

Self-Amplifying RNA of Non-Cartilage-Permeating TGF- β Inhibitors as a Targeted Arthrofibrosis Treatment

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INTRODUCTION: Transforming growth factor beta (TGF- β) is an essential mediator of synovial joint homeostasis. In healthy joint tissues, TGF- β activity is tightly regulated via the mechanobiological activation of the latent TGF- β complex, yielding low activity in response to joint motion [1]. Arthrofibrosis (AF) is a debilitating joint disorder associated with excessive TGF- β activation following surgery, infection, or trauma [2,3]. Excessive TGF- β in synovial fluid induces the conversion of synovial fibroblasts to myofibroblasts, which in turn elicits a fibrotic cascade of excessive ECM deposition and contraction, leading to joint contracture and limited joint motion (**Fig.1A**). While targeting TGF- β to prevent AF is achievable, prior attempts to treat synovial fibrosis through TGF- β inhibition have been limited because of the off-target effect of blocking TGF- β from chondrocytes, requisite for cartilage homeostasis, which resulted in GAG loss and cartilage thinning [4,5]. Thus, differentially blocking TGF- β to maintain a healthy pool in cartilage for tissue homeostasis but blocking the TGF- β induced conversion of synovial fibroblasts to myofibroblasts is required to prevent AF, without provoking cartilage degeneration.

We recently developed a novel targeted TGF- β inhibition approach that capitalizes on the size-exclusion properties of the anionic, sulfated GAG-rich cartilage ECM, which limits the penetration of large, anionic macromolecules into the tissue (**Fig.1B**). Previously, we demonstrated in an *ex vivo* bovine tissue co-culture model that the administration of large, anionic recombinant TGF- β soluble receptor proteins (SolR2 [15 kDa, net charge -4] and SolR3 [85kDa, net charge -2]) can inhibit synovial fibrosis while mitigating off-target degeneration of cartilage explants [6]. To further advance this concept towards a clinical AF therapy, we examined the efficacy of self-amplifying RNA (saRNA) delivery to synovial fibroblasts to express TGF- β SolR antagonist proteins (**Fig.1C**). Due to the presence of RNA-dependent-RNA polymerase in the sequence, saRNA constructs can efficiently and persistently scale up the cargo gene expression of SolR2 or SolR, thus providing long-term sustained protection of the synovium against aberrant TGF- β activation [7]. In this study, we hypothesize that: 1) SolR proteins will exhibit low partition coefficients (PC) in human cartilage, 2) cells transfected with SolR-saRNA will produce sustained high levels of SolR proteins, and 3) SolR-saRNA delivery to synovial fibroblasts will provide protection from fibrosis in response to pathogenic TGF- β exposure.

METHODS: SolR PC in cartilage: Devitalized patellar cartilage explants (\varnothing 2x2mm) were procured from 7 donors (age/sex; 13M, 27F, 32M, 43F, 47F, 51F, 80F) and condylar explants (\varnothing 4x2mm) were procured from 2 donors (21F and 30F). Explants were cultured with SolR2 (1 μ g/mL) or SolR3 (5 μ g/mL) for 3 days. Condylar explants were axially sectioned and SolRs were extracted and assayed via SolR2/3 ELISAs (R&D Systems) for depth-dependent PC measures. All other donors were analyzed for full-thickness bulk PC measures. **SolR secretion from saRNA:** The SolR-saRNA was designed and constructed based on commercial recombinant protein sequences (R&D system). The SolR-saRNA constructs were initially transfected into HEK293T cells to validate the transfection platform. The secretion of SolR2 and SolR3 proteins were monitored over 3 days via ELISA. **SolR-saRNA fibrosis inhibition:** Synoviocytes isolated from immature bovine condylar synovium were plated and transfected with or without SolR2/3-saRNA via Lipofectamine Messengermax (Thermo). After 3 days of culture, cells were encapsulated in 0.2% type I collagen hydrogel constructs (\varnothing 6x1.5mm) at a 2×10^6 cells/mL density. Constructs were cultured in DMEM, supplemented with 0.25X amino acid, 100 nM dexamethasone, 1mg/mL BSA, and a range of pathologic TGF- β 1 doses (50-1000 pg/mL) [8,9]. For comparison, constructs were cultivated in the presence of a small molecular TGF- β antagonist (LY364947), which exhibits strong TGF- β inhibition efficacy and significant penetration into articular cartilage [5,6]. As a surrogate measure of fibrosis, contraction of synovium constructs in the collagen hydrogel, portraying the conversion of synovial fibroblasts to myofibroblasts, was monitored over 3 days by imaging changes in construct cross-sectional area (ImageJ).

RESULTS: SolR PC in cartilage: SolR proteins exhibited low PCs in the cartilage superficial zone (0.22 ± 0.08 & 0.13 ± 0.10 for SolR2 & SolR3, respectively), which decreased further with tissue depth, following an inverse relationship with increasing GAG content in the deeper zones (**Fig.1D**). Similar low PCs were observed for full-thickness bulk measures of all other human cartilage donors. **SolR secretion from saRNA:** HEK293T transfected with SolR2-saRNA secreted high levels of SolR2 protein levels (7.5-33 ng/mL per day; **Fig.2A**). SolR3-saRNA transfection yielded lower SolR3 protein levels (0.02-0.71 ng/mL per day; **Fig.2B**). **SolR-saRNA fibrosis inhibition:** No viability loss was observed in synoviocytes from SolR-saRNA transfection (**Fig.3A**). Pathogenic doses of TGF- β 1 induced significant contraction of synovium constructs, which increased over time and with TGF- β 1 dose ($p < 0.05$; **Fig.3B,C**). Similar to LY364947, SolR2-saRNA and SolR3-saRNA significantly reduced synovial construct contraction at all time points and across all TGF- β 1 doses ($p < 0.05$). Further, TGF- β 1 increased expression of α -SMA, which was mitigated by LY364947, SolR2-saRNA, and SolR3-saRNA (**Fig.2D**).

DISCUSSION: These results support that the use of saRNA achieves the sustained delivery of SolR proteins by synoviocytes following a provocative insult to the synovial joint, enabling targeted inhibition of TGF- β in the synovium to prevent AF while preserving a pool of TGF- β in articular cartilage. This targeted pharmacologic approach capitalizes on the unique biophysical properties of the cartilage ECM, which partially excludes large, anionic proteins from the tissue due to steric/electrostatic interactions with its sulfated GAG network, critical for interstitial fluid load support. Despite, documented GAG loss in cartilage with age [5], we observe low penetration of SolRs in human cartilage over a large age range, supporting the clinical efficacy of this target approach. The *in vitro* culture model provides initial validation of SolR-saRNA efficacy in inhibiting synovial fibrosis, as measured by prevention of fibroblast-to-myofibroblast conversion, construct contraction, and α -SMA expression in collagen hydrogels. While LY364947 is a potent inhibitor of synovial fibrosis, its low molecular weight allows off-target depletion of TGF- β from cartilage [5,6], limiting its clinical utility. Alternatively, the delivery of non-cartilage-permeating SolRs via saRNA achieves similar suppression of synovial fibrosis without depleting the cartilage ECM, supporting its use as a targeted AF therapy.

SIGNIFICANCE: The study supports the feasibility of using saRNA to express non-cartilage-permeating TGF- β inhibitors as a novel therapy for AF. The sustained delivery of inhibitors allows for long-term, targeted depletion of TGF- β -mediated synovial fibrosis in the aftermath of synovial joint insult, while minimizing the off-target effects on articular cartilage.

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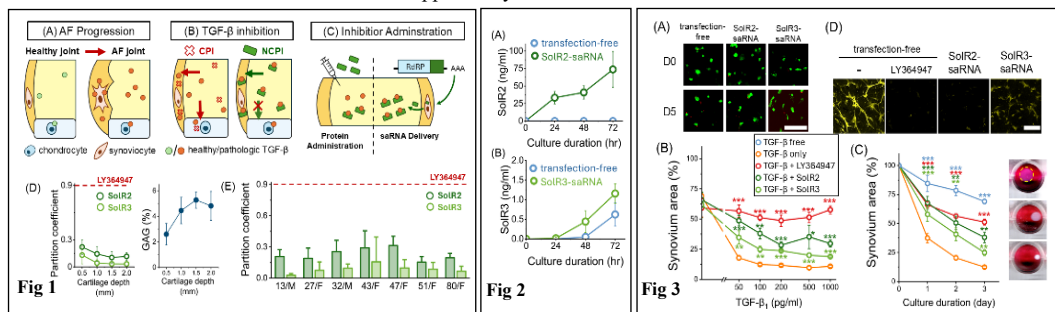


Fig 1: (A) Excessive TGF- β activation in SF leads to AF. (B) TGF- β inhibition via small molecular cartilage permeating inhibitors (CPI) elicit off-target effects on cartilage while large, anionic non-cartilage-permeating inhibitor (NCPI) proteins mitigate off-target effects. (C) NCPIs can be administered via protein injection or saRNA synovial transfection. (D) Measured PC of SolR2/3 through cartilage depth and accompanying GAG content. (E) Full-thickness PC of SolR2/3 in cartilage of human donors. **Fig 2:** Temporal secretion of (A) SolR2 and

(B) SolR3 proteins from HEK293T cells after SolR-saRNA delivery. n=3 for each group. **Fig 3:** (A) Cell viability (Green: live, Red: dead). Contraction of synovium constructs in response to pathogenic TGF- β dosing and inhibition therapies for (B) variable TGF- β 1 doses at day 3, and (C) over time for 100ng/mL TGF- β 1 dose. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs TGF- β -only groups. n=4 (D) α -SMA expression for inhibitor groups. Scale bars = 100 μ m.