Follistatin Antagonizes Myostatin-mediated Promotion of Tenocyte Differentiation in Mesenchymal Stem Cells

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INTRODUCTION: Myostatin, also known as growth differentiation factor (GDF-8), is an inhibitor of myoblast proliferation and skeletal muscle growth. We previously reported that myostatin negatively regulate myogenesis and promotes tenogenic differentiation of myoblast cell line C2C12 through Smad3.[1] Follistatin also antagonizes myostatin; however, its effects on tenogenic differentiation are unclear. This study aimed to examine whether myostatin can promote tenogenic differentiation of murine pluripotent mesenchymal stem cell line C3H10T1/2 more efficiently than other growth factors and if follistatin can inhibit tenogenic differentiation by antagonizing myostatin.

METHODS: *Cell culture:* The Murine pluripotent mesenchymal stem cells C3H10T1/2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fatal bovine serum (FBS) and antibiotics. To induce tenocyte differentiation, the cells were starved for 12 h in DMEM supplemented with 1% FBS and then treated with 25 ng/ml of recombinant mouse GDF-7, myostatin, transforming growth factor (TGF-β) 1, 2, 3 and basic fibroblast growth factor (bFGF) (R&D Systems) in starving media. To examine the effects of follistatin on tenocyte differentiation, recombinant mouse follistatin (R&D Systems) was added tenocyte differentiation media at various concentrations.

siRNA Transfections: siRNA transfections were carried out using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. C3H10T1/2 cells were transfected with Foristatin-siRNA (Silencer Select Pre-Designed siRNA s201362, Thermo Fisher Scientific). The effects of target-specific siRNA were confirmed by real-time PCR for foristatin at 24 h after siRNA transfection. Cells were subsequently treated with 25 ng/ml GDF-8 for 7 days prior to harvest for RT-qPCR.

Real-time PCR quantification: Cells were collected on day 7. RNA was isolated from samples by using RNeasy Mini kit (Qiagen) and quantified using a NanoDrop 2000 (ThermoFischer Scientific). RNA was reverse-transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR was carried out on the StepOnePlus real-time PCR system (Applied Biosystems) using TaqMan Gene Expression Master Mix (Applied Biosystems) to compare the relative expression levels of Scrélaxis (SCX), Mohawk (MKX) Tenomodulin (TNMD), Collagen Type I alpha 1 (COL1A1), and Tenascin-C (TN-C). The relative mRNA expression levels of target genes were calculated as fold changes of threshold cycle (Ct) value relative to reference using the $2^{-\Delta\Delta Ct}$ method. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as the reference gene.

Immunofluorescence: The cells were fixed with 4% paraformaldehyde in PBS. The fixed cells were incubated overnight at 4℃ with anti-tenomodulin antibody (Santa Cruz Biotechnology). Subsequently, cells were stained with Alexa Fluor 488 and 588, goat anti-rabbit IgG secondary antibody (Life Technologies) at room temperature for 1 h. Then, cells were mounted with the Vectashield HardSet Mounting Medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Statistical analysis: The data are presented as means ± standard errors of the means (SEM). Comparisons were made using a t test to determine the significance of differences between two groups. For multiple comparisons, differences between means were determined by a one-way ANOVA coupled with Dunnet's, Williams' or Tukey's post hoc tests.

RESULTS SECTION: To examine whether myostatin induces differentiation of C3H10T1/2 into tenogenic lineage cells more efficiently than other growth factors, C3H10T1/2 cells were treated with GDF-7, myostatin, TGF-β 1, 2, 3, and bFGF. Although the expressions of SCX and MKX in the cells treated with TGF-β 1, 2, 3 were higher than the cells treated with myostatin, increase in expression of TNMD was only seen in the myostatin treated cells (Figure 1). Immunofluorescence showed that TNMD was highly expressed when treated with myostatin (Figure 2). Next, we investigated the effect of follistatin on tenogenic induction by myostatin. When C3H10T1/2 cells were treated with myostatin in the presence of follistatin, the expression of tenogenic markers were inhibited in dose dependent manner (Figure 3). On the contrary, follistatin-siRNA increased TNMD expression in C3H10T1/2 cells treated with myostatin.

DISCUSSION: This study revealed that myostatin induces C3H10T1/2, which is even more undifferentiated than C2C12, into tendon cells more efficiently than other growth factors and that tendon cell differentiation is inhibited by the antagonistic action of follistatin. Since both myostatin and follistatin have been confirmed to be expressed in tendon,[2-4] it is possible that both play an important role in the tendon healing process. The limitation of our study is that all of our experiments were conducted only *in vitro* cell culture. Further *in vivo* experiments are needed to verify the actual roles of myostatin and follistatin, especially in tendon healing.

SIGNIFICANCE/CLINICAL RELEVANCE: Further development of this research will clarify the roles of myostatin and follistatin in tendon healing, which may lead to the development of new tendon healing promotion technologies that control these growth factors.

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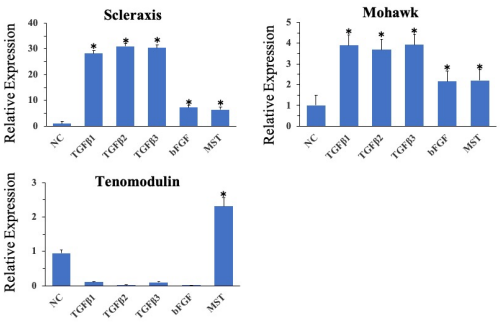

Figure1. Rerative expression of tenogenic makers. MST: myostatin, ＊: p<0.05

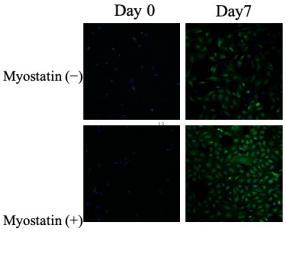

Figure 2. Immunofluorescence staining of tenomodulin

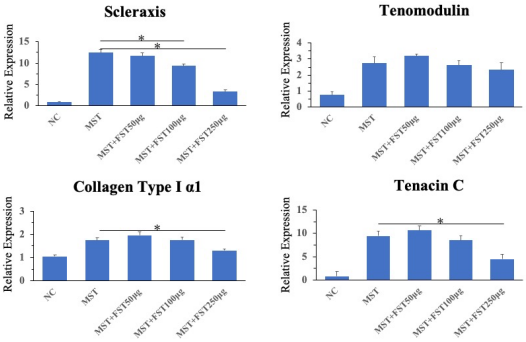

Figure3. Rerative expression of tenogenic makers. MST: myostatin, FST: follistatin. ＊: p<0.05