

Physiological Dosing of MRL-Derived Recombinant Protein Therapeutic Improves Patellar Tendon Healing After Acute Injury

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INTRODUCTION: Tendon ruptures heal by forming scar, a mechanically inferior tissue characterized by hypercellularity and disorganized matrix, which impedes tendon function. The MRL/MpJ (MRL) mouse has been shown to be a model of improved patellar tendon (PT) healing compared to canonical healer C57/B6 (B6) mice.¹ Further investigation showed that the local tissue environment of the MRL tendon drives its improved healing response,² motivating its use as a therapeutic. More specifically, exogenous addition of decellularized MRL provisional extra-cellular matrix (ECM) harvested 1 week after PT midsubstance punch injury, known as M7, improved healing of midsubstance punched B6 tendons when compared to addition of decellularized B6 ECM.³ We then digested this M7 therapeutic and combined it with the soluble factors removed by decellularization. *In vitro* analysis showed that the combination of M7 digest with secretome more effectively promoted MRL cell behavior in B6 cells.⁴ Proteomic analysis identified 29 proteins (POIs) that were particularly enriched in these MRL homogenates² which we have shown can alter mechanical properties in a dose dependent manner.⁵ The promising results obtained suggest that a meaningful and substantial therapeutic improvement can be gained from optimizing the dosage of the entire panel in conjunction with refinement of the concentrations of each protein based on physiological data. In the present study, we determined the physiological concentrations of each of the 29 proteins and sought to establish the efficacy of physiologically dosed POIs on an acute PT injury. We hypothesize that delivery of these proteins at a physiologically relevant dose and duration in an *in vivo* acute PT midsubstance punch injury will improve structural properties as manifested by improved alignment and organization.

METHODS: To determine the physiologically relevant amounts of the POIs, the absolute amounts in M7 were first determined by normalizing abundance values for all proteins of interest (POIs) in the M7 samples by dividing by the sum of normalized abundances of all peptides found in the sample to get a relative amount of each POI within the sample. An ELISA was then run on the M7 to determine the absolute concentration of TGFβhigh3 and fibronectin (FN). These concentrations were then scaled to the therapeutic dose in our *in vivo* study³ (200 μg total protein) to get absolute amounts of TGFβhigh3 and FN which were used to calculate the amounts of the other POIs. Subsequently, relative concentrations of secretomic POIs were normalized to the newly calculated concentration of mimecan, which is found in both the M7 and the MRL secretome and has a low coefficient of variation, thus determining every POI's physiologically relevant dosage. Subsequently, with IACUC approval, we conducted an *in vivo* study to evaluate the efficacy of 3 day delivery period for early healing, as measured by tendon structure at 14 dpi (days post injury). Briefly, sixteen- to nineteen-week-old male B6 mice underwent a 1.0 mm PT midsubstance punch injury. Immediately following injury, 100 μL 3-day Alzet osmotic pumps were placed subcutaneously on the back of the mouse with a catheter winding down to the lateral side of the injury site. The osmotic pumps delivered M7 digest (200 μg), PBS, or POIs at physiologically relevant dosages (n=4/group). The pumps were excised at 5 dpi per manufacturer's instructions. Mice were sacrificed at 14 dpi and the patellar-tibial complex was removed and processed for toluidine blue stain and analysis. IHC analysis was conducted by two blinded graders who assigned a score summing the following criteria: matrix organization (0-2), GAG content (0-1), cellularity (0-2), cell alignment (0-2), cell distribution (0-1) and cell nucleus morphology (0-2), where a high score was indicative of better healing. Treatment groups were compared using a one-way ANOVA with Kruskal-Wallis comparisons.

RESULTS: The two separately run ELISAs for TGFβhigh3 and PN led to POI absolute amounts within 10% of each other, and thus these amounts were averaged for use in the *in vivo* study. For the *in vivo* study conducted to evaluate the efficacy of the physiologically determined POI concentrations, two samples were discarded for poor embedding orientation. Pumps were emptied as verified by BCA protein quantification assay. Using the 3-day pumps, we have found that patellar tendons treated with POI showed significantly higher tissue organization than PBS controls at 14 dpi, but still significantly lower than the positive control M7 group at 14 dpi (Fig 1).

DISCUSSION: Supporting our hypothesis, M7 digest delivered via pump improved healing compared to the PBS controls, showing that solubilized M7 retains its therapeutic capacity. Excitingly, the physiological dose of POIs showed an increase in organization compared to PBS controls. However, the POI treatment still resulted in worse organization than M7 digest. The 3-day pump was chosen because of previously published work in which our therapeutic M7 laden PEG-4MAL gel released all protein by 3 dpi. However, important healing processes such as inflammation, cell recruitment, and matrix deposition all peak at various points after injury generally within 2 weeks.⁶ Because we have seen a dose dependent effect of POI on mechanics using a 14-day pump and the M7 POIs are derived from a 7 dpi timepoint, we believe that this 3-day delivery may not be enough time to affect changes to the relevant biological processes. For example, in an acute injury to the MRL mouse, relative MMP3 gene expression levels jump ten-fold between 1 dpi and 7 dpi.⁷ Therefore, to recapitulate the contribution to healing of this POI, one would want to sustain the delivery through at least 7 dpi which may not be achieved with a 3-day pump. In future work, we will deliver the therapeutic M7 and POIs using longer duration pumps or a PEG-4MAL gel with tunable, degradable crosslinkers.

SIGNIFICANCE/CLINICAL RELEVANCE: We have shown that recapitulating the physiologically relevant dosages of recombinant proteins induce improved healing at an early timepoint. Additionally we have shown that the digested M7 therapeutic maintains its therapeutic potential.

REFERENCES: ¹Sereysky 2013 *Int J Exp Path*, ²Paredes+ 2020 *FASEB*, ³Paredes+ 2020 *JOR*, ⁴Marvin+ 2023 *JOR*, ⁵Pacheco+ 2023 *ORS*, ⁶Chamberlain+ 2009 *Wound Rep Regen*, ⁷George+ 2019 *JOR*

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