The Regulation of Tenocyte Differentiation and Morphological Maturation by mTORC1

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Introduction

Tenocytes drive postnatal tendon growth and healing via extracellular matrix (ECM) production and organization. Tenocyte differentiation is a multistep process that requires specific gene expression and unique morphological maturation. Tendon progenitors are marked by the expression of *Scleraxis* (*Scx*), a basic helix-loop-helix (bHLH) transcription factor and differentiated tenocytes express a high level of type I collagen.[1-4] Besides molecular changes, tenocytes undergo unique morphological maturation. Specifically, tendon cells in early postnatal mice are rounder whereas cells in adult mice are elongated to align longitudinally between dense and highly organized collagen fibers.[5,6] Although cellular maturation process of tenocytes is pivotal for tendon ECM maturation and the structural/mechanical properties of tendon, the current paradigm underlining cellular maturation of tenocytes is not fully understood. The objective of this study is to determine the function of mTORC1 signaling in cellular maturation of tenocytes.

Methods

All procedures were approved by UPenn's IACUC.The assessment of morphological changes of tendon cells is challenging because tendon cells assemble into a complex cellular and ECM network that is difficult to quantify accurately without time-consuming electron microscopy. To overcome this limitation, we developed a novel method by which we can measure cell density, cell size, cell shape, and protrusion numbers at a single cell level by using a high-resolution confocal imaging technique with Zo-1 and Phalloidin double immune staining. To further measure the morphological changes in tendon cells, we measured the nuclear aspect ratio (ratio of long and short axes of the nucleus) that indicates the longitudinal shape of tendon cells. To test the effect of genetic manipulation of mTORC1 signaling on cellular maturation, we generated a tendon-specific mTORC1 loss-of-function mouse (*Scx-Cre;Raptor$^{fl/fl}$*) and gain-of-function (*Scx-Cre; Tsc1$^{fl/fl}$*) mouse model.To exam the function of mTORC1 in the differentiation of tendon progenitors into the *Col1(2.3)-GFP-positive cells*, we generated *Scx-Cre; Raptor$^{fl/fl}$; Col1 (2.3)-GFP* (loss-of-function) and *Scx-Cre; Tsc1$^{fl/fl}$; Col1 (2.3)-GFP* (gain-of-function) mouse models. To perform pharmacological rescue experiment, rapamycin, an inhibitor of mTORC1 signaling, was injected into the control and gain-of-function mouse from day 22 to 29 after birth with the dosage of 4mg/kg. All quantitative data were analyzed using students t-test.

Results

Fluorescent imaging analysis showed that loss of mTORC1 increased Col1-2.3GFP-positive cells in patellar tendon at all postnatal developmental stages (Figure 1A) and this phenotype was verified by quantification (Figure 1B). This result suggests that inhibition of mTORC1 enhances the differentiation of tendon cells into the Col1a1-expressing tenocyte population. This enhanced differentiation phenotype in loss-of-function mouse model prompted us to investigate morphological maturation of tenocytes in mTORC1 loss-of-function mouse model. To investigate the morphological maturation of tendon cells, we analyzed tenocyte morphology at a single-cell level using Zo-1 and Phalloidin double immune staining (Figure 2A). Tendon cells displayed the largest cell area at P30, and the

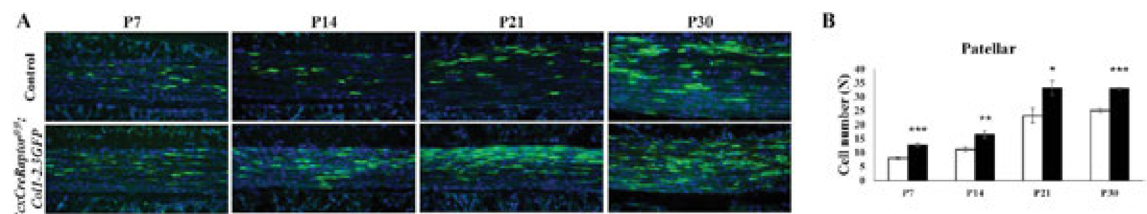

Figure 1. (A) Fluorescent microscopy images for Col1 (2.3)-GFP positive cells in the mid-substance of Patellar tendon from control (Col1 (2.3)-GFP) and mTORC1 loss-of-function (*Scx-Cre; Raptor$^{fl/fl}$*; Col1 (2.3)-GFP) mouse**. (B)** Quantification of Col1 (2.3)-GFP positive cells in Mid-substance of Patellar tendon from each genotype at various stages. Scale bar indicates 100μm *** indicates $P < 0.005$, **** indicates $P < 0.001$ n=3

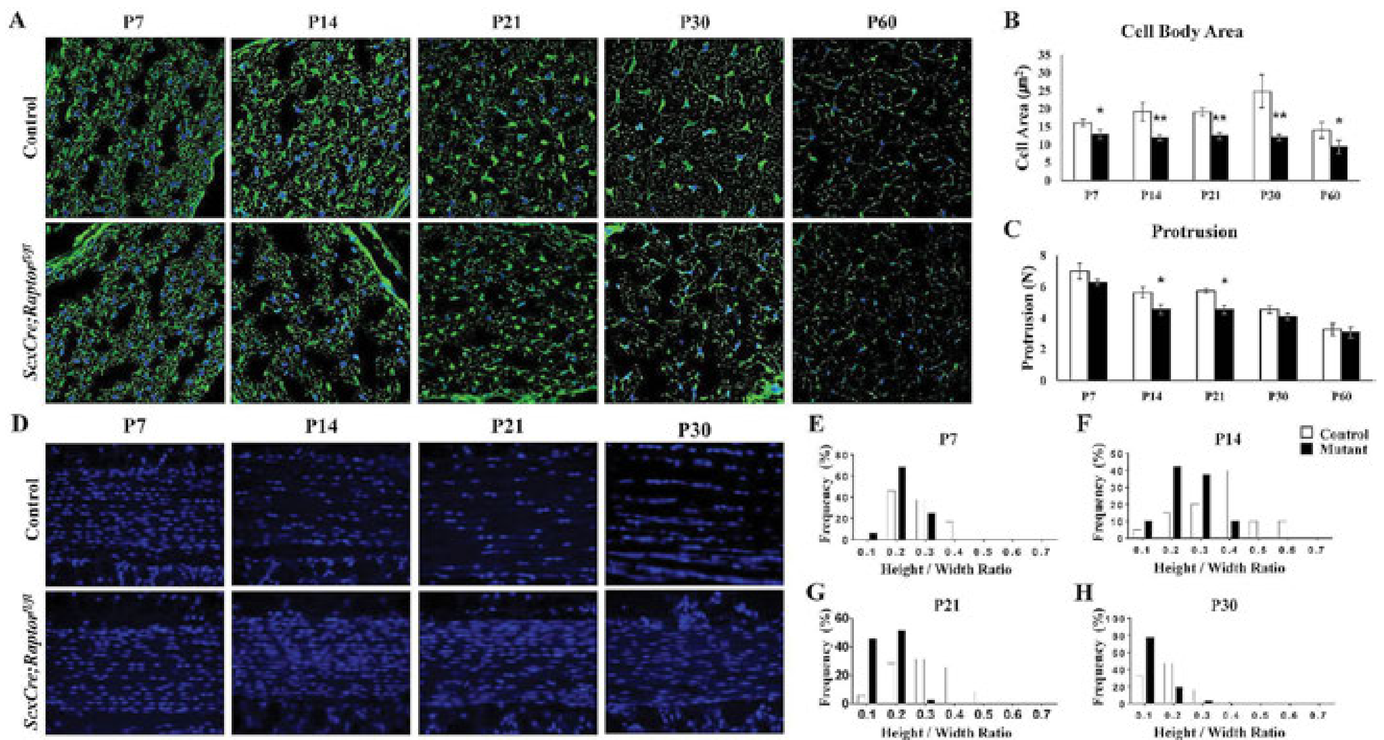

Figure 2. (A) High resolution confocal microscopy with Zo1 and Phalloidin double-stained cross-sections from patellar tendons of control and mTORC1 loss-of-function (*Scx-Cre; Raptor*$^{fl/fl}$) mouse at various stages. **(B and C)** Quantification results of protrusion number, cell body area, and cell density using high resolution confocal images. **(D)** Nuclear imaging with DAPI staining using from control and mTORC1 loss-of-function mouse model at various stages. (E-H) Quantification results of nuclear aspect ratio. Scale bar indicates 10μm **(A)** * indicates $P < 0.05$, ** indicates $P < 0.001$, and *** indicates $P < 0.005$ n=3

cell area was dramatically decreased at P60 in wildtype mice (Figure 2B, white bar), which indicates terminally matured tenocyte population at P60, respectively. Tendon cells in loss-of-function mice exhibited decreased cell area at all stages (Figure 2B, black bar) compared to wildtype. The protrusion is the projections from the cell body that is critical for cell-cell and cell-matrix interaction. The protrusion number was gradually decreased during postnatal tendon development in wildtype mice (Figure 2C, white bar). The *ScxCre;Raptor*$^{fl/fl}$ mice showed significantly reduced protrusion numbers compared with those of wildtype mice at P14 and P21 (Figure 2C, black bar). These morphological analyses suggest that the loss of mTORC1 signaling enhanced the maturation of tendon cells. To further confirm the enhanced morphological maturation, we measured the nuclear aspect ratio (ratio of long and short axes of the nucleus) that indicates the longitudinal shape of tendon cells (Figure 2D). At the early developmental stage (P7), most tendon cells in wildtype were relatively elongated, which was indicated by a lower nuclear aspect ratio (Figure 2E, white bar). And then, tendon cells gradually become rounder at P14 and P21 (Figure 2F and 2G, white bar). Eventually, terminally matured tendon cells become elongated again, as indicated by a low nuclear aspect ratio at P30 (Figure 2H, white bar). Interestingly, tendon cells in loss-of-function mice became very elongated even at P14 and P21 (Figure 2F and 2G, black bar). These data suggest that loss of mTORC1 enhanced the cellular maturation of tendon cells. Our loss-of-function study suggests that mTORC1 signaling negatively regulates differentiation of tendon progenitor cells into col1a1-expressing cells and cellular maturation of tenocytes. To verify this inhibitory function of mTORC1 signaling, we performed a gain-of-function study by generating *Scx-Cre;Tsc1*$^{fl/fl}$ *;Col1*

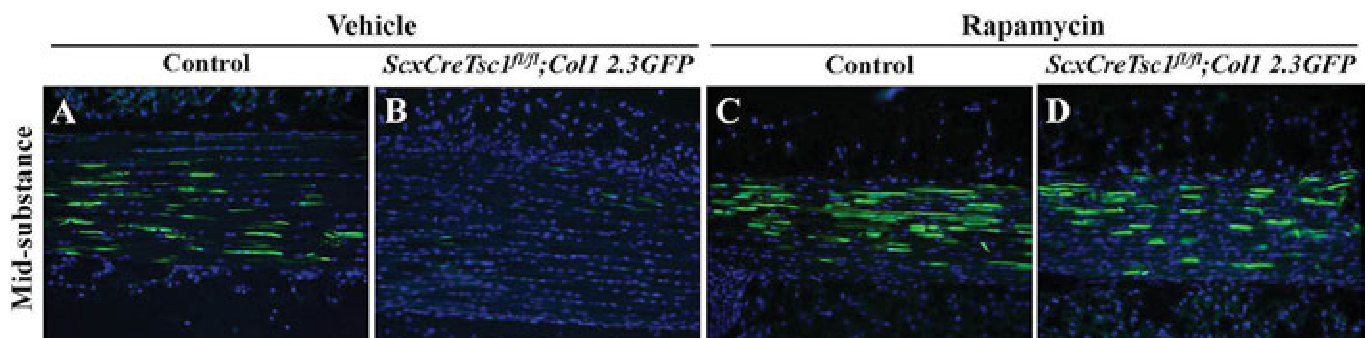

Figure 3. Fluorescent microscopy images for Col1 (2.3)-GFP positive cells in the mid-substance of Patellar tendon from control (Col1 (2.3)-GFP, **(A and C)** and mTORC1 gain-of-function (*Scx-Cre; Tsc1*$^{fl/fl}$; Col1 (2.3)-GFP, **(B and D)** mouse with vehicle **(A and B)** or rapamycin **(C and D)** treatment.

(2.3)-GFP mouse models. As expected, *Scx-Cre; Tsc1*$^{fl/fl}$ (gain-of-function) mice showed dramatically decreased Col1 (2.3)-GFP-positive cells in the Patellar tendon (Figure 3B) compared with control mice. To test if the inhibition of mTORC1 signaling can rescue the reduced number of Col1 (2.3)-GFP-positive cells in *Scx-Cre;Tsc1*$^{fl/fl}$ mice, rapamycin, a mTOR inhibitor, was injected in both control and *Scx-Cre;Tsc1*$^{fl/fl}$ mice. Col1-2.3GFP-positive cells were increased by rapamycin in *Scx-Cre;Tsc1*$^{fl/fl}$ mice (Figure 3D). These results verify that mTORC1 signaling inhibits early differentiation of tendon progenitors into Col1a1-expressing tenocytes.

Discussion

Our results showed that mTORC1 signaling is a critical regulator of early tendon differentiation and morphological maturation. Our current studies suggest that mTORC1 negatively regulates the differentiation of tendon progenitor cells into Col1a1-expressing cells and cellular maturation of tenocytes. Further morphological analysis using this gain-of-function mouse model will be required to verify the function of mTORC1 in cellular maturation of tenocytes. Further studies with other reporter mice are needed to investigate that the function of mTORC1 in tendon differentiation at various time points.

Significance/Clinical relevance

This study will fill the knowledge gap in tenocyte biology by establishing the function of mTORC1 signaling in tenocyte differentiation and cellular maturation.

Acknowledgements

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