Myostatin-Null Mice Exhibit Delayed Skin Wound Healing through The Blockade of
Transforming Growth Factor-β Signaling by Decorin

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Abstract

Myostatin (Mstn) is a secreted growth and differentiation factor that belongs to the transforming growth factor-β (TGF-β) superfamily. Mstn has been well characterized as a regulator of myogenesis, and has been shown to play a critical role in postnatal muscle regeneration. Herein, we report for the first time that Mstn is expressed in both epidermis and dermis of murine and human skin and that Mstn-null mice exhibited delayed skin wound healing due to a combination of effects resulting from delayed epidermal re-epithelialization and dermal contraction. In epidermis, reduced keratinocyte migration and protracted keratinocyte proliferation were observed, which subsequently led to delayed recovery of epidermal thickness and slower re-epithelialization. Furthermore, primary keratinocytes derived from Mstn-null mice displayed reduced migration capacity and increased proliferation rate as assessed through in vitro migration and adhesion assays, as well as BrdU incorporation and Western blot analysis. Moreover, in dermis, both fibroblast-to-myofibroblast transformation and collagen deposition were concomitantly reduced, resulting in a delayed dermal wound contraction. These decreases are due to the inhibition of TGF-β signaling. In agreement, the expression of decorin, a naturally occurring TGF-β suppressor, was elevated in Mstn-null mice; moreover, topical treatment with TGF-β1 protein rescued the impaired skin wound healing observed in Mstn-null mice. These observations highlight the interplay between TGF-β and Mstn signaling pathways, specifically through Mstn regulation of decorin levels during the skin wound healing process. Thus, we propose that Mstn agonists might be beneficial for skin wound repair.

Keywords: Myostatin, Wound healing, Decorin, Skin, TGF-β
Introduction

Skin wound healing is critical for the maintenance of skin homeostasis after acute skin injury. The skin consists of two major layers: epidermis, the upper layer consisting of numerous cell types which include keratinocytes, melanocytes, Langerhans, and Merkel cells; and dermis, the lower layer of fibroblasts and connective tissue, including collagen and elastic fibers. After deep skin injury, wound healing can be categorized into four processes (i.e. inflammation, migration, proliferation, and maturation) which overlap spatio-temporally. During the inflammatory phase, a blood clot is formed at the wounded area to prevent excessive blood loss, followed by vasodilation and secretion of key growth factors such as Transforming Growth Factor-β (TGF-β) and platelet-derived growth factor (PDGF) from inflammation-responsive cells; which further facilitate the influx of macrophages, neutrophils, and mesenchymal stem cells to the site of injury. Next, peri-wound keratinocytes become activated to form a migratory tongue from each side of the wound edge. This involves the migration of cells from the unwounded area of the stratum basale to form a repair bridge covering the underlying regenerating dermis, where the activated myofibroblasts migrate to, and the formation of granulation tissue takes place to regenerate the wound bed. During the proliferative and maturation phases, the activated and alpha-smooth muscle actin (α-SMA)-expressing myofibroblasts undergo augmented proliferation and increase the production of collagen and vessels that eventually fill the wound area.

Growth factors play a major role in skin wound healing. Several studies have unravelled the important function of TGF-β1 in skin wound healing. It is reported that TGF-β1 stimulates collagen deposition and extracellular matrix growth, resulting in the accumulation of fibrotic tissue, suggesting that TGF-β as an important mediator during fibrosis (33). This is further
supported by the observation that Smad3-null mice (in which TGF-β signaling is perturbed) exhibit accelerated wound healing with reduced scarring (10). Decorin, a small leucine-rich proteoglycan, is mainly expressed in connective tissue (18). Being a crucial regulator of extracellular matrix assembly, decorin is able to bind to type I collagen fibrils (12, 29), participating in the regulation of collagen fibril formation (20), thus modulating connective tissue formation, skeletal muscle cell differentiation, and migration (5, 7, 46). Decorin has been reported to be a binding partner of TGF-β receptors, thereby it is an important regulator of TGF-β bioavailability and subsequent downstream signaling (9). It has also been demonstrated that collagen-bound decorin can still interact with TGF-β (34), indicating that TGF-β can be immobilized to the ECM and prevented from interacting with its receptor at the cell membrane. Therefore decorin, by regulating bioavailability of TGF-β, plays a role in skin wound healing. It is noteworthy to mention that in addition to TGF-β, decorin can also sequester, and thus regulate the activity of, another TGF-β superfamily member, Myostatin (Mstn) (28). Mstn, also known as growth and differentiation factor 8 (GDF-8) is primarily expressed in skeletal muscle, but relatively low expression of Mstn has also been detected in adipose tissue (27) and heart (38). In mice and humans, loss of Mstn leads to increased muscle growth due to both hypertrophy and hyperplasia (27, 35). Therefore, Mstn functions as a negative regulator for skeletal muscle growth. Functionally, Mstn has been found to regulate not only the proliferation and differentiation of myoblasts (23, 44), but also the activation and proliferation of muscle stem cells, also known as satellite cells (25). In a muscle regeneration study, Mstn-deficient mice demonstrated accelerated muscle healing accompanied by reduced fibrosis (26) due to enhanced activation of satellite cells, accelerated migration of macrophages and myoblasts into the injured area, and less fibrotic tissue formation (26). Although a number of reports have previously
demonstrated that Mstn plays an important role in muscle regeneration through TGF-β signaling, its expression and role in skin wound healing is not known. Herein, we demonstrate for the first time that Mstn is expressed in skin and its deficiency leads to delayed skin closure due to a delay in epidermal re-epithelialization and dermal contraction. We find that the delay in skin wound healing in Mstn-null mice is attributed to blockade of TGF-β signaling due to increased decorin expression during skin wound repair. These findings reveal Mstn as a novel regulator of skin wound healing, and suggest that Mstn agonists may be a potential therapeutic solution for the treatment of chronic and diabetic wounds.
Materials and Methods

Animals and human biopsy

Mstn-null mice (C57BL/6) were kindly gifted by Dr. Se-Jin Lee (The Johns Hopkins University, Baltimore, MD, USA). Wild-type mice (C57BL/6) were obtained from the Centre for Animal Resources (National University of Singapore, Singapore). Taking into consideration that the anagen (active growth) phase of the hair follicle cycle promotes wound healing and re-epithelization (2), six-week-old mice were chosen for our studies, as mouse skin reaches the second and longest telogen (resting) phase during this age (4). Therefore, the hair follicle cycle effect on wound healing can be eliminated as a confounding factor in data analysis. All experiments were performed according to the approved protocols of the Institute Animal Ethics Committee (IACUC), Singapore. The human biopsy was kindly provided by Dr. Cleo Choong Swee Neo (Nanyang Technological University, Singapore).

Skin Wounding Experiment and Biopsy Procurement

Mstn-null and wild-type female mice were first anaesthetised and the area assigned for wounding was shaved. Two 0.25 cm$^2$ (0.5 cm x 0.5 cm) excisional wounds were generated on the dorsal skin. Prior to the procurement of wound biopsies at specific time points (non-wounded and post-wounding day 1, 3, 5, 7, 10, 12, and 15), the wound area was measured using callipers. A subset of Mstn-null mice was treated topically with either vehicle (3% methylcellulose in phosphate-buffered saline and 4mM HCl) or 100 ng of recombinant TGF-β1 (42). Mouse wound biopsies and the human biopsy were embedded in OCT compound before freezing in liquid nitrogen. Sections (6 µm thickness) were subsequently cut using the Leica CM3050S cryostat microtome. The slides were stored at -20 ºC until further use.
**Reagents**

Details of the antibodies used in this manuscript are provided below: Anti-Ki67 and anti-cytokeratin 6 (K6) antibodies were purchased from Novocastra; anti-α-smooth muscle actin (α-SMA), anti-GDF8 (Mstn), anti-Phospho-Smad2/3 (p-Smad2/3) and anti-PCNA antibodies were from Santa Cruz Biotechnology; anti-decorin antibody was obtained from Developmental Studies Hybridoma Bank; anti-Cyclin D1 antibody was from Thermo Lab Vision; AlexaFluor® 488 goat anti-rabbit IgG, AlexaFluor® 594 goat anti-rabbit IgG, AlexaFluor® 594 goat anti-mouse IgG and streptavidin-conjugated AlexaFluor® 488 antibodies were from Molecular Probes, Invitrogen; biotinylated goat anti-rat IgG antibody was from GE Healthcare Life Sciences; goat anti-rabbit HRP-, goat anti-rat HRP-, and goat anti-mouse HRP-conjugated antibodies were from Santa Cruz Biotechnology.

The recombinant TGF-β1 protein used in this study was obtained from PeproTech.

**Histology**

Sections were fixed with 4% paraformaldehyde for 10 min, rinsed with water, and immersed in 0.1% HCl for 2 seconds. The sections were then stained with eosin for 27 seconds and hematoxylin for 2.5 min. Finally, sections were dehydrated and mounted with DPX. Images were captured using MIRAX MIDI slide scanner microscope (Carl Zeiss) with a 10x objective and MIRAX Scan software.

Van Gieson staining was performed as previously described (40). Briefly, sections were stained with Weigert’s iron hematoxylin and rinsed with water, followed by staining in Van Gieson solution for 5 min. Then the sections were immersed in 90% and 100% ethanol with 6
drops of saturated picric acid for 1 min respectively. Finally, sections were immersed twice in xylene (5 min each) and mounted with DPX. Images were captured using MIRAX MIDI slide scanner microscope (Carl Zeiss) with a 10x objective and MIRAX Scan software.

**Immunohistochemistry**

Cryosections were fixed in 100% acetone at -20 ºC for 5 min and then rinsed with 1x PBS for 5 min. Antigen retrieval was performed only for Ki67 immunostaining. For antigen retrieval, sections were immersed in 95-100 ºC citrate buffer (pH 6.0) for 10 min and then cooled for 30 min. Next, sections were blocked with their respective blocking buffer for a specific incubation time in a humid chamber. Sections were then incubated with the respective primary and secondary antibodies as described above, with the exception of Mstn, which was detected using a streptavidin-conjugated tertiary antibody. Finally, sections were mounted with DAPI-containing mounting media (Vector Laboratories).

**Quantitative Analysis of Collagen Content**

Collagen content was measured using the Sirius Red/Fast Green Collagen Staining Kit (Chondrex, Inc.) as per the manufacturer’s instruction, with minor modifications.

**Isolation and Culture of Primary Mouse Keratinocyte**

Primary mouse epidermal keratinocytes were isolated from neonates (0–2 days old) according to previously described protocols, with slight modifications (30). Briefly, pups were decapitated with limbs and tail removed. A ventral cut was made from neck to tail and the whole skin was peeled off. Each skin was then submerged in 3 ml of cold dispase solution (0.5 U/ml)
overnight at 4 °C. Epidermis and dermis were separated the next day with fine forceps. Epidermis extracted from 5–8 pups were pooled and transferred to a 50-ml tube containing 10 ml of low-calcium (0.06 mM) and SFM keratinocyte medium (Invitrogen), supplemented with recombinant epidermal growth factor (0.4 ng/ml) and bovine pituitary extract (40 μg/ml). Epidermal keratinocytes were mechanically separated by vigorous shaking for 20 seconds. Cell clumps were then removed by passing the cell suspension through a 100-μm cell strainer. Cultures were maintained in a 5% CO₂ humidified incubator at 37°C. Medium was changed every 2 days and cells were subcultured upon reaching 70% confluence. For subculture, medium was removed followed by washing the cells with PBS. Trypsin (0.25%)/EDTA (1 mM) in PBS was added to the culture and incubated at 37 °C for 10 min, after which the flask was rapped gently to dislodge cells from the surface. To neutralize the action of trypsin, PBS containing 1% dialyzed fetal bovine serum was added, and the cells were collected by centrifugation at 160 × g for 5 min. The cell pellet was resuspended with fresh medium and subcultured in a new flask at 2.5 × 10³ cells/cm².

**In Vitro Scratch-Wound Assay**

The scratch-wound assay was performed as previously described (13). Briefly, when primary cultured keratinocytes reached confluence, a pipette tip was used to scratch a line followed by washing with PBS to remove scratched cells. Mitomycin C was added at a concentration of 2 μg/ml to inhibit cell proliferation prior to image capture at 5-min intervals over a total period of 12 h using a Carl Zeiss live imaging microscope.

**Cell Proliferation Assay**
To determine the cell proliferation rate of wild-type and Mstn-null primary keratinocytes, isolated keratinocytes were seeded at a density of $2.5 \times 10^3$ cells/cm$^2$. Cells were pulsed with BrdU (10 µl of 1 mM BrdU per 1 ml of medium) for 16 h, after which the cells were stained using the FITC BrdU Flow kit and quantified by flow cytometry (FACS), as per the manufacturer’s protocol (Becton Dickinson).

**Cell Adhesion Assay**

Primary keratinocytes of similar passage number (3-5 passages) were seeded onto a 96-well plate at a density of $2 \times 10^4$ cells/well. At the indicated time points, cells that were not adhered to the surface were removed by three washes with 1 × PBS. Attached cells were then fixed with 1% glutaraldehyde for 15 min followed by incubation with 0.05% crystal violet for 10 min at room temperature. After staining, excess dye was removed and the cells were then washed three times with 1 × PBS. The plate was subsequently dried at room temperature overnight, after which, 50 µl of 1% triton-X/PBS was added to each well to solubalize the dye. The absorbance of the crystal violet was read at OD$_{590nm}$.

**RNA Extraction and Quantitative Real-time PCR (qPCR)**

Total RNA was extracted from the skin wound biopsy using TRIzol reagent, as per the manufacturer’s instructions (Invitrogen). RNA was further purified using the RNeasy mini kit (Qiagen), prior to cDNA synthesis using the Superscript II First strand synthesis system (Invitrogen) from 1 µg of total RNA. qPCR was performed in duplicate in 10 µl reactions using Sso Fast Evagreen (Bio-Rad) in the CFX96 real-time PCR machine (Bio-Rad). Primer sequences used in this study are listed in Table 1.
Immunobloting (Western Blot)

Skin wound biopsies were homogenized in protein lysis buffer containing 50 mM Na$_2$H$_2$PO$_4$, 250 mM NaCl, 1% Triton X-100, and 0.1% SDS. This was followed by centrifugation at 12,000 × g for 15 min. The supernatant was collected and 10 µg of the extracted proteins were resolved by 4–12% NuPAGE (Invitrogen) followed by transfer onto a nitrocellulose membrane. Membranes were blocked with polyvinylpyrrolidone (PVP) blocker (1% PVP, 1% polyethylene glycol, and 0.3% bovine serum albumin in TBST) for 1 h. Membranes were incubated with primary and secondary antibodies in the PVP blocking solution overnight and for 1 h, respectively. Immunoreactivity was detected through enhanced chemiluminescence.

Statistical Analysis

The Mann-Whitney test was used for statistical analysis of cell migration and the adhesion assay. Two-tailed Student’s $t$-test was used for all other statistical significance determinations. $P < 0.05$ was considered statistically significant. Error bars represent ± SEM.
Results

Expression of Mstn is Restricted to the Epidermis, Dermis and Hair Follicles in Skin

As a first step towards understanding the role of Mstn in skin wound healing, we determined the expression of Mstn in skin. Our qPCR results showed that Mstn was expressed in skin, and that the expression level was relatively lower than in muscle (Figure 1A). Immunohistochemical analysis in mouse (Figure 1C) and human skin (Figure 1D) showed that Mstn was highly expressed in the epidermal layer while lower levels of Mstn expression were seen in the dermis and hair follicles. Consistent with the result from qPCR and immunohistochemical analysis, western blot analysis also revealed a detectable level of Mstn protein during skin wound healing (Figure 1B). The distribution of Mstn in skin suggests that Mstn may play an important role in epidermis and dermis during skin wound repair.

Delayed Wound Healing in Mstn-Null Mice

A significant difference in wound healing rate was observed between wild-type and Mstn-null mice (Figure 2A). Following full-thickness excisional wounding, the rate of wound healing was delayed in Mstn-null mice when compared to the wild-type mice as indicated by the wound area (Figure 2B). In particular, the wound area of Mstn-null mice was 1.78-, 5.56-, 23.39- and 5.13-fold larger than that of the wild-type mice at post-wounding days 3, 5, 7 and 10 respectively ($P < 0.05$). An approximate delay of 8 days in complete wound closure was observed in the Mstn-null mice as compared to the wild-type mice, with similar wound healing observed between Mstn-null mice on day 15 and wild-type controls on day 7 post-wounding.
To determine the reasons for delayed wound healing in Mstn-null mice, we performed histomorphometric analysis on the wounded skin biopsies at various time points (Figure 3A). As the rate of wound healing is mainly determined by the rate of re-epithelialization and the contractile action of myofibroblasts, we measured several parameters; including area and length of migratory tongues, thickness of epidermis, and the distance between the peri-wound hair follicles. The initial proliferation rate of keratinocytes is indicated by the area of migratory tongues. The area of migratory tongues on day 3 post-wounding in Mstn-null mice was significantly smaller than wild-type mice (wild-type versus Mstn-null: 73752.63 ± 5542.14 μm² versus 47978.37 ± 338.35 μm², \( P < 0.05 \)) (Figure 3B), implying slower proliferation of migratory tongues (keratinocytes) at the initial stage of wound healing. The length of migratory tongues in Mstn-null mice was also found to be significantly reduced when compared to wild-type mice on day 3 post-wounding (wild-type versus Mstn-null: 194.03 ± 21.7 μm versus 70.88 ± 2.06 μm, \( P < 0.05 \)) (Figure 3C). Smaller area and shorter length of migratory tongues in Mstn-null mice, during the initial stage of wound healing, is indicative of decreased migration of keratinocytes in Mstn-null mice. In addition to reduced keratinocyte proliferation, we also observed that the wounded epidermal region in the Mstn-null mice was significantly thicker than wild-type mice after 10 days post-wounding (wild-type versus Mstn-null: 68.82±2.39 μm versus 108.72±4.94 μm respectively, \( P < 0.01 \)) (Figure 3D). Taken together, these data suggest that there is delayed re-epithelialization in Mstn-null mice during skin wound regeneration. Next we assessed the extent of dermal wound contraction, as quantified by the distance between peri-wound hair follicles. As shown in Figure 3E, we observed a significant increase in the distance between
peri-wound hair follicles in Mstn-null mice (wild-type versus Mstn-null: \(1266.76 \pm 26.59 \, \mu m\) versus \(1560.57 \pm 7.03 \, \mu m, \, P < 0.01\)) at day 10 post-wounding (Figure 3E), which is indicative of reduced dermal wound contraction. In summary, we conclude that the slower wound healing observed in Mstn-null mice is a result of delayed re-epithelialization and wound contraction.

**Delayed Recovery to Normal Epidermal Thickness is due to Protracted Keratinocyte Proliferation in Mstn-Null Mice**

To determine whether Mstn affects the proliferation of keratinocytes, resulting in the observed delay in recovery to normal epidermal thickness, we next stained sections with anti-K6 antibody, which is used as a marker of the hyperproliferative regions of the wound biopsies. On day 7 post-wounding, the enhanced K6 expression coincided with the increase in epidermis thickness in Mstn-null mice. On day 10, K6 expression was dramatically higher in Mstn-null mice when compared to wild-type mice (Figure 4A). Ki67 is a molecular marker for proliferating cells, therefore, to detect whether or not there is protracted proliferation of cell populations in the wound site of Mstn-null mice, we performed Ki67 staining. As shown in Figure 4B, Mstn-null mice exhibited increased proliferative cells on day 15, whereas few Ki67 positive cells were detected in wild-type mice (Figure 4B). To further strengthen our findings, we performed qPCR analysis of Ki67 mRNA expression. On day 10, nearing the end of the skin wound healing period, we found relatively higher Ki67 expression in Mstn-null mice as compared with wild-type controls (Figure 4C); moreover, increased PCNA protein abundance was also observed in Mstn-null mice on day 10 post-wounding (Figure 4D), supporting the presence of increased numbers of proliferating cells in Mstn-null mice. To analyze keratinocyte proliferation *in vitro*, we assessed the proliferation rate of primary keratinocytes extracted from
wild-type and Mstn-null mice through BrdU staining and FACS analysis and also assessed the expression of markers associated with proliferation via Western blot. Consistent with enhanced proliferation, we observed a significant increase in the number of keratinocytes positive for BrdU (81.1% versus 55.7% of the 20,000 cells sampled using FACS) from Mstn-null mice when compared to wild-type keratinocytes, indicating that Mstn-null keratinocytes exhibit prolonged proliferation when compared to wild-type controls (Figure 5A). Furthermore, we also observed more than 2.5-fold increase in both PCNA and Cyclin D1 protein expression in Mstn-null keratinocytes when compared to wild-type (Figure 5B). Altogether, Mstn-null keratinocytes displayed protracted proliferation both in vitro and in vivo, which we suggest attenuates recovery of the epidermal layer to a normal thickness.

Delayed Migration and Impaired Cell Adhesion in Keratinocytes Isolated from Mstn-Null Mice

To analyze keratinocyte migration in further detail, we performed an in vitro scratch-wound assay (13) which mimics the in vivo keratinocyte migration during wound healing. As shown in Figure 5C, a significant delay in keratinocyte migration can be seen in Mstn-null cultures when compared to wild-type counterparts. Next we quantified the extent of keratinocyte migration by measuring the area of the cell culture dish remaining uncovered (analogous to the in vivo wound gap), at each time point, and expressing this as a percentage of 0 h. Subsequent quantification revealed a significant delay in keratinocyte migration in Mstn-null cultures, which was apparent at 3 h post-scratch wounding (with 80% and 95% of the gap area remaining, when compared to 0 h, for wild-type and Mstn-null respectively; P<0.05), and further exaggerated at 6, 9 and 12 h post-scratch wounding (Figure 5C&5D).
Keratinocyte adhesion is an important marker for migration, whereby reduced adhesion is indicative of slower migration and increased apoptosis. Keratinocytes isolated from Mstn-null mice displayed decreased cell adhesion, in an in vitro cell adhesion assay, with a significant reduction in cell adhesion initially observed at 30 min ($P<0.05$), and a sustained comparable reduction in cell adherence observed from 60 min onwards ($P<0.05$) (Figure 5E). Taken together these data suggest that Mstn-null keratinocytes exhibit a decreased migratory rate, which we suggest results in delayed re-epithelialization.

Elevated Decorin and Reduced TGF-β Expression in Mstn-Null Mice

It has been previously shown that TGF-β stimulates collagen deposition and extracellular matrix growth, leading to the accumulation of fibrotic tissue (33), fibroblast activation, and subsequent myofibroblast transformation (8, 39). Activated myofibroblasts are essential for wound contraction by pulling together the wound edges to complete the skin wound healing process. It has been reported that decorin can compete with TGF-β by binding to the TGF-β receptor or through directly interacting with TGF-β to restrict its bioavailability, therefore inhibiting TGF-β signaling (9, 34). To determine the interplay between TGF-β and decorin proteins and how they regulate wound healing in Mstn-null mice, we examined the mRNA and protein expression profiles of these proteins during wound healing. We found that TGF-β gene expression was reduced in Mstn-null mice at all time points during wound healing (Figure 6A), particularly at critical times when extracellular matrix deposition and myofibroblasts activation should normally occur. Moreover, Mstn-null mice showed consistently elevated decorin expression, as compared to wild-type mice, throughout the entire healing period, with the exception of day 15 post-wounding, where decorin levels were comparable between wild-type
and Mstn-null mice (Figure 6B). To confirm that the observed 43kDa protein band is decorin, we performed an additional western blot using samples in which high (M. gastrocnemius muscle) and low (serum) levels of decorin are present. As expected, we find reduced levels of decorin (43kDa) protein in serum when compared to skeletal muscle confirming antibody specificity (Data not shown). Coinciding with the increased expression of decorin, phospho-Smad2/3 (p-Smad2/3) expression decreased in Mstn-null mice during skin wound repair, which is indicative of reduced TGF-β signaling (Figure 6B).

### Mstn Deficiency Attenuated Dermal Contraction and Collagen Deposition

As described above, Mstn-null mice exhibit impaired dermal contraction during wound healing. To delineate the underlying mechanisms, we performed myofibroblast-specific staining using an antibody against α-smooth muscle actin (α-SMA), as well as Van Geison staining of collagen. During wound healing, fibroblasts are activated and are transformed into α-SMA-expressing myofibroblasts. Myofibroblasts are involved in both wound contraction, through intracellular contraction via integrin-mediated collagen fiber alignment (11, 14, 16), and extracellular collagen fiber deposition to strengthen the wound site (19, 48). As shown in Figure 7A, myofibroblasts were readily detected in the skin wound area of wild-type mice, as indicated by the intense α-SMA staining. However, in contrast, the level of α-SMA staining in the skin wound of Mstn-null mice was greatly reduced (Figure 7A).

In addition to analyzing α-SMA expression histologically, we next analysed the protein levels of α-SMA via western blot. As shown in Figure 7B, reduced α-SMA protein expression was observed in Mstn-null mice on day 7 post-wounding (Figure 7B), which is further consistent with reduced myofibroblast formation. Next we performed Van Geison staining to detect the
presence of collagen, and consistent with reduced α-SMA staining and reduced myofibroblast number, we also observed reduced collagen (indicated by the decrease in red colour observed in the dermis of the wound area, as well as through quantitative analysis of collagen content using Sirius Red/Fast Green collagen staining) in Mstn-null mice (Figure 7C&7D), implicating reduced wound contraction strength. Considering the crucial roles of myofibroblasts in dermis wound healing, these results may partially explain the delayed wound contraction in Mstn-null mice.

**Treatment of Mstn-Null Mice with TGF-β1 Improved Skin Wound Healing**

To further analyze the involvement of TGF-β signaling in Mstn-null mice skin wound healing, either recombinant TGF-β1 protein or vehicle control were topically applied onto the skin wound area immediately upon excisional wounding. As can be seen in Figure 8A, a significant difference in wound healing was observed between vehicle and TGF-β1 treated Mstn-null mice, with topical application of TGF-β1 leading to accelerated skin wound healing in Mstn-null mice. To determine the cause of the accelerated skin wound healing, histomorphometric analysis was performed on the wounded skin biopsies at various time points (Figure 8B). During wound healing, the recovery rate is primarily determined by re-epithelialization and the contractile action of myofibroblasts, therefore, we analyzed the thickness of the epidermis and the distance between the peri-wound hair follicles as parameters for assessing skin wound healing. On day 10 post-wounding, we observed that in Mstn-null mice treated with TGF-β1, the wounded epidermis was significantly thinner, when compared to vehicle treated Mstn-null mice (Vehicle versus TGF-β1: 920.53±38.33 μm versus 381.25±16.42 μm respectively, P < 0.001) (Figure 8C). In addition, a significant reduction in the distance between peri-wound hair follicles was
detected in Mstn-null mice treated with TGF-β1 (vehicle versus TGF-β1: 6918.19 ± 187.51 μm versus 5991.56 ± 61.16 μm, P < 0.01), at day 10 post-wounding (Figure 8D), which is indicative of increased dermal wound contraction in TGF-β1 treated Mstn-null mice, when compared with the vehicle treated mice. Taken together, these data suggest that TGF-β1 treatment during Mstn-null skin wound healing ameliorates the delayed epidermal re-epithelization and dermal wound contraction.
Here, we show for the first time that mice lacking Mstn exhibit delayed skin wound healing due to impaired epidermal re-epithelialization and dermal contraction. These two events are caused by a decrease in TGF-β signaling through elevated expression of decorin, which can either compete with TGF-β for binding to its receptor or physically bind to TGF-β itself, sequestering it in the ECM and preventing TGF-β/membrane receptor interaction. The present study provides a novel insight into the involvement of Mstn in skin wound healing, thus highlighting Mstn as a potential candidate involved in the pathology behind delayed wound healing.

We demonstrated that decreased re-epithelialization in Mstn-null mice was due to a reduction in TGF-β signaling. TGF-β1 has been shown to inhibit keratinocyte proliferation (1, 24, 37), which is further supported by the finding that keratinocytes exhibit enhanced proliferation in Smad3-null mice during wound healing (3). Interestingly, in vitro and in vivo studies have indicated that TGF-β1 promotes keratinocyte migration through up-regulation of integrin expression and enhanced integrin-mediated signaling (47). These findings support the hypothesis that TGF-β1 exhibits two disparate functions (i.e., inhibition of keratinocyte proliferation and promotion of keratinocyte migration) to regulate skin wound healing. In agreement, we show that by inhibiting TGF-β signaling, Mstn-null mice exhibit delayed epidermal thickness recovery as a result of protracted proliferation of keratinocytes. This was further validated by the abundance of proliferating cells in Mstn-null primary keratinocyte cultures. Protracted keratinocyte proliferation has also been shown to cause delayed wound healing in a previous publication (45). In addition, Mstn-null primary keratinocytes also exhibit a reduced migration rate during the initial stages of wound healing, which we suggest is due to
reduced TGF-β signaling. In agreement with our hypothesis, topical addition of exogenous TGF-β1 protein to the Mstn-null skin wound site resulted in enhanced epidermal thickness recovery, indicating rescue of protracted keratinocyte proliferation. Activation of TGF-β signaling has previously been shown to lead to increased integrin expression and enhanced integrin-mediated signaling in migrating epithelial tongues at the wound edge (47). Therefore, the observed decrease in keratinocyte migration in Mstn-null mice may be partly due to blockade of integrin expression/signaling through the repression of TGF-β1. Despite an increase in keratinocyte proliferation rate, Mstn-null mice exhibit a delay in wound healing primarily due to a slower keratinocyte migration rate, indicating that keratinocyte migration, as opposed to keratinocyte proliferation alone, is critically important for timely skin re-epithelialization.

An increase in decorin expression has previously been reported in the skeletal muscle of Mstn-null mice (26). Interestingly, we demonstrated that elevated decorin expression is also detected in the skin of these mice. Elevated levels of decorin, as well as reduced fibrosis have been reported in regenerated skeletal muscle of Mstn-null mice (26). McCroskery et al further suggested that this reduced fibrosis was due to decorin sequestering TGF-β1 (26). In agreement with this, the presence of decorin in the microenvironment can sequester the TGF-β latency complex to the ECM, thus preventing TGF-β-mediated signaling (21). Furthermore, our results demonstrate that Mstn deficiency in mice results in decreased expression of TGF-β and reduced phosphorylation of its downstream signal transducers (Smad2/3), concomitant with increased decorin expression. Moreover, we show here that topical administration of TGF-β1 to the Mstn-null skin wound site is able to overcome the delayed wound healing in these mice. Thus, we speculate that the delayed epidermal re-epithelialization and dermal contraction observed in Mstn-null mice may be due to decorin-mediated inhibition of TGF-β1. The inhibitory effect of decorin
on TGF-β1 signaling has been previously reported in human heart, where decorin is involved in reverse cardiac remodeling by directly inhibiting the TGF-β pathway and its pro-fibrotic effects on the failing heart (22). It is now well established that decorin and other small proteoglycans are able to form complexes with TGF-β (17). Moreover, ultrastructural studies have previously shown that the small proteoglycan decorin can also bind to collagen fibrils (32, 36). Purified decorin has also been demonstrated to bind to collagen type I with high affinity and specificity during in vitro fibrillogenesis (6), and in a solid phase assay (15), via an interaction between its core protein and collagen (31). The collagen binding site was later found to be located within two leucine-rich repeats comprising amino acids 152 & 201 of the decorin core protein (41). More importantly, the decorin core protein fragment Leu155-Val260 has been shown to interact with TGF-β but does not compete for decorin binding to type I collagen, thereby allowing the existence of a tripartite complex of TGF-β1, decorin, and collagen (34).

Myofibroblasts have been shown to have two main functions during wound healing. Firstly, myofibroblasts exert contractile strength and align collagen via integrins to contract wound edges (11, 14, 16), and secondly, deposit ECM collagen fibers to strengthen the wound (19, 48), thus, myofibroblast activation and differentiation is a crucial step in wound contraction. As shown in the present manuscript we found reduced myofibroblasts, as assessed through α-SMA expression, in Mstn-null mice when compared to wild-type mice during skin wound healing. TGF-β has been shown to be a critical factor in myofibroblast activation via its stimulatory action on fibroblast proliferation, myofibroblast differentiation, and matrix deposition (8, 33). Therefore, reduced TGF-β signaling may be responsible for the diminished myofibroblast differentiation and delayed dermal wound contraction observed in Mstn-null mice. To date, however, the mechanism by which TGF-β1 regulates myofibroblast activation
and differentiation remains unclear. Nonetheless, it has been proposed that TGF-β1 regulates these events in myofibroblasts via cell adhesion-dependent integrin signaling-mediated regulation of focal adhesion kinase (FAK). Specifically, it has been demonstrated that TGF-β stimulates the expression of integrin and fibronectin, thus leading to autophosphorylation/activation of FAK, which is essential for myofibroblast activation and differentiation (43). Thus we propose that reduced FAK, as a result of reduced TGF-β, may be responsible for the diminished myofibroblast activation and differentiation in Mstn-null mice during wound healing; current work is underway to confirm this.

While we find that Mstn-null mice show delayed skin wound healing due to slower re-epithelialization and dermal wound contraction, previously published data from our laboratory has revealed that there is enhanced skeletal muscle regeneration in Mstn-null mice (26). This contrasting wound healing response needs to be addressed in the context of tissue-specific differences. Enhanced skeletal muscle regeneration in Mstn-null mice was found to be due to increased satellite cell activation and proliferation, accelerated myoblast and macrophage migration and reduced fibrosis. The decreased fibrosis in regenerated Mstn-null muscles was due to increased expression of decorin and inhibition of TGF-β signaling (21, 26). Similar to skeletal muscle, increased decorin levels and reduced TGF-β signaling were also seen in the skin of Mstn-null mice undergoing regeneration. However, unlike the positive effect conferred by reduced TGF-β on skeletal muscle regeneration, reduced TGF-β signaling has a negative impact on skin wound healing, leading to reduced myofibroblast activation and differentiation as well as reduced collagen deposition, ultimately resulting in impaired wound contraction in skin.

Herein, we propose a mechanism whereby lack of Mstn in mice leads to increased decorin expression during skin wound healing. We propose that elevated decorin may either bind
to TGF-β, or compete with TGF-β to bind to its receptor, to block TGF-β downstream signaling.

Subsequent inhibition of TGF-β signaling will thus result in decreased keratinocyte migration, protracted keratinocyte proliferation, delayed re-epithelialization in the epidermis; as well as aberrant myofibroblast formation, reduced collagen protein secretion and finally delayed wound contraction in the dermis. Altogether, we propose that these events result in the delayed wound healing observed in Mstn-null mice, thereby highlighting a potential therapeutic role for Mstn agonists in the treatment of chronic and diabetic wounds (Figure 9).
Acknowledgments

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Disclosures

The authors declare no conflict of interest.


Figure Legends

Figure 1. Expression of Mstn is Restricted to the Epidermis, Dermis and Hair Follicles in Skin.

(A) Relative fold change of Mstn mRNA expression levels normalized to ribosomal protein L27 mRNA expression levels in the skin and muscle of wild-type mice as measured by qPCR. The normalized qPCR result for Mstn expression in skin was adjusted to a value of 1, with fold change in skeletal muscle Mstn expression calculated with respect to skin expression. (B) Mstn protein level in the skin of wild-type mice at specific time points during skin wound healing as determined by Western blot. Both full-length and mature Mstn are expressed in skin during wound healing. Representative images of Mstn immunostaining on wild-type skin in mouse (C) and human (D). Mstn (green)-immunostained cryosections were counterstained with DAPI (nuclei; blue). Arrows indicate Mstn positive cells on the epidermis, hair follicles and dermis. Negative control (Non-primary antibody control) images indicate unspecific staining. White dashed lines demarcate the epidermis from the dermis. Scale bar: 100µm. **P < 0.01. Error bars represent ± SEM, n = 5.

Figure 2. Delayed Wound Healing in Mstn-Null Mice.

(A) Images of skin wounds at different time points after excisional wounding. Mstn-null mice exhibited delayed wound healing. Small divisions of ruler = 1mm. (B) Wound healing analysis of Mstn-null mice and their wild-type littermates. Average wound area was presented as percentage of the wound area on day 0 (100%). *P < 0.05, **P < 0.01. Error bars represent ± SEM, n = 5.
Figure 3. Slower Wound Healing in Mstn-Null Mice is Due to Delayed Re-Epithelialization and Wound Contraction.

(A) H&E staining of wild-type and Mstn-null mice wound biopsies on day 3 and day 10 post-wounding. White dashed lines demarcate the epidermis from the dermis. Scale bar: 100µm. (B) Area of migratory tongues on day 3 post-wounding, presented in µm². (C) Length of migrating tongue on day 3 post-wounding. (D) Epidermis thickness on day 10 post-wounding. (E) Distance between the peri-wound hair follicles on day 10 post-wounding, as a measure of the extent of wound contraction. *P < 0.05, **P < 0.01. Error bars represent ± SEM, n = 5.

Figure 4. Delayed Recovery to Normal Epidermal Thickness is Due to Protracted Keratinocyte Proliferation in Mstn-Null Mice.

(A) K6 immunofluorescence on wild-type and Mstn-null mice wound biopsies on day 7 and day 10 post-wounding. Higher magnification images are presented on the right panel for each time point. K6 (red)-immunostained cryosections were counterstained with DAPI (nuclei; blue). Negative control (Non-primary antibody control) images show unspecific staining of the stratum corneum. (B) Ki67 immunofluorescence on wild-type and Mstn-null mice wound biopsies on day 15 post-wounding. Higher magnification images are presented on the right panel. Unmerged DAPI and Ki67 stainings are also displayed in higher magnification. Ki67-positive cells are indicated by white arrows. Ki67 (green)-immunostained cryosections were counterstained with DAPI (nuclei; blue). Area with non-specific staining is indicated by negative control (Non-primary antibody control). White dashed lines demarcate the epidermis from the dermis. Scale bar: 50µm (20 µm in higher magnification images). White arrowheads indicate wound edges and regions between arrows represent wounded area. White boxes indicate the regions presented in
higher magnification. (C) Relative fold change of Ki67 mRNA expression levels normalized to ribosomal protein L27 mRNA expression levels in the skin at day 10 post-wounding, as measured by qPCR in wild-type and Mstn-null mice. (D) PCNA protein expression levels in the skin of wild-type and Mstn-null mice at day 10 post-wounding as determined by Western blot. Numbers under images indicate the fold changes of band density normalized to the wild-type, which was assigned the value 1. The levels of α-tubulin were assessed to ensure equal loading of samples. *$P < 0.05$. Error bars represent ± SEM, $n = 5$.

**Figure 5. Increased Proliferation, Delayed Migration and Impaired Adhesion in Keratinocytes Isolated from Mstn-Null Mice**

(A) Cell proliferation analysis as measured through BrdU incorporation and FACS analysis in wild-type and Mstn-null primary keratinocytes. (B) Western blot analysis of the indicated proliferation markers from wild-type and Mstn-null primary keratinocytes. Values below the bands represent mean fold-difference in protein expression levels when compared with wild-type, which was assigned the value 1. Beta-tubulin was assessed to ensure equal loading of samples. (C) Representative time-lapsed images of scratch-wounded primary keratinocyte cultures treated with mitomycin C (2 μg/ml), showing the cell migration kinetics of wild-type and Mstn-null primary keratinocytes at the indicated time points after scratch-wounding. Yellow dotted lines demarcate the scratch gap at the time of wounding. (D) The graph shows the area remaining to be covered by the migrating keratinocytes expressed as a percentage of 0 h (100%) in vitro wound gap area. (E) Absorbance of crystal violet stain (OD$_{590nm}$) at the indicated time points after $2 \times 10^4$ wild-type and Mstn-null primary keratinocytes were seeded. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent ± SEM, $n = 4$. 

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Figure 6. Elevated Decorin and Reduced TGF-β Expression in Mstn-Null Mice.

(A) Relative fold change of TGF-β1 mRNA expression normalized to ribosomal protein L27 mRNA expression in the skin at specific time points during skin wound healing, as measured by qPCR in wild-type and Mstn-null mice. (B) Decorin and p-Smad2/3 protein expression levels in the skin of wild-type and Mstn-null mice at specific time points during skin wound healing, as determined by Western blot. The 49kDa molecular weight marker of the decorin Western blot is indicated. Numbers under images indicate the fold changes of band density normalized to the first band (Non-wounded, wild-type). The levels of α-tubulin were assessed to ensure equal loading of samples. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars represent ± SEM, n = 5.

Figure 7. Mstn Deficiency Attenuated Dermal Contraction and Collagen Deposition.

(A) α-SMA immunofluorescence on wild-type and Mstn-null mice wound biopsies on day 7 after wounding. Higher magnification images are presented on the right panel. α-SMA (red)-immunostained cryosections were counterstained with DAPI (nuclei; blue). White dashed lines demarcate the epidermis from the dermis. Scale bar: 50µm (20 µm in higher magnification images). White arrowheads indicate wound edges and regions between arrows represent wounded area. White boxes indicate the regions of higher magnification images. (B) α-SMA protein expression levels in skin of wild-type and Mstn-null mice on day 7 post-wounding, as determined by Western blot. Numbers under images indicate the fold changes of band density normalized to wild-type, which was assigned the value 1. The levels of α-tubulin were assessed to ensure equal loading of samples. (C) Van Geison staining of wild-type and Mstn-null mice wound biopsies on day 7 after wounding. Higher magnification images are presented on the right.
panel. White dashed lines demarcate the epidermis from the dermis. Scale bar: 200µm (20 µm in higher magnification images). Black arrowheads indicate wound edges and regions between arrows represent wounded area. Black boxes indicate the regions of higher magnification images. (D) Quantitative analysis of collagen content in skin wound biopsy sections on day 7 post-wounding. **P < 0.01. Error bars represent ± SEM, n = 5.

Figure 8. Treatment of Mstn-Null Mice with TGF-β1 Improved Skin Wound Healing

(A) Images of skin wounds at day 7, 10 and 15 after excisional wounding. Mstn-null mice treated topically with exogenous TGF-β1 exhibited improved wound healing. Small divisions of ruler = 1mm. (B) H&E staining of TGF-β1 or vehicle treated Mstn-null mice wound biopsies on day 10 post-wounding. Black dashed lines demarcate the epidermis from the dermis. Scale bar: 100µm. (C) Epidermis thickness on day 10 post-wounding. (D) Distance between the peri-wound hair follicles on day 10 post-wounding, as a measure of the extent of wound contraction. **P < 0.01, ***P < 0.001. Error bars represent ± SEM, n = 5.

Figure 9. Proposed Mechanism.

In Mstn-null mice, increased levels of decorin bind to TGF-β and either sequesters it into the ECM, preventing TGF-β receptor binding, or competes with TGF-β for binding to its receptor. In the epidermis, reduced TGF-β signaling results in reduced keratinocyte migration, protracted keratinocyte proliferation, and delayed epidermis thickness recovery, leading to delayed re-epithelialization. In the dermis, impaired TGF-β signaling results in less myofibroblast formation and collagen deposition, leading to delayed wound contraction in the dermis. Overall, absence of Mstn results in delayed epidermis and dermis wound healing.
Table 1. Primer sequences used in qPCR

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Figure 1

A. Bar graph showing the relative fold change of Mstn expression in skin and muscle.

B. Western blot analysis of Mstn expression over time (days post-wounding) for non-wounded and wounded conditions.

C. Immunofluorescence images of Mstn expression in mouse and negative control tissues.

D. Immunofluorescence images of Mstn expression in human and negative control tissues.

Legend:
- **: Significant difference
- skin, muscle
Figure 2

(A) Wound area (% of day 0) over different days post-wounding for wild-type and Mstn-null mice. The graphs show a statistically significant decrease in wound area over time, with Mstn-null mice having a faster healing rate.

(B) Graphs showing the decrease in wound area over time for wild-type and Mstn-null mice. The y-axis represents wound area (% of day 0), and the x-axis represents days post-wounding. The graphs indicate a faster healing rate for Mstn-null mice compared to wild-type mice.
Figure 3

A

Day 3

Day 10

wild-type

Mstn-null

B

C

D

E

Area of migratory tongues (µm²)

Migratory tongue length (µm)

Epidemis thickness (µm)

Wound contraction (Distance between the periwound hair follicles in µm)

**

**

**
Relative fold change (Ki67)
Figure 5

A

B

C

D

E

Figure 5

A

B

C

D

E
Relative fold change (TGF-β1)

Days post-wounding

B

days post-wounding

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49 kDa
Figure 7

A

Day 7

wild-type

Mstn-null

B

days post-wounding

7

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D

Collagen content (μg/section)

[bar chart with wild-type and Mstn-null comparisons]

C

Day 7

wild-type

Mstn-null
Figure 8

A

Mstn-null+vehicle  Mstn-null+TGFβ

Day7

Day10

Day15

B

Mstn-null+vehicle  Mstn-null+TGFβ

Day10

C

Epidemis thickness (μm)

0 200 400 600 800 1000 1200

Mstn-null + vehicle  Mstn-null + TGFβ

***

D

Wound contraction (Distance between the Peri-wound hair follicles in μm)

5400 5600 5800 6000 6200 6400 6600 6800 7000 7200 7400

Mstn-null + vehicle  Mstn-null + TGFβ

**
Proposed mechanism of skin regeneration in Mstn-null mice

- TGF-β and Decorin
  - Reduced migration rate and protracted keratinocyte proliferation
  - Delayed epidermis thickness recovery
  - Delayed re-epithelialization

- Dermis wound healing
  - Less myofibroblast formation and collagen deposition
  - Delayed wound contraction
  - Delayed wound healing