1   **Cell non-autonomous functions of S100a4 drive fibrotic tendon healing**

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25

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27

28 **Abstract**

29

30   Identification of pro-regenerative approaches to improve tendon healing is of critical importance given

31   the diminished quality of life and physical function that accompanies the typical fibrotic response to

32   tendon injury. S100a4 modulates fibrosis through tissue-type dependent mechanisms, and the role of

33   S100a4 in fibrotic, scar-mediated tendon healing has not been established. In the present study we

34   tested the hypothesis that inhibition of S100a4 improves tendon function following acute injury and

35   surgical repair. We demonstrate cell non-autonomous functions of S100a4 as S100a4

36   haploinsufficiency promotes regenerative tendon healing, including decreased scar formation and

37   improved mechanical properties. Moreover, inhibition of S100a4 via antagonism of its putative

38   receptor, the Receptor for Advanced Glycation Endproducts (RAGE), also decreases scar formation.

39   Mechanistically, knock-down of S100a4 decreases myofibroblast and macrophage content at the site of

40   injury, with both cell populations being key drivers of fibrotic progression. In contrast, S100a4^{+} cell

41   depletion displays time-dependent effects on scar formation, and consistent impairments in restoration

42   of mechanical properties, indicating a critical role for these cells in re-establishing tendon strength after

43   injury. Finally, we demonstrate, that S100a4-lineage cells become α-SMA^{+} myofibroblasts, via loss of

44   S100a4 expression. Using a combination of genetic mouse models, small molecule inhibitors and in

45   vitro studies we have defined S100a4 as a novel, promising therapeutic candidate to improve tendon

46   function after acute injury.

47 **Introduction**

48 Tendons are composed primarily of a dense, highly aligned collagen extracellular matrix (ECM), and

49 connect muscle to bone to transmit mechanical forces throughout the body. Following injury, tendon

50 demonstrates limited regenerative potential and heal through a scar-mediated fibrotic process involving

51 abundant, disorganized ECM deposition. While scar tissue can impart some mechanical strength to the

52 healing tissue, it is also mechanically inferior to native tendon and dramatically impairs normal tendon

53 function resulting in substantial morbidity. In addition, scar tissue increases tendon bulk and forms

54 adhesions to the surrounding tissues, impeding normal range of motion (ROM). This pathological

55 response to injury represents a major clinical burden considering there are over 300,000 surgical

56 tendon repairs in the United States annually (1), and up to 40% of primary flexor tendon repairs heal

57 with function limiting scar tissue (2). Despite this burden, there is currently no consensus biological or

58 pharmacological approach to improve tendon healing, due in large part to a paucity of information on

59 the cellular and molecular components involved.

60

61 S100a4 (also known as *Fsp1*, *Mts1*, *Pk9a*) is a member of the S100 family of EF-hand Ca^{2+}-binding

62 proteins, and is a potent regulator of fibrosis in many tissues including the liver (3, 4), lung (5), heart (6)

63 and oral submucosa (7). An increase in the proportion of S100a4^{+} cells is characteristic of many

64 fibrotic conditions (5, 8), and elevated serum S100a4 levels positively correlate with fibrosis clinically

65 (4). Moreover, the therapeutic potential of S100a4 inhibition is suggested by S100a4-cell depletion

66 studies and S100a4 RNAi treatments in which fibrosis was halted, or effectively reversed (4, 9, 10).

67 While depletion of S100a4^{+} cells can inhibit fibrotic progression, S100a4 can also function as an intra-

68 and extracellular signaling molecule to impact cellular processes including motility, survival,

69 differentiation, and contractility (11, 12). Additionally, the effects of S100a4 are cell and tissue-type

70 dependent. We have previously shown that *S100a4*-Cre efficiently targets resident tendon cells (13),

71 however, the specific function of S100a4 and whether that function is cell-autonomous or cell-non-

72 autonomous during scar-mediated fibrotic tendon healing is unknown.

73

74 In the present study we delineate the relative contributions of S100a4 expression and S100a4⁺ cells to

75 scar-mediated tendon healing and investigated both the cell non-autonomous extracellular signaling

76 function of S100a4, as well as the fate and function of S100a4-lineage cells. We have identified

77 S100a4 haploinsufficiency as a novel model of regenerative tendon healing and define a requirement

78 for S100a4⁺ cells in the restoration of mechanical properties during tendon healing. These data identify

79 S100a4 as a novel target to improve tendon healing and demonstrate the efficacy of pharmacological

80 inhibition of S100a4 signaling to improve functional outcomes during healing.

81

82 **Methods**

83 *Ethics statement:* All animal studies were approved by the University of Rochester Committee for

84 Animal Resources.

85

86 *Mouse strains:* S100A4-GFP^promoter mice (#012893), S100a4-TK (#012902), S100a4^GFP/+ (#012904),

87 S100a4-Cre (#012641), ROSA-Ai9 (#007909), and C57BL/6J (#000664) were acquired from The

88 Jackson Laboratory (Bar Harbor, ME).

89 S100a4-GFP promoter mice contain a construct encoding EGFP under control of the S100a4 promoter

90 sequence, resulting in green fluorescence in cells actively expressing S100a4 (14).

91 S100a4-TK mice contain a viral thymidine kinase gene downstream of the S100a4 promoter, and

92 treatment with the nucleoside analog ganciclovir (GCV) halts DNA replication in proliferating cells

93 expressing S100a4, resulting in apoptosis and S100a4⁺ cell ablation (9). Mice were treated twice per

94 day (i.p) with 75mg/kg GCV. S100a4^GFP/+ mice contain a GFP-encoding gene knocked into the exons

95 2-3 of the S100a4 gene, resulting in a 50% reduction in S100a4 protein expression (15). For RAGE

96 Antagonist Peptide (RAP) studies, C57BL/6J mice were treated with either 100μg RAP or vehicle (0.5%

97 bovine serum albumin in saline) via i.p. injection on D5-10 post-surgery.

98

99 *Murine model of tendon injury and repair:* Male and female mice aged 10-12 weeks underwent

100 complete transection and surgical repair of the flexor digitorum longus (FDL) tendon as previously

101 described (16). Mice were monitored and given analgesics post-operatively as needed.

102

103 *RNA extraction and qPCR*: For in vivo studies, the tendon repair site was excised from the hind paw at

104 D10 following injury, along with 1-2mm of native tendon on either side. Three repairs were pooled, and

105 RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad CA). cDNA was generated with

106 500ng of RNA using an iScript cDNA synthesis kit (BioRad, Hercules CA). Quantitative PCR was

107 carried out with gene specific primers, and expression normalized to *β-actin*. All experiments were done

108 in triplicate and repeated twice.

109

110 *Assessment of gliding function and mechanical properties:* Following sacrifice, the hindlimb was

111 harvested at the knee. The medial side of the hindlimb was carefully dissected to free the FDL, and the

112 proximal end was secured between two pieces of tape with cyanoacrylate. The distal tendon was

113 loaded via the tape with weights ranging from 0 to 19g, with digital images taken upon application of

114 each weight. MTP Flexion Angle and Gliding Resistance were calculated as previously described (13,

115 17, 18), with lower MTP Flexion Angle and higher Gliding Resistance corresponding to restricted range

116 of motion and impaired gliding function. Following gliding testing, the FDL was released from the tarsal

117 tunnel, and the proximal end of the tendon and the digits were secured in opposing custom grips on an

118 Instron 8841 uniaxial testing system (Instron Corporation, Norwood, MA). The tendon was loaded until

119 failure at a rate of 30mm/minute (17).

120

121 *Histology, Immunohistochemistry and Immunofluorescence:* Following sacrifice, hind paws were

122 dissected just above the ankle, and underwent routine processing for paraffin or frozen sectioning.

123 Paraffin samples were fixed for 72h in 10% NBF, then decalcified for 2 weeks in 14% EDTA before

124 processing. Three-micron sagittal sections were stained with Alcian Blue Hematoxylin / Orange G

125 (ABHOG) or picrosirius red stain (Polysciences Inc, Warrington PA). Frozen samples were fixed

126 overnight, decalcified for 4 days, incubated in 30% sucrose (in PBS) overnight, and embedded in

127 Cryomatrix (#6769006, ThermoFisher, Waltham MA). Eight-micron sagittal sections on Cryofilm tape

128 (Section-lab, Hiroshima, Japan) were cut on a Leica CM1860UV cryostat, and adhered to slides with

129 1% chitosan in 0.25% acetic acid.

130   Chromogen immunohistochemistry was performed on paraffin sections for S100a4 (1:20000, #197896,

131   Abcam, Cambridge MA), with a rabbit polymer kit (MP-7401, Vector Laboratories, Burlingame CA).

132   Immunofluorescence was carried out with the following primary and secondary antibodies: RAGE

133   (1:100, #sc-365154, Santa Cruz Biotechnology, Dallas TX), with goat anti-mouse AlexaFluor488

134   secondary (1:1000, #A11029, ThermoFisher, Waltham MA);   F4/80 (1:500, #sc-26643, Santa Cruz

135   Biotechnology, Dallas TX) with a donkey anti-rabbit AlexaFluor594 secondary (1:200, #705-546-147,

136   Jackson ImmunoResearch, West Grove PA); GFP (1:5000, #ab6673, Abcam, Cambridge MA), with a

137   donkey anti-goat 488 secondary (1:200, #705-546-147, Jackson ImmunoResearch, West Grove PA);

138   RFP (1:500, #ab62341, abcam, Cambridge MA), with a donkey anti-rabbit 647 secondary (1:200, #711-

139   606-152, Jackson ImmunoResearch, West Grove PA); and α-SMA-Cy3 (1:250, #C6198 Sigma-Aldrich,

140   St Louis MO). All slides were imaged with the Olympus slide scanner and processed with Olyvia

141   software (Olympus, Waltham MA). Images were pseudo-colored using ImageJ software (v1.51j8, NIH).

142   At least three animals were evaluated per genotype per time point.

143

144   *In Vitro Studies:*

145   Bone marrow derived primary macrophages (BMDM) were grown from the bone marrow of C57BL/6J

146   mice. Following sacrifice, femurs were flushed with ice-cold phosphate buffered saline (PBS, without

147   Ca^{2+}/Mg^{2+}). The cell suspension was strained through a 70μm filter, resuspended in differentiation

148   medium(19) and plated at a concentration of 3×10^6 cells per 10cm plate. At D7 of differentiation,

149   primary macrophages were re-plated as needed for experimental use.

150

151   Migration assay: Primary BMDMs were seeded at confluence in 96-well Oris 96-well plates (Platypus

152   Technologies, Madison WI) with silicon stoppers inserted and incubated overnight. Plugs were

153   removed, and cells washed once with PBS (with Ca^{2+}/Mg^{2+}) prior to addition of treatment. Vehicle or

154   recombinant S100a4 protein was added to wells at 20, 50, 200, 500, and 1000ng/mL in quadruplicate,

155   and cells allowed to migrate for 24h. Following migration, cells were gently washed with PBS and

156   stained with NucBlue Live ReadyProbe (Life Technologies, Carlsbad CA). A detection mask was affixed

157   to the bottom of the plate to obscure cells that had not migrated, and the plate read on a Synergy 2

158 plate reader (Biotek, Winooski VT) at 360ex/480em, and fluorescence normalized to vehicle treated

159 cells.

160

161 Polarization: Primary BMDMs were seeded into 6-well plates at 85% confluence overnight. Cells were

162 treated with either S100a4-RP (20-1000ng/mL) or vehicle for 24h. Cells were lysed in Trizol (Life

163 Technologies, Carlsbad CA), and RNA extracted using the RNeasy mini kit (Qiagen, Germantown MD).

164 Quantitative PCR was performed for markers of M1 (*iNOS*, *TNFα*, *CD86*, *CD64*) and M2 (*CD206*, *Arg1*,

165 *IL-1ra*, *CD163*) polarization, and data normalized to *β-actin* expression.

166

167 *Statistical analyses:* Statistically significant differences between genotypes or treatments in in vitro and

168 in vivo studies were assessed by unequal variance unpaired t-test, with the exception of the S100a4

169 qPCR time-course, BMDM migration and polarization experiments, which were analyzed by one-way

170 ANOVA, followed by Tukey's multiple comparisons test. All analyses were conducted using GraphPad

171 Prism software (v8.0.0, La Jolla CA). p values ≤ 0.05 were considered significant, with the following

172 conventions: * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

173

174 **Results**

175 *S100a4 is expressed by resident tendon cells and the S100a4+ population expands during healing*.

176 Spatial localization of S100a4 was examined before and after tendon repair surgery in S100a4-Cre;

177 ROSA-Ai9 reporter mice to trace S100a4-lineage cells (S100a4[lin+]; Fig. 1A & B), and S100a4-

178 GFP[promoter] mice to identify cells actively expressing S100a4 (S100a4[active+]; Fig. 1C & D). Most resident

179 tendon cells were S100a4[lin+] in the uninjured tendon. Following tendon repair, the S100a4-lineage

180 population expanded with S100a4[lin+] cells in the native and bridging scar tissue at D7 and D14 post-

181 surgery (Fig. 1B). Many resident tendon cells were actively expressing S100a4 at baseline, however

182 many S100a4^{-} cells were also present (Fig. 1D). Following injury, the S100a4[active+] population also

183 expanded, with abundant S100a4[active+] cells in the bridging scar tissue from D3 to D14, with a marked

184 decrease by D28 (Fig. 1D). Notably, the expansion of the S100a4[active+] population was also observed in

185 the healing Achilles tendon (Supplemental Fig. 1), suggesting potential conservation of S100a4 function

186   between tendons. Consistent with changes in spatial expression over time, *S100a4* mRNA expression

187   increased from D3 to a peak at D10, followed by a progressive decline through D28 (Fig. 1E).

188

189   *S100a4 haploinsufficiency promotes regenerative, mechanically superior tendon healing*

190   To determine the functional implications of decreasing S100a4 expression during tendon healing (Fig.

191   2A), we utilized S100a4 haploinsufficient mice (S100a4$^{GFP/+}$), which results in a 50% reduction in

192   *S100a4* mRNA expression in the tendon (Fig. 2B), as well as a robust decrease in S100a4 protein

193   expression during tendon healing (Fig. 2C). Decreased S100a4 expression did not noticeably alter the

194   spatial localization of S100a4$^+$ cells in either the un-injured tendon or at D14 post-surgery

195   (Supplemental Fig. 2). At D14 post-surgery, functional outcomes of scar formation in healing

196   S100a4$^{GFP/+}$ tendons were significantly improved compared to WT. A significant 36% increase in MTP

197   Flexion Angle was observed in S100a4$^{GFP/+}$ repairs, relative to WT (p=0.04) (Fig. 2D). Gliding

198   Resistance was significantly decreased by 43% in S100a4$^{GFP/+}$ repairs, relative to WT (p=0.028) (Fig.

199   2E), suggesting a reduction in scar formation in S100a4$^{GFP/+}$ repairs. In addition, maximum load at

200   failure was significantly increased (+35%) in S100a4$^{GFP/+}$ repairs relative to WT (p=0.003) (Fig. 2F),

201   while stiffness was increased 28% in S100a4$^{GFP/+}$ repairs, relative to WT, however this increase was not

202   statistically significant (p=0.08) (Fig. 2G). Taken together, these data suggest that S100a4

203   haploinsufficiency improves functional outcomes, while also improving tendon strength.

204

205   *S100a4 knockdown improves tendon morphology and decreases myofibroblast content.*

206   Morphologically, both ABHOG and picrosirius red staining demonstrate more highly aligned, mature

207   collagen fibers bridging the tendon ends in S100a4 haploinsufficient mice (blue arrows, Fig. 3A).

208   Consistent with this, *Col1a1* expression was significantly increased 5.6-fold (p=0.0095) in S100a4$^{GFP/+}$

209   repairs, relative to WT repairs (Fig. 2B), while no change in *Col3a1* or *Scx* were observed between

210   groups (Fig. 2C & D). Expression of the myofibroblast marker *α-SMA* was decreased 2.4-fold in

211   S100a4$^{GFP/+}$ repairs, relative to WT (p=0.02) (Fig. 2E). Consistent with this, a marked decrease in *α-*

212   *SMA* protein was also observed in S100a4$^{GFP/+}$ repairs, relative to WT (white arrows, Fig. 3F). These

213 data suggest that S100a4 haploinsufficiency promotes regenerative tendon healing via deposition of a

214 mature Col1 ECM and a decrease in pro-fibrotic myofibroblasts.

215

216 *S100a4 modulates macrophage function.*

217 Given the fibrotic nature of scar-mediated tendon healing, and the ability of macrophages to modulate

218 multiple aspects of the fibrotic process(20-23), we examined changes in macrophage content during

219 healing in S100a4 haploinsufficient mice, and changes in migration and polarization in response to

220 S100a4 in vitro. A substantial decrease in F4/80$^+$ macrophages were observed in S100a4$^{GFP/+}$ repairs,

221 relative to WT (white arrows, Fig. 4A), suggesting that knock-down of S100a4 can suppress

222 macrophage recruitment or retention during tendon healing. S100a4-RP was shown to drive

223 macrophage migration relative to vehicle, with a significant increase at the highest dose of 1000ng/mL

224 (Fig. 4B). S100a4-RP treatment of primary macrophages appeared to have a variable impact on

225 polarization. M1 markers *iNOS* and *CD64* were significantly upregulated in a dose-dependent manner

226 following S100a4-RP treatment, while *TNFα* was downregulated and no change observed in *CD86*

227 expression (p < 0.05) (Fig. 4C). M2 markers *Arg1* and *IL1ra* were significantly upregulated with higher

228 doses of RP-S100a4, *CD163* was downregulated and no change seen in *CD206* (p<0.05) (Fig. 4D).

229 Collectively this data supports a role for extracellular S100a4 in regulating macrophage migration and

230 polarization.

231

232 *Inhibition of S100a4 signaling, via antagonism of RAGE improves tendon healing.*

233 Considering that knockdown of S100a4 expression improves healing, and S100a4 can function as an

234 extracellular signaling molecule to drive fibrotic progression (24-26), we next examined expression of

235 the putative S100a4 receptor, RAGE (Receptor for Advanced Glycation Endproducts) (27-29). RAGE

236 expression was observed throughout the scar tissue during tendon healing, with abundant co-

237 localization of S100a4 and RAGE (Fig. 4A). Consequently, we investigated the feasibility of inhibiting

238 S100a4 signaling via disruption of S100a4-RAGE interaction using RAGE antagonist peptide

239 (RAP)(30). In vivo, RAP treatment (Fig. 5B) significantly improved measures of gliding function relative

240 to vehicle treated controls, with a 41% increase in MTP Flexion Angle (p=0.008) (Fig. 5C), and a 39%

241 decrease in Gliding Resistance (p=0.007) (Fig. 5D). No differences in maximum load at failure (p=0.57)

242 and stiffness (p=0.30) were observed between groups (Fig. 5E, F). These data suggest that inhibition of

243 S100a4-RAGE recapitulates the improvements in gliding function seen with S100a4 haploinsufficiency

244 but is insufficient to improve mechanical properties.

245

246 *S100a4$^+$ cell ablation results in aberrant matrix deposition during tendon healing.*

247 While knock-down of S100a4 expression and inhibition of S100a4 signaling improves tendon healing,

248 S100a4 can also function in a cell-autonomous manner (31). To determine the effects of S100a4$^+$ cell

249 ablation on tendon healing, we depleted proliferating S100a4$^+$ cells from D5-10 post-surgery

250 (immediately preceding peak *S100a4* expression) (Fig. 6A). Depletion of S100a4$^+$ cells from D5-10

251 resulted in a significant 91% reduction in *S100a4* mRNA expression (p<0.001) (Fig. 6B), and a

252 substantial reduction in S100a4 protein expression, relative to WT (Fig. 6C). Functionally, slight but

253 non-significant improvements in gliding function were observed in S100a4-TK (D5-10), relative to WT

254 (Fig. 6D & E). However, max load at failure was significantly decreased by 43% (p=0.02) (Fig. 6F),

255 while stiffness was unchanged (Fig. 6G). Morphologically, S100a4-TK (D5-10) tendons healed with

256 thinner, more acellular bridging scar tissue between the native tendon ends, compared to the larger,

257 more cellular granulation tissue in WT repairs (Figure 6H). Picrosirius staining demonstrated a

258 substantial reduction in bridging ECM in S100a4-TK (D5-10) repairs, relative to WT (Fig. 6I). In contrast

259 to this, qPCR revealed significant increases in ECM proteins *Col1a1* (3.9-fold, p=0.04) (Supplemental

260 Figure 3A) and *Col3a1* (1.9-fold, p=0.033) (Supplemental Figure 3B) mRNA expression in S100a4-TK

261 (D5-10) mice. Additionally, significant decreases in the tenogenic transcription factor *Scx* (1.75-fold,

262 p=0.04), and the myofibroblast marker α-SMA (9-fold, p=0.0003) were observed in S100a4-TK (D5-10),

263 relative to WT (Supplemental Figure 3C & D). Consistent with this, and the phenotype in S100a4$^{GFP/+}$

264 repairs, α-SMA staining was markedly reduced in S100a4-TK (D5-10) repairs, relative to WT

265 (Supplemental Figure 4), as was total macrophage content (Supplemental Figure 5). Taken together,

266 S100a4-cell depletion alters normal matrix deposition during tendon healing, leading to pronounced

267 morphological changes in the scar tissue and a loss of overall strength, while recapitulating the

268 changes in myofibroblast and macrophage populations observed in S100a4$^{GFP/+}$ repairs.

269

270   We then investigated the effect of continuous S100a4 cell depletion (D1-14) on healing (Figure 7A). In

271   contrast to depletion from D5-10, depletion from D1-14 significantly reduced MTP Flexion Angle (-53%,

272   p=0.0003) (Fig. 7B), and increased gliding resistance (+187%, p<0.001) (Fig. 7C), indicating

273   impairment of normal gliding function with sustained S100a4$^+$ cell depletion. Consistent with D5-10

274   depletion, depletion from D1-14 reduced mechanical properties, with a 43% decrease in max load

275   (p=0.025) (Fig. 7D), and a 49% decrease in stiffness (p=0.0078) (Fig. 7E).

276

277   *S100a4-lineage cells represent differentiated a-SMA myofibroblasts in the scar tissue of healing*

278   *tendon.*

279   Examination of S100a4$^{GFP/+}$ and S100a4-TK (D5-10) healing tendons demonstrate dramatically reduced

280   myofibroblast content, suggesting potential interplay between these populations. The relationship

281   between S100a4 and pro-fibrotic myofibroblasts is controversial and likely tissue-dependent, with

282   conflicting reports of myofibroblast fate for S100a4$^+$ cells(32-35). To understand the relationship

283   between these cell populations during tendon healing we examined α-SMA expression in both S100a4-

284   lineage cells and cells actively expressing S100a4. S100a4-Cre; Ai9 mice demonstrate that most α-

285   SMA$^+$ myofibroblasts at D14 post-surgery are derived from S100a4-lineage, as shown by co-

286   localization (Fig. 8A, arrows). In contrast, very few S100a4$^{active+}$ cells demonstrated co-localization with

287   α-SMA (Fig. 8B). Taken together these data suggest that S100a4-lineage cells lose S100a4 expression

288   during the transition to α-SMA$^+$ myofibroblasts.

289

290   **Discussion**

291   Consistent with the role of S100a4 as a key driver of fibrosis (5, 14, 26, 36), we have demonstrated that

292   S100a4 promotes scar-mediated tendon healing via cell-non-autonomous extracellular signaling.

293   Further, we have shown that knockdown of S100a4 expression drives regenerative tendon healing and

294   that depletion of S100a4$^+$ cells disrupts re-acquisition of mechanical properties. Interestingly, the

295   effects of S100a4$^+$ cell depletion on functional metrics were time-dependent, suggesting a period of

296   optimal S100a4 inhibition. Mechanistically, S100a4 interacts with RAGE and modulates macrophage

297   content and function, suggesting the ability of S100a4 to regulate the inflammatory milieu during tendon

298   healing. In addition, S100a4-lineage cells lose expression of S100a4 to become α-SMA$^+$ pro-fibrotic

299   myofibroblasts, which are likely involved in both restoration of matrix integrity and deposition of excess

300   ECM. Taken together, these data establish S100a4 haploinsufficiency as a novel model of

301   regenerative, mechanically superior tendon healing, and identify S100a4 as a potent anti-fibrotic

302   therapeutic candidate to improve tendon healing.

303

304   S100a4 lacks enzymatic activity and functions predominantly through the regulation and interaction with

305   other proteins. While the intracellular functions of S100a4 are not well-characterized, the extracellular

306   signaling functions of S100a4 include regulation of multiple cellular processes important in fibrosis

307   including motility (37, 38), differentiation (39-41) and survival (39). S100a4 protein levels are strongly

308   correlated with idiopathic pulmonary fibrosis(34), and S100a4 has been suggested as a potential

309   fibrotic biomarker in the liver (4). Consistent with this, we see highest S100a4 expression immediately

310   prior to the period of peak scar formation during tendon healing. The therapeutic potential of targeting

311   S100a4 has been well established in the lung, with decreased fibrotic progression following treatment

312   with an S100a4 neutralizing antibody (34), and pharmacological inhibition of S100a4 (42). Moreover,

313   Tomcik *et al*., demonstrated that deletion of S100a4 prevented bleomycin-induced skin fibrosis(26).

314   Consistent with these studies we demonstrated the knock-down of S100a4 is sufficient to attenuate

315   scar-mediated tendon healing and promote a more regenerative response. More specifically,

316   S100a4$^{GFP/+}$ repairs demonstrate suppression of multiple components of the fibrotic cascade including

317   macrophage content, myofibroblasts, as well as an altered ECM balance toward a mature tendon

318   composition (Col1a1> Col3a1).

319

320   Macrophages play an essential role in wound healing, and S100a4 is a potent chemokine and regulator

321   of macrophage chemotaxis. Bone marrow-derived macrophages from S100a4$^{-/-}$ mice display defects in

322   chemotactic motility and impaired recruitment to the site of inflammation (43). While we have not

323   assessed the effects of S100a4 knockdown in macrophages, we have demonstrated that S100a4 alters

324   macrophage migration and polarization in vitro and have shown that macrophage content is markedly

325 reduced in S100a4 haploinsufficient and S100a4$^+$ cell-depleted repairs, suggesting a potential role for

326 extracellular S100a4 in macrophage recruitment during tendon healing. Moreover, while RAGE can be

327 expressed on a variety of cell types, macrophages have been shown to be the primary source of RAGE

328 in the context of pathology (44-47). Therefore, in addition to decreasing the macrophages content, and

329 thereby RAGE$^+$ cells, S100a4$^{GFP/+}$ may also suppress signaling via direct down-regulation of RAGE.

330 RAGE expression is highly dependent on ligand concentration (48), and we have decreased *S100a4*

331 expression by 50%, implying down-regulation of the entire S100a4-RAGE-macrophage axis in

332 S100a4$^{GFP/+}$ mice. Interestingly, although inhibition of S100a4-RAGE with RAP recapitulates the

333 improvements in gliding function observed in S100a4$^{GFP/+}$ repairs, it is insufficient to improve

334 mechanical properties. This suggests that S100a4 may regulate restoration of mechanical properties

335 through mechanisms independent of RAGE. In addition to RAGE, S100a4 can interact with EGFR (49)

336 , Heparan Sulfate Proteoglycans (50), TLR4 (51) and Annexin 2 (52) receptors. However, these

337 S100a4-receptor interactions have been primarily observed in metastasis and neurological disorders.

338 Understanding the potential RAGE independent mechanisms of S100a4 signaling will be an important

339 aspect of future research.

340

341 S100a4$^+$ cell depletion has shown great efficacy in managing fibrotic progression in the peritoneum

342 (10), and kidney (9). In contrast, depletion of S100a4$^+$ cells impairs tendon healing, particularly when

343 S100a4$^+$ cells are depleted from D1-14. These opposing effects may be due to fundamental

344 differences in the functions of affected tissues, with tendon being a mechanical, load-bearing tissue.

345 Interestingly, the more pronounced negative effect of cell depletion from D1-14, relative to D5-10

346 depletion may be explained by suppression of the acute inflammatory phase. Anti-inflammatory

347 administration during the acute inflammatory phase of tendon healing is effective at reducing scar

348 formation, but causes marked reductions in mechanical properties (53-55). Taken together, these data

349 further support the importance of timing treatment to modulate fibrotic tendon healing, particularly as it

350 relates to S100a4 inhibition.

351

352   One of the main controversies surrounding S100a4$^+$ cells is their potential to become α-SMA$^+$

353   myofibroblasts. Osterreicher *et al*., demonstrated that neither actively expressing S100a4$^+$ cells or

354   S100a4^{lin+} cells express α-SMA during lung fibrosis (56), and additional work demonstrates that

355   S100a4$^+$ bone marrow cells do not express α-SMA (57). In contrast, dermal fibroblasts are positive for

356   expression of both S100a4 and α-SMA (56) and Chen *et al*., demonstrate that S100a4 treatment

357   increases α-SMA expression, while α-SMA$^+$ cells decrease with S100a4-cell depletion in a model of

358   liver fibrosis (4). Using a similar combination of S100a4-lineage tracing and active S100a4 expression

359   analyses as in (56), we demonstrate that S100a4^{lin+} cells become α-SMA$^+$, and that the α-SMA$^+$

360   population is largely negative for S100a4. These data not only define a terminal myofibroblast fate for

361   many S100a4-lineage cells during tendon healing, but further support the concept of exquisite cell and

362   tissue specificity of S100a4 signaling. In addition to terminal cell fate, the origin of S100a4$^+$ cells are

363   unclear. Osterreicher *et al*., demonstrate that bone marrow derived cells (BMDCs) represent the main

364   source of S100a4 during liver fibrosis (56), and S100a4$^+$ BMDCs have been shown to migrate to the

365   site of neointima formation following vein grafting (57). While we have not traced S100a4$^+$ BMDCs in

366   this study, we have previously shown specific recruitment of BMDCs to the healing tendon (58), and we

367   also see S100a4^{Lin+} and S100a4$^{active+}$ populations in the tendon without injury. Future studies will seek

368   to delineate the relative contribution of S100a4$^+$ cells from different origins to scar-mediated tendon

369   healing.

370

371   While we clearly identify S100a4 haploinsufficiency as a model of regenerative tendon healing, there

372   are several limitations that must be considered. First, we have not investigated whether this signaling

373   paradigm is conserved in other tendons. However, we do observe S100a4$^+$ cells in the Achilles tendon

374   at homeostasis and expansion of this population following injury, and since scar-mediated healing is

375   consistent between tendons(59), this reinforces the potential application of this approach to improve

376   healing in multiple tendons. Second, the long-term effects of inhibiting S100a4-RAGE signaling are

377   unknown, as we have only examined healing at D14. However, peak expression of S100a4 at D10

378   post-surgery suggests the prime effects of S100a4 may occur during the early inflammatory/matrix

379   deposition phases of healing. Moreover, the time-dependent effects of S100a4-cell depletion suggest

380   there is likely an optimal therapeutic window for S100a4 inhibition, which will be examined in future

381   studies. Finally, we have not delineated between the effects of S100a4 expression in resident tendon

382   cells relative to expression in extrinsic cells, and how the cell origin of S100a4 may dictate the effects

383   on healing.

384

385   Restoring satisfactory function following tendon injury has remained an intractable clinical problem for

386   decades (60). To our knowledge this is the first model of regenerative tendon healing in transgenic

387   mice, defined by improvements in range of motion and mechanics. These data will inform future work to

388   define the pathways down-stream of S100a4-RAGE, rigorously determine the time-dependent effects of

389   S100a4 inhibition and identify the cues that drive the transition of S100a4-lineage cells to

390   myofibroblasts. More directly however, these studies define the tremendous potential of inhibition of

391   S100a4 signaling as a therapeutic approach to promote regenerative tendon healing.

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397   **Figure Legends**

398

399   **Figure 1. S100a4 is expressed by resident tenocytes and the S100a4$^+$ cell population expands**

400   **during tendon healing**. (A & B) S100a4-Cre; Rosa-Ai9 reporter mice demonstrate efficient targeting of

401   resident tendon cells. Following injury, the S100a4-lineage population expands, with S100a4^{Lin+} cells in

402   the native tendon stubs and the bridging scar tissue at D7 and D14 post-surgery. Tendons are outlined

403   in white, and bridging granulation tissue outlined in blue. (C) The S100a4-GFP^{promoter} construct identifies

404   cells actively expressing S100a4 (S100a4$^{active+}$). (D) A subpopulation of resident tenocytes is

405   S100a4$^{active+}$ at baseline, and the S100a4$^{active+}$ population increases following injury, with S100a4$^{active+}$

406   cells observed in the bridging scar tissue and native tendon ends through D28 post-surgery. Tendons

407   are outlined in white, and bridging granulation tissue outlined in orange, (*) identifies sutures. (E) qPCR

408   analysis of S100a4 during tendon healing demonstrates peak S100a4 expression at D10, followed by a

409   progressive decline through D28 (n=3 per time-point). (*) indicates p<0.05 vs. D3 repair (1-way

410   ANOVA). Data were normalized to expression in D3 repairs, and the internal control β-actin.

411

412   **Figure 2. S100a4 knockdown promotes regenerative, mechanically superior tendon healing.** (A)

413   S100a4$^{GFP/+}$ haploinsufficient and wild type (WT) littermates underwent transection and repair of the

414   FDL tendon, and tendons were harvested at D14 post-surgery. (B) *S100a4* mRNA expression was

415   reduced by 50% in S100a4$^{GFP/+}$ tendon repairs, relative to WT (n=3 per group). (C) A substantial

416   reduction in S100a4 protein expression was observed in S100a4$^{GFP/+}$ tendon repairs, relative to WT.

417   Tendon ends are outlined in blue and bridging scar tissue outlined in black (n=3-4 per group). (D-G) At

418   D14, MTP Flexion Angle was significantly increased in S100a4$^{GFP/+}$ (D), and Gliding Resistance was

419   significantly decreased in S100a4$^{GFP/+}$ repairs (E). Max load at failure was significantly improved in

420   S100a4$^{GFP/+}$ repairs (F), while no change in Stiffness was observed between genotypes (G) (n=7-10 per

421   group). (*) indicates p<0.05, (**) indicates p<0.01 between genotypes, n=7-10 for (D-G) (un-paired t-

422   test).

423

424   **Figure 3. S100a4 haploinsufficiency enhances deposition of a mature Collagen matrix and**

425   **reduced myofibroblast content.** (A) ABHOG and picrosirius red staining demonstrate an increase in

426   mature collagen fibers (blue arrows) bridging the tendon ends in S100a4$^{GFP/+}$ repairs compared to WT

427   littermates (n=3-4 per group) (*) indicate sutures. (B-D) S100A4$^{GFP/+}$ tendons expressed significantly

428   more *Col1a1* mRNA (B), while transcript levels of *Col3a1* (C) and *Scx* (D) were unaffected by S100a4

429   haploinsufficiency. (*) indicates $p < 0.05$ (un-paired t-test), n=3 per group. (E & F) *α-SMA* mRNA

430   expression was significantly decreased in S100a4$^{GFP/+}$ repairs (E) (n=3 per group), while a substantial

431   reduction in *α-SMA* protein expression was observed in S100a4$^{GFP/+}$, relative to WT, using

432   immunofluorescence. White arrows indicate areas of α-SMA$^+$ cells in the healing tissue, while yellow

433   arrowheads denote α-SMA staining of vessels (F).

434

435   **Figure 4. S100a4 alters macrophage function and response to tendon injury.** (A) F4/80 staining

436   demonstrates decreased macrophage content in the healing tendon of S100a4$^{GFP/+}$ repairs. White

437   arrows identify concentrated areas of macrophages. Tendon ends are outlined in white, while scar

438   tissue is outlined in yellow (n=3-4 per group). (B) S100a4 promotes BMDM migration (n=3 per

439   treatment). (****) Indicates $p < 0.0001$ vs. vehicle treated cells (1-way ANOVA). (C & D) Following

440   treatment with S100a4-RP (20-1000ng/mL), significant increases in M1 polarization markers (*iNos*,

441   CD64) were observed relative to vehicle treated primary macrophages, a significant decrease in *TNFα*,

442   and no change in *CD86* expression (C). Significant increases in M2 markers *Arg2* and *IL1ra* were seen

443   with S100a4-RP treatment, a decrease in *CD163* expression, and no change in *CD206* (D) (n=3 per

444   treatment). (*) indicates $p < 0.05$ between vehicle and S100a4-RP treatment (1-way ANOVA).

445

446   **Figure 5. Inhibition of S100a4 signaling via RAGE antagonism improves tendon healing.**

447   (A) Co-immunofluorescence demonstrated co-localization of S100a4 and its putative receptor RAGE in

448   the healing tendon (n=3). (B) C57Bl/6J mice were treated with either RAP or vehicle, via i.p. injection

449   from D5-10 post-surgery, and harvested at D14 for functional testing. (C-F) At D14 RAP treatment

450   significantly improved measures of gliding function relative to vehicle, with a (C) significant increase in

451   MTP Flexion Angle, and (D) a significant decrease in Gliding Resistance. No change in (E) Max load at

452 failure, or (F) Stiffness was observed between treatments (n=13 per group). (**) indicates p<0.01

453 between treatments (un-paired t-test).

454

455 **Figure 6. Delayed depletion of S100a4+ cells impairs restoration of mechanical properties and**

456 **alters matrix deposition.** (A) WT and S100a4-TK mice were treated twice daily with ganciclovir (GCV)

457 from D5-10 post-surgery. (B) S100a4+ cell depletion results in a 91% reduction in S100a4 mRNA at

458 D10 post-surgery (n=3). (C) A substantial reduction in S100a4 protein expression was observed

459 S100a4-TK repairs, relative to WT. Tendon is outlined in blue, scar tissue is outlined in black and (*)

460 identify sutures (n=4). (D-G) At D14 no change in MTP Flexion Angle (D) and Gliding Resistance (E)

461 were observed between WT and S100a4-TK repairs. (F) Max load at failure was significantly reduced

462 following S100a4-cell depletion, while no change in Stiffness was observed (G) (n=7-10), (**) indicates

463 p<0.01 (un-paired t-test). (H & I) Morphologically, (H) ABHOG and (I) Picrosirius staining demonstrate

464 reduced matrix deposition bridging the tendon ends in the S100a4-TK repairs, relative to WT. (*)

465 Indicates sutures.

466

467 **Figure 7. Sustained ablation of S100a4+ impairs restoration of gliding function and mechanical**

468 **properties.** (A) WT and S100a4-TK mice were treated with GCV from D1-14 post-surgery to ablate

469 proliferating S100a4+ cells. At D14 (B) MTP Flexion Angle was significantly reduced, and (C) Gliding

470 Resistance was significantly increased in S100a4-TK repairs, relative to WT. (D) A non-significant

471 decrease in Max load at failure and (E) a significant reduction in Stiffness were observed in S100a4-TK

472 repairs (n=8-11). (*) indicates p<0.05, (**) indicates p<0.01 (un-paired t-test).

473

474 **Figure 8. S100a4-lineage cells lose S100a4 expression during the transition to α-SMA+**

475 **myofibroblasts.** (A) Co-localization of Red Fluorescent Protein (S100a4[lin+] cells; red) and the

476 myofibroblast marker α-SMA (white) demonstrated abundant co-localization (yellow arrows) during

477 tendon healing. (B) Minimal co-localization of α-SMA (white) and cells actively expressing S100a4

478 (S100a4[active+]; green) was observed during healing (n=3). (C) Schematic representation of the cell non-

479   autonomous signaling functions of S100a4 in fibrotic healing, as well as cell fate of S100a4-lineage

480   cells.

481   **Supplemental Figures**

482

483   **Supplemental Figure 1.   S100a4⁺ cells are found in the healthy and healing Achilles tendon.**

484   S100a4-GFP$^{Promoter+}$ (S100a4$^{active+}$) cells are observed in the native Achilles tendon, and a S100a4$^+$

485   population persists following complete transection and repair of the Achilles tendon at D14 post-

486   surgery.

487

488   **Supplemental Figure 2. S100a4$^{GFP/+}$ knock-in mice permit tracing of S100a4 haploinsufficient**

489   **cells.** To determine if S100a4 haploinsufficiency altered the S100a4$^+$ population during healing,

490   fluorescent imaging of uninjured and D14 repairs from S100a4$^{GFP/+}$ and S100a4$^{+/+}$ (WT) mice were

491   analyzed. No GFP expression was observed in S100a4$^{+/+}$ WT mice either at baseline or at D14 post-

492   surgery. In contrast, knock-down of S100a4 does not alter the S100a4^{GFP+} resident tendon cell

493   population, or the expansion of the S100a4^{GFP+} population at D14 post-surgery. Orange insets identify

494   high-power magnification images.

495

496   **Supplemental Figure 3. S100a4$^+$ cell depletion alters expression of matrix, tenogenic and**

497   **myofibroblast-associated genes.** qPCR analyses demonstrated significant increases in (A) *Col1a1*,

498   and (B) *Col3a1* expression, while (C) *Scx* and (D) α-*SMA* expression levels were significantly reduced

499   in S100a4-TK, relative to WT. Data were normalized to expression in WT samples and the internal

500   control β-actin. (*) Indicates $p<0.05$, (***) indicates $p<0.001$, (n=3 per group)(un-paired t-test).

501

502   **Supplemental Figure 4. S100a4$^+$ cell depletion reduces α-SMA$^+$ myofibroblast content during**

503   **healing.** At D14 post-surgery, abundant α-SMA$^+$ myofibroblasts (red) were observed in WT repairs. In

504   contrast, α-SMA$^+$ myofibroblast content was markedly reduced in S100a4-TK (D5-10) repairs. White

505   arrows indicate areas of α-SMA$^+$ cells in the healing tissue, while yellow arrowheads denote α-SMA

506   staining of vessels. Tendons are outlined in white and scar tissue outlined in red.

507

508 **Supplemental Figure 5. S100a4$^+$ cell depletion reduces macrophage content during healing.** At

509 D14 post-surgery abundant F4/80$^+$ macrophages (red) were observed in WT repairs. In contrast,

510 F4/80$^+$ macrophage content was markedly reduced in S100a4-TK (D5-10) repairs. White arrows

511 identify concentrated areas of macrophages. Tendon ends are outlined in white, while scar tissue is
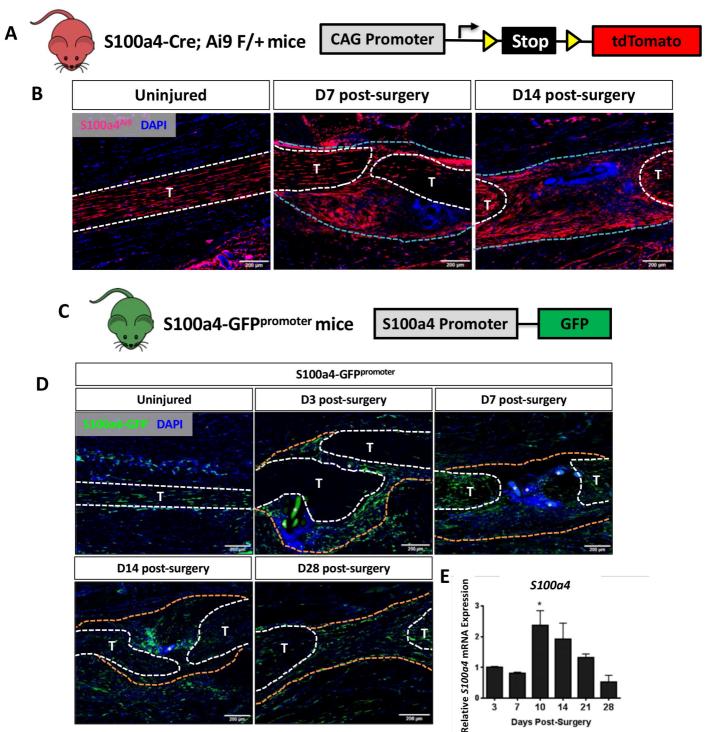
512 outlined in red. (*) indicates sutures.

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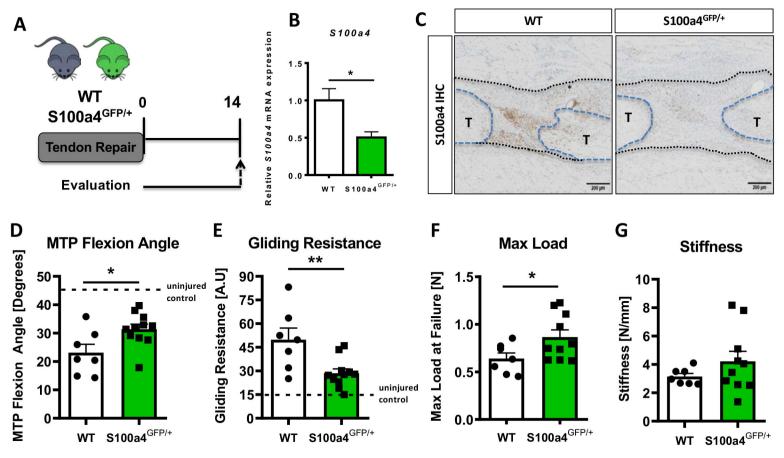
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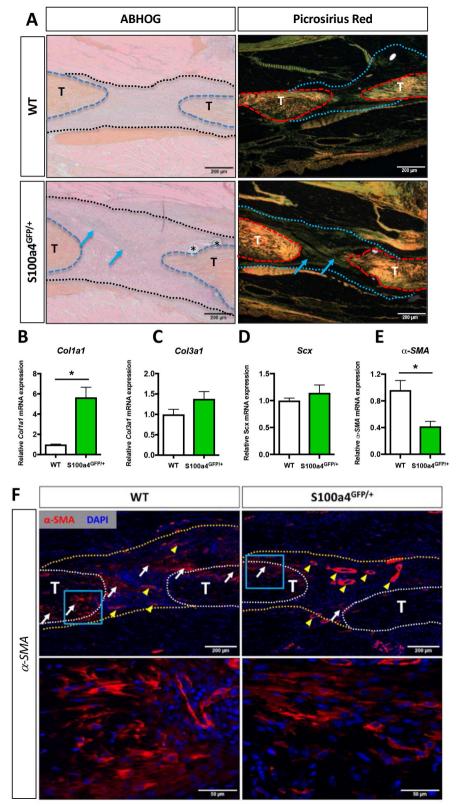
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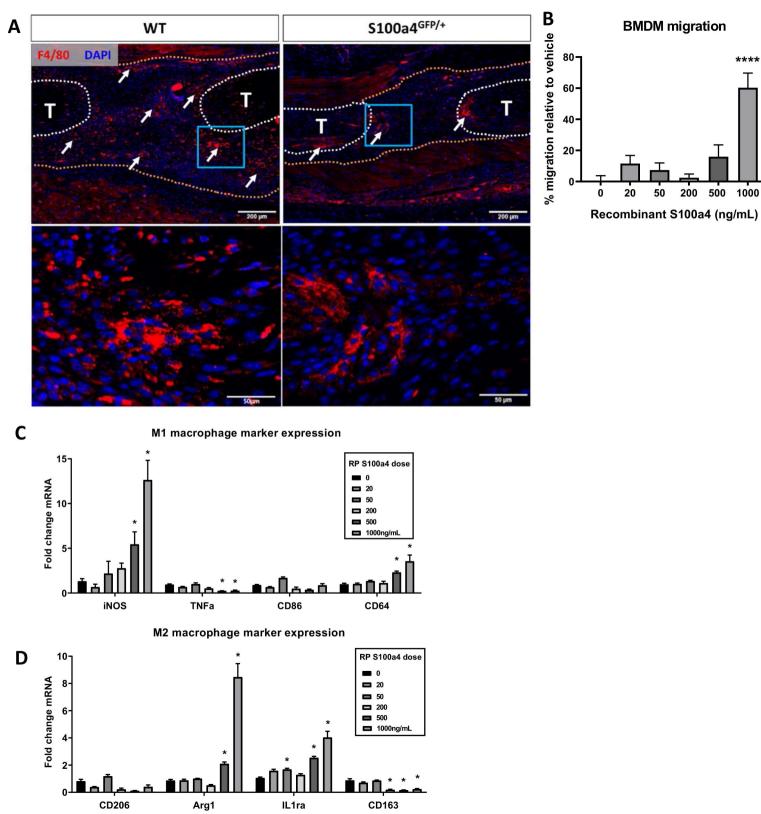
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A S100a4-Cre; Ai9 F/+ mice

CAG Promoter → Stop → tdTomato

B

Uninjured	D7 post-surgery	D14 post-surgery

S100a4^Ai9 DAPI

T

C S100a4-GFP^promoter mice

S100a4 Promoter — GFP

D

S100a4-GFP^promoter

Uninjured	D3 post-surgery	D7 post-surgery

S100a4-GFP DAPI

D14 post-surgery	D28 post-surgery

E

S100α4

Relative *S100α4* mRNA Expression

Days Post-Surgery

A WT S100a4^GFP/+

Tendon Repair

0 — 14

Evaluation

B *S100a4*

Relative *S100a4* mRNA expression

C S100a4 IHC

WT   S100a4^GFP/+

T   T   T   T

200 μm   200 μm

D MTP Flexion Angle

MTP Flexion Angle [Degrees]

uninjured control

WT   S100a4^GFP/+

E Gliding Resistance

Gliding Resistance [A.U]

uninjured control

WT   S100a4^GFP/+

F Max Load

Max Load at Failure [N]

WT   S100a4^GFP/+

G Stiffness

Stiffness [N/mm]

WT   S100a4^GFP/+

A

	ABHOG	Picrosirius Red
WT		
S100a4^{GFP/+}		

B *Col1a1*

C *Col3a1*

D *Scx*

E α-*SMA*

F α-*SMA*

WT	S100a4^{GFP/+}
α-SMA DAPI	

A

WT — S100a4^GFP/+

F4/80 DAPI

B BMDM migration

C M1 macrophage marker expression

D M2 macrophage marker expression

A C57BL/6J D14 post-surgery

S100a4 RAGE DAPI
200 μm
50 μm

B RAP or vehicle
D5-10

C57BL/6J

Tendon Repair

0   7   14

Evaluation

C MTP Flexion Angle

D Gliding Resistance

E Max Load

F Stiffness

A (mouse diagram) WT / S100a4-TK, Tendon Repair, GCV D5-10, timeline 0 3 7 10 14, Evaluation

B *S100a4*
Relative *S100a4* mRNA expression
WT ~ 1.0, S100a4-TK ~ 0.0, ***

C S100a4 IHC — WT | S100a4-TK (T, *, scale bar 200 µm)

D **MTP Flexion Angle**
MTP Flexion Angle [Degrees]
uninjured control
WT | S100a4-TK

E **Gliding Resistance**
Gliding Resistance [A.U.]
uninjured control
WT | S100a4-TK

F **Max Load at Failure**
Max Load at Failure [N]
**
WT | S100a4-TK

G **Stiffness**
Stiffness [N/mm]
WT | S100a4-TK

H ABHOG — WT | S100a4-TK (T, *)

I Picrosirius Red — WT | S100a4-TK (T, *, scale bar 200 µm)

A — WT / S100a4-TK mice; GCV D1-14; Tendon Repair; Evaluation

B MTP Flexion Angle — MTP Flexion Angle [Degrees]; WT vs S100a4-TK; ***

C Gliding Resistance — Gliding Resistance [A.U]; WT vs S100a4-TK; ****

D Max Load at Failure — Max Load at Failure [N]; WT vs S100a4-TK; *

E Stiffness — Stiffness [N/mm]; WT vs S100a4-TK; **

A D14 S100a4-Cre; Ai9 D14 post-surgery

T

S100a4^lineage+ α-SMA DAPI

200 μm

50 μm

B D14 S100a4^promoter D14 post-surgery

T   T

S100a4^active+ α-SMA DAPI

200 μm

50 μm

C

S100a4^lineage+

S100a4^lineage+; S100a4^active-

αSMA+ Myofibroblasts

S100a4^lineage+; S100a4^active+;

S100a4 Signaling

MΦ Recruitment

RAGE

Fibrotic Tendon Healing