The Role of Prostanoid Receptor EP4 on Adhesion Formation in Flexor Tendon Healing -
Differential Effects of Tendon-Specific Deletion Versus Systemic Antagonism

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Introduction: Despite advances in surgical technique and rehabilitation protocols, primary repair of flexor tendon (FT) injury remains frequently complicated by postoperative scarring and adhesion formation. Significant increases in PGE2 production are observed during the inflammatory phase of tendon healing, with signaling mediated predominantly through the EP4 receptor [1]. Previous studies have shown that matrix metalloproteinase 9 (Mmp9), a proteinase induced through EP4 signaling, increases tendon catabolism and impairs gliding function [2]. We investigated the hypothesis that inhibiting PGE2-EP4 signaling attenuates adhesion formation by blunting the inflammatory response. This hypothesis was tested at both the tendon-specific and systemic level.

Methods: Murine FT healing model: Mice underwent surgical transection and repair of the flexor digitorum longus (FDL) tendon as previously described [2]. Fsp1-Cre;EP4
fx/fx mice (Tendon specific EP4cKO) and EP4
fx/fx (WT) were used to determine the effects of local EP4 signaling inhibition. Systemic antagonism was achieved in C57BL/6 mice through i.p. injection of EP4 antagonist L161,982 (10mg/kg) on days 5-8 post-surgery. Delayed EP4 inhibition is preferable to immediate post-repair inhibition, since negative remodeling and inflammation is inhibited without disrupting initial healing [3].

Biomechanical Testing: Adhesion formation was quantified using the ‘Gliding Coefficient’, a measure of the resistance to metatarsophalangeal (MTP) joint flexion upon incremental loading of the proximal FDL tendon [4], as well as MTP range of motion (MTP ROM), defined as the change in flexion angle from 0g to 19g loading. A lower gliding coefficient indicates fewer adhesions, while a higher MTP ROM represents less resistance to flexion. Maximum tensile force and stiffness were determined in EP4cKO and WT mice, as well as mice treated with the EP4 antagonist, or vehicle.

Statistical Analysis: Biomechanical data were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons with a significance level of α=0.05.

Results: EP4cKO results in attenuated adhesion formation and decreased Mmp9 expression without compromising strength

The gliding coefficient was significantly decreased in EP4cKO tendons relative to WT repairs at 10 days (2.7-fold decrease, p<0.01), a trend maintained at 14 days. The significant decrease in gliding coefficient was maintained at 21 (2.1-fold decrease, p<0.05) and 28 days (3.2-fold decrease, p<0.01) post-repair compared to WT tendons (Figure 1A). Relative Mmp9 expression was significantly decreased at day 7 in EP4cKO tendons compared to WT (5.3-fold decrease, p=0.016) (Figure 2A). The maximum load and stiffness of EP4cKO tendons was not significantly different relative to WT repairs between 10 and 28 days.

Systemic EP4 antagonism impairs early gliding function and increases Mmp9 expression in injured FTs
The gliding coefficient was significantly increased at day 14 in tendons treated with systemic EP4 antagonist relative to control repairs (11.1-fold increase, p<0.0001), and remained significantly elevated at 21 days (7.2-fold increase, p<0.001) (Figure 1B). There was no significant difference observed between gliding coefficients of the two groups at 28 days post-repair. MTP ROM was significantly decreased at days 14 (11.9-fold decrease, p<0.05) and 21 (3.9-fold decrease, p<0.05) in tendons treated with systemic EP4 antagonist compared to control repairs. Relative Mmp9 expression was significantly increased at days 14 (2.6-fold increase, p<0.001) and 21 (3.1-fold increase, p<0.0001) compared to control repairs (Figure 2B). No significant differences in maximum load and stiffness were observed between control and systemic EP4 treated tendons.

Systemic EP4 inhibition alters tendon homeostasis
Uninjured FDL tendons in the systemic EP4 antagonist group demonstrated a gradual increase in gliding coefficient (6.2-fold increase at 28 days) with a concomitant decrease in MTP ROM (3.1-fold decrease at 28 days) from day 10 to day 28 post-repair compared to baseline biomechanics in uninjured, control tendons (Table 1). No change in gliding function was observed in EP4cKO contralateral tendons relative to WT contralateral tendons.

Discussion: PGE2-EP4 signaling has been identified as a dominant mediator of prostanoid-driven inflammation [1]. This study reports on flexor tendon healing following inhibition of EP4 signaling at both the tendon-specific and systemic level. EP4cKO tendons had improved gliding function in the early stages of repair, with significantly lower gliding coefficients relative to WT repairs at days 10, 21, and 28 post-repair (Figure 1A), suggesting that tendon-specific loss of EP4 signaling attenuates the inflammatory response and decreases adhesion formation. This observation is consistent with significant decreases in relative Mmp9 expression at day 7 in EP4cKO tendons compared to WT (Figure 2A), since it has been shown that loss of Mmp9 decreases adhesion formation [2]. The maximum load at failure and stiffness of EP4cKO tendons was not significantly different than WT repairs, suggesting that the strength of repair is not compromised in the absence of EP4 signaling. Taken together, this suggests that local loss of EP4 signaling reduces adhesion-forming inflammation without a concomitant reduction in repair strength. This last observation is especially important, since most approaches that decrease adhesions do so at the expense of repair strength, increasing rates of rupture and gapping at the surgical site [5].

Systemic EP4 antagonism impairs healing in injured flexor tendons, suggested by the early and significant increase in adhesion formation (Figure 1B) as well as the significant decrease in MTP ROM compared to control repairs. These findings are corroborated by molecular analysis, which demonstrates elevated Mmp9 expression in EP4 antagonist treated repairs, an expression pattern associated with impaired gliding function [2]. Since the EP4 antagonist is administered on days 5-8 post-repair, the changes observed on days 14 and 21 may be secondary to a compensatory increase in PGE2-EP4 signaling. This is further supported by the increase in gliding coefficient and decrease in MTP ROM in contralateral tendons exposed to systemic EP4 antagonism (Table 1). In these uninjured tendons, we observed a gradual and sustained increase in the resistance to flexion, suggesting a disruption in tendon homeostasis.

Consistent with the complex nature of PGE2-EP4 signaling, we have demonstrated that local and systemic loss of EP4 signaling imparts contrasting phenotypes during flexor tendon healing. Future
studies will identify the mechanisms through which EP4 differentially regulates FT repair in the context of both tissue specific and systemic inhibition.

**Significance:** EP4 has been identified as novel target in flexor tendon repair, shown by decreasing adhesions without reducing strength in tendon-specific EP4cKO. However, cell-specific treatment may be needed to achieve the desired healing response, given our observation that systemic EP4 antagonism negatively affects repair.

<table>
<thead>
<tr>
<th>Day Post-Repair</th>
<th>Increase in Gliding Coefficient</th>
<th>Decrease in MTP ROM</th>
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<tbody>
<tr>
<td>Day 10</td>
<td>2.7-fold</td>
<td>1.8-fold</td>
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<tr>
<td>Day 14</td>
<td>5.0-fold</td>
<td>2.5-fold</td>
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<td>Day 21</td>
<td>5.6-fold</td>
<td>2.7-fold</td>
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<tr>
<td>Day 28</td>
<td>6.2-fold</td>
<td>3.1-fold</td>
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Figure 1. Adhesion formation in tendon-specific EP4cKO and EP4 antagonist treated repairs. EP4cKO results in attenuated adhesion formation (Fig. 1A), while systemic EP4 antagonism impairs early gliding function (Fig. 1B). Fig. 1A The gliding coefficient was significantly decreased in EP4cKO tendons relative to WT repairs at 10 days, a trend maintained at 14 days. The significant decrease in EP4cKO tendons was maintained at 21 and 28 days post-repair compared to WT tendons. As a lower gliding coefficient indicates better gliding function, our data suggests an improvement in early gliding function in EP4cKO tendons. Fig. 1B The gliding coefficient was significantly increased at day 14 in tendons harvested from repairs treated with systemic EP4 antagonist relative to control repairs and remained elevated at 21 days. Given that higher a gliding coefficient indicates impaired gliding function, our data suggests that systemic EP4 antagonism increases the inflammatory response and adhesion formation in the early stages of repair. (*) Indicates p<0.05 between EP4cKO and WT repairs, and between Control and EP4 antagonist treated mice.
Figure 2. Relative Mmp9 mRNA expression in EP4cKO and EP4 antagonist treated repairs. EP4cKO decreases relative Mmp9 expression (Fig. 1A), while systemic EP4 antagonism increases relative Mmp9 expression (Fig. 1B). Fig. 2A Relative Mmp9 expression was significantly decreased at day 7 in EP4cKO tendons compared to WT, corresponding to fewer adhesions and improved gliding function. Fig. 2B Repairs treated with systemic EP4 antagonist have increased relative Mmp9 expression at days 14 and 21 compared to control, consistent with impaired gliding function at the same time points. (*) Indicates p<0.05 between EP4cKO and WT repairs, and between Control and EP4 antagonist treated mice.