Nano-layered Tendon Patch Orchestrates Timely Interplay between Macrophages and Tendon Stem/Progenitor Cells

Md Sarker, Anh Hoang, Emma Wang, Hun Jin Jeong, Chang H Lee
Center for Dental and Craniofacial Research, Columbia University, New York, NY

DISCLOSURES: Md Sarker (N), Anh Hoang (N), Emma Wang (N), Hun Jin Jeong (N), Chang H. Lee (N)

INTRODUCTION: The immune system plays essential roles in tendon pathology, repair, and regeneration. Macrophages are actively involved in tendon healing from the early inflammatory phase to the late remodeling phase through M1 and M2 polarization, respectively. We previously reported a novel combination of pharmacokinetic small molecules (SMs), Oxotremorine M (Oxo-M) and PPBP maleate (4-PPBP), which leads to tendon regeneration by promoting endogenous tendon stem/progenitor cells (TSCs). Given the newly discovered roles of Oxo-M and 4-PPBP, attenuating M1 polarization and promoting M2 polarization, respectively, we developed a sequential delivery system for these SMs to guide regenerative healing of tendons while modulating inflammation and tissue remodeling regulated by M1 and M2 macrophages. To enable a precisely controlled sequential delivery of Oxo-M and 4-PPBP, we implemented layer-by-layer (LbL) nanocoating with anionic and cationic polymers, where small molecules are loaded in between nanolayers connected through galvanic force. LbL fabrication technique is advantageous in controlling the release sequence, loading amount, and thickness of constructs. Our recent data supports the potential of the LbL tendon patch sequentially releasing Oxo-M and 4-PPBP in attenuating M1 polarization in the early healing phase and promoting M2 polarization in the later healing phase, consequently promoting tendon healing. In addition, our previous *in vitro* co-culture study suggested interactions between TSCs and macrophages under the timely stimulation of Oxo-M and 4-PPBP. This study was designed to investigate the effects of LbL tendon patch with sequential delivery of Oxo-M and 4-PPBP on gene expression profiles at the single cell level, potentially associated with interactions between macrophages and TSCs in the promoted tendon healing.

METHODS: LbL tendon patches were prepared per our established methods on a 3D-printed polycaprolactone (PCL) patch. Briefly, cationic polymer poly-l-lysine (PLL) and anionic polymer methacrylated hyaluronic acid (HAMA) were sequentially applied at a concentration of 0.5 mg/mL to build the $(PLL/HAMA)_{40}$ nanolayers. Oxo-M or 4-PPBP was loaded between the PLL (positively charged) and HAMA (negatively charged). 4-PPBP was loaded in the first half of the layers, and Oxo-M was loaded in the other half of the layers for the release of Oxo-M, followed by 4-PPBP (**Fig. 1A**). PCL barriers with 4 μm pores were added in between the 4-PPBP-loaded and Oxo-M-loaded layers to achieve a prolonged, sequential release of Oxo-M and 4-PPBP. A release test was conducted by incubating the SMs-loaded PCL patch into PBS for 6 wks, measuring Oxo-M and 4-PPBP concentrations by spectrophotometry at 230 and 208 nm, respectively. The SM-loaded LbL PCL patch was then applied to our well-established patellar tendon repair model. Briefly, after creating a full-thickness rat patellar tendon (PT) incision, a cerclage suture was applied, and then LbL PCL patch was attached on top of the repaired tendons. PCL patch without SM served as a control. At 1 wk and 2 wks post-op, cells were isolated from the harvested tendons for single-cell RNA sequencing (scRNA-seq) with Next Generation Sequencing (NGS). We utilized 10X Genomics analysis software to yield >1,000 unique molecular identifiers (UMI) and genes per cell, followed by producing the t-SNE graphs. At 4 wks, tensile tests were performed for functional evaluation of tendon healing.

RESULTS: The LbL tendon patch was prepared in a micro-thin membrane (**Fig. 1B**) showing 6,000 nm-thick 40 layers of PLL/HAMA with 75 nm of each polymer layer (**Fig. 1C**). Also, two distinct stacks of positive and negative polymers suggest that layer-by-layer nanocoating can maintain the coating uniformity. The $(PLL/HAMA)_{40}$ layered PCL patch with sequential loading of 4-PPBP and Oxo-M showed a fast release of Oxo-M by 7 - 10 days, followed by slow release of 4-PPBP up to 42 days (data not shown). Application of SM-releasing LbL tendon patches in full-transected rat patellar tendons (**Fig. 1D**) resulted in notable improvements in tendon healing with significantly enhanced collagen reorganization (data not shown) and functional properties (**Fig. 1F**) compared to controls with PCL patches without SMs. Immunofluorescence showed attenuated M1-like macrophages at 1 wk and promoted M2 polarization at 2 wks (data not shown). scRNA-seq data revealed multiple cell populations in the tendons, including but not limited to tenocytes, macrophages, T cells, B cells, and endothelial cells (**Fig. 2A**). At both time points, LbL tendon patch-treated tendons showed higher numbers of M2 macrophages and TSCs as compared to control (**Fig. 2A**). Consistently, IL-1β expression levels in macrophage clusters were lower in the treated group at 1 wk (**Fig. 2B**). Similarly, IL-10 expression levels were robust in the treated group at 2 wks in comparison with control (**Fig. 2B**). These findings are consistent with our previous *in vitro* co-culture data, suggesting early released Oxo-M attenuates M1 polarization and prolong-release 4-PPBP promotes M2 polarization. In addition, the numbers of TSCs (Tppp3+/Pdgfra+) and matured tenocytes (Tnmd+/Mkx+) were higher in LbL patch-treated tendons (data not shown). Our initial analysis of single cell-level gene profiles with intercellular signaling atlas suggested active interactions between TSCs and macrophages via IL6 and PDGFA signaling at early and later time points, respectively (data not shown).

DISCUSSION: Our data suggest the sequential release of Oxo-M and 4-PPBP via LbL tendon patch orchestrates tendon healing by regulating the polarization of macrophages and their interactions with TSCs in a timely manner. Consistently with our previous findings, the early healing phase with Oxo-M release showed attenuated expressions of M1-associated genes, and the later healing phase with 4-PPBP release showed elevated levels of M2-associated genes. Interestingly, the LbL tendon patch increased the numbers of matured tenocytes and TSCs during tendon healing, suggesting a likely connection between macrophage polarization and tenogenic differentiation, as our previous *in vitro* findings support. scRNA-seq data also suggested the inter-cellular communications between macrophages and TSCs via IL6 and PDGFA signaling. Further investigation is warranted to describe communications among other types of cells. We are currently implementing CellChat, a novel tool to infer and analyze intercellular communications from scRNA-seq data.

SIGNIFICANCE: Our findings indicate that the timely released Oxo-M and 4-PPBP from the LbL tendon patch guide regenerative tendon healing by regulating macrophage polarization, TSC differentiation, and intercellular interactions. Timely controlled delivery of Oxo-M and 4-PPBP with LbL patch has significant potential to enhance the clinical outcomes of tendon repair.

IMAGES AND TABLES:

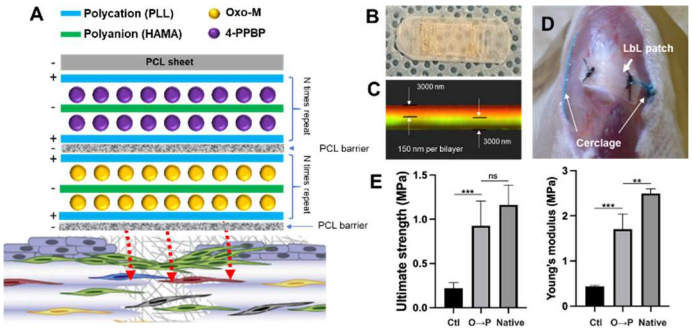

Fig. 1. LbL nanocoating on PCL patch to apply sequential delivery of Oxo-M and 4-PPBP during tendon healing (**A**). Prepared nano-layered tendon patch (**B**) with nanocoated film shown in confocal image (**C**). Nano-layered tendon patch was applied to full-transected rat PT (**D**). At 4 wks, sequential delivery of Oxo-M and 4-PPBP through nano-layered tendon patch significantly improved mechanical properties at the level of native tendon (**E**) ($p<0.001$; $n = 5$ per group).

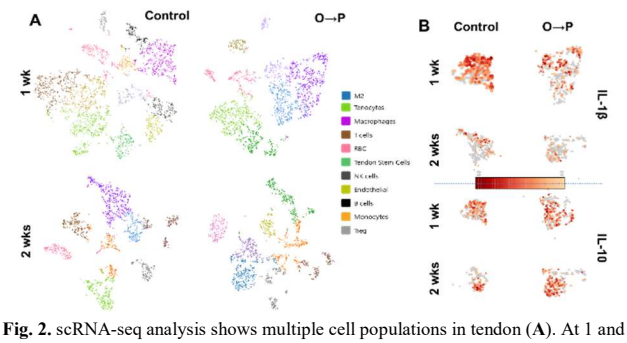

Fig. 2. scRNA-seq analysis shows multiple cell populations in tendon (**A**). At 1 and 2 wks post-op, more M2-like macrophages were identified with the O→P treatment via LbL patch than control. TSCs populations were robust in the O→P group at both time points. IL-1β expressions in the macrophage cluster were higher than control at 1 wk (**B**). IL-10 expressions in macrophages were higher in the treatment group at 2 wks (**B**).