Synthetic gene circuits for autonomously regulated circadian- and inflammatory-driven drug delivery to target flares in arthritis

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INTRODUCTION Rheumatoid arthritis (RA) is a debilitating autoimmune disease estimated to affect 1% of the global population. RA often impacts multiple joints throughout the body, leading to cartilage destruction, bone damage, and severe pain [1,2]. Notably, this chronic inflammation is not constant over time; instead, it can gradually increase over the course of disease, with unpredictable periods of worsened symptoms that occur over the scale of weeks to months [1,3]. Additionally, inflammation fluctuates in daily, or circadian, patterns, corresponding to changes in physical symptoms like morning joint stiffness and serum cytokine levels [4]. Most current RA therapies, including recent advances in biologics, require repeated high doses to achieve clinical impact. Moreover, biologic treatment regiments rarely consider the flare-like nature of RA at either daily or long-term scales. Chronotherapy, or the delivery of therapeutics to coincide with circadian fluctuations, is an emerging approach that has demonstrated promising results in diseases with circadian patterns of inflammation, such as RA [4]. However, chronotherapy is not designed to address non-circadian, long-term flares, which may ultimately contribute to circadian dysregulation [4,5]. Therefore, the goal of this work is to investigate the therapeutic potential of circadian- and inflammatory-responsive gene circuits that autonomously produce interleukin-1 receptor antagonist (IL-1Ra) daily and/or on-demand and develop a system that concurrently integrates both approaches to target flares.

METHODS To generate circadian expression, three tandem repeats of E'-box elements, which cyclically bind circadian transcriptional regulators, were cloned upstream of a minimal CMV element to drive either GFP or IL-1Ra production in addition to a luciferase reporter in lentiviral vectors (E'box-GFP-LUC or E'box-IL1Ra-LUC, respectively) or into a vector with only IL-1Ra output (E'box-IL1Ra) [6]. iPSCs were differentiated towards the mesenchymal lineage; then, prior to the final-stage pellet culture, pre-differentiated iPSCs (PDiPSCs) were transduced with lentiviral vectors [7]. Output was monitored by luminescence over 72 hours. For normalized detrended samples recorded over 72 hours, period was quantified based on a sine fit. Luminescence by E'box-GFP-LUC prior to and following an IL-1β inflammatory challenge at 1 ng/mL at the 24-hour timepoint of recording was assessed in PDiPSCs with and without the therapeutic generating circuit (E'box-IL1Ra) to mimic response in the presence of a flare. The therapeutic effect on circadian outcomes was quantified based on the normalized peak difference pre- and post-challenge and the ratio of post/pre-challenge as a metric to estimate relative amplitude dampening. Previous work demonstrated that five tandem NF-κB response elements (REs) activate downstream gene expression in response to inflammation and can drive IL-1Ra production (NFκB-IL1Ra) that protects iPSC-derived cartilage pellets [7]. To compare the functional protection of E'-box- or NF-κB-driven therapeutic production, PDiPSCs were transduced with respective gene circuits, differentiated into pellets, and then subject to 1 ng/mL IL-1β as an *in vitro* model of RA, in comparison to non-transduced (NT) controls. 24 and 72 hours following inflammatory challenge, media and pellet RNA were collected for protein analysis by ELISA and gene expression analysis by qPCR. At the 72-hour time point, samples were collected for histological and biochemical analysis. To generate a promoter responsive to both circadian and inflammatory cues, NF-κB REs were cloned directly upstream upstream of E'-box elements to generate the combined NFκB.E'Box-GFP-LUC circuit. Luminescence was recorded prior to and following an IL-1β inflammatory challenge at 1 ng/mL at the 24-hour timepoint and phase was assessed. T-tests were used to assess peak differences and phase; ANOVAs with Tukey's post hoc test were used to assess all other metrics.

RESULTS E'-box-driven gene circuits demonstrated circadian luminescence in PDiPSCs and pellets over 72 hours with periods of approximately 24 hours, supporting this as a potential chronotherapy (Fig. 1). Inflammation is a known disruptor of circadian rhythms, but IL-1Ra has been shown to protect circadian rhythms during IL-1 challenge [8]. Following inflammatory challenge, circadian-driven IL-1Ra (E'box-IL1Ra) mitigated amplitude peak dampening, suggesting that this chronotherapy protects circadian output (Fig. 2). In pellets, IL-1Ra production was significantly increased under inflammatory conditions for NFκB-IL1Ra samples in comparison to E'Box-IL1Ra-LUC or NT control samples (Fig. 3). Pellets transduced with NFκB-IL1Ra demonstrated protection by reduced inflammatory *Ccl2* and *Il6* gene expression and increased cartilage-associated *Acan* and *Col2a1* gene expression. This paralleled biochemical and histological results for glycosaminoglycans (GAGs) characteristic of healthy cartilage. Lower-level IL-1Ra production in pellets transduced with E'Box-IL1Ra-LUC corresponded to comparatively reduced levels of protection under IL-1β conditions but demonstrated a significant reduction in inflammatory *Il6* gene expression in comparison to NT controls. Therefore, NFκB-driven therapeutic output may better support unpredictable, high level inflammatory flares while E'-box-driven therapeutic output can target smaller-scale daily flares. To address the ultimate aim of this research, a circuit responsive to both inflammatory and circadian cues was designed, and PDiPSCs and pellets transduced with NFκB.E'Box-GFP-LUC demonstrated circadian luminescence output under baseline conditions that peaked approximately four- or three-times higher in magnitude than circadian-alone output, respectively, when challenged with 1 ng/mL IL-1β (Fig. 3). There was no significant difference in phase between E'box and NFκB.E'Box luminescence under basal conditions, suggesting that the chronotherapeutic contribution is maintained in the absence of an inflammatory flare.

DISCUSSION We developed an autoregulated system that senses and responds to inflammatory and time-of-day cues to drive biologic production on-demand and on a 24-hour schedule. When gene circuits were introduced into an iPSC-derived model of articular cartilage, they demonstrated protection when challenged with IL-1β. The combined promoter design supports a proof-of-concept model that circadian and inflammatory-driven therapeutic production will provide a baseline chronotherapeutic output plus a peak in response after a flare. Future work aims to assess functional protection of the NFκB.E'box-driven therapeutic circuit in comparison single cue-responsive circuits. Together, this has the potential to concurrently address long-term and daily-scale RA flares.

CLINICAL RELEVANCE This biologic delivery system has the potential to provide therapeutics when they are most efficacious, reducing the need for a constant high dose. This can minimize off-target side effects and immunosuppression while also protecting tissue-level circadian rhythms and RA outcomes.

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REFERENCES [1] Akram *et al* 2021, [2] Neidhart *et al* 2019, [3] van Steenbergen *et al* 2018, [4] Cutolo 2019, [5] Guo *et al* 2015, [6] Ukai-Tadenuma *et al* 2008, [7] Pferdehirt *et al* 2019. [8] Pferdehirt & Damato *et al* 2022.

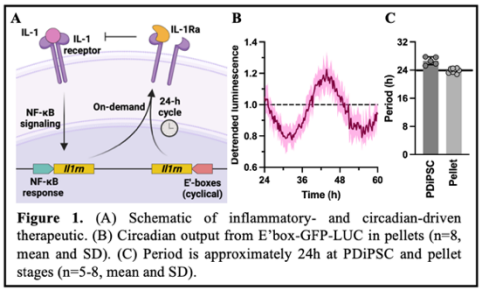

Figure 1. (A) Schematic of inflammatory- and circadian-driven therapeutic. (B) Circadian output from E'box-GFP-LUC in pellets (n=8, mean and SD). (C) Period is approximately 24h at PDiPSC and pellet stages (n=5-8, mean and SD).

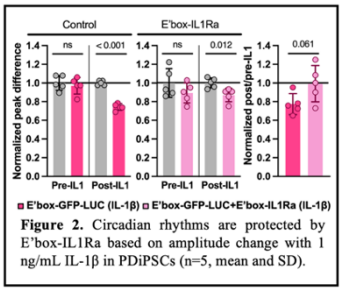

Figure 2. Circadian rhythms are protected by E'box-IL1Ra based on amplitude change with 1 ng/mL IL-1β in PDiPSCs (n=5, mean and SD).

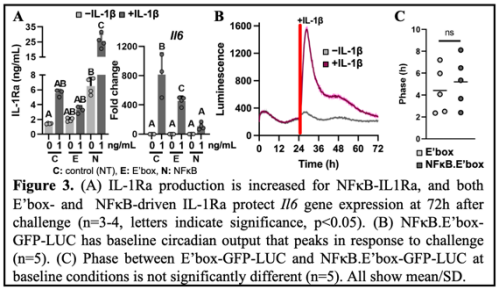

Figure 3. (A) IL-1Ra production is increased for NFκB-IL1Ra, and both E'box- and NFκB-driven IL-1Ra protect *Il6* gene expression at 72h after challenge (n=3-4, letters indicate significance, p<0.05). (B) NFκB.E'box-GFP-LUC has baseline circadian output that peaks in response to challenge (n=5). (C) Phase between E'box-GFP-LUC and NFκB.E'box-GFP-LUC at baseline conditions is not significantly different (n=5). All show mean/SD.