A Multi-functional Small Molecule 4-PPBP Enhances Healing of Meniscus Tears and Functional Outcomes

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INTRODUCTION: Meniscus injuries lead to knee joint pain and weakened knee joint function, frequently progressing to cartilage thinning and osteoarthritis. Simultaneously, the endogenous repair capacities of the avascular meniscus are limited. The local delivery of small molecules and growth factors to the injured meniscus site could be challenging due to the physical barrier posed by the extracellular matrix. Fibrin, an irreplaceable constituent that forms a 3-dimensional biocompatible meshwork comprised of minuscule fibers, serves as a biodegradable material to deliver small molecules. Notably, this intricate structure attracts endogenous SMSCs, but also demonstrates distinctive viscoelastic characteristics defined by its mechanical reaction to varying frequencies of loading. Our previous studies demonstrated the efficacy of a bioactive glue that sequentially releases connective tissue growth factor (CTGF) and transforming growth factor beta 3 (TGF-β3) in meniscus healing and harnessing endogenous synovial mesenchymal stem/progenitor cells (SMSCs). However, an approach involving multiple growth factors has faced several translational challenges. To address these challenges, we repurposed a small molecule, 4-PPBP as an agonist for the Sigma 1 receptor (σ1R) to maintain endoplasmic reticulum homeostasis. Our previous studies suggested that 4-PPBP induces proliferation, migration, and differentiation of SMSCs, reduces local inflammation *in vitro* and promotes meniscus explant healing *ex vivo* (Fig. 1A). In this study, we investigated the efficacy of a genipin-crosslinked bioactive glue (FibGen) to deliver 4-PPBP/BD1047 for meniscus repair in a rodent model. This preclinical study helps researchers and clinicians better understand the biological mechanisms of σ1R signaling in regulating meniscus homeostasis and functional repair *in vivo*.

METHODS: *FibGen glue preparation*: 200 mg/mL fibrinogen, 200U/mL thrombin and 2.5 mg/mL genipin at the final concentration was mixed in equal volumes. 10 μM 4-PPBP (antagonist) or 200nM BD1047 (σ1R-antagonist) was injected into the torn meniscus site through a syringe injection. A total of 20 μl 4-PPBP/BD1047-laden bioglue was injected into the injured site. *Meniscus explant model*: Per our established methods, a longitudinal/radial tear was created in the inner one-third zone of fresh bovine meniscus explants (1.0cm × 0.8cm × 0.6cm) and treated with 4-PPBP/FibGen. *Animal model:* This study was approved by the IACUC (project #AC-AACB1704). 78 male SD rats (body weight: 300-350g) were used in this study. Longitudinal tears were established in the anterior horn of medial meniscus as we reported previously. *Video-based functional assessment* was performed using a custom-built CatWalk system. Animals were allowed to walk at least 5 times through a one-meter-long treadmill. Average walking speed, stance length, and stance width were evaluated for gait analysis. *Clinical and quantitative histological assessment:* The full range of motion of the knee joint was measured prior to euthanizing the rats at 2 and 4 weeks. Isolated medial meniscal was evaluated by histological staining and quantitative Pauli scoring. *Gene expression*: Total RNA was isolated from the knee joint by Qiagen, and qPCR was conducted to detect the anabolic and catabolic markers. scRNA-seq analysis and CellChat analysis: Cells were isolated from knee joint tissue at 2 wks post-op by 5.0mg/ml collagenase enzymatic digestion for 2 h. scRNA-seq was performed followed by cell-cell communication analysis using CellChat system. Statistical analysis: GraphPad Prism 5.0 with Student's *-t* test was used for comparisons. $p<0.05$ was considered as statistically significant.

RESULTS: In our meniscus explant healing model, a single application of 4-PPBP via FibGen enhanced healing of longitudinal tears in the inner avascular zone, demonstrated by minimal tissue gapping and newly formed collagen fibers (**Fig. 1A & B**). In addition, we observed highly proliferative cells at the peripheral region of meniscus tears with 4-PPBP application, likely confirming the role of 4-PPBP in stimulating endogenous cellular proliferation. Similarly, 4-PPBP-laden glue enhanced healing of radial tears in the deep zone, while SMSC-only treatment failed to bridge the meniscus gapping, suggesting that FibGen provided viscoelastic scaffolding which allow the SMSCs to localize and proliferate (**Fig. 1C& D**). Our animal model data showed that a single injection of 4-PPBP via FibGen enhanced functional recovery including increased average walking speed, paw contact pressure, stance length, and decreased stance width of right hindlimbs (data not shown). It also improved the range of motion within 14 days, compared to bioglue-only and non-treated defects with poor activity performance (**Fig. 2 A-C**). BD1047 treatment impaired walking ability and delayed meniscus healing of longitudinal tears in the inner avascular zone and limited the range of motion at 2 wks and 4 wks. In addition, we noticed early healing in the avascular zone of meniscus tears after 4-PPBP application (**Fig. 2D&E**). Lastly, 4-PPBP bioglue treatment inhibited synovial hyperplasia and protected cartilage against degeneration compared to non-treated group at 4 wks. The BD1047-treated group progressed to cartilage thinning, as the thickness of hyaline cartilage relative to calcified cartilage decreased (**Fig. 3A & B**). Our further isolated RNA from the synovium, medial meniscus, cartilage from the medial compartment of knee joint at 2 wks. The PCR results further showed that 4-PPBP/bioglue inhibited *mmp3* mRNA expression, while upregulated the expression of sox9 and col2 compared to non-treated and BD1047 treated meniscus defect groups (**Fig. 3C**).

DISCUSSION: In this study, FibGen bioglue was activated by a single small molecule, 4-PPBP. It serves as an artificial matrix to enhance healing and seal meniscus gaps within a torn meniscus. Our results suggest that 4-PPBP induces multiple functions in SMSCs, including stimulating proliferation and reducing inflammatory responses. However, BD1047 inhibited the stimulating effects. It indicates that 4-PPBP bioglue application stimulates endogenous SMSCs to proliferate and migrate to the injury site, leading to meniscus repairing. In addition, 4-PPBP inhibits synovial inflammatory responses and promotes CTGF synthesis in the acute meniscus tear site through σ1R signaling. As 4-PPBP also inhibits *mmp3* expressions, our approach has significant potential as an intra-articular therapeutic in an early intervention setting following an acute meniscus injury. Moreover, a notable advantage in the use of a single small molecule rather than multiple growth factors in our previous work is its significant translational potential by minimizing regulatory barriers and the cost of developing a clinical grade product. A limitation of this study is the incomplete characterization of the mechanism of 4-PPBP at the cellular level. To better understand the biological mechanisms and pathways regulating meniscus homeostasis and repair *in vivo*, we are currently investigating cellular crosstalk in meniscus, synovium and infrapatellar fat tissue by applying single cell RNA-sequencing data.

SIGNIFICANCE/CLINICAL RELEVANCE: This viscoelastic, biodegradable FibGen glue, combined with the σ1R agonist 4-PPBP, significantly enhances avascular meniscus healing, improves overall functional performance, and reduces cartilage damage associated with inflammatory responses. The 4-PPBP-laden bioglue may provide sufficient meniscal stability at the tear site to tolerate load bearing and minimize injured meniscal motion, thereby enabling early functional recovery to pre-injury levels.

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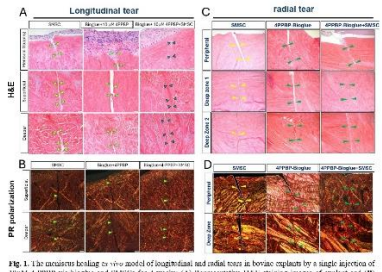

Fig. 1. The meniscus healing *ex vivo* model of longitudinal and radial tears in bovine explants by a single injection of 10xM 4-PPBP via bioglue and SMSCs for 4 weeks. (A) Representative H&E staining images of explant and (B) Picrosirius red staining under polarized microscopy for longitudinal tears (n=6 each group, scale bar=100 μm). (C) H&E and (D)PR staining under polarized microscopy of radial tears. 1million SMSC was seeded. Scale bar=100 μm.

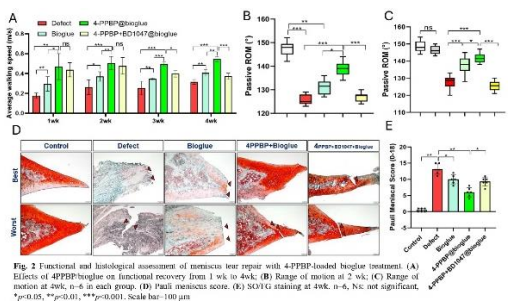

Fig. 2 Functional and histological assessment of meniscus tear repair with 4-PPBP-loaded bioglue treatment. (A) Effects of 4PPBP/bioglue on functional recovery from 1 wk to 4wk; (B) Range of motion at 2 wk; (C) Range of motion at 4wk, n=6 in each group. (D) Pauli meniscus score. (E) SO/FG staining at 4wk. n=6, NS: not significant, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Scale bar=100 μm

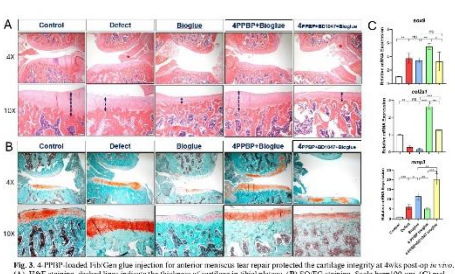

Fig. 3. 4-PPBP-loaded FibGen glue injection for anterior meniscus tear repair protected the cartilage integrity at 4wks post-op *in vivo*. (A) H&E staining, dashed lines indicate the thickness of cartilage in tibial plateau. (B) SO/FG staining, Scale bar=100 μm. (C) real time PCR results of anabolic markers *sox9*, *col2a1* and catabolic marker *mmp3*. n=6 in each group. ns: not significant, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.