Promoting Tendon Regeneration by Activating AMPK and Decreasing α-SMA⁺ Cell Migration

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INTRODUCTION: Current strategies for enhancing tendon repair after injury consist mainly of applying stem cells, growth factors, natural and artificial biomaterials, and surgical treatments [1]. However, such treatments typically result in scar tissue formation or fibrosis, resulting in compromised tendon function. High levels of α-smooth muscle actin (α-SMA) and collagen III are two markers of scar tissue formation. High mobility group box1 (HMGB1), a potent inflammatory mediator, plays a key role in hypertrophic scar formation in skin and scar-forming wound in fetal mice [2]. Adenosine monophosphate-activated protein kinase (AMPK) has been linked to the regulation of tissue inflammatory signaling. AMPK activation can inhibit scar tissue formation [3]. Metformin (Met), a drug commonly used for the treatment of type-2 diabetes, is known to inhibit HMGB1 and activate AMPK [4, 5]. Met has been shown to prevent lung fibrosis previously [6]. In this study, we investigated the effect of Met on wounded tendon healing using SMA-Ai9-Scx-GFP transgenic mice that express both Scx (a tendon marker) and α-SMA (progenitor cell marker) in tendon cells.

METHODS: The tamoxifen-inducible α-SMA-CreERT2 mice were crossed with Scx-GFP mice, and then crossed with Ai9 Cre reporter mice to generate triple transgenic α-SMA-Ai9-Scx-GFP mice (referred to as Ai9 mice hereafter). Five intraperitoneal (IP) injections of tamoxifen (100 mg/kg) were administered consecutively over five days to a total of 24 Ai9 mice before surgery. A window defect was created in the Achilles tendon of all mice by a 0.5 mm diameter biopsy puncher. The wounded animals were divided into four groups with 6 mice/group and treated with IP injections of saline and Met (160 mg/kg) daily for total 6 weeks as follows: <u>Group 1</u>: **Saline** for 6 weeks; <u>Group 2</u>: Met 2 weeks **before** surgery and saline 4 weeks after surgery (**Met-B**); <u>Group 3</u>: Saline 2 weeks before surgery and Met for 4 weeks **after** surgery (**Met-A**); and <u>Group 4</u>: Met 2 weeks **before** surgery and 4 weeks **after** surgery(**Met-B&A**). The animals were sacrificed at day-30 post-surgery. The Met effect on wounded tendon healing was investigated by ELISA for HMGB1 levels in mouse serum and histological analysis on mouse tendon tissue sections. ***Statistical analysis*** – Data were analyzed by One-way ANOVA followed by Fisher's LSD test for multiple comparisons. A p-value less than 0.05 was considered to be a significant difference between two groups. This study was approved by IACUC.

RESULTS: The wounded tendon treated with saline at 30 day post-surgery formed scar tissue with high density cells (red arrow in **Fig. 1A**) and poorly organized, loose collagen fibers (green box area in **Fig. 1A**) stained with blue (black arrow in **Fig. 1E**). In contrast, Met IP injection resulted in well-organized tendon structure with dense collagen fibers (white arrows in **Fig. 1B-D**) stained with red (green arrows in **Fig. 1F-H**). The healed tissue in saline treated tendon was formed by collagen III as evidenced by green fluorescence under polarized light microscopy (**Fig. 1M**). However, the healed tendon tissues in the wound areas of the mice treated with IP injection of Met were collagen I as evidenced by yellow fluorescence under bright field (**Fig. 1I-L**) and polarized light microscopy (**Fig. 1N-P**). The saline-treated mice serum had high levels of HMGB1 which was significantly decreased in Met-A and Met-B&A groups (**Fig. 1Q**). Under fluorescent microscope, the frozen tendon tissue sections of the mice showed that Met IP injection inhibited the migration of α-SMA⁺ cells (less red fluorescence) in the wounded mouse Achilles tendons (**Fig. 2F-H; J-L; N-P**) compared to saline (**Fig. 2E**). There was no significant difference of the Scx⁺ cells (green fluorescent cells) in the wound areas of four groups (**Fig. 2A-D**). Immunostaining showed increased number of α-SMA⁺ cells (brown in **Fig. 3A**), low level of AMPK staining (**Fig. 3E**), high level of TGF-β1 (**Fig. 3I**) and HMGB1 (**Fig. 3M**) in the wound area of saline injection mice. However, in Met treated groups, less α-SMA⁺ cells (**Fig. 3B-D**), and high level of AMPK staining (**Fig. 3F-H**) were found in the wounded tendons of mice compared to saline treated group. Met injection also inhibited the expression of TGF-β1 (**Fig. 3J-L**) and HMGB1 (**Fig. 3N-P**) in mouse tendons.

DISCUSSION: This study set up a useful animal model to investigate the wounded tendon healing using transgenic Ai9 mice which can track cell migration during healing. Using this model, we have demonstrated that Met injection reduces scar formation in wounded tendon by reducing HMGB1 levels, inhibiting α-SMA⁺ cell migration, decreasing collagen III but increasing collagen I production, activating AMPK, and decreasing TGF-β1 expression. With longer treatment of Met injections, scar tissue formation was reduced at a larger extent, as evidenced by the wound treated with **Met-B&A** healed better than **Met-B** and **Met-A** only. In response to injury, tissue remodeling and formation of fibrotic scars are initiated by TGF-β1, which stimulates anabolic metabolism in activated myofibroblasts. Our findings indicated that AMPK activation may inhibit myofibroblast differentiation by TGF-β1, supporting a preventive role of AMPK in the development of fibrosis.

SIGNIFICANCE/CLINICAL RELEVANCE: Metformin may be used to promote tendon regeneration by reducing scar tissue formation.

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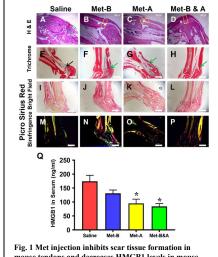
Fig. 1 Met injection inhibits scar tissue formation in mouse tendons and decreases HMGB1 levels in mouse sera in a treatment time-dependent manner. Bars: 500 μm. *p<0.05 compared to saline.
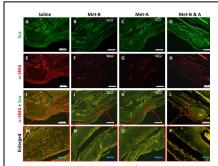

Fig. 2 Met injection inhibits α-SMA⁺ cell migration. A-D: Scx-GFP⁺ cells; **E-H:** α-SMA-Ai9⁺ cells; **I-L:** Merged images of A-D and E-H. **M-P:** Enlarged red box areas in the images of **I-L.** Red cell numbers are increased in the wound area of saline injection mice (**E, I, M**). However, less red cell numbers are found in the wounded tendons of mice with Met injection (**F, G, H**) compared to saline treated group. White bars: 500 μm; Blue bars: 125 μm.

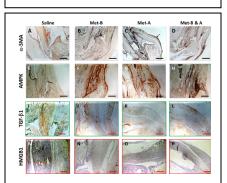**Fig. 3 Met injection inhibits α-SMA⁺ cell migration, increases AMPK activity, decreases HMGB1 and TGF-β1 expression in mouse tendons assessed by IHC staining.** Black bars: 500 μm; red bars: 200 μm.