122 (J) transfection group compared with control were detected by real-time qPCR *in vitro*. (D) In either normal condition or with TGF-β stimulation, the relative protein level of Smad2, the phosphorylated Smad2, i.e., p-Smad2, was detected by western blot in miR-24 transfection group compared with control *in vitro*. (E) The relative protein level of Smad2 and the phosphorylated Smad2, i.e., p-Smad2, was detected by western blot *in vitro* when miR-24 was artificially elevated or prohibited. (F and M) The protein level of target genes Smad2 or Tgfbr2 in uninjured muscle (control group), injured skeletal muscle with control agomir injection, and injured sample with agomir-24 (F) or agomir-122 (M) injection were detected by western blot. (G and N) The relative mRNA expression of (G) Smad2 and (N) Tgfbr2 and other components of TGF-β/Smad signaling pathway, i.e., Smad3, Smad4, and TGF-β, were measured *in vivo*. (K) In either normal condition or with TGF-β stimulation, the relative protein level of Tgfbr2 and its downstream target Smad3, as well as phosphorylated Smad3, i.e., p-Smad3, were detected by western blot in miR-122 transfection group compared with control *in vitro*. (L) The relative protein level of Tgfbr2 and its downstream target Smad3, as well as phosphorylated Smad3, i.e., p-Smad3, were detected by western blot *in vitro* when miR-122 was artificially elevated or prohibited. Data are expressed as mean ± SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DISCUSSION

Current available treatments for skeletal muscle injury are mainly physiotherapy, such as modifications in activity modes, electoral stimulation, message, ultrasound, or stretching.[36–38] Although patients may return to sports, the fibrotic tissue does not vanish and lacks the elasticity of the native muscle, rendering the muscle susceptible to re-injury.[39] Repeated injury can cause heterotopic ossification, leading to pain and dysfunction of skeletal muscle.[40] In the current study, miR-24 and miR-122 have been proven to be regulators for alleviating skeletal muscle fibrosis, providing foundation for novel treatment development.

The anti-fibrotic effect of miRs, especially that of miR-24 and miR-122, has been increasingly discovered recently. In CCl4-induced liver

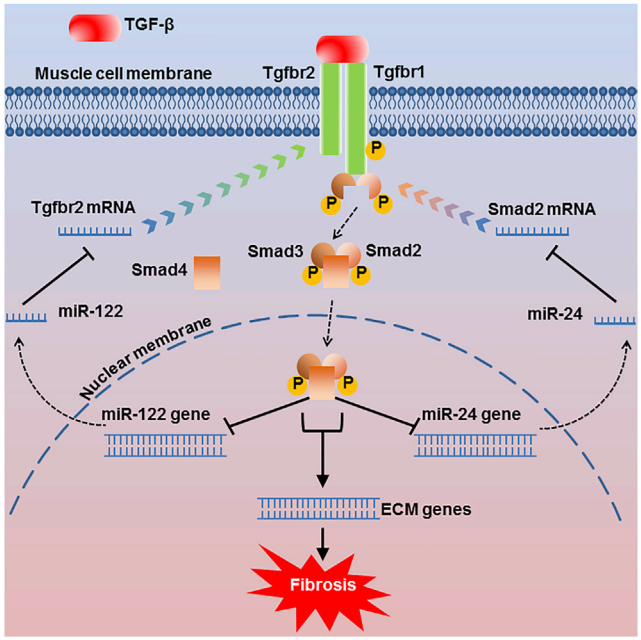

Figure 5. Diagram of the Inhibitory Role of miR-24 and miR-122 in the TGF-β/Smad Signaling Pathway in Skeletal Muscle Fibrosis after Contusion

After Smad4 translocates into nucleus, it inhibits the expression of miR-24 and miR-122 expression, which correspondingly prohibits the expression of Smad2 and Tgfbr2 in the cytoplasm.

fibrosis in a rodent model, the expression of miR-122 declined. Supplementing miR-122 was noticed to be able to suppress the expression of prolyl 4-hydroxylase subunit alpha-1 (P4HA1) in hepatic satellite cells, thus reducing collagen production.[41] The relationship between miR-122 and liver fibrosis is also confirmed in patients with hepatitis C virus, endowing it a potential to be a bio-marker for the severity of disease.[42] Similar to the findings of miR-122 in liver, miR-24 was noticed to be able to inhibit cardiac fibrosis subsequent to infarction in a rat model by downregulating the expression of furin, a subtilisin-like proprotein convertase that can facilitate the activation of TGF-β.43 However, the role of miR-24 and miR-122 in skeletal muscle after injury is unclear. According to our findings, miR-24 and miR-122 were downregulated in the fibrotic process of injured skeletal muscle. Excessive deposition of interstitial substance makes skeletal muscle stiffer and fragile,[44] and these pathological changes will weaken the contractile force and increase the risk of re-injury.[26] When the levels of miRs were artificially upregulated both *in vitro* and *in vivo*, the level of fibrotic and mesenchymal markers, i.e., Col1a1, α-SMA, and VIM, was attenuated and immunohistochemically positive area decreased. These findings indicated that upregulation of these miRs may facilitate the recovery of injured skeletal muscle, though further studies with more functional evaluations are needed.

An effective manner for reducing skeletal muscle fibrosis is to interfere with TGF-β/Smad signaling pathway. In the TGF-β/Smad signaling pathway, TGF-β binds with Tgfbr2 and activates Tgfbr1, by which Smad2 and Smad3 are recruited and activated, and promote the synthesis of ECM after being transported into nucleus with Smad4.[45] Previously, we found interferon-γ an agent with treating potential in mice skeletal muscle fibrosis because of a prohibitory effect on Smad2 and Smad3.[46] Terada et al.[47] administered losartan to mice with skeletal muscle contusion and found that the regenerated tissue had less fibrotic area with lower Smad2/3 expression. According to our bioinformatic analysis and tests *in vitro* and *in vivo*, miR-24 lowered the expression of Smad2 with binding area in 3′ UTR. Interestingly, the binding site of miR-122 in the current study was located in the CDS region instead of the UTR. Experiments proved that, other than 3′ UTR, which was the most common binding site, miRs could function by binding with CDS[48] or 5′ UTR.[49] We found that, by binding with the CDS of Tgfbr2, miR-122 declined the expression of target gene. Moreover, a lower level of activated Smad3 was noticed with miR-122 interference, indicating that TGF-β/Smad-mediated fibrotic process is down-graded by miRs.

Smad proteins, especially Smad4, play a central role in the TGF-β-mediated fibrotic process. By using siRNA to specifically knock down the expression of Smad4, several reports have suggested the significant pro-fibrogenic role of Smad4 in TGF-β-mediated pathological process. Chen et al.[50] found that, after Smad4 was silenced by siRNA, the fibrotic characteristics of C2C12 cells decreased. Similar to reports *in vitro*, researchers notified the anti-fibrotic effect of Smad4 interference on joint capsule fibrosis and renal fibrosis.[51,52] However, as a co-Smad, Smad4 has a role in several pathways, including TGF-β pathway, bone morphogenic protein pathway, and activin pathway.[17] Cross-talking among pathways makes it more difficult to outline the function of Smad4 in fibrosis and may induce side effects that were yet to be discussed. The deeper understanding of miRs, on the other hand, provides us another point of view into exploring the fibrogenic property of Smad4, as miR directly influences the level of targets by post-transcriptional regulation.[19] On ground of this, we questioned whether Smad4 was responsible for the decrease of miR-24 and miR-122 under TGF-β stimulation. Transfection of Smad4 significantly reduced the level of miR-24 and miR-122, whereas silencing of Smad4 led to a reverse change pattern of miRs. To ensure that Smad4 transferred into nucleus, co-transfection of Smad3+Smad4 was performed, because Smad4 generally transports into nucleus in combination with Smad3. Similarly, the expression of miR-24 and miR-122 decreased, indicating a relationship between Smad4 and miRs. Strengthened by observations in ChIP and luciferase assay, it was Smad4 that repressed the level of both miRs. In skeletal muscle fibrosis, the role of Smad4 in transcription remains poorly studied. According to our previous research, miR-146, an inhibitor of skeletal muscle fibrosis, could be downregulated by Smad4 transfection too.[29]

To sum up, we revealed a positive feedback loop in the fibrotic process of injured skeletal muscle: when TGF-β/Smad pathway is overactivated, the upregulated Smad4 suppresses the level of pathway inhibitors (miR-24 and miR-122), exacerbating signaling activation and subsequently leading to skeletal muscle fibrosis (Figure 5).

Interrupting this feedback loop by targeting these miRs may have therapeutic potentials for this condition.

Interestingly, after artificially upregulating the miR level, the transcription level of TGF-β, Smad3, and Smad4 also decreased significantly. As both proteins are not the predicted target of either miR-24 or miR-122, we suppose that this change may be a result of other potential target genes. Besides, even in physiological environment *in vitro*, miR-24 and miR-122 were noticed to downregulate the transcription and expression ECM gene; this phenomenon suggests that the involvement of the two miRs in anti-fibrotic process may be deeper than our current findings. The discovered feedback loop is established in an acute contusion model. Whether it is the same in other skeletal muscle injury models, such as laceration or strain, needs verification.

Unavoidably, the current study has several limitations. First, a rescue of target genes lowered by miRs was not performed. However, given the targets are located in TGF-β/Smad pathway, it is reasonable to conclude that the anti-fibrotic role of the two miRs is based on its interaction with this pathway. Besides, it is well acknowledged that skeletal muscle participates in many physiological and biochemical activities, among which the most important is its contraction and metabolic profile.[53] In the current study, albeit a proven anti-fibrotic property, what happened to muscle regeneration and the function of skeletal muscle remain unveiled. Finally, in the current study, we did not detect the influence of different time points of miR intervention on the injured skeletal muscle, although we chose to deliver agomir at the time when the miR level was lowest *in vivo*. Recently, it has been proposed that a timed progression of microenvironment can be either beneficial or harmful to skeletal muscle regeneration;[54] therefore, it is reasonable to further delineate the role of miR-24 and miR-122 in skeletal muscle throughout the post-injury time course. Moreover, miR is mediated by several transcriptional factors with several target genes.[19] For example, miR-24 was reported to enhance cell growth by targeting metallothionein 1M and facilitate metastasis by targeting p53[55] in hepatocellular carcinoma but was proven to inhibit metastasis by targeting Ack1 in human osteosarcoma cells.[56] The exact role of miR-24 and miR-122 in the post-injury period of skeletal muscle requires further verification.

In conclusion, we have identified, in skeletal muscle fibrosis, a pathologically positive feedback loop in TGF-β/Smad signaling pathway. miR-24 and miR-122 act as fibrogenic inhibitors that reduce the level of Smad2 and Tgfbr2, respectively, whereas Smad4 suppresses the expression of two miRs.

MATERIALS AND METHODS

Cell Culture

Mouse C2C12 myoblasts and HEK293T cells were purchased from the Shanghai Institutes for Biological Sciences. Cells were cultured in DMEM supplemented with 10% FBS (fetal bovine serum) (Gibco), 100 units/mL penicillin, as well as 100 mg/mL streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2 atmosphere. For induction of myotube from myoblast, DMEM with 2% HS (horse serum) (Gibco) and 1% penicillin/streptomycin was used. To induce fibrosis *in vitro*, TGF-β was added into the culture medium with different final concentration (0 ng/mL, 5 ng/mL, 10 ng/mL, and 15 ng/mL; Sigma, St. Louis, MO) for 12 hr or with different culturing time (0 hr, 4 hr, 8 hr, 12 hr, and 24 hr with a concentration of 10 ng/mL). To block the effect of TGF-β, SB431542, with a concentration of 10 nM dissolved in DMSO, was co-administered to cells with TGF-β for 8 hr. Then DMEM with 10% FBS was replaced by medium with 2% HS for 48 hr for myotube induction prior to sample harvesting.

In Vitro Transfection and Luciferase Reporter Assay

Construction of luciferase reporter plasmids, the fragment of Smad2 3′ UTR, and Tgfbr2 CDS was amplified from mouse cDNA by PCR, respectively (primers used listed in Table S1). The PCR product was inserted into pRL-TK vector. Reporter plasmids, including the constructed vectors and pGL-3 basic vectors, were co-transfected with miR-24 or miR-122 mimics into HEK293T cells with or without TGF-β stimuli using Lipofectamine 2000 reagent (Invitrogen, USA).

For promoter assay, a genomic fragment upstream of the transcriptional start site of miR-24 precursor and miR-122 precursor were amplified by PCR using mouse genomic DNA as a template (primers used were listed in Table S1). The PCR product was cloned into pGL3-basic vector (Promega, USA). The constructed vectors and pRL-TK vectors were co-transfected with smad4 vector or smad4 siRNA into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, USA).

At 48 hr after transfection, a luciferase assay was performed with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Firefly and Renilla luciferase activities were measured on a luminometer (Berthold Technologies).

Previously, the construction and transfection of several lentivirus-based Smad4-siRNAs into myoblast were reported by us.[50] In the current study, the siRNA with highest interference efficiency and control siRNA was used (sequences were listed in Table S2). For the expression of Smad4 and Smad3 in mouse C2C12 myoblasts, plasmids (Hanbio Biotechnology, Shanghai, China) transfection were performed using Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. miR mimics (10 nM), control mimics (10 nM), miR inhibitor (10 nM), and inhibitor control (10 nM) were transfected into myoblasts using Lipofectamine 2000 (Invitrogen) following the protocol. The sequence of the control mimics, control inhibitor, miR-24 mimics, miR-24 inhibitor, miR-122 mimics, and miR-122 inhibitor were listed in Table S2. After transfection for 8 hr, 2% HS culture medium was used to induce myotube from myoblasts. Cells were harvested 48 hr after induction.

ChIP

The ChIP Assay Kit was purchased from Millipore (Boston, MA, USA). In brief, the nuclei were extracted from C2C12 cells cultured

in 10-cm dish with or without TGF-β stimuli and then sonicated into fragments. Precleared chromatin was incubated with anti-Smad4 antibody and control immunoglobulin G (IgG) antibody (both from Cell Signaling Technology). After the removal of the protein and RNA, the precipitated DNA was purified and subjected to PCR. The sequences of primers specific for the Smad binding element (SBE) in miR-24 and miR-122 promoters were listed in Table S3. PCR-amplified products were then resolved by 1.5% agarose gel electrophoresis.

Animal Experiments

Male mice ~10 weeks old were used in our research. All animal experiments were conducted according to the Declaration of Helsinki and the Guiding Principle in care and use of animals. The experimental protocols were approved by the Animal Care Committee of Fudan University. The acute contusion model of the right tibialis anterior (TA) was established by a steel ball according to our previous method.[18] A total of 70 mice were maintained in the current study, of which 10 were normal control, 10 were injured, 10 were injured with miR-24 agomir injection, and 10 were injured with miR-122 agomir injection. Seven days after contusion, animals in all four groups were sacrificed and the right TAs were harvested. Another 30 mice were also harmed as three injury groups with 10 in each and executed at 3rd, 7th, and 14th days after contusion, respectively. In each group, 5 were used for western blot and real-time PCR test and 5 were used for histological test. miR agomir was injected via tail vein at the third day post-injury. The sequence of agomir-24, agomir-122, and agomir nonsense control were listed in Table S2.

RNA Isolation and Real-Time qPCR Analysis

Total RNA (cell or tissue derived) was extracted by the Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by Nanodrop. Total RNA was reverse transcribed by using a PrimeScript RT reagent kit (Takara Bio). For small RNAs, stem loop real-time qPCR (TaqMan) was used to detect difference in the level of miRNA as previously described.[29] Real-time PCR was performed on an ABI7900 Real-Time PCR System (Applied Biosystems). The primer sequences used were shown in Table S3.

Western Blot

Protein was extracted according to the previous method.[57] Primary antibodies against Col1a1, α-SMA, VIM, Hsp-90, GAPDH (all from Cell Signaling Technology, MA), Smad2, p-Smad2, Smad3, p-Smad3, Tgfbr2 (all from Cell Signaling Technology, MA), and anti-tubulin (Sigma) antibodies were used as primary antibodies.

Histological Analysis

Samples collected were fixed in 10% neutral buffered formalin for 24 hr and then sectioned perpendicular to the direction of the muscle fiber with 5 μm in thickness using a microtome (SM2500; Leica Microsystems, Wetzlar, Germany). These sections were stained with H&E stain. Col1a1, α-SMA, and VIM antibody (Sigma) was used in immunohistochemical analysis. The histological slices were visualized by inverted light microscopy (IX71SBF2; Olympus, Tokyo, Japan), with DP72 Manager (Olympus) to capture digital images.

Statistical Analysis

A statistical significance of the differences between the means of groups is indicated if the p value is less than 0.05. The significance is shown as *$p < 0.05$, **$p < 0.01$), and ***$p < 0.001$. Student's t test was performed when only two groups were compared, whereas one-way ANOVA with a post hoc Bonferroni multiple comparison test was performed when no less than three groups were compared. GraphPad Prism 5.0 (GraphPad Software) was used for all statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.04.005.

AUTHOR CONTRIBUTIONS

Y.S. and H.W. conducted *in vitro* experiments and image quantification. Y.L. and S.L. conducted *in vivo* experiments. J.C. and H.Y. organized the work.

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