INHIBITION OF MYOSTATIN: A NEW APPROACH TO IMPROVE MUSCLE HEALING AFTER INJURY

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Introduction:

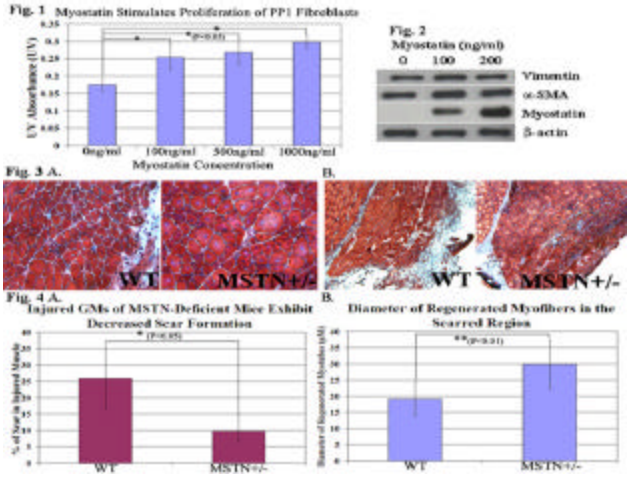
Skeletal muscle injuries are the most common injuries encountered in sports medicine. Muscle injuries can heal spontaneously via regeneration, but fibrosis impedes this process and results in incomplete functional recovery. We have demonstrated that TGF-β1 plays a significant role in both the initiation of fibrosis and the inducement of myofibroblastic differentiation by myogenic cells in injured muscle [1]. Additionally, we have shown that the use of antifibrosis strategies that neutralize TGF-β1 production can enhance muscle regeneration and, consequently, improve muscle healing [2,3]. The recent identification of myostatin (MSTN), a member of the TGF-β superfamily, may yield new avenues for therapeutic intervention to improve muscle regeneration [4]. MSTN is found almost exclusively in skeletal muscle [4]. Genetic manipulations of MSTN expression (e.g., via knockout, mutation, and blockade of signaling pathways) have significantly increased the skeletal muscle mass of transgenic mice, resulting in either hypertrophy or a combination of hypertrophy and hyperplasia [4,5]. Research has shown that MSTN acts primarily to inhibit skeletal muscle development, but the possible role of MSTN in fibrosis after injury remains unclear. We hypothesized that MSTN stimulates fibrosis during skeletal muscle healing. We performed this study to investigate the effects of MSTN on fibroblasts *in vitro* and to evaluate if partial loss of MSTN expression *in vivo* would improve injured muscle healing by enhancing regeneration and reducing fibrosis.

Methods:

Proliferation assay and Western blot analysis: Muscle-derived fibroblasts (PP1) were plated onto collagen-coated 96-well plates for analysis of cell proliferation and onto 6-well plates for evaluation of α-smooth muscle actin (α-SMA), vimentin and MSTN expression. The cells then were cultured for 48 hours in serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) containing serum replacement (Sigma) and different concentrations of human recombinant MSTN (0ng/ml, 100ng/ml, 500ng/ml, or 1000ng/ml for proliferation assay; 0ng/ml, 100ng/ml, or 200ng/ml for Western blot; Lenico Technologies, Inc.). An MTT cell proliferation assay kit (Roche Diagnostics) was used to measure cell proliferation, and Western blot was used to examine α-SMA, vimentin, and MSTN expression. *Injury model:* All experiments in this study were in accordance with research protocols approved by the ARCC of Children's Hospital of Pittsburgh. MSTN-deficient mice (heterozygotes [$MSTN^{+/-}$]) and C57BL/6 wild-type mice (control) were used to establish an injury model of skeletal muscle laceration. The mice were anesthetized, and a surgical blade (no. 11) was used to lacerate the gastrocnemius muscle (GM) of each leg at the largest diameter through the lateral 50% of the muscle width and 100% of the muscle thickness. GMs of both $MSTN^{+/-}$ and wild-type mice were harvested 2 weeks after laceration. Masson's Trichrome staining (nuclei [black], muscle [red], collagen [blue]) was performed to identify fibrotic scar tissue in the injured muscles. Northern Eclipse software (Empix Imaging, Inc.) was used to measure areas of fibrotic tissue and regenerated muscle within the scar tissue. Student's *t*-test was used to determine significance ($p<0.05$).

Results:

MSTN stimulates muscle-derived fibroblasts (PP1) to proliferate and express fibrosis-related proteins: In comparison with non-stimulated PP1 fibroblasts, PP1 fibroblasts stimulated with MSTN exhibited significant dose-dependent increases in proliferation (Fig. 1). Moreover, Western blot revealed that MSTN treatment of PP1 fibroblasts elevated their expression of fibrosis-related proteins, including α-SMA and vimentin (Fig. 2). *Autocrine expression of MSTN*: PP1 fibroblasts cultured in normal medium did not express detectable levels of MSTN. However, PP1 fibroblasts incubated with medium containing various concentrations of MSTN secreted high levels of MSTN protein. This finding suggests that MSTN stimulation may lead to autocrine expression of MSTN by PP1 fibroblasts (Fig. 2). *Improved muscle healing in MSTN-deficient mice after GM laceration:* Figure 3 shows regenerating myofibers, identifiable by their centralized nuclei, in wild-type and $MSTN^{+/-}$ mice. The healing muscles of the $MSTN^{+/-}$ mice contained larger myofibers than did those of wild-type mice (Fig. 3A). The significantly larger mean diameter of fibers observed within the muscles of the $MSTN^{+/-}$ mice (Fig. 4B) suggests enhanced muscle regeneration in these mice compared with wild-type mice. Moreover, Masson's Trichrome histochemistry showed significantly less fibrotic scar tissue in the GMs of $MSTN^{+/-}$ mice than in the GMs of wild-type mice (Figs. 3B and 4A).



Discussion:

Prior research has demonstrated that MSTN has a negative effect on muscle growth. Researchers have observed that the muscles of *mdx* mice (an animal model for Duchenne muscular dystrophy) with MSTN gene knockout ($MSNT^{-/-}$/*mdx*) contain regenerating myofibers with larger diameters than those of myofibers found in the muscles of *mdx* mice ($MSNT^{+/+}$/*mdx*). It is particularly important to note that these $MSNT^{-/-}$/*mdx* mice exhibited decreased fibrosis [6]. These results strongly suggest that MSTN plays an important role in fibrosis after skeletal muscle injury.

Our study demonstrates that MSTN stimulation induced greater proliferation and the expression of fibrotic proteins (vimentin and α-SMA) by PP1 fibroblasts. Furthermore, PP1 fibroblasts cultured in the presence of MSTN exhibited autocrine expression of MSTN. These results suggest that fibroblasts activated after muscle injury exhibit autocrine expression of MSTN, which in turn promotes myofibroblastic differentiation of myogenic cells. Our *in vivo* studies revealed that the muscles of $MSTN^{+/-}$ mice displayed significantly less fibrotic scar tissue and significantly more hypertrophic regenerating myofibers 2 weeks after laceration than did normal controls. These findings are consistent with results from previous studies showing that either blocking MSTN or neutralizing TGF-β1 results in similar beneficial effects on muscle healing after injury [4]. Our future studies will investigate if TGF-β1 causes fibrosis by elevating MSTN expression and if TGF-β1 and MSTN share certain signaling pathways. Results of these studies will improve our understanding of the mechanisms of fibrosis in injured skeletal muscle.

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