Identification of the Prostanoid Receptor EP4 as a Novel Target to Enhance Flexor Tendon Healing

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Introduction: Flexor tendon (FT) injuries represent a significant clinical challenge due to the formation of fibrous adhesions between the tendon and synovial sheath. Adhesions impair tendon excursion and limit digit range of motion, which can impair the function of the entire hand. An optimal therapeutic approach would decrease adhesions while maintaining or improving the biomechanical properties of the healing tendon. Adhesions are resolved earlier in Mmp9−/− mice relative to wild type (WT), and bone marrow cells are the predominant source of Mmp9 in the healing FT [1]. Here we show that PGE2, an inflammatory mediator during tendon healing [2], stimulates Mmp9 expression and activity in bone marrow stromal cells (BMSCs), specifically through the EP4 receptor. Fibroblast specific protein-1 (Fsp1) is expressed by fibroblasts in remodeling tissues [3]. In this study we demonstrate that abundant Fsp1+ cells are localized to the granulation tissue and have generated mice with conditional deletion of EP4 using Fsp1-Cre. FTs from Fsp1-Cre++; EP4fx/fx mice heal with a dramatic reduction in adhesions while maintaining the biomechanical properties of the FT relative to WT. As such, we have identified EP4 as a novel target to enhance FT healing.

Methods: In vitro studies: Primary tenocytes and BMSCs were isolated from WT and EP2+/− mice. Cells were stimulated with either 1μM PGE2, 1μM EP4 antagonist (L161,982) for 1hr, followed by 1μM PGE2, or vehicle control (DMSO). RNA and conditioned media were isolated between 30 minutes and 20 hours after PGE2 stimulation for qPCR and gelatin zymographic analysis.

Murine FT healing model: 8-week old EP4fx/fx (WT) and Fsp1-Cre++; EP4fx/fx (EP4cKO) mice underwent surgical transection and repair of the flexor digitorum longus (FDL) tendon as previously described [1]. Healing tendons were isolated between 3-28 days post-surgery. X-gal staining of LacZ was used to determine the spatial localization of Fsp1+ cells during FT healing, using Fsp1-Cre++; Rosa26Reporterfx/+ (Gtrosa26tm1Sor) mice.

Biomechanical testing: Adhesion formation was quantified using the ‘Gliding Coefficient’, a measure of the degree of metatarsophalangeal joint range of motion (MTP ROM) when incremental loads are applied to the tendon. A higher gliding coefficient is indicative of more adhesion formation [1]. FDL tendons were released from the tarsal tunnel and tested in tension in displacement control at a rate of 30 mm/minute until failure. Force-displacement data were plotted to determine the maximum load at failure and stiffness.

Statistical analysis: Data are presented ± SEM. A two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used to determine significance.

Results: PGE2 stimulates Mmp9 in BMSCs specifically through the EP4 receptor: BMSCs had a significant 894-fold increase in Mmp9 expression relative to tenocytes under basal conditions (p<0.0001). PGE2 treatment resulted in a 809-fold increase in Mmp9 expression in BMSCs compared to PGE2 treated tenocytes (p<0.0001), and a 4-fold increase versus vehicle treated BMSCs (p<0.0001). No change in Mmp2, Mmp3 or Mmp14 expression levels were observed in BMSCs upon PGE2 treatment (Figure 1A). The potential role for EP1 and EP3 signaling in Mmp9 induction was not assessed due to the minimal expression levels of these receptors during FT healing. No significant differences in Mmp9 expression or activity were observed in EP2+/−. BMSCs upon PGE2 stimulation relative to WT. EP4 antagonism, using L161,982 significantly decreased Mmp9 expression within 30 minutes of PGE2 treatment, and with suppression persisting to 6 hours. Mmp9 gelatinolytic activity was decreased with EP4 receptor antagonism 12 and 24 hours after treatment (Figure 1B).

Fsp1+ cells are localized to granulation tissue during FT healing: To determine the localization of cells in which the Fsp1 promoter was active during FT healing, Fsp1-Cre+ mice were crossed to Rosa26Reporterfx/+ mice. At 14 and 21 days post-surgery LacZ+ cells were present in the granulation tissue between the tendon ends and interspersed between the collagen fibers within the tendon (Figure 1C). LacZ+ tenocytes were observed in contralateral control tendons of Fsp1-Cre++; Rosa26Reporterfx/+ mice, while no LacZ- tenocytes were found in control tendons of Fsp1-Cre++; Rosa26Reporterfx/+ mice.

EP4cKO results in attenuated adhesion formation: EP4 expression was significantly increased relative to EP1, EP2 and EP3 at all time-points post-surgery in WT FTs. Peak EP4 expression occurred at seven days post-repair with a 3.3-fold increase relative to day 3 expression. Expression of EP4 decreased on day 10 and was not significantly different than day 3 for the remainder of the time-course. EP4cKO tendons had a significant 81% decrease in EP4 expression at day 7 relative to WT (p=0.006), with no significant changes in EP4 expression after this point relative to WT (Figure 2A). Deletion of EP4 resulted in a significant 83% decrease in Mmp9 expression at day 7 (p=0.016)(Figure 2B), while no change in Mmp2 or Mmp14 expression levels were observed in EP4cKO tendons.
Histologically, a dramatic reduction in catabolism of tendon at the injury site was observed in EP4cKO, concomitant with a marked decrease in granulation tissue at 7 and 10 days post-surgery, relative to WT. By 28 days the morphology of EP4cKO tendons was comparable to un-injured control tendons while progressive remodeling of a disorganized tendon ECM occurred in WT mice.

The gliding coefficient, a measure of adhesion formation, was significantly increased in WT tendons at day 14 (151.1 ± 63.9) relative to control (9.9 ± 0.8, p=0.02), and remained significantly elevated through 28 days. The gliding coefficient of EP4cKO FTs was significantly increased relative to EP4cKO control FTs at 14 days (Ctrl: 11.7±2.5, D14: 42.0±3.7, p=0.01), however, adhesions were significantly decreased relative to WT tendons at this time (p=0.02). At 21 and 28 days the gliding coefficient of EP4cKO FTs was no longer significantly increased relative to EP4cKO controls (Figure 2C). The maximum load at failure of EP4cKO tendons was not significantly different than WT tendons at any time post-surgery (Figure 2D).

**Discussion:** In this study we demonstrate that minimal Mmp9 expression and activity are observed in primary tenocytes relative to BMSCs and that PGE2 stimulation of Mmp9 occurs specifically through activation of the EP4 receptor in BMSCs. As Mmp9−/− mice have a dramatic decrease in adhesions during FT healing, understanding the mechanism of Mmp9 induction may suggest alternative strategies to improve FT healing. Based on our in vitro data we have identified EP4 as one such target. EP4cKO tendons heal with decreased adhesion formation at 14 days relative to WT, while no decrements in biomechanical properties are observed, indicating that decreased adhesion formation does not compromise the strength of the healing tendon upon EP4 deficiency. Moreover, a decreased granulation tissue response and tendon catabolism occurs in EP4cKO tendons, possibly due to decreased Mmp9 expression and activity. EP4 deficiency did not alter express of other Mmps, suggesting a specific effect on Mmp9. These studies identify a previously unknown mechanism for Mmp9 regulation during FT healing, and suggest EP4 as a novel target to improve healing of FT injuries.

**Significance:** We have demonstrated that deletion of the PGE2 receptor EP4 improves FT gliding function, without impairing mechanical properties relative to WT. As EP4 antagonists are currently in clinical trials [4], there is a high degree of clinical translation for EP4 as a therapeutic target to decrease scarring and improve healing following FT injury.

**Acknowledgments:**

**References:**
Figure 1. [A] Mmp9 expression in primary WT tenocytes and BMSCs at baseline and in response to PGE2 stimulation. (*) indicates p<0.05 vs. vehicle control treatment, (#) indicates p<0.05 vs PGE2 treated tenocytes. [B] Mmp9 expression in EP2^-/- BMSCs treated with PGE2 or the EP4 specific antagonist L161,982, followed by PGE2 stimulation. (*) indicates p<0.05 vs. vehicle treated cells, (#) indicates p<0.05 vs. L161,982 treated cells. [B’] gelatin zymography showing decreased Mmp9 activity at 12 and 24 hours after PGE2 stimulation in EP2^-/- BMSCs treated with L161,982. [C] X-gal staining of a healing FT from Fsp1-Cre^+; Rosa26Reportertox^+ mice showing abundant Fsp1^+ cells (blue) in the granulation tissue at 14 days.
Figure 2. [A & B] qPCR analysis of [A] EP4 and [B] Mmp9 expression in WT and EP4cKO flexor tendons isolated between 3-28 days post-surgery. mRNA was isolated for 4 tendons per genotype per time-point. (*) p<0.05 vs. WT day 3 expression. [C] Quantification of adhesions, using the Gliding coefficient and [D] Max Load at failure in WT and EP4cKO tendons between 10-28 days post-surgery. (*) indicates p<0.05 vs un-injured genotype control tendons, (#) indicates p<0.05 vs EP4cKO tendons at 14 days post-surgery. N=8 per genotype per time-point.