Received: 3 November 2021 | Revised: 28 January 2022 | Accepted: 10 February 2022

DOI: 10.1096/fj.202101699R

THE FASEB JOURNAL

RESEARCH ARTICLE

The functionality and translatability of neocartilage constructs are improved with the combination of fluid-induced shear stress and bioactive factors

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Funding information
HHS | NIH | National Institute of Dental and Craniofacial Research (NIDCR), Grant/Award Number: R01 DE015038; HHS | NIH | National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), Grant/Award Number: R01 AR078389 and R01 AR067821; California Alliance for Minority Participation

Abstract

Neocartilage tissue engineering aims to address the shortcomings of current clinical treatments for articular cartilage indications. However, advancement is required toward neocartilage functionality (mechanical and biochemical properties) and translatability (construct size, gross morphology, passage number, cell source, and cell type). Using fluid-induced shear (FIS) stress, a potent mechanical stimulus, over four phases, this work investigates FIS stress' efficacy toward creating large neocartilage derived from highly passaged minipig costal chondrocytes, a species relevant to the preclinical regulatory process. In Phase I, FIS stress application timing was investigated in bovine articular chondrocytes and found to improve the aggregate modulus of neocartilage by 151% over unstimulated controls when stimulated during the maturation stage. In Phase II, FIS stress stimulation was translated from bovine articular chondrocytes to expanded minipig costal chondrocytes, yielding a 46% improvement in aggregate modulus over nonstimulated controls. In Phase III, bioactive factors were combined with FIS stress to improve the shear modulus by 115% over bioactive factor-only controls. The translatability of neocartilage was improved in Phase IV by utilizing highly passaged cells to form constructs more than 9-times larger in the area (11 × 17 mm), yielding an improved aggregate modulus by 134% and a flat morphology compared to free-floating, bioactive factor-only controls. Overall, this study represents a significant step toward generating mechanically robust, large constructs necessary for animal studies, and eventually, human clinical studies.

KEYWORDS

articular cartilage, fluid-induced shear stress, mechanical stimulation, neocartilage, tissue engineering

Abbreviations: ACK, ammonium-chloride-potassium; ANOVA, analysis of variance; C-ABC, chondroitinase ABC; CHG, chondrogenic medium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DW, dry weight; ECM, extracellular matrix; FBS, fetal bovine serum; FDA, Food and Drug Administration; FIS, fluid-induced shear; H&E, hematoxylin and eosin; LOXL2, lysyl oxidase-like 2; OA, osteoarthritis; PBS, phosphate-buffered saline; Picro Red, picrosirius red; PSF, penicillin-streptomycin-fungizone; Saf O, safranin O; TGF-β1, transforming growth factor beta 1; WW, wet weight.

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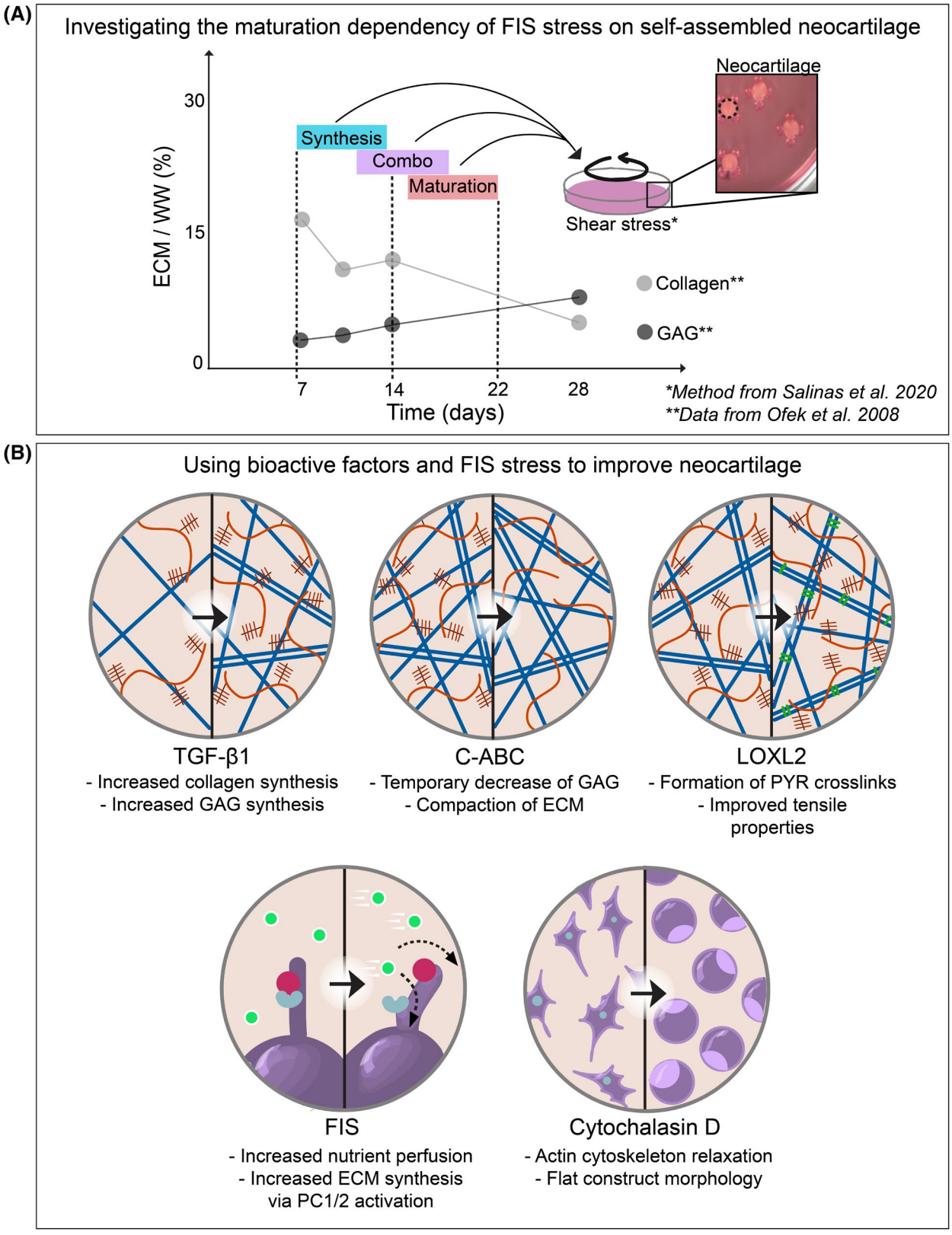
1 | INTRODUCTION

Articular cartilage is a stiff and resilient tissue that protects bones and distributes forces during movement. Native articular cartilages vary widely in compressive aggregate modulus values, ranging from about 250 kPa to about 1400 kPa depending on the location and species.[1–3] Cartilage trauma and wear can cause defects that do not naturally heal.[4] Currently, the most common treatments for articular cartilage lesions are chondroplasty and microfracture, neither of which are considered effective for more than 5 years post-treatment.[5,6] If cartilage lesions are not properly treated, they can progress to osteoarthritis (OA). OA currently affects 32 million people in the U.S. and is projected to rise to 60% in prevalence over the next 20 years.[7,8] Compared to the current short-term solutions applied to cartilage lesions, an emerging solution for articular cartilage defects is tissue engineering, which aims to provide a long-term regenerative solution.[4] Additionally, tissue-engineered cartilage derived from expanded cells has the potential to provide an inexhaustible amount of implant material, addressing the major clinical problem of donor tissue scarcity.[9] Although several advances in cartilage tissue engineering have been developed over the last several years, including the self-assembling process,[9–12] certain improvements must still be made before the successful implantation of neocartilage in humans, such as (1) finding the ideal timing of neocartilage stimulation regimens toward functional improvement, (2) establishing the reproducibility of the effects of mechanical stimuli across multiple species and cell types, (3) exploring the additive effects of multiple types of stimuli (i.e., biochemical and mechanical), and (4) scaling-up of constructs.

Certain magnitudes of mechanical stimulation regimens improve neocartilage tissue properties toward those of native tissue, but the timeline of application is not typically scrutinized. For example, fluid-induced shear (FIS) stress has been previously shown by our group and others to mechano-regulate ion channels on the primary cilia of chondrocytes, leading to enhancements in neocartilage extracellular matrix (ECM) content and mechanical properties.[11,13–16] A previous study investigating the use of FIS stress found that applying 0.05–0.21 Pa of shear stress on neocartilage improved both bovine and human constructs;[11] however, the timeline of application has not been previously investigated. Determining an ideal application window for neocartilage mechanical stimulation is critical because timed mechanical cues help maintain tissue health in native cartilages. For example, during embryonic development, chondrocytes are stimulated by mechanical loading to synthesize ECM,[17,18] and during postnatal development, mechanical loading regulates cartilage thickness and maturation.[19,20] Certain tissue engineering methods, such as the neocartilage self-assembling process, have also been shown to follow similar developmental steps and may exhibit the same maturation-dependent mechanical signaling needs.[21–23] For example, self-assembled neocartilage undergoes the synthesis stage (days 7–14), when the chondrocytes produce ECM, and the maturation stage (days 15–22), when production of glycosaminoglycans is increased and collagen content is decreased (Figure 1A).[23,24] The maturation-dependency of mechanical stimulation regimens, such as FIS stress, should be investigated toward maximizing mechanical properties of neocartilage.[25]

To translate mechanical stimulation technologies to the clinic, the reproducibility of tissue engineering techniques across species and sources must be examined. This is a necessary design criterion because the Food and Drug Administration (FDA) requires analogous products to be tested in animal studies prior to use in human clinical trials. For neocartilages, exploratory experiments may be conducted using bovine articular chondrocytes since they are inexpensive and easily obtainable. Eventually, the species and source will need to be translated to a commonly used preclinical animal model for in vivo testing. The Yucatan minipig is considered a suitable animal model for most preclinical work because of its similarity to humans in weight, anatomy, immunology, physiology, and bone biology.[26–30] Specifically, it is widely used for cartilage therapeutic testing in articular cartilage, knee meniscus, and temporomandibular joint disc investigations.[12,28,30,31] Another consideration is the cell source. For example, costal chondrocytes may be advantageous over articular

FIGURE 1 Maturation dependency and modes of action. (A) The design of the experimental groups of Phase I follows the stages of the self-assembling process of neocartilage. The synthesis stage occurs from day 7 to day 14 of culture when glycosaminoglycan content increases slowly, and collagen content decreases slowly. During the maturation stage, from day 15 to day 22, glycosaminoglycan content continues to increase, and collagen content continues to decrease, but the total matrix production increases. The neocartilage constructs were either nonstimulated controls or stimulated with FIS stress during the synthesis stage, the maturation stage, or a combination stage (days 11–18). *Shear stress refers to fluid-induced shear stress, described previously in Salinas et al. (2020).[11] **Data obtained from Ofek et al. (2008).[23] (B) Schematic representation of the modes of action for bioactive and mechanical (FIS stress) signaling factors are shown. All the bioactive and mechanical signaling factors shown were used to create the neocartilage constructs in Phase IV. C-ABC, chondroitinase ABC; Combo, combination; ECM, extracellular matrix; FIS, fluid-induced shear; GAG, glycosaminoglycan; LOXL2, lysyl oxidase-like 2; PC1/2, polycystin 1/2; PYR, pyridinoline; TGF-β1, transforming growth factor beta 1; WW, wet weight



chondrocytes due to their ability to be harvested autologously and allogeneically without further damaging diseased joints that require treatment. Costal chondrocytes are also advantageous because they regain their chondrogenic phenotype via redifferentiation methods after expansion.[32–34] To eventually translate a mechanical stimulus for human use, analogous sources should be tested with an appropriate species that will be used in preclinical studies to satisfy regulatory guidance toward eventual human use.

To improve neocartilage functional properties, exogenous growth factors, enzymes, and other small molecules (i.e., bioactive factors) have been extensively studied to optimize their dosage and timeline of application.[35–40] Nevertheless, how bioactive factors work in conjunction with mechanical stimulation is of interest because of the potential for additive improvements in neocartilage functional properties. For example, transforming growth factor beta 1 (TGF-β1) alone has lead to enhanced collagen and glycosaminoglycan synthesis during the self-assembling process (Figure 1B)[37] and in scaffold-based neocartilages.[41] TGF-β signaling has been shown to be regulated through the primary cilia,[42] the main mode of action of FIS stress.[11] This is significant because exogenous TGF-β1 addition and FIS stress might act in a similar manner to improve ECM content. Other bioactive factors, such as chondroitinase ABC (C-ABC), lysyl oxidase-like 2 (LOXL2), and insulin-like growth factor 1, have been studied in conjunction with TGF-β1 toward further improving functional properties of neocartilage,[43] including self-assembled neocartilage (Figure 1B).[37] Additionally, the use of bioactive factors independently or together with tensile and compressive stimulation has been explored,[10,23,35] motivating further exploration and combination with other mechanical stimulation regimens, such as FIS stress.

To repair larger cartilage defects, large neocartilage constructs (e.g., 11 × 17 mm) that are mechanically robust and have a flat morphology must be generated, and more cells will be necessary to create these large constructs. While the process of further expanding chondrocytes is simple, recent studies have shown that maintaining a chondrogenic phenotype in highly passaged cells is exceedingly complex due to dedifferentiation[44,45] and leads to the creation of neocartilage constructs that are not flat.[9] For example, protocols for the creation of flat, robust neocartilages have been developed using aggregate rejuvenation combined with the use of bioactive factors, such as TGF-β1, C-ABC, and LOXL2. This work has yielded small constructs that maintain flat morphology and mechanical robustness despite using cells that have been passaged up to 11 times.[9] However, further attempts at increasing the size of self-assembled constructs in the past have yielded constructs that fold or become wavy due to the actin cytoskeleton exerting internal tensile forces within the construct.[46] In large constructs, the addition of cytochalasin D, a potent inhibitor of actin polymerization, has been shown to yield flat neocartilage construct morphology (Figure 1B).[46] Moreover, cytochalasin D addition has also been known to restore the primary cilia on extensively passaged chondrocytes, which could further enhance the effects of FIS stress in this phase.[42] For addressing articular cartilage indications of larger sizes, it will be critical to develop protocols for larger constructs with both biochemical and mechanical stimulation which generate mechanically robust and flat neocartilages.

Toward successful implantation of neocartilage in humans, the global objective of this work was to improve the functional and translational aspects of neocartilage constructs using FIS stress and bioactive factors. In particular, the functional properties investigated were mechanical properties and ECM content, while the translational aspects investigated were construct size (5-mm diameter circular vs. 11 × 17-mm rectangular), gross morphology (flat neocartilage), passage number (passages 0, 3, and 6), cell source (bovine vs. minipig), and cell type (articular chondrocytes vs. costal chondrocytes). We hypothesized that the combination of FIS stress and bioactive factors would yield flat, large, neocartilage constructs that are mechanically robust. This series of studies was divided into four phases. In Phase I, the objective was to determine if the application of FIS stress during the synthesis stage (days 7–14) or during the maturation stage (days 15–22) of the self-assembling process was most beneficial for biochemical and mechanical properties (Figure 1A). In Phase II, a clinically relevant and widely accepted animal model and cell source (i.e., costal chondrocytes from the Yucatan minipig) were used with the appropriate timing derived from Phase I.[28,30] In Phase III, bioactive factors previously shown to improve functional aspects of neocartilage constructs were used in conjunction with FIS stress with the goal of increased functionality (Figure 1B). Finally, in Phase IV, the combination of FIS stress timing and bioactive factors discovered in Phases I to III was applied to constructs derived from highly passaged minipig costal chondrocytes to create large, flat neocartilage constructs for larger articular cartilage indications.

2 | METHODS

2.1 | Overview of experimental phases

In Phase I, the ideal FIS stress application window was determined by creating neocartilage constructs from bovine articular chondrocytes and stimulating them with FIS stress during the synthesis stage (days 7–14), maturation stage (days 15–22), or a combination of both stages (days 11–18) (Figure 1A). In Phase I, nonstimulated neocartilage created from bovine articular chondrocytes served as a control group. Next, in Phase II, Yucatan minipig costal chondrocytes were expanded to passage 3 and used to create neocartilage constructs that were either stimulated with FIS stress during the optimal period or not stimulated with FIS stress. In

Phase III, the combination of FIS stress and bioactive factors (TGF-β1, C-ABC, and LOXL2) was investigated to further improve the functionality of neocartilage constructs created from Yucatan minipig costal chondrocytes. Bioactive factors were included in the creation of all the neocartilage constructs for Phase III, including controls. Finally, in Phase IV, the results from all previous phases were used to create large (11 × 17 mm), neocartilage constructs from highly passaged Yucatan minipig costal chondrocytes.

2.2 | Isolation of bovine articular chondrocytes

For Phase I of this study, bovine articular chondrocytes were isolated by mincing cartilage from the femoral condyles and trochlear grooves of the knees of six, 2-month-old Jersey calves. Minced pieces from each leg were stored in 30 ml of wash medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) and 1% penicillin-streptomycin-fungizone (PSF). The minced tissue from each leg was washed 2–3 times with wash medium and digested in a petri dish using collagenase II solution (0.2% w/v, Worthington Biochemical's Collagenase type II in wash medium, 3% fetal bovine serum (FBS)) for 18 h on an orbital shaker at 37°C at 60 RPM. Following this, the solution with cells was filtered through 70 μm cell strainers and centrifuged for 5 min at 400 *g* to remove the collagenase. The resulting cell pellet was washed with phosphate-buffered saline (PBS). The chondrocytes were then washed with ammonium-chloride-potassium (ACK) lysis buffer, followed by several washes with PBS.[47] Finally, chondrocytes were counted and frozen in 90% FBS and 10% dimethyl sulfoxide (DMSO) for downstream self-assembly.

2.3 | Isolation of minipig costal chondrocytes

For Phases II, III, and IV of this study, minipig costal cartilage was obtained from three, 6-month-old Yucatan minipigs. Costal cartilage was minced into ~1 mm^3 pieces. The cartilage pieces were then digested with pronase solution (0.4% w/v, in wash medium, 3% FBS) for 1 h followed by 18 h in collagenase II solution on an orbital shaker at 60 RPM. Finally, the cells were strained, treated with ACK lysis buffer, washed several times, counted, and frozen in 90% FBS and 10% DMSO either immediately as primary cells or after one passage, as previously described.[12]

2.4 | Passaging and aggregate rejuvenation of minipig costal chondrocytes

Cell vials were thawed by placing in a 37°C water bath and adding cells dropwise to wash medium to ensure high viability. The tubes were spun down at 400 *g* for 5 min, and cells were resuspended in a pre-warmed chondrogenic medium (CHG), which consisted of DMEM, 1% PSF, 1% nonessential amino acids, 1% insulin-transferrin-selenous acid, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate, and 100 nM of dexamethasone. The cells were seeded in 27 ml per flask of CHG, plus 2% FBS and growth factors (1 ng/ml TGF-β1 + 5 ng/ml basic fibroblast growth factor + 10 ng/ml platelet-derived growth factor), at 2.5 million cells per flask. Finally, the flasks were checked for confluence every 1–2 days, and the cells were fed every 3–4 days with CHG, plus 2% FBS and growth factors.

The cells were passaged every two weeks or until the cells were confluent, whichever came first. Cells for Phase II and III were expanded to passage 3, while Phase IV utilized passage 6 cells. Wash medium was added to each flask to rinse, and, subsequently, 0.05% Trypsin-EDTA was added to each flask. The flasks were placed in an incubator for 8–9 min, and a wash medium, plus 10% FBS, was added to the growth surface of the flask to neutralize the Trypsin-EDTA. The suspension of cells was spun down, and the supernatant was discarded. The cell pellet was resuspended in collagenase II solution and placed in a 37°C water bath. The cell suspension was pipetted up and down every 10–15 min for 20–30 min. The cell suspension was spun down to remove the supernatant, and the cells were counted in a wash medium. The cells were plated for the next passage until the terminal passage when they were placed into aggregate rejuvenation. The three individual costal chondrocyte donors were passaged individually until the last passage when they were combined for the remainder of culture.

Finally, the cells were then placed into aggregate rejuvenation, which allowed the cells to recover their chondrogenic phenotype.[48] Petri dishes were coated with molten 1% agarose to create a nonadherent surface. The cells were seeded at a final density of 750 thousand cells per ml of medium in 30 ml (22.5 million cells per dish) of CHG plus growth factors (10 ng/ml TGF-β1 + 100 ng/ml growth differentiation factor 5 + 100 ng/ml bone morphogenetic protein 2). The Petri dishes were placed on an orbital shaker for 24 h at 50 RPM, then subsequently cultured under static conditions. The aggregates were fed every 3–4 days for 14 or 11 days for Phases II/III or IV, respectively. It has been previously shown that expanded chondrocytes that are placed in aggregate rejuvenation maintain a chondrogenic phenotype throughout the self-assembling process, exhibiting high expression of aggrecan, collagen type II, and SRY-box transcription factor 9 and minimal expression of osteocalcin and collagen type I.[49]

TABLE 1 Timeline of FIS stress and bioactive factor application

Treatment	Time					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Phase I:						
Synthesis						
Combination						
Maturation						
Phase II:						
Maturation						
Phase III:						
Maturation						
TGF-β1						
C-ABC						
LOXL2						
Phase IV:						
Maturation						
TGF-β1						
C-ABC						
LOXL2						
Cytochalasin D						

Note: The specific timeline of application for FIS stress, TGF-β1, C-ABC, LOXL2, and cytochalasin D is shown.

Abbreviations: C-ABC, chondroitinase ABC; LOXL2, lysyl oxidase-like 2; TGF-β1, transforming growth factor beta 1.

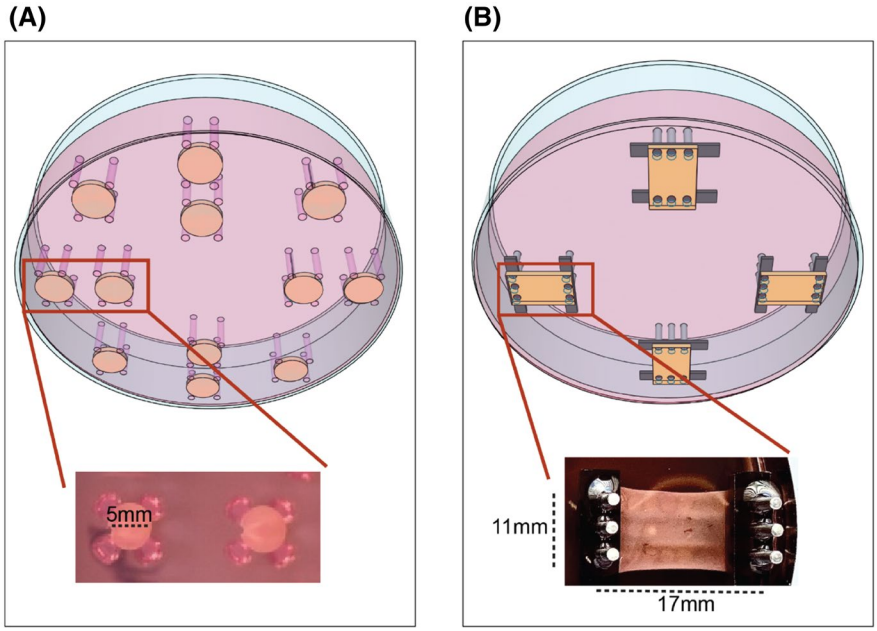


FIGURE 2 The two FIS stress devices are used for the stimulation of circular and rectangular neocartilage constructs. (A) The FIS stress device for 5 mm diameter neocartilage constructs was created using a 3% agarose base inside a 100 mm diameter petri dish. Neocartilage constructs of 5 mm diameter were placed within four agarose posts to be held in place during FIS stress stimulation. (B) A novel design of the FIS stress device was necessary to stimulate 11 × 17 mm neocartilage constructs. The new FIS stress device was created using an acrylic base and stainless-steel metal rods. The acrylic base was placed inside a 100 mm diameter petri dish, and the 11 × 17 mm neocartilage constructs were held in place by the six metal rods and acrylic weights. Previous studies have shown that the changes created in the new design for rectangular constructs do not significantly alter the fluid flow or FIS stress applied to the neocartilage.[50–53] For both designs, previous computational fluid dynamic modeling studies have shown that a speed of 50 RPM on an orbital shaker achieves 0.05–0.21 Pa of FIS stress on neocartilage constructs[11]

2.5 | Self-assembly of neocartilage constructs

Custom-made well-makers were used to make negative molds in 2% agarose wells. CHG was added and exchanged twice before seeding chondrocytes. Wells were 5 mm diameter cylinders for Phases I, II, and III. For Phase IV, wells were scaled up from prior studies to 11 × 17 mm rectangular wells.[10,24] As described previously, chondrocytes were seeded into the 2% agarose wells at densities of 4 million bovine chondrocytes per well for Phase I, 2 million minipig chondrocytes per well for Phases II and III, and 15 million minipig chondrocytes per well for Phase IV to begin the self-assembling process. After seeding, 0.5 ml of

CHG was added to each well in Phases I, II, and III, while 5 ml of CHG was added to each well for Phase IV, at the 4 h time point. Seven or two days after seeding for small circular or large rectangular constructs, respectively, the self-assembled neocartilage constructs were unconfined from the agarose wells and cultured in 24- or 6-well plates. The small circular and large rectangular constructs received changes of medium (0.5 ml or 5.0 ml) every day up until unconfining, and 1 ml or 10 ml of the medium on alternating days for the remainder of the 28 day culture period.

2.6 | FIS stress stimulation and devices

FIS stress was applied to the treatment groups by placing the neocartilage constructs in a FIS stress device at the previously specified stages of maturation (Table 1). The device for 5 mm diameter neocartilage constructs was created by adding 25 ml of 3% agarose to a 100 × 25 mm petri dish, placing the device mold to create small protruding agarose poles, and removing the mold once the agarose solidified, as described previously.[11] Each of the neocartilage constructs was positioned between four surrounding poles to keep the constructs in place, and 20 ml of CHG was added to the device (Figure 2A). A new device was created for the stimulation of 11 × 17 mm neocartilage constructs using an acrylic base and stainless-steel metal rods (Figure 2B). As with the original device, the new device was designed to be placed inside a petri dish (100 × 25 mm), and the neocartilage constructs were loaded into the device. Acrylic weights were also used to hold the neocartilage constructs in place (Figure 2B). Three constructs were loaded per FIS stress device, and 30 ml of CHG was added to the device. The FIS stress device was then placed on an orbital shaker at 50 RPM, and, as the orbital shaker rotated, it allowed the medium in the FIS stress device to flow over the neocartilage constructs, thereby applying 0.05–0.21 Pa of FIS stress, as previously characterized.[11] Since both devices were designed to have the same radius and are placed on the orbital shaker at the same rotational speed, the fluid flow and resulting FIS stress applied to the neocartilage constructs are not altered, as is shown by previous computational fluid dynamic modeling studies.[50–53]

2.7 | Bioactive factor treatment

The timeline of application for bioactive factor treatment is summarized in Table 1. The use of these bioactive factors was used in accordance with previous studies showing that (1) bioactive factors improved the functional properties of self-assembled constructs[9,10,12,24,35,37,46,54,55] and (2) bioactive factors improved the flatness of large constructs derived from highly passaged cells.[35,46] For Phase III, TGF-β1 (10 ng/ml) was applied for the entire duration of the 28 days of self-assembly, while C-ABC (2.0 U/ml) was applied on day 7 for 4 h to temporarily deplete glycosaminoglycan content, as previously described.[55] LOXL2 was applied at 0.15 μg/ml in conjunction with copper sulfate (1.6 μg/ml) and hydroxylysine (0.146 mg/ml) from days 7–21. For Phase IV, the culture time was extended to 42 days to maximize construct thickness and mechanical properties; LOXL2 treatment was extended until the end of culture, and C-ABC was treated as described above. TGF-β1 was modified for large constructs in Phase IV to be applied after unconfining (day 2) for the remaining duration of the culture, and cytochalasin D (2 μM) was applied from days 1 to 3, as previously described.[46]

2.8 | Analysis of mechanical properties

After completion of the culture, mechanical testing of the neocartilage constructs was performed. To determine the compressive properties, a circular 2 mm diameter punch was taken from the center of the construct, and a creep indentation test was performed to determine aggregate modulus and shear modulus, as previously described.[56] For Phase IV, 3 mm diameter circular punch specimens were also examined in a compressive stress-relaxation test at 10% strain on a uniaxial Instron machine (Model 5665) to determine the instantaneous modulus and relaxation modulus, as determined from a standard linear solid model using MATLAB software.[30]

Tensile testing was conducted using an uniaxial Instron machine, as previously described.[57] Neocartilage constructs were cut into dog bone-shaped samples and were glued to paper tabs with a predefined gauge length of 1.55 mm. The thickness and width of the dog bone-shaped samples were measured using ImageJ, and a subsequent cross-sectional area was calculated. A uniaxial strain-until-failure test was conducted with a strain rate of 1% per second. Load–displacement curves were normalized to the cross-sectional area and gauge length of each sample. Finally, Young's modulus and ultimate tensile strength were calculated using MATLAB software.

2.9 | Analysis of biochemical properties

For ECM content, wet weight (WW), and dry weight (DW) of the samples were measured, and specific assays were used to quantify collagen content and glycosaminoglycan

content. First, the samples were frozen to allow for sublimation during a 72 h lyophilization cycle. After lyophilization, DWs were measured, and the tissue was digested in a buffered papain solution for 18 h at 65°C. The glycosaminoglycan and collagen contents were normalized per WW and are reported in percentage. Glycosaminoglycan content was measured using a BioColor Blyscan glycosaminoglycan assay kit according to the manufacturer's directions. The total collagen content was measured using a modified chloramine T hydroxyproline assay and a Sircol collagen standard, as previously described.[58]

2.10 | Histology

Construct samples from Phase IV were fixed in 10% neutral buffered formalin for at least 48 h immediately after culture completion. Samples were then processed, embedded in paraffin, and sectioned at 5 μm for subsequent staining with hematoxylin and eosin (H&E), safranin O (Saf O) with fast green counterstain, and picrosirius red (Picro Red).

2.11 | Statistics

For Phase I, one-way analysis of variance (ANOVA) and Tukey's *post hoc* tests were performed using $p < .05$ to determine statistically significant differences among groups. Groups deemed significantly different by Tukey's *post hoc* tests are denoted using alphabetical letters via a connecting letters report, where groups not sharing the same letter are statistically significant. For Phases II, III, and IV, Student's *t*-test was used at $p < .05$ to determine statistically significant differences between groups.

3 | RESULTS

3.1 | Phase I: FIS stress stimulation during the maturation stage yields the largest functional increases over nonstimulated controls

To determine which stimulation time would lead to the most mechanically robust neocartilage constructs, FIS stress was applied at the different stages of neocartilage development (Figure 1A). For the compressive stiffness, the neocartilage constructs stimulated during the maturation stage improved 2.51-times over the nonstimulated control in aggregate modulus values ($p < .0001$) (Figure 3A). They also trended higher than the neocartilage constructs stimulated during the combination stage (Figure 3A). When considering tensile properties, neocartilage stimulated during the maturation and combination stages saw improvements in ultimate tensile strength over nonstimulated controls, showing a 100% increase ($p = .0185$ and $p = .0144$, respectively) (Figure 3B). In parallel, the collagen content of constructs stimulated during the maturation stage was improved over the collagen content of nonstimulated neocartilage ($p < .0001$), exhibiting an 82% increase (Figure 3C). Toward achieving the maximal improvement in neocartilage biochemical and mechanical properties, FIS stress stimulation during the maturation stage was selected to move forward to Phases II through IV.

3.2 | Phase II: FIS stress yields similar neocartilage functional increases using expanded and rejuvenated minipig costal chondrocytes

To evaluate the translatability of FIS stress stimulation across chondrocytes from different sources and species, Phase II explored the use of Yucatan minipig costal chondrocytes to produce neocartilage constructs. As in Phase I, neocartilage derived from Yucatan minipig costal chondrocytes was stimulated during the maturation stage. Compressive properties were examined, and it was found that the aggregate modulus values of neocartilage stimulated with FIS stress during the maturation stage were 46% higher than nonstimulated neocartilage ($p = .0020$) (Figure 4A). Furthermore, tensile properties were also improved; the ultimate tensile strength of neocartilage stimulated with FIS stress during the maturation stage was 78% higher than that of nonstimulated neocartilage ($p = .0002$) (Figure 4B). Similarly, Young's modulus of neocartilage stimulated with FIS stress during the maturation stage was 78% higher than nonstimulated neocartilage ($p = .0041$) (Figure 4B). An improvement in Young's modulus had not been previously seen in self-assembled neocartilage constructs made with bovine articular chondrocytes stimulated with FIS stress. As in neocartilage created with bovine neocartilage constructs in Phase I, an increase in glycosaminoglycan content was observed in minipig neocartilage stimulated during the maturation stage compared to the nonstimulated control (Figure 4C); a 136% increase in glycosaminoglycan content was observed ($p = .0008$). Interestingly, although collagen content trended higher, a significant increase in collagen content was not observed in the FIS stress-stimulated neocartilage created with minipig costal chondrocytes (Figure 4C). As the Yucatan minipig is a widely used preclinical model for the treatment of cartilage lesions,[12,28,30,31] this phase showed that FIS stress can be applied across various sources and species for similar functional improvements.

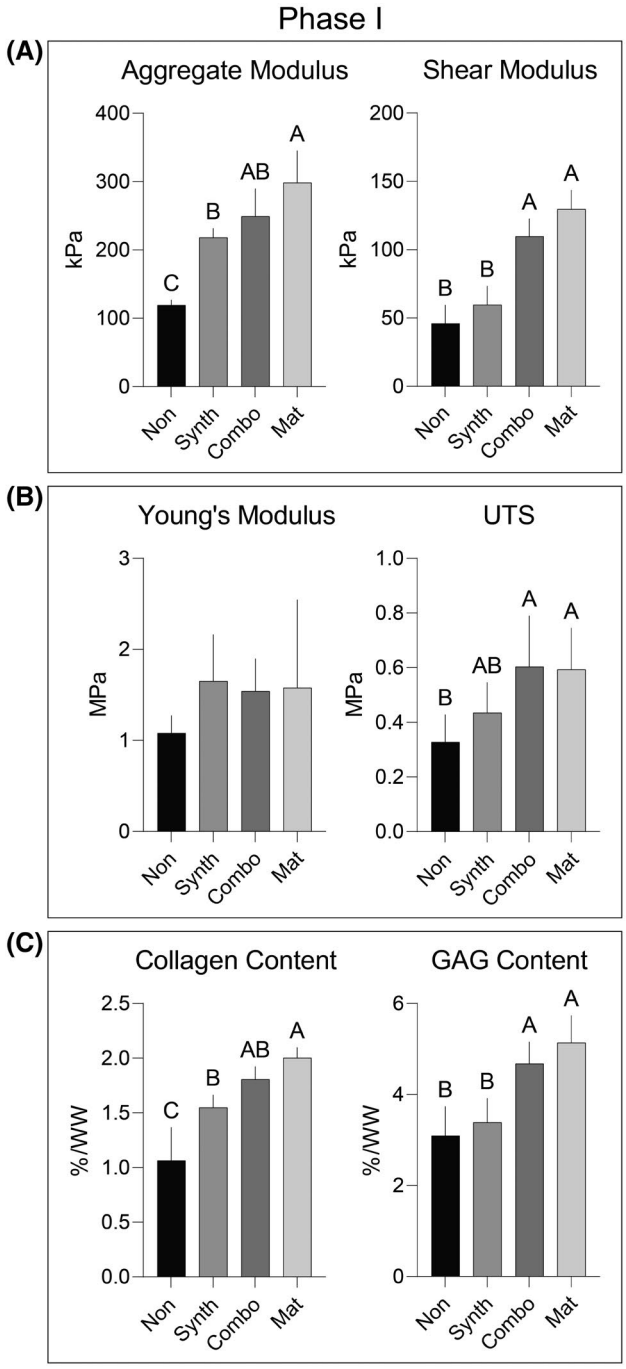


FIGURE 3 Identifying the best time of application for FIS stress. Phase I constructs were derived from bovine articular chondrocytes in small 5 mm diameter circular shapes and included four groups to determine an optimal FIS stress stimulation stage: nonstimulated, stimulated with FIS stress during the synthesis stage, stimulated with FIS stress during the maturation stage, and stimulated with FIS stress during the combination of synthesis and maturation stages. (A) The aggregate modulus and shear modulus under compressive conditions, (B) Young's modulus and the ultimate tensile strength under tensile conditions, (C) and the collagen content and the glycosaminoglycan content of neocartilage constructs are shown. Bars not sharing the same letter are statistically different when evaluated at $p < .05$ using a one-way ANOVA and Tukey's *post hoc* test. Combo, combination; GAG, glycosaminoglycan; kPa, kilopascals; Mat, maturation stage; MPa, megapascals; Non, nonstimulated; Synth, synthesis stage; %/WW, percent by wet weight

3.3 | Phase III: Bioactive factors in conjunction with FIS stress yield further functional improvements over bioactive factors alone

Bioactive factors have been previously found to further enhance neocartilage constructs,[37] and they were applied here in conjunction with FIS stress toward further functional improvement. FIS stress in combination with bioactive factor treatment significantly increased aggregate modulus ($p = .0006$) and shear modulus ($p < .0001$) values by 48% and 115%, respectively, over bioactive factor-only controls (Figure 5A). Both measures of tensile properties, Young's modulus and ultimate tensile strength, were also significantly higher with the application of FIS stress and bioactive factors compared to bioactive factor-only controls ($p = .0047$ and $p = .0014$, respectively) (Figure 5B). In terms of ECM content, glycosaminoglycan and collagen content were measured (Figure 5C); glycosaminoglycan content was significantly higher ($p < .0001$) with the application of FIS stress and bioactive factors compared to bioactive factor-only controls. The combination of bioactive factors and FIS stress led to improved neocartilage properties when compared to bioactive factors alone. However, when comparing the magnitudes of aggregate modulus and Young's modulus values of the FIS stress plus bioactive factor group of Phase III to those from the FIS stress-stimulated group of Phase II, the neocartilage properties did not further improve when stimulated with bioactive factors. However, the application of bioactive factors is important for examining large constructs as it has been previously demonstrated that rejuvenated constructs stimulated with bioactive factors yield mechanically robust and flat constructs,[9] an important clinical feature for tissue-engineered cartilage therapeutics. Despite this, stimulation with bioactive factors and FIS stress yielded improved functionality when compared to neocartilage stimulated with only bioactive factors.

3.4 | Phase IV: Large constructs derived from highly passaged cells are mechanically robust and flat

Phase IV examined the effect of FIS stress in conjunction with bioactive factors on large rectangular 11 × 17 mm neocartilage constructs derived from passage 6 costal chondrocytes. Although the functional

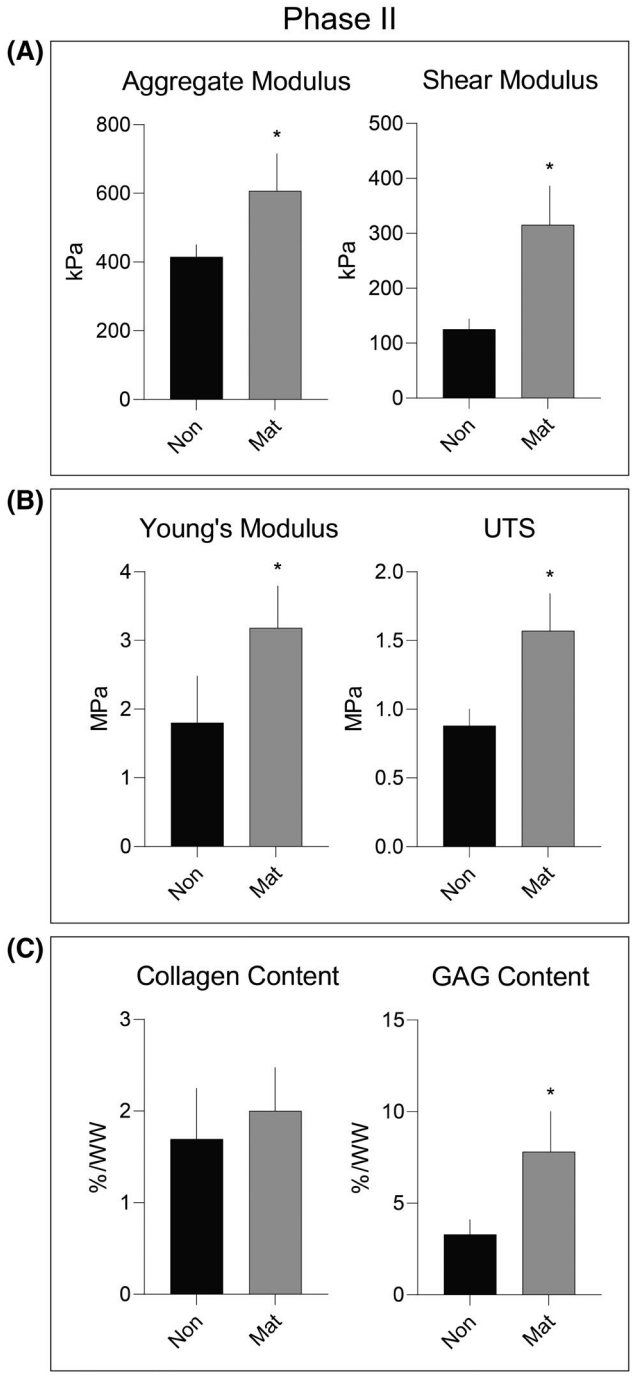


FIGURE 4 Translating FIS stress stimulation to costal chondrocytes from the Yucatan minipig. Phase II constructs were derived from passage 3 minipig costal chondrocytes in small 5 mm diameter circular shapes and included two groups to assess the translatability of the FIS stress stimulation regimen across cell sources and species: nonstimulated and stimulated with FIS stress during the maturation stage. (A) The aggregate modulus and the shear modulus under compressive conditions, (B) Young's modulus and the ultimate tensile strength under tensile conditions, (C) and the collagen content and the glycosaminoglycan content of neocartilage constructs are shown. The asterisk (*) above the bars indicates statistically different groups when evaluated at $p < .05$ using Student's *t*-test. GAG, glycosaminoglycan; kPa, kilopascals; Mat, maturation stage; MPa, megapascals; Non, nonstimulated; %/WW, percent per wet weight

properties of neocartilage stimulated with a combination of FIS stress and bioactive factors in Phase III did not significantly improve over FIS stress-stimulated groups of Phase II, the addition of bioactive factors and cytochalasin D has previously been necessary to create neocartilage constructs larger than 5 mm diameter, specifically when using highly passaged chondrocytes.[9,46] Compressive properties were examined using both creep indentation and stress relaxation. Aggregate modulus increased 2.34-times over bioactive factor-only controls ($p = .0251$), while shear modulus increased 2.72-times over controls ($p = .0064$) (Figure 6A). Under 10% strain, the relaxation modulus significantly increased by 100% ($p = .0251$) and the instantaneous modulus did not significantly differ (Figure 6A). The constructs were also measured under uniaxial tension in both axes (i.e., short and long) (Figure 2B). The only significant difference was in the short axis Young's modulus, which increased significantly when stimulated with FIS stress over bioactive factor-only controls ($p = .0216$). For biochemical content, collagen increased when treated with FIS stress compared to bioactive factor-only controls ($p = .0209$) (Figure 6C). While glycosaminoglycan trended upward with the application of FIS stress, it was not significant (Figure 6C). Compared to free-floating constructs stimulated only with bioactive factors, those to which FIS stress was applied exhibited a flatter morphology and less curling (Figure 6D). Histologically, samples stimulated with FIS stress and bioactive factors had increased staining intensity in Saf O (Figure 7A), indicative of neocartilage constructs with more glycosaminoglycan content, but similar staining intensities under H&E (Figure 7B). Picro Red staining shows the spatial organization of collagens within the matrix. The groups seem to have similar overall intensities, but FIS stress increased the peripheral staining, indicating stronger collagen deposition on the outer edges of the neocartilage constructs (Figure 7C). These staining trends from Figure 7 follow those quantitative metrics for glycosaminoglycan and total collagen content presented in Figure 6C. Combined, these results indicate that large constructs derived from highly passaged cells respond in a similar manner to the combination of bioactive factors and FIS stress during the maturation stage compared to small constructs from Phases I through III. Additionally, mechanical confinement in the form of the novel FIS stress device also improves construct flatness. This represents a significant step toward treating larger articular cartilage defects using large constructs.

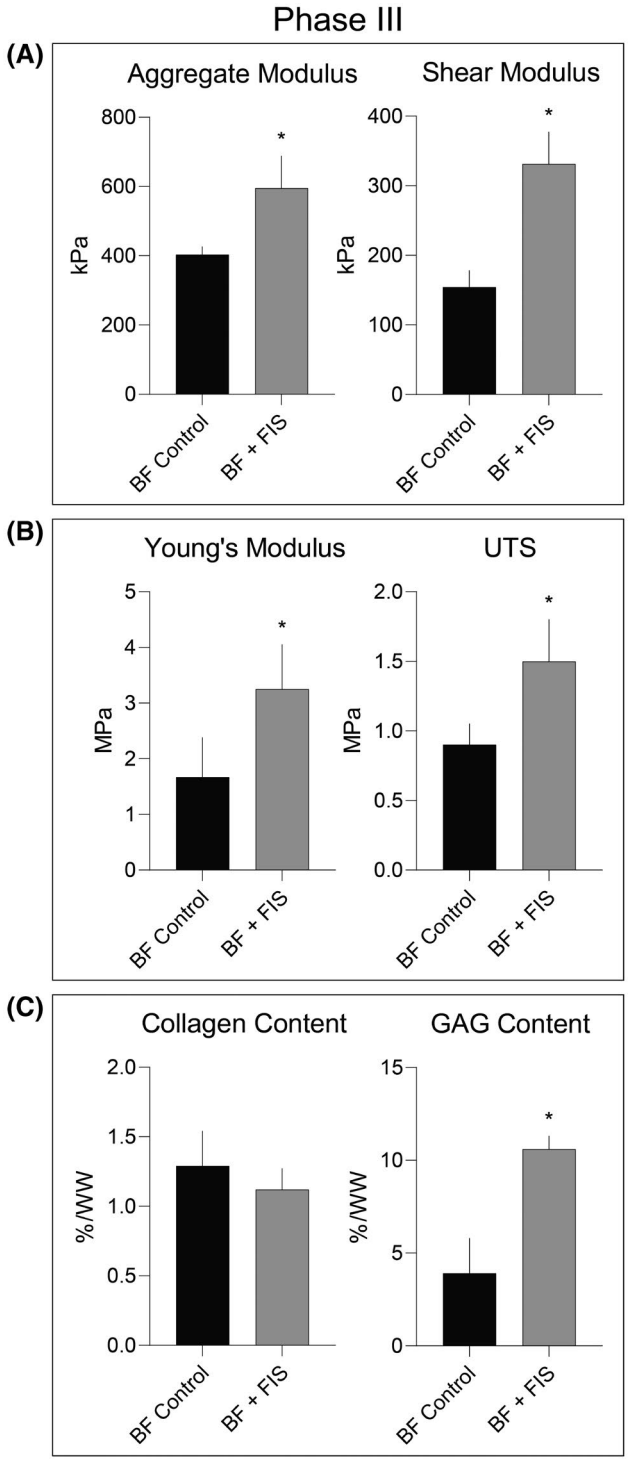


FIGURE 5 Combining bioactive factors and FIS stress to further improve the functional properties of neocartilage constructs. Phase III constructs were derived from passage 3 minipig costal chondrocytes in small 5 mm diameter circular shapes and included two groups: treated with bioactive factors alone and treated with bioactive factors and FIS stress stimulation during the maturation stage. (A) The aggregate modulus and the shear modulus under compressive conditions, (B) Young's modulus and the ultimate tensile strength under tensile conditions, (C) and the collagen content and the glycosaminoglycan content of neocartilage constructs are shown. The asterisk (*) above the bars indicate statistically different groups when evaluated at $p < .05$ using Student's *t*-test. BF, bioactive factors; FIS, fluid-induced shear; GAG, glycosaminoglycan; kPa, kilopascals; MPa, megapascals; %/WW, percent per wet weight

4 | DISCUSSION

In this series of studies, the global objective of this work was to improve the functional and translational aspects of neocartilage constructs. Specifically, this work tackled four critical aspects of neocartilage engineering across the four phases presented, including (1) finding the ideal application window of FIS stress stimulation for biochemical and mechanical improvement, (2) establishing the reproducibility of FIS stress stimulus across bovine and minipig cell sources, (3) exploring the beneficial effects of combining FIS stress and bioactive factors, and (4) scaling-up the size of neocartilage constructs using highly passaged cells while maintaining a flat morphology and other improvements in functional properties seen with application of FIS stress and bioactive factors. Overall, it was hypothesized that the appropriate combination of FIS stress and bioactive factors would yield flat, large neocartilage constructs with improved mechanical properties and ECM content. Indeed, both the functional characteristics (mechanical properties and ECM content) and the translational aspects (construct size, gross morphology, passage number, cell source, and cell type) were improved. Namely, in Phase I, FIS stress applied during the maturation stage improved compressive stiffness by 151%, tensile stiffness by 45%, and collagen content by 82% in self-assembled neocartilage constructs engineered from bovine articular cartilage cells. In Phase II, the FIS stress stimulation regimen from Phase I was implemented using costal chondrocytes from the Yucatan minipig and also led to similar increases in compressive stiffness (46%) and tensile stiffness (78%). Then, in Phase III, the combination of FIS stress with bioactive factors improved the compressive stiffness (48%) and tensile stiffness (94%) of neocartilage constructs over bioactive factor-only controls. Finally, in Phase IV, the methods from Phases I through III were combined to engineer large neocartilage constructs derived from highly passaged cells, while maintaining the earlier increases seen via the application of FIS stress and bioactive factors.

Toward achieving native tissue functionality, the ideal window of FIS stress time of application was identified. This objective is significant because, in native articular cartilage, chondrocytes depend on mechanical loading during embryonic development to synthesize ECM,[17,18] and, during postnatal development, timed mechanical signaling regulates cartilage thickness and maturation for

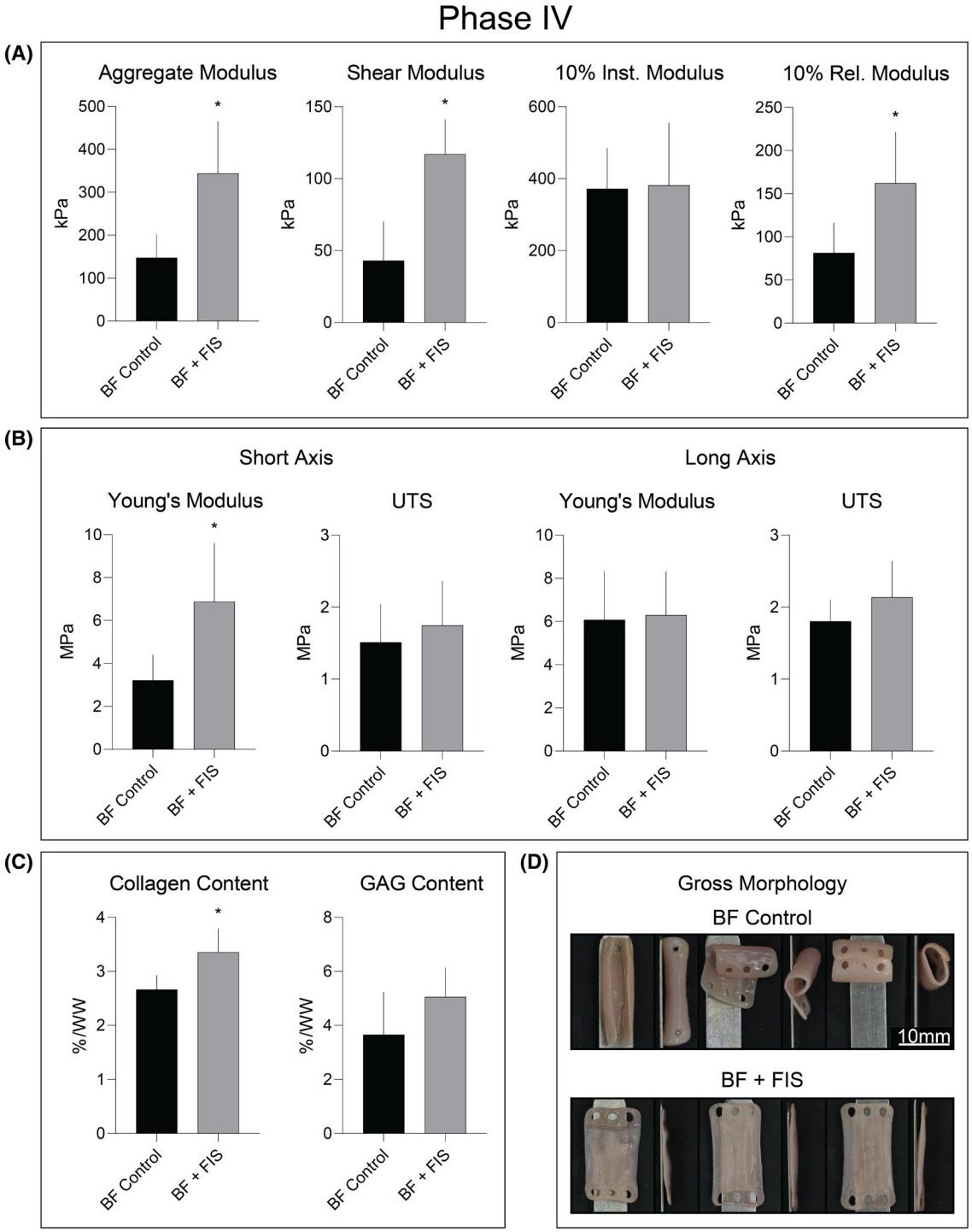


FIGURE 6 Scaling-up the size of neocartilage constructs created from highly passaged chondrocytes using FIS stress and bioactive factors. Phase IV constructs were derived from passage 6 minipig costal chondrocytes in 11 × 17 mm rectangular shapes and included two groups: treated with bioactive factors plus cytochalasin D, and treated with bioactive factors, cytochalasin D, plus FIS stress stimulation during the maturation stage. (A) The aggregate modulus, the shear modulus, and the 10% instantaneous and relaxation moduli under compressive conditions, (B) and Young's modulus and the ultimate tensile strength for both the long and short axis of neocartilage constructs under tensile conditions are shown. (C) The collagen content and the glycosaminoglycan content, and (D) images of the gross morphology of neocartilage constructs are shown. The asterisk (*) above the bars indicates statistically different groups when evaluated at $p < .05$ using Student's *t*-test. BF, bioactive factors; FIS, fluid-induced shear; GAG, glycosaminoglycan; Inst., instantaneous; kPa, kilopascals; MPa, megapascals; %/WW, percent per wet weight; Rel., relaxation

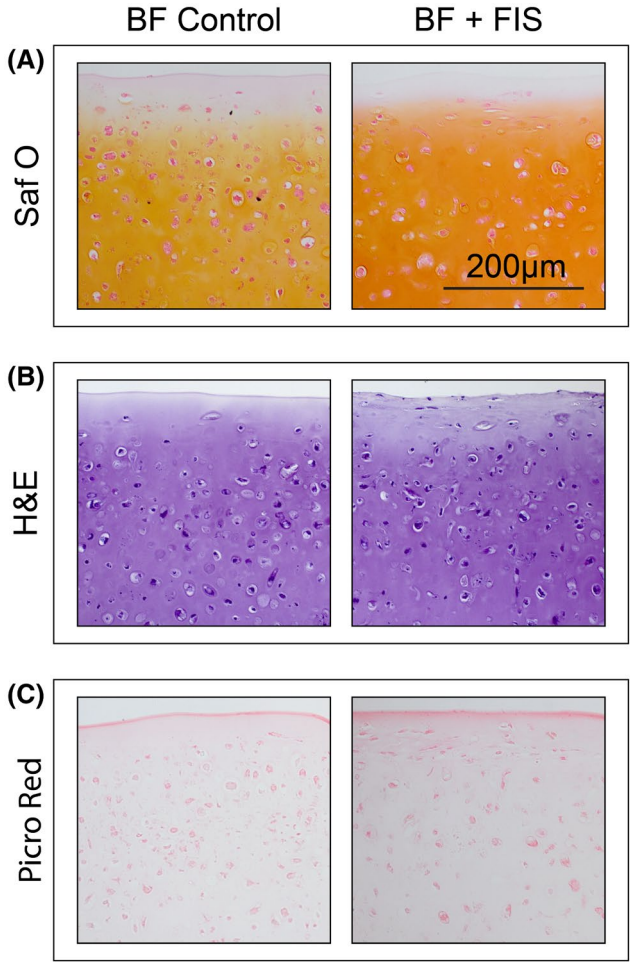


FIGURE 7 Combining bioactive factors and FIS stress increases intensity for glycosaminoglycan staining. Phase IV constructs were derived from passage 6 minipig costal chondrocytes in 11 × 17 mm rectangular shapes and included two groups: treated with bioactive factors plus cytochalasin D, and treated with bioactive factors, cytochalasin D, plus FIS stress stimulation during the maturation stage. Representative images of (A) safranin O staining for glycosaminoglycan content, (B) hematoxylin and eosin staining for general cellular and tissue morphology, and (C) picrosirius red staining for general collagen content are shown. BF, bioactive factors; FIS, fluid-induced shear; H&E, hematoxylin and eosin; Picro Red, picrosirius red; Saf O, safranin O

proper function.[19,20] Similarly, previous studies showed that self-assembled neocartilage also follows chronological steps and may exhibit maturation-dependent mechanical signaling needs.[22,23] For example, when nonstimulated neocartilage constructs enter the maturation stage, the production of glycosaminoglycan is increased, whereas collagen content shows a stark decrease.[23] In contrast, when FIS stress is applied during the maturation stage (days 15–22) in Phase I of this work, collagen content increased by 82% over nonstimulated constructs and by 33% over constructs stimulated during the synthesis stage (days 7–14). The neocartilage constructs also improved in compressive and tensile properties. These results indicate that, like native cartilage, carefully timed mechanical signaling is crucial to the development of robust neocartilage. Although further study is necessary to determine the precise pathways leading to these results, FIS stress has been previously shown to activate ECM producing protein pathways via the perturbation of the PC1/PC2 complex on the primary cilia of chondrocytes.[11,15,16,59] Inasmuch as previous studies on self-assembled cartilage have seldom investigated the time of application of mechanical stimulation, this study showed that stimulation during the maturation stage is optimal in terms of producing a mechanically robust neocartilage suitable for preclinical in vivo implantation toward eventual FDA approval for human studies.

As specified by the FDA, tissue-engineered therapeutics must undergo preclinical studies in an appropriate animal model prior to human clinical studies, and, for these, an analogous animal product should be investigated. Therefore, an important translational feature of this study is the demonstration that the FIS stress stimulus is efficacious in an animal model, such as the Yucatan minipig. Furthermore, transferring the FIS stress stimulus to a different cell type, namely, costal chondrocytes, is significant for translation because of their ability to be harvested autologously and allogeneically without further damaging diseased joints that require treatment. For example, in a recent study using bioactive factor-stimulated constructs derived from costal cartilage, the Yucatan minipig was used as a model for temporomandibular joint disc cartilage repair.[12] Similarly, several researchers are performing in vivo meniscus and articular cartilage repair in the minipig.[60–62] Here, constructs were generated from the costal cartilage of the Yucatan minipig and stimulated during the maturation stage. Similar to constructs derived from bovine articular chondrocytes, compressive stiffness and tensile strength and stiffness of minipig-derived neocartilage significantly increased by 46%, 78%, and 78%, respectively, when FIS stress was applied. Although increases in shear modulus are similar between Phases I and II, aggregate modulus increases were larger in Phase I compared to Phase II. These differences are most likely due to changes in the ECM-producing capacity of the different cell types (i.e., articular vs. costal chondrocytes) when stimulated with FIS stress. For example, the increases in collagen are also different between the two phases. Despite these small differences, these are significant results because (1) FIS stress has not been previously investigated in the costal cartilage cell source and (2) the Yucatan minipig is a widely used preclinical model. Previous studies have also corroborated the findings shown here that FIS stress stimulation, as well as tensile stimulation, of self-assembled constructs, can be translated across cell passage numbers

and species (e.g., bovine and human articular chondrocytes).[10,11] Although additional studies examining FIS stress during the maturation stage in human costal chondrocytes will eventually need to be performed, the work done here shows promise that the use of FIS stress on neocartilage would be feasible and beneficial across different cell passage numbers, cell sources, and cell types.

Previously identified cocktails of bioactive factors have been shown to individually improve the functionality and morphology of neocartilage constructs,[9,37] but their interactions with FIS stress have not been previously investigated. This is of scientific interest because healthy cartilage, whether native or engineered, is dependent on a variety of signals which include both mechanical and biochemical cues.[19,20,22,23,35–40] Phases III and IV relied on a plethora of past studies which used bioactive factors, employing the regimens described here, to improve the functional properties of self-assembled cartilage compared to nonstimulated controls.[35–39,46,63] Here, the addition of bioactive factors (TGF-β1, C-ABC, and LOXL2) was investigated in combination with FIS stress stimulation to further improve the functional properties of neocartilage constructs and, eventually, keep constructs derived from high passage cells flat. Indeed, it was found that similar to Phase II, increases were observed in Phase III when FIS stress was combined with bioactive factors, increasing compressive stiffness (by 48%) and tensile stiffness (by 94%). It appears that FIS stress dominates the functional increases seen in constructs regardless of bioactive factor stimulation. As previously shown,[9,10,12,24,35,37,46,54,55] future studies should include a direct comparison between bioactive factor-stimulated and nonstimulated groups to ensure that the beneficial effects of bioactive factors are maintained in the Yucatan minipig costal cartilage source. The increases in mechanical properties seen here may be due to increased perfusion of growth factors in neocartilage by FIS stress, but this is unknown as the perfusion rates in FIS stress-stimulated neocartilage constructs have not been explored.[11] However, previous studies have shown that the primary cilia are implicated in both TGF-β signaling and FIS stress stimulation. Future studies should determine whether perfusion, primary cilia perturbation, or a combination is the exact cause of further improved functional properties of neocartilage constructs.[42] The findings of this study mirror the results of previous studies which used these bioactive factors in conjunction with other mechanical stimuli, such as tension and compression.[10,24] For example, a combination of passive axial compression and bioactive factors yielded significantly higher relaxation modulus values compared to bioactive factor-only controls.[24] Additionally, previous studies have shown that bioactive factors are an important element for maintaining mechanical robustness for highly passaged cells and large constructs.[9,46] Thus, the combination of FIS stress and bioactive factors is important when considering the generation of self-assembled neocartilages larger than 5 mm in diameter.

Toward addressing larger cartilage lesions, this study examined the creation of large, flat, mechanically robust constructs generated from highly passaged costal chondrocytes stimulated with bioactive factors and FIS stress. This phase represents a significant step toward functional and translational improvements for implantation by increasing the tissue-engineered implant in size from 5 mm diameter to 11 × 17 mm, representing more than a 9-times increase in construct area. This increase in size also increased the number of cells needed by-7.5 times. Therefore, in Phase IV, in order to accommodate for the high number of cells needed (i.e., 15 million per construct), the number of passages costal chondrocytes undergo, compared to Phases II and III, was doubled from three to six. Although this may seem straightforward, as passage number increases, it has been shown that the cells undergo more dedifferentiation toward a fibroblastic phenotype.[64] Using the expansion and aggregate rejuvenation process described (i.e., no bioactive factors), constructs up to passage 5 have exhibited flat morphologies, but then started to display unwanted morphological characteristics at passage 7 (e.g., decreased diameter, biconcave structure).[9] This same study further examined bioactive factor use, as described here, during self-assembly of small 5 mm diameter constructs and noted additional increases in functional properties, especially for higher passage constructs, and maintenance of a flat morphology.[9] While 5 mm diameter constructs remained relatively flat, larger constructs of 25 mm diameter did not remain flat due to internal stresses through the actin cytoskeleton, applying forces to the ECM.[46] Previous studies noted that the application of bioactive factors, cytochalasin D (an actin polymerization inhibitor), and mechanical confinement in the form of an agarose coverslip are necessary to maintain flatness.[46] Cytochalasin D was applied here in a similar regimen toward keeping constructs flat, but it has also been shown to recover primary cilia of chondrocytes after passaging,[65] thus, potentially making cytochalasin D-treated cells here more sensitive to FIS stress to yield further increases in functional properties. Similarly, this study showed that mechanical confinement in the form of the novel FIS stress device was necessary to keep constructs flat. As expected, the FIS stress system and other bioactive factors also maintained the functional properties seen in earlier phases of this study. Thus, the combination of highly passaged cells with FIS stress and bioactive factors was successful in generating mechanically robust neocartilage constructs toward improving the range of

cartilage lesion indications that can be potentially addressed by self-assembled neocartilage.

This work represents substantial progress toward generating a tissue-engineered neocartilage solution for addressing articular cartilage lesions. Using FIS stress, functional properties, such as aggregate modulus and collagen content, of constructs derived from primary bovine articular chondrocytes and passaged and rejuvenated minipig costal chondrocytes were improved toward native tissue values. For example, compressive aggregate modulus values reported here for neocartilages range from approximately 120–600 kPa. These values are within the range reported for native articular cartilages (250–1400 kPa).[1–3] Additionally, bioactive factors have been previously used in conjunction with other forms of mechanical stimuli (i.e., passive axial compression and tension) but have not been examined in combination with FIS stress, as performed here. The addition of bioactive factors with FIS stress stimulation did not adversely affect functional improvements when compared to bioactive factor-only controls. Finally, combining all the previous phases, large constructs derived from highly passaged costal chondrocytes exhibited mechanical robustness and flatness, important translational features. Combined, the four phases of this study represent significant steps toward generating mechanically robust, flat, large neocartilage constructs necessary for a wide range of preclinical animal studies and, eventually, human clinical studies for various articular cartilage indications.

ACKNOWLEDGMENTS

This work was funded by the National Institutes of Health (R01 DE015038, R01 AR078389, R01 AR067821). Evelia Y. Salinas was also in part supported by a National Institutes of Health Diversity Supplement (on R01 AR067821). Jessica M. Herrera was also in part supported by the California Alliance for Minority Participation.

DISCLOSURES

Kyriacos A. Athanasiou and Jerry C. Hu are scientific consultants for Cartilage Inc.

AUTHOR CONTRIBUTIONS

Evelia Y. Salinas, Ryan P. Donahue, and Jessica M. Herrera designed the experiments, analyzed the data, drafted the manuscript, and critically edited the manuscript. Jerry C. Hu and Kyriacos A. Athanasiou oversaw and guided the experiments, drafted the manuscript, and critically edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the article.

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How to cite this article: Salinas EY, Donahue RP, Herrera JM, Hu JC, Athanasiou KA. The functionality and translatability of neocartilage constructs are improved with the combination of fluid-induced shear stress and bioactive factors. *FASEB J*. 2022;36:e22225. doi:10.1096/fj.202101699R