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Stat3 Mediates the Function of mTORC1 in Fibrovascular Scar Formation During Postnatal Tendon Development

Introduction

Tendon injuries are challenging clinical problems due to slow, incomplete healing with fibrovascular scar formation, which reduces tendon function and causes chronic complications such as pain and tendon ruptures. The limited understanding of the regulatory mechanisms underlying fibrovascular scar formation hinders the development of effective treatment modalities for tendon diseases. Our recent study showed that constitutive activation of mTORC1 signaling during postnatal tendon development caused fibrovascular scar-like phenotypes in tendons, including disorganized extracellular matrix (ECM), high cellularity, and neovascularization¹. However, the downstream mechanism mediating mTORC1 function in fibrovascular scar formation is not clear.

Stat3 is a transcription factor and plays a crucial role in fibrosis and inflammation via the regulation of cell proliferation and ECM organization². Interestingly, a previous study showed that Stat3 can be activated by mTORC1 signaling³. This study aims to determine if Stat3 is a mediator of mTORC1 function in fibrovascular scar formation in tendons.

Methods

All procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. To genetically determine Stat3 as a mediator of mTORC1 function in fibrovascular scar formation in tendons, we performed a genetic rescue experiment by generating three types of the tendon-specific deficient mouse: 1) *Scx-Cre; Tsc1^{fl/fl}* (tendon-specific mTORC1 gain-of-function mouse model), 2) *Scx-Cre; Stat3^{fl/fl}* (tendon-specific Stat3 knockout mouse model), and 3) *Scx-Cre; Tsc1^{fl/fl}; Stat3^{fl/fl}* (tendon-specific Tsc1 and Stat3 double knockout mouse model for rescue experiment). Histological analyses were conducted on patellar and Achilles tendons at one month of age. RNA sequencing analysis was used to examine gene expression changes in Achilles tendons of wildtype and *Scx-Cre; Tsc1^{fl/fl}* mice. Primary tenocytes were isolated from tail tendon to perform *in vitro* molecular studies using monolayer cell culture. A western

blotting experiment was performed to examine the alteration in phosphorylated protein *in vitro*. All quantitative data were analyzed using student's t-test.

Results

Fibrovascular scar-like phenotypes in *Scx-Cre; Tsc1^{fl/fl}* (mTORC1 gain-of-function mouse model) prompted us to examine the transcriptional changes of fibrotic markers and metalloproteinases (*Mmps*). Consistent with histological data, *Scx-Cre; Tsc1^{fl/fl}* mouse exhibited an increased expression of *Col3a1*, *Fibronectin1* (*Fn1*), *Tenascin C* (*Tnc*), and *Metalloproteinases* (*Mmps*) in tendon, which are highly expressed in pathogenic tendon conditions such as tendon repair and tendinopathy (Figure 1). These data suggest the involvement of mTORC1 in the transcriptional gene regulation during fibrovascular scar formation.

Stat3 is involved in transcriptional gene regulation, and mTORC1 can activate Stat3 via phosphorylation of serine-727 (S727). We performed western blot analysis using primary tendon cells from wildtype and *Scx-Cre; Tsc1^{fl/fl}* to determine if mTORC1 can activate Stat3 in tendons. The serine-727 phosphorylation of Stat3 (Stat3 S727), which is mTOR dependent, was significantly increased in tendon cells from *Scx-Cre; Tsc1^{fl/fl}* mouse. The mTORC1 independent tyrosine-705 phosphorylation of Stat3 (Stat3 Y705) tended to increase in *Scx-Cre; Tsc1^{fl/fl}* cells (Figure 2). These results suggest that Stat3 can be a downstream target of mTORC1 in tendons.

To genetically confirm that Stat3 is a downstream mediator of mTORC1 function in fibrovascular scar formation, we performed a genetic rescue experiment in which we tested if the deletion of Stat3 can rescue fibrovascular scar-like phenotypes caused by constitutive activation of mTORC1 signaling. We first generated the Stat3 loss-of-function mouse model (*Scx-Cre; Stat3^{fl/fl}*) to determine the function of Stat3 in tendon development. *Scx-Cre; Stat3^{fl/fl}* mouse showed normal growth in tendons (Figures 3A and 3B, second panel). We then generated a conditional double knockout mouse (*Scx-Cre; Tsc1^{fl/fl}; Stat3^{fl/fl}*) model to perform a

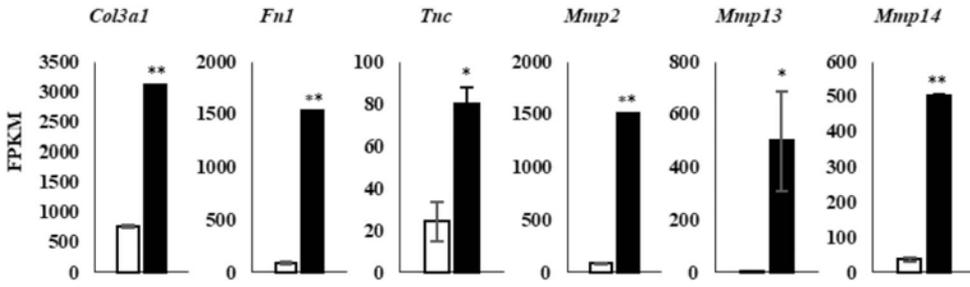


Figure 1. Transcriptome analysis of tendon from Gain-of-mTOR (*Scx-Cre; Tsc1^{fl/fl}*) mouse. FPKM (Fragment Per Kilobase of transcript per Million mapped reads) values of RNA-seq data from Achilles tendon of wildtype and *Scx-Cre;Tsc1^{fl/fl}* mice in *Col3a1*, *Fibronectin1 (Fn1)*, *Tenascin C (Tnc)*, and *Metalloproteinases (Mmps)*. (White bar indicates wildtype mice, Black bar indicates *Scx-Cre; Tsc1^{fl/fl}* mice, *indicate $P < 0.05$ and **indicate $P < 0.001$ between genotypes, n = 3).

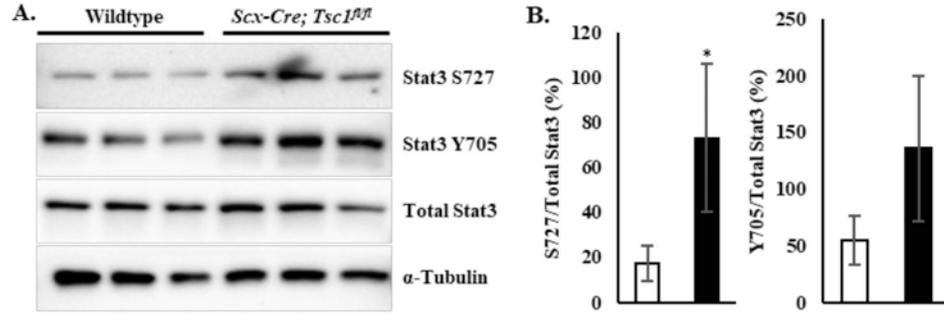


Figure 2. Gain-of-mTOR activated Stat3 signaling in primary tendon cells. Western blots to assess Stat3 signaling, phosphorylation of S727 is mTOR dependent and phosphorylation of Y705 is mTOR independent, activation in *Scx-Cre; Tsc1^{fl/fl}* cells (A). Quantification of the western blot bands (B). (White bar indicates wildtype cells, Black bar indicates *Scx-Cre; Tsc1^{fl/fl}* Cells, *indicate $P < 0.05$ between genotypes, n = 3).

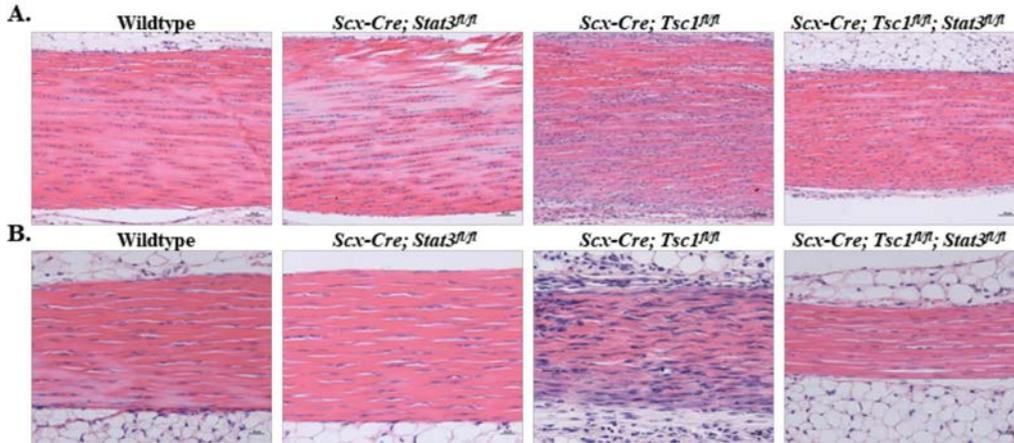


Figure 3. Tendon phenotype of wildtype, *Scx-Cre; Stat3^{fl/fl}*, *Scx-Cre; Tsc1^{fl/fl}*, and *Scx-Cre; Tsc1^{fl/fl}; Stat3^{fl/fl}* mouse at 1 month old. H&E stained Achilles tendon section (A) and patellar tendon section (B).

genetic rescue experiment. Very interestingly, our histological analysis showed that the deletion of Stat3 noticeably rescued the fibrovascular scar-like phenotypes in the mTORC1 gain-of-function mouse (Figures 3A and 3B, third and fourth panel). This data strongly supports our hypothesis that Stat3 mediates mTORC1 function in tendon fibrovascular scar formation.

Discussion

Our study suggests that mTORC1 can be a major biological mechanism regulating fibrovascular scar formation in pathogenic tendon conditions. Our cell and mouse genetic data strongly support that Stat3 is a mediator of mTORC1 function in fibrovascular scar formation. Further molecular study will be required to confirm that the direct molecular interaction between mTORC1 and Stat3. We only tested our hypothesis using the developmental model. Further investigations with healing or repair models will be necessary to confirm the precise function of mTOR/Stat3 signaling in fibrovascular scar formation during tendon healing.

Significance

This study will contribute to the understanding of regulatory mechanisms for fibrovascular scar formation, which may provide the basis and therapeutic approach for pathogenic tendon conditions such as tendon injury repair and tendinopathy.

Acknowledgements

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References

1. Lim+, 2017
2. Kasembeli+, 2018
3. Saleiro+, 2015