Innovative Scaffold Delivery for Localized Hh Pathway Activation in Tendon-to-Bone Repair

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INTRODUCTION: Anterior cruciate ligament (ACL) injuries are a leading cause of training and sports-related injuries. Even with improved surgeries and rehabilitation, fewer than half of patients recover fully due to post-op complications such as graft failure, knee instability, and bone tunnel widening [1]. Integration between the tendon graft and adjacent bone is critical for restoring function following ACL reconstruction (ACLR). We previously demonstrated that the hedgehog (Hh) signaling pathway, a critical mediator of enthesis maturation [2], promotes the zonal, fibrocartilaginous tendon-to-bone attachments in the bone tunnels after ACLR [3]. Given the importance of Hh in various tissues and organs, there is an unmet need to localize delivery of Hh signaling drugs to promote tunnel integration while not eliciting off-target effects. Hence, we devised two scaffold delivery systems to localize release of smoothened agonist (SAG), a small molecule Hh signaling agonist, in different concentrations to support tendon-to-bone integration. SAG was infused into polycaprolactone (PCL) electrospun nanofibers in the first scaffold, whereas it was infused into poly(lactic-co-glycolic acid) (PLGA) microspheres that were sintered between PCL fibers in the second scaffold, known as the Bilayer Delivery System (BiLDS). The advantage of the BiLDS is that the SAG microspheres can be positioned directly in the bone tunnels, avoiding potential off-target effects from SAG release into the joint space [4]. The primary objectives of this study are to incorporate SAG into these scaffolds and analyze the release pattern *in vitro* to determine if SAG release can locally trigger the Hh pathway to ultimately promote tunnel integration.

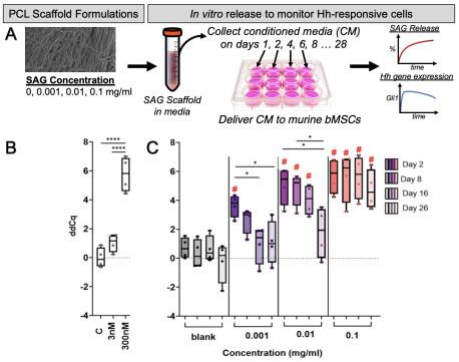

Figure 1. SAG release study experimental design (A). CM released from SAG PCL scaffolds was collected every other day for 28 days. Murine bMSCs were given either SAG directly to the media (B) or CM from the scaffolds at different time points (C) and Gli1 expression was measured via qPCR. * $p < 0.05$; # $p < 0.05$ vs. blank

METHODS: All animals and procedures were IACUC approved. PCL Scaffold Fabrication: PCL solution (35 wt%) was prepared in DMF/THF with SAG at different concentrations (0, 0.001, 0.01, 0.1 mg/ml) and electrospun on a rotating mandrel. SAG In Vitro Release Study: Blank and SAG-infused scaffolds were placed in culture media in tubes on a shaker at 37ºC (Fig. 1A) and conditioned media (CM) was collected over 28 days. CM samples from days 2, 8, 16, or 26 were applied to bone marrow stromal cell (bMSC) cultures for 4 days. Gli1 gene expression was assessed by qPCR. BiLDS Fabrication: The microspheres (MS) were loaded with SAG (0.1mg/ml) and created as previously described [5]. They were resuspended in PBS, and the BiLDS were created by heat sintering 20 µL (0.17 mg/µL) of the solution between two aligned PCL electrospun nanofibrous sheets in a region that is 20mm long (average length of the rabbit bone tunnel). Our pocket design featured several diamond-shaped pockets (Fig. 2A) instead of a single pocket to increase durability during surgical transplantation. Rabbit ACL Reconstruction Implant Feasibility Study: Flexor digitorum longus (FDL) tendons were harvested from two cadaveric rabbits. The FDL was doubled over and the BiLDS was sutured between the tendons. The tunnels were drilled, and the graft was inserted such that the microspheres were only in the bone tunnels and not the joint space (Fig. 3). We then fixed and sectioned the tissue immediately after implantation.

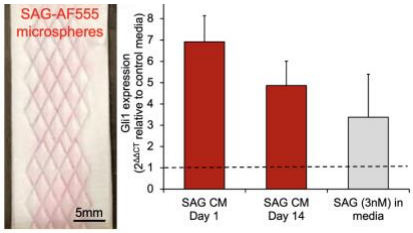

Figure 2. BiLDS Scaffold (A). Gli1 expression of bMSCs treated with conditioned media (CM) from SAG loaded BiLDS on day 1 and day 14 compared to SAG (3nM) given directly to media, normalized to fresh media (C, n=2)

RESULTS: Delivery of conditioned media from the PCL scaffold to bMSCs increased Gli1 expression in a dose- and time-dependent manner: As expected, there was a marked increase in Gli1 expression with the direct addition of 3nM (2-fold) and 300nM (70-fold) of SAG directly to the media ($p<0.05$, Fig. 1B). Furthermore, there was a dose- and time-dependent effect of scaffold SAG release on Gli1 expression ($p<0.05$, Fig. 1C). Time did not alter Gli1 expression in the empty scaffolds, maintaining levels comparable to the control media (C in Fig. 1B). Notably, all SAG concentrations (0.001, 0.01, and 0.1 mg/ml) showed significantly higher Gli1 expression compared to empty scaffolds in CM collected on day 2, with levels akin to the 300nM media for the 0.01 and 0.1 mg/ml scaffolds. Intriguingly, while Gli1 expression remained high in the 0.1 mg/ml SAG scaffold for CM collected up to day 26 ($p<0.05$), both the 0.01 and 0.001 mg/ml groups exhibited reduced Gli1 expression over time in CM (Fig. 1C). These findings suggest that the 0.1mg/ml group released enough SAG over 26 days to sustain activated Gli1 expression in bMSCs. Delivery of conditioned media from the BiLDS to bMSCs increased Gli1 expression in time-dependent manner: We conducted a similar study where we delivered conditioned media collected on days 1 and 14 to murine bMSC cultures for 3 days. There was a 6.9-fold and 4.9-fold increase in Gli1 expression in the bMSCs, respectively, which compared to a 3.4-fold increase from SAG (3nM) added to the media (normalized to control media) (Fig. 2B). SAG infused BiLDS/graft construct remained intact during ACLR: In a surgical feasibility study, we implanted the BiLDS into cadaveric rabbit bone tunnels. We found that the SAG microspheres (dyed with red Alexa Fluor 555 in Fig. 3) sintered between PCL sheets could be positioned within the bone tunnels between the tendon graft as visualized by sagittal and axial micrographs (Fig. 3).

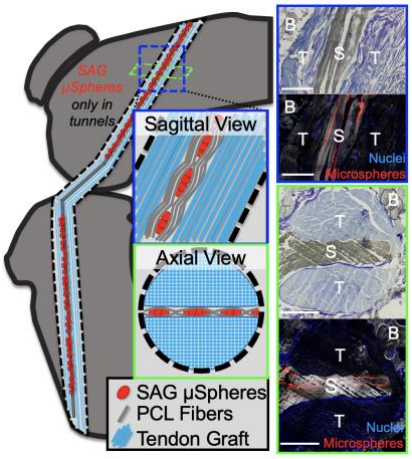

Figure 3. BiLDS scaffold sutured between tendon graft such that SAG microspheres are only in bone tunnels (left). Histology images of rabbit femur showing BiLDS containing AF-555 (red) SAG microspheres (right). B: bone, T: FDL tendon, S: BiLDS scaffold.

DISCUSSION: This study offers crucial insights into the release of SAG from different scaffold systems and its effect on downstream Gli1 expression. The *in vitro* release profile was dose dependent with CM from the 0.1 mg/ml concentration inducing sustained, elevated Gli1 expression over 26 days. Moreover, to localize the delivery to the bone tunnel and avoid release in the joint space, BiLDS with SAG infused MS were fabricated and implanted in a rabbit femur as a proof of concept for a future localized therapy.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates promising drug delivery systems to leverage the therapeutic potential of the Hh pathway to promote tendon-to-bone integration.

REFERENCES: 1. Rizer et al., Skeletal Radiol., 2017; 2. Dyment et al., *Dev Biol*, 2015; 3. Kamalitdinov et al., Osteoarthritis Cartilage. 2023; 4. Lin et al., Nat Med., 2009; 5. Kim et al., Acta Biomater., 2020

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