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Tension stimulation drives tissue formation in scaffold-free systems

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Abstract

Scaffold-free systems have emerged as viable approaches for engineering load-bearing tissues. However, the tensile properties of engineered tissues have remained far below the values for native tissue. Here, by using self-assembled articular cartilage as a model to examine the effects of intermittent and continuous tension stimulation on tissue formation, we show that the application of tension alone, or in combination with matrix remodelling and synthesis agents, leads to neocartilage with tensile properties approaching those of native tissue. Implantation of tension-stimulated tissues results in neotissues that are morphologically reminiscent of native cartilage. We also show that tension stimulation can be translated to a human cell source to generate anisotropic human neocartilage with enhanced tensile properties. Tension stimulation, which results in nearly 6-fold improvements in tensile properties over unstimulated controls, may allow the engineering of mechanically robust biological replacements of native tissue.

Introduction

During embryonic development, tight regulation of chemical gradients drives the formation of specialized tissues. Similar to chemical factors, biomechanical stimuli are critical during embryogenesis. Beyond development, biomechanics is also essential for maturation, maintenance, and pathophysiological processes. To study the role of biomechanical forces

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Author contributions: JKL, LWH, JCH, and KAA were responsible for the design and execution of InTenS and CoTenS studies. NP and JKL together conducted all animal work, while NP, AA, and LWH performed the human articular chondrocyte experiments. CAG assisted in the design and fabrication of the tensile loading device. JKL, LWH, NP, AA, and CAG collected all data. AA performed finite element modeling and analysis. JKL and LWH performed the data analysis. JKL, LWH, JCH, and KAA prepared the manuscript.

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on tissue formation from the tissue level to the cellular level, we used scaffold-free articular cartilage, formed using a self-assembling process, as a model. In articular cartilage, it is known that biomechanical forces similarly drive development, maturation, maintenance, and pathophysiology[1]. Moreover, since articular cartilage is considered to function primarily in compression and shear, application of these stimuli dominates the cartilage regeneration field. Tensile forces also play a role in cartilage homeostasis, but the use of tension stimulation is largely understudied.

In addition to exploring biomechanics in a model system where the use of tension is understudied, we selected cartilage tissue formation because of the clinical impact it can have for those with osteoarthritis. Worldwide, approximately 240 million cases of osteoarthritis were reported in 2013, a 72% increase from 1990[2]. With the aging population and increasingly effective methods of diagnosis, the prevalence of osteoarthritis will continue to increase. The degeneration of articular cartilage—the smooth tissue that lines the articulating surfaces within a joint—can be a result of acute trauma or long-term overuse. Healthy articular cartilage functions as a load-bearing tissue, self-lubricated to provide a frictionless surface for joint movement. When cartilage is damaged, the joint compartments no longer translate smoothly, leading to increased tissue wear and, ultimately, degeneration.

Though the difficulty of repair and regeneration of articular cartilage has been recognized as early as the 4th century BCE, when Aristotle stated that “Cartilage…when once cut off, [does not]' grow again”[3], only in the last five decades have tissue regeneration strategies specifically sought to replace damaged cartilage by creating implants *in vitro*[4,5]. These implants must be able to withstand the mechanically strenuous joint environment; as such, the aim is to generate tissues with properties matching those of native cartilage[6,7]. Chemical stimulation methods have been widely employed, as have mechanical stimulation regimens in the form of compression, hydrostatic pressure, or shear[8-10]. In contrast, tension stimulation has not been examined commonly. A few representative studies include tension stimulation of chondrocytes in monolayer[11], of chondrocytes or fibroblasts seeded in scaffold[12,13], and of chondrocytes seeded in fibrin gel[14,15]. However, no studies have identified an efficacious tensile loading regimen for improving tensile properties of tissue-engineered cartilage; in particular, tension has not been examined at all for scaffold-free cartilage formation. While the compressive properties of native articular cartilage have been attained in engineered cartilage[16,17], achieving tensile properties akin to native tissue remains a major challenge. Motivated by the lack of studies examining tension as a potential stimulus in scaffold-free systems, we first sought to examine the effects of tension stimulation on tissue formation. To do so, we used self-assembling neocartilage as a model system of tissue formation in the absence of exogenous scaffolds. This system allows tensile forces to be directly applied to matrix and cells, without stress-shielding from scaffolds. Furthermore, to address the disparity between engineered and native tissue tensile properties, an additional goal of this study was to create neocartilage with tensile modulus and strength mimicking native articular cartilage via the application of Intermittent or Continuous Tension Stimulation (InTenS and CoTenS, respectively).

Toward achieving these objectives, we executed a series of studies involving the use of tension stimulation alone or in combination with matrix remodeling and synthesis agents

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known to enhance tissue formation and organization. Previous studies have shown that bioactive factors transforming growth factor-β1 (TGFβ1), chondroitinase-ABC (CABC), and LOXL2 (lysyl oxidase-like protein 2 with copper sulfate and hydroxylysine) can increase the tensile properties of scaffold-free neocartilage[18-20]. Here, we hypothesized that tension stimulation would improve neocartilage tensile properties to native tissue-like levels —beyond what is achieved by bioactive factors—as well as produce an anisotropic extracellular matrix (ECM) to mimic native cartilage structure. We also explored cellular mechanisms by which tension stimulation may affect the neotissue, identifying potential pathways via which mechanotransduction occurs to initiate matrix remodeling. Since the ion channel transient receptor potential vanilloid-4 (TRPV4) channel has been shown to modulate cellular response to compressive mechanical loading[21], it was also hypothesized that TRPV4 channel function is responsible for cellular response to InTenS. The CoTenS regimen was developed to further build on the effects seen with InTenS, aiming to fully explore the role that tension as a stimulus may play in the development of neocartilage. To determine the stability of the enhanced properties when subject to the *in vivo* milieu, we implanted these constructs in a subcutaneous environment. Finally, to highlight the clinical potential of these tensile and bioactive regimens, we applied these regimens to self-assembled, human neocartilage.

Design and validation of tension stimulation devices

We report the design and fabrication of tensile loading devices capable of InTenS and CoTenS (Figures 1a and 1b). Custom well-makers were fabricated to create rectangular neocartilage constructs, generated using the self-assembling process[22], with a surface area of 80mm^2 (Figure 1c). To predict the strain distribution in the neocartilage during tension stimulation, finite element modeling using the biphasic mixture theory was used. The model predicted uniform strain distribution through the center portion of neocartilage (Figure 1d). For InTenS, 12-15% strain was applied each day for 1 hr for 5 days. For CoTenS, a continuous strain of 12-15% strain was applied initially, followed by an additional 4-6% strain per day for 5 days. The highest strains were predicted in regions around the loading posts (i.e., openings in the neocartilage), reaching approximately 18%. Despite these areas of higher strain, mechanical and biochemical samples were easily portioned from the center region experiencing uniform strain (Figure 1e). Indeed, a topographical analysis across neocartilage samples demonstrated uniform tensile properties, with InTenS-stimulated neocartilage exhibiting enhanced tensile properties as compared to untreated tissues (Supplemental Figure 1a and 1b). It was demonstrated that just 2 days of InTenS was sufficient to induce significant increases in tensile properties (Supplemental Figure 1e). Finally, the model was validated by confirming that the predicted deformation matched the actual deformation of neocartilage after tension stimulation (Figure 1f). We, thus, built and employed devices that could successfully apply tension stimulation to neocartilage.

Neocartilage engineering with tension stimulation

Regimens have been established for the application of growth factors, matrix remodeling enzymes, and matrix synthesis agents to enhance the material properties of neocartilage; these regimens of soluble stimuli are amenable to the addition of mechanical stimuli such as

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InTenS and CoTenS. The soluble stimuli, alone or in combination, can enhance the tensile properties of scaffold-free and scaffold-based neocartilage. TGFβ1 has been shown to enhance collagen production[18,23,24]. CABC, a catabolic enzyme that cleaves glycosaminoglycans (GAGs), removes excess GAGs within developing neocartilage and helps to reorganize the collagen matrix[25]. In this study, we showed that, once the collagen matrix is free of hydrophilic GAGs, the neocartilage is most responsive to tension stimulation, which also serves to align the collagen fibers. Finally, LOXL2 increases neocartilage tensile properties through crosslinking of nearby collagen molecules[20]. This study explored the use of tension alone and in combination with bioactive agents TGFβ1, CABC, and LOXL2 for engineering neocartilage. The various combinations of these agents were applied with InTenS. With CoTenS, an optimized bioactive regimen, or OBR, combining all three of these stimuli (TGFβ1, CABC, and LOXL2), was used (see Methods).

Tension stimulation had a significant effect on neocartilage formation. Tension influenced matrix synthesis and remodeling to increase matrix density and organization (Figure 2a), resulting in functional enhancements in the tensile properties (Figure 2b). Specifically, we demonstrated that InTenS alone could enhance the tensile properties up to 1.3-times those of untreated neocartilage, as measured in dumbbell-shaped specimens cut out from the neocartilage constructs (Figure 1e). The effects of tension stimulation were impressive when combined with bioactive factors, increasing tensile modulus and strength. The individual contribution of each of these stimuli to improving neocartilage material properties was also examined. InTenS, in combination with TGFβ1, elicited tensile Young's modulus and ultimate tensile strength (UTS) 2.4- and 2.7-times those of untreated controls, respectively (Figure 2b, and Supplemental Table 1). InTenS, in combination with TGFβ1, LOXL2, and CABC (or InTenS + TGFβ1/LOXL2/CABC), increased both the tensile modulus and UTS to 3.9-times untreated control values (Figure 2b). The Young's modulus reached 8.4±0.9MPa with InTenS + TGFβ1/LOXL2/CABC, in the range of reported values for native tissues[6,7] (Supplemental Table 1), and is reflective of the developmental stage of the cell source (i.e., juvenile bovine cartilage)[26]. These values are the highest achieved for this scaffold-free, cell-based neocartilage system using primary cells, which previously reached values up to 2.3MPa for tensile modulus[20]. To achieve greater benefits from tension stimulation, the magnitude and duration of tension stimulation in InTenS were extended in CoTenS. Indeed, additional gains in material properties were observed. Overall, we discovered that while the contributions from bioactive factors were significant, tension stimulation was critical to bring the neocartilage material properties to native tissue-like levels, as CoTenS + OBR treatment increased tensile modulus 2.3-times that of OBR alone. Overall, tension stimulation resulted in neocartilage with tensile properties 5.8-times of untreated controls (Figure 2b). Therefore, tension as a stimulus can generate significant increases in scaffold-free neocartilage properties.

Concurrent with the increases in tensile values, the compressive properties of treated neocartilages also approached native tissue values. Interestingly, LOXL2 application in the presence of TGFβ1 and InTenS resulted in a significant decrease in compressive stiffness and shear modulus compared to TGFβ1 and InTenS; this reduction was not previously seen with LOXL2 use alone[20] (Supplemental Table 1). LOX family members are known to possess growth factor binding domains and are able to modulate TGFβ1 activity via direct

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inhibition, processing alteration, and enzyme activation[27]. The simultaneous use of TGFβ1 and LOXL2 in this study may therefore result in their undesirable interaction. Further examination of timing regimens of TGFβ1 and LOXL2 may optimize their respective effects on neocartilage properties. Favorably, despite a decrease in compressive stiffness with InTenS + TGFβ1/LOXL2 treatment compared to InTenS + TGFβ1, the InTenS + TGFβ1/LOXL2 treatment was not significantly different than untreated neocartilage. Similarly, InTenS and bioactive treatment yielded neocartilage with comparable compressive modulus and shear modulus as neocartilage treated with bioactive agents alone (Supplemental Table 2). Moreover, use of CoTenS abrogates the decreases in compressive properties possibly induced by LOXL2, increasing the compressive stiffness to 199±33kPa from 148±22kPa with OBR alone. Overall, tension stimulation does not sacrifice other properties in favor of enhancing tensile values (Supplemental Table 1a).

In our study, we successfully engineered fully biologic tissues in the absence of exogenous scaffolds. In other tissue engineering studies using scaffold-based systems where mechanical properties were reported, native tissue-like properties can be achieved. However, it should be noted that a significant portion of those properties can be derived from the scaffold[28]. Besides preventing stress-shielding of cells, a benefit of scaffold-free systems is the ability to control fiber alignment to generate anisotropy. In addition to eliciting native tissue-like properties, tension stimulation—especially CoTenS—was highly effective in driving anisotropy in neocartilage (Figure 2c); the tensile modulus in the direction parallel to tension stimulation was 1.7-times that of the perpendicular direction. Moreover, this anisotropy is driven by tension stimulation because anisotropy was not present in non-tension stimulated groups. These studies, thus, demonstrate that tension stimulation, when combined with matrix enhancing agents, can produce neocartilage constructs that recapitulate native tissue properties in the absence of scaffolds. In particular, anisotropy was successfully engineered in neocartilage without the use of exogenous materials.

Tension stimulation modes of action

The enhanced functional properties of tension-stimulated neocartilage constructs are the result of complex cellular signaling and matrix remodeling events. Microarray analysis (2 hours post-tension) revealed that tension stimulation results in 99/9 up-/down-regulated genes, as compared to unloaded controls (Figure 3a, Supplemental Table 6). The major gene categories that appeared to be altered in response to tension stimulation relate to matrix remodeling enzymes, cellular signaling pathways, and membrane-bound receptors and channels. These findings suggest that tension stimulation up-regulates mRNA expression of molecules related to matrix remodeling (ADAMTS1, LOXL4), cell-matrix interactions (ITGA2), and signaling pathways (BMP2, SMAD7) (Supplemental Figure 3). ADAMTS1 plays a role in the maintenance of articular cartilage and is not associated with pathological changes[29,30]; up-regulation of ADAMTS1 in this study likely served to enhance matrix remodeling. LOXL4, like LOXL2, serves to cross-link collagen fibers within the ECM. Though lysyl oxidase-like protein 2 has been shown to be the major isoform of cartilage[31], thus motivating our exogenous use of LOXL2 specifically, LOXL4 is also widely expressed in cartilage[32]. As in studies exposing cartilage cells to direct strain[33], tension stimulation in these studies caused significant up-regulation of integrin α2, which may enable enhanced

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cell-matrix interaction. Finally, signaling of TGFβ1 and SMAD7 is known to function in a negative regulatory feedback loop[34], while the balance of BMP2 and SMAD7 is known to regulate proper development and maturation of cartilage[35]. Tension stimulation may induce a similar BMP2/SMAD7 equilibrium to modulate matrix development. Though additional studies are necessary to examine thoroughly the mechanism of action of tension stimulation, the preliminary gene expression data suggest that the early response to tension stimulation involves changes in cellular signaling, cell-matrix interactions, and matrix remodeling, which, ultimately, may lead to neocartilage with enhanced properties.

In this study, we demonstrated that TRPV4 channel function plays a role in the mechanotransduction of tension stimulation. TRPV4 has previously been shown to be an important mechanosensitive ion channel that mediates the cellular response to compressive stimulation and ultimately affects neotissue functional properties[21]; without a functioning TRPV4 channel, compressive stimulation did not increase the compressive modulus. In our hands, TRPV4 agonism resulted in up to a 153% increase in tensile properties[36,37]. Here we showed that TRPV4 channel function is needed to sense tension stimulation and initiate signal transduction to result in functional property increases (Young's modulus), as GSK205 inhibition of TRPV4 during tension stimulation eliminated the emergent tissue-level response to tension (Figure 3b). While GSK205 alone appeared to cause a slight increase in tensile modulus, this effect was not significant (one-way ANOVA across groups, $p = 0.77$). In contrast to GSK205, non-specific inhibition of stretch-activated channels via gadolinium chloride did not affect the response to tension stimulation (Figure 3b), suggesting that the TRPV4 ion channel is more likely responsible for mechanotransduction. TRPV4 function and mechanosensation of tensile forces did not result in an increase in TRPV4 gene expression (Supplemental Figure 3). With the present study, the role of TRPV4 mechanotransduction is emerging more clearly. Prior literature has shown that acute inhibition of TRPV4 during compressive stimulation abrogates the effect of the stimulus[21], implicating it in mechanotransduction. A TRPV4 agonist in the absence of tension leads to increased mechanical properties[30]. Finally, we show that applying a TRPV4 antagonist in combination with tension abolishes tension stimulation's effects. Thus, these studies further contribute to the role of TRPV4 as an essential element in sensing mechanical forces, specifically of tensile forces, to elicit cellular responses that enhance functional neotissue properties.

To assess matrix-level organizational changes resulting from tension stimulation, we used second harmonic generation to image the collagen fiber network. Second harmonic generation demonstrated that tension stimulation enhanced collagen fiber density and organization within neocartilage, resulting in alignment of fibers (Figure 3c). This remodeling process occurred over the final 2 weeks of culture to elicit significant differences in tensile properties between tension-stimulated and untreated neocartilage. Neotissues were also assessed immediately after loading to avoid detecting cellular responses to tensile loading. After 1 day of loading, neotissues were not significantly different from unloaded controls, suggesting that a permanent matrix response was not induced at this time scale (data not shown). Permanent deformation was present 2 days after tension stimulation (Supplemental Figure 1c, 1d). Following the tension stimulation regimen and after 2 additional weeks of culture, 28-day-old neocartilage treated with tension stimulation—either

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InTenS or CoTenS—were permanently elongated as compared to neocartilage without tension stimulation (Supplemental Figure 2c, 2d). This result was likely due in part to physical deformation of the matrix during the time course of tension stimulation; however, the cellular responses induced by tension stimulation, as discussed above, likely played a larger role in driving matrix remodeling. Continued development of neocartilage properties —leading to anisotropy and increased tensile modulus and strength—was initiated by tension stimulation, but required the remainder of tissue culture to develop and for the properties to emerge. At the matrix level, InTenS served to generate anisotropy within the collagen network, ultimately contributing to increases in neocartilage properties.

Collectively, our cell- and tissue-level analyses suggest that tension stimulation works primarily at the cellular level, inducing production of matrix enzymes to initiate matrix remodeling, up-regulation of BMP2/SMAD7 signaling, and expression of cell-matrix interaction proteins to improve mechanotransduction. Moreover, TRPV4 channel function was found to be a component of the cellular response to tension stimulation. Finally, InTenS served to align matrix collagen, resulting in substantial increases in tensile properties. These tension-induced changes, when combined with up-regulation of collagen synthesis by TGFβ1, led to synergistic increases in functional properties. Specifically, TGFβ1 increased the matrix density, while tension stimulation enhanced the cell's ability to sense mechanical forces through increased integration with the matrix. Ultimately, the integration of these cell- and tissue-level responses to tension led to functional changes in tensile modulus and strength toward those of native tissues.

In vivo implantation of InTenS-treated neocartilage

Subcutaneous implantation of neocartilage constructs treated with InTenS + TGFβ1/LOXL2/CABC and with TGFβ1/LOXL2/CABC in athymic mice allowed for assessment of the long-term stability of tensile properties in an *in vivo* environment. Before implantation, all neocartilage groups exhibited properties similar to those obtained in the other experiments of this study (Figure 4). After 4 weeks *in vivo*, neocartilage shape and structure were maintained, and growth was limited, as compared to constructs grown *in vitro* (free-swelling culture controls) (Figure 4a). Histological analysis revealed substantial differences between free-swelling controls and constructs implanted *in vivo* (Figure 4b). In both free-swelling and *in vivo* conditions, the InTenS + TGFβ1/LOXL2/CABC treatment induced richer deposition of GAGs compared to neocartilage treated with TGFβ1/LOXL2/CABC. Interestingly, the *in vivo* environment elicited changes in the cellular morphology of stimulated constructs, with cells in these tissues appearing to orient themselves in a columnar fashion perpendicular to the tissue surface. This organization is reminiscent of native articular cartilage. The use of scaffold-free method in tissue engineering led to a slightly higher cellularity in engineered neocartilage compared to native articular cartilage (Figure 4b). The high cellularity is reminiscent of juvenile articular cartilage, which has a higher regeneration potential[38]. As the implanted cartilage matures the cellularity is expected to decrease. The biochemical and mechanical properties of implanted neocartilage reflected values closer to those of native articular cartilage, as compared to those in free-swelling culture (Figure 4c). The free-swelling control neocartilage treated with InTenS + TGFβ1/LOXL2/CABC possessed the highest tensile properties as compared to the TGFβ1/

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LOXL2/CABC-treated and untreated control groups, despite receiving no tension stimulation for the remaining 6 of 8 weeks of culture. This result suggests that properties attained by tension stimulation are persistent and can be maintained in culture. We previously reported that the subcutaneous environment leads to increased neocartilage properties[19]. In this study specifically, collagen content, Young's modulus, and UTS of *in vivo* implanted constructs reach 90%, 94%, and 60% of native articular cartilage values, respectively, as compared to those grown in free-swelling culture (54%, 102%, and 38%, respectively). Thus, the subcutaneous *in vivo* environment not only maintains or further enhances the properties of treated neocartilage toward those of native articular cartilage, but also results in neocartilage that is morphologically reminiscent of native tissues.

Translation of tension stimulation to a human cell source

The increases in neocartilage tensile properties described in the preceding sections were achieved using a bovine cell source. Inasmuch as the ultimate goal of engineering mechanically robust neocartilage is clinical translation to human patients, we examined the effectiveness of the newly developed CoTenS regimen in passaged human articular chondrocytes. Importantly, without tension stimulation, human neocartilage of the large dimensions employed here could not be engineered. Specifically, unstimulated and OBR-treated neocartilage contracted and folded dramatically, whereas tension-treated constructs maintained their flat morphology and dimensions (Figure 5a). Similar to the effects seen with bovine cells, tension stimulation, in combination with OBR, drove significant increases in human neocartilage tensile and compressive stiffness, reaching 4.0-times and 3.0-times of unstimulated control values, and even 1.9-times and 2.2-times of OBR values alone, respectively (Figure 5b and 5c). The absolute values achieved for human-derived neocartilage are not yet on par with those of native human adult cartilage[39,40], but are the highest properties of scaffold-free, human cell-based engineered cartilage achieved to-date. In addition to increases in tensile properties, human neocartilage constructs also exhibited statistically significant tensile anisotropy in response to tension stimulation, with the direction parallel to tensile loading achieving a Young's modulus of 2.3±0.7MPa versus 1.4±0.7MPa in the perpendicular direction. Since human articular cartilage is anisotropic—as evidenced by split lines at the surface of the tissue[41]—the generation of anisotropy in our human cell-derived neocartilage supports the translational potential of tension stimulation in the self-assembling process to generate neocartilage similar to the native tissue in terms of functional properties.

Conclusions and future directions

Despite the existence of tensile loading capabilities for musculoskeletal tissue engineering, successful achievement of native tissue-like tensile properties has not yet been obtained. Indeed, static and dynamic tensile loading has been applied in tendon and ligament engineering[42-44], but these fall short of achieving native tissue-like properties[45]. The successful application of static or dynamic tensile loading to neocartilage is significantly more limited, with only a handful of studies on dynamic tensile load application demonstrating the potential of tension application.[46-48] It should be noted that no tension stimulation studies have been performed in scaffold-free systems; also, no effective tension

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stimulation regimens have been identified in cartilage tissue engineering. Therefore, it is significant that this body of work has generated scaffold-free musculoskeletal tissues with tensile properties on the order of native tissue values, using custom-fabricated stimulation devices and intermittent and continuous tension stimulation regimens.

The scaffold-free tissue formation process used in this study further contributes to the successful application of tension stimulation. Scaffold-based methods can result in discontinuities in matrix distribution and stress-shielding of resident cells[49-51]. Application of tensile load to a scaffold-based neocartilage, then, may not allow for appropriate force transfer from the bulk tissue to the cellular level. As a result, a scaffold-free neocartilage system, where cells synthesize and integrate intimately with their ECM and do not rely on migration, integration, or remodeling as may be needed with scaffolds, may be more amenable to tensile loading. Enhanced matrix integration allows for direct mechanotransduction of the applied tensile load from the bulk tissue level to the matrix and cellular levels without dampening via an exogenous scaffold. Unique aspects of the current system include the combination of tension stimulation with a scaffold-free approach, the avoidance of stress shielding resulting in direct application of mechanical stimulation to cells, and the use of an articular cartilage model system since tension is not thought to be the prevailing stimulus in cartilage. Additional studies on the use of tensile loading in both scaffold-free and scaffold-based musculoskeletal tissue formation efforts will elucidate tensile loading regimens that can recapitulate native tissue-like properties in a variety of musculoskeletal tissues. Similarly, continued exploration of the mechanisms by which tension stimulation induces the significant enhancements in scaffold-free neocartilage tensile properties achieved in these studies would further improve the field's understanding of mechanotransduction.

Here, we report the design and use of tensile loading devices that successfully apply intermittent and continuous tension stimulation regimens to engineer neocartilage with greater tensile properties. The effects of tension stimulation and its interactions with TGFβ1, CABC, and LOXL2—agents specifically selected for their role in enhancing the collagen network—are multifaceted and are the topic of continued exploration by our group. The scaffold-free, cell-based neocartilage constructs generated herein possess the highest tensile properties to-date by rationally targeting matrix synthesis and collagen organization via the combination of tension and matrix enhancing agents. We showed that the mechanosensitive ion channel TRPV4 is implicated in the successful application of tension stimulation, resulting in increased functional properties. The *in vivo* microenvironment was shown to enhance the tissues' biochemical and mechanical properties and to mimic native cartilage's morphological structure. Finally, we translated tension stimulation to human neocartilage to enhance the tissue's mechanical properties, toward those of native tissues. These loading devices may be amenable to the scaffold-free or scaffold-based engineering of other musculoskeletal tissues such as ligament, tendon, muscle, or bone. In the case of articular cartilage, we demonstrated increases in tensile modulus and strength nearly 6-times those of untreated neocartilage, generating scaffold-free neocartilage with native tissue-like properties. Similarly, tension stimulation of human neocartilage reached tensile properties over 4-times those of untreated controls, demonstrating the translational nature of the regimen. Achievement of these mechanical properties suggests better graft survival and

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function when translated to the clinic. Our examination of tension stimulation regimens in tissue formation enhances our understanding of the importance of biomechanics in driving tissue formation in a biomimetic fashion.

Methods

Application of tension stimulation

Design of tension stimulation devices—To create custom devices capable of applying tension stimulation to neocartilage, the open source MakerBot was modified to produce the devices shown in Figure 1a and 1b. The device designs incorporate stainless steel rods, onto which neocartilage constructs can be loaded. The rationale for using rod systems was to avoid directly gripping and potentially damaging the tissues.

Shape-specific mold design—Toward applying tensile strains to neotissues, a rectangular construct was desired. To be compatible with the tensile loading devices, four openings were engineered into the neocartilage to avoid generation of stress concentrations that may result from the use of a punch. A positive mold was designed and 3D printed with biocompatible materials to generate a well-maker, which was placed in molten 2% agarose to generate wells for forming the self-assembled constructs. Each well was rinsed with four exchanges of Dulbecco's Modified Eagles Medium prior to use.

Self-assembling process for bovine and human neocartilage—Articular chondrocytes were harvested from the distal femur of juvenile bovine joints (Research 87, Boston, MA) as previously described[52]. Neocartilage was formed using the self-assembling process[53] at a seeding density of 8 million cells in 100μL of chondrogenic medium (CHG)[52] per 13×8mm construct. Human articular chondrocytes were obtained from three Caucasian, male donors, ages 19, 21, and 43, with no known musculoskeletal pathology (Musculoskeletal Transplant Foundation, Kansas City, MO). The isolated cells were passaged and chondrotuned as described previously [54]. Briefly, human articular chondrocytes were expanded in TGFβ1, bFGF, and PDGF-bb-containing (all from Peprotech, Rocky Hills, NJ) CHG to passage 3. Then, the cells were cultured in 3D aggregates to redifferentiate them into chondrocytes, after which cells were self-assembled at 7 million cells per 13x8mm construct.

Applied tension stimulation—The InTenS regimen involved subjecting neocartilage to 18-20% tensile strain for 1 hour per day, during days 10-14 of the 28-day culture period. Each treatment group was subjected to InTenS with or without bioactive agents. The CoTenS regimen involved the application of strain as follows: 18% on day 7, followed by approximately 4.9-6.5% additional strain applied each day from days 8-12 and then held constant until day 28; tensile strain in this group was, thus, applied continuously from days 7-28.

Matrix enhancing treatment regimens—TGFβ1 at 10ng/mL was used from days 1-28 of the 28-day culture period. In the InTenS regimen, CABC (Sigma-Aldrich, St. Louis, MO) at 2U/mL CHG was applied for 4 hours on day 7. After CABC treatment, neocartilage was thoroughly rinsed with CHG. From days 15-28, 0.15μg/mL lysyl oxidase-like protein 2

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(SignalChem, Richmond, British Columbia) with 1.6μg/ml copper sulfate (Sigma-Aldrich, St. Louis, MO) and 0.146μg/ml hydroxylysine (Sigma-Aldrich, St. Louis, MO), defined as LOXL2, were applied. In studies employing CoTenS, CABC was applied at day 8, and LOXL2 was used from days 10-28, with both factors applied at the same concentrations as in InTenS. This regimen, referred to as OBR, was logistically necessary to accommodate continuous tension stimulation. Medium was changed every other day.

Ion channel inhibition—For channel inhibitor studies, GSK205 (Calbiochem, San Diego, CA) and gadolinium chloride (GadChl; Sigma-Aldrich, St. Louis, MO), were used to block the function of the TRPV4 channel and non-specifically block stretch-activated channels, respectively[55,56]. Neocartilage was transferred to CHG containing 10μM GSK205, 10μM GadChl, or no drug and allowed to equilibrate for 30 minutes. InTenS was then applied to neocartilage in a bath of GSK205 or GadChl to inhibit channel function while neocartilage was subjected to tensile strain.

Neocartilage characterization

Mechanical and biochemical analyses—Tensile and compressive tests (n=6 per group) and biochemical evaluation (n=6 per group) for collagen, sulfated GAG, and DNA content were performed as previously described[52]. Dumbbell-shaped tensile test samples were obtained in the direction parallel and perpendicular to the axis of tension stimulation and as indicated in Figure 1e and Supplemental Figure 1a. Tensile Young's modulus and UTS are reported (although structural properties are also provided in Supplemental Table 7). In each experiment of the study, untreated group was included as control. When comparing data across multiple experiments in the study, Young's modulus and UTS from treated groups were normalized to those of untreated control, and fold-changes with respect to untreated control were presented. Collagen and GAG data were normalized to tissue wet weight, by dividing the weight of collagen or GAG in sample by sample wet weight, and reported as collagen/WW and GAG/WW. Cellularity values in constructs are reported from measuring DNA content.

Microarray analysis—RNA was isolated 2 hours post-InTenS on day 10 using an RNAqueous-micro kit (Thermo Fisher, Waltham, MA); untreated samples were also prepared on day 10 of culture. Reverse transcription was performed to generate cDNA. InTenS-treated and untreated cDNA were hybridized to Bovine Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA) based on the manufacturer's instructions (n=3 per group). Expression results were analyzed using the Affymetrix Transcriptome Analysis Console. Results were filtered based on a minimum 2-fold change and $p<0.05$.

Gene expression—Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on genes of interest that were up-regulated via microarray analysis. Total RNA was reverse transcribed using random primers (Amersham Biosciences, Little Chalfont, UK), with GAPDH primers used to control for cDNA concentration in separate PCR reactions for each sample. Primers for GADPH, ADAMTS1, LOXL4, BMP2, SMAD7, ITGA2, and TRPV4 were designed using Primer3 and are shown in Supplemental Table 5. To each PCR reaction (triplicates), LightCycler Fast Start DNA Master SYBR Green Mix

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(Roche, Basel, Switzerland) was added, along with cDNA and 1pmol primer in a total reaction volume of 10μl. C_t values were converted to fold expression changes ($2^{-\Delta\Delta C_t}$ values) after normalization to GAPDH expression levels.

Histological analysis—Free-swelling and explanted neocartilage samples were fixed in 10% neutral buffered formalin before paraffin-embedding and sectioning at 5μm. Slides were stained with H&E, picrosirius red, and Safranin-O/Fast Green.

Second harmonic generation—For two-photon imaging, whole neocartilage samples were fixed in 4% neutral buffered formalin for 3 days before storing in 1% sodium azide. Second harmonic imaging was performed as previously described[57]. Z-stacks were captured at 3μm increments through the neocartilage thickness. Stacks were Z-projected at maximum intensity using ImageJ software to normalize across samples.

Finite element modeling—Finite element analysis was performed using FEBio 2.1 (febio.org) to appropriately model the biphasic behavior of engineered neocartilage[58]. Due to the symmetry in loading and geometry, a quarter of the system was modeled and analyzed. The neocartilage geometry was modeled using Abaqus 6.14 (3ds.com) and meshed with 2104 solid elements. FEBio was then used to model the tissue as a biphasic material, based on experimental measurements of neocartilage properties at day 10. The stainless steel loading pole through the neocartilage opening was modeled as a rigid body. A strain of 18% was applied to the model.

Statistical analysis—Statistical analysis was performed using JMP Pro 12 (jmp.com) statistical package. Analysis of variance, followed by Tukey's *post hoc* test, was performed for multiple group comparisons, with statistical significance set at $p<0.05$. Student's t-test was performed to compare two groups, with statistical significance also set at $p<0.05$. Specific p-values were also indicated where appropriate. Data were presented as mean±SD. Samples were excluded from analysis only if a box plot deemed samples outliers. N=8 was used for all InTenS experiments, including animal work, while N=6 was used in CoTenS experiments. N=3 was used for microarray analysis. A power analysis confirmed n=6 sufficient to detect statistical significance in studies employing the self-assembling process for neocartilage.

Animal studies

Athymic male mice (Charles River Laboratories, Wilmington, MA), aged 6-8 weeks, were used under the approval of the Institutional Animal Care and Use Committee (IACUC) of UC Davis (IACUC protocol #18612). Under general anesthesia, one 1.5cm-long incision was made along the dorsal surface of 12 animals. Bilateral pouches were formed on either side; one neocartilage sample (untreated, TGFβ1/CABC/LOXL2-treated, or InTenS + TGFβ1/CABC/LOXL2-treated) was randomly inserted per pouch (n=8 per group), with no animal receiving two samples from the same experimental group. No blinding of investigators was used. Wounds were closed with surgical clips. Mice were humanely sacrificed 4 weeks after implantation. Mechanical, biochemical, and histological analyses were performed on explanted constructs (as described above).

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Data Availability

The datasets generated during and/or analyzed during the current study are available as Supplemental Tables or from the corresponding author on reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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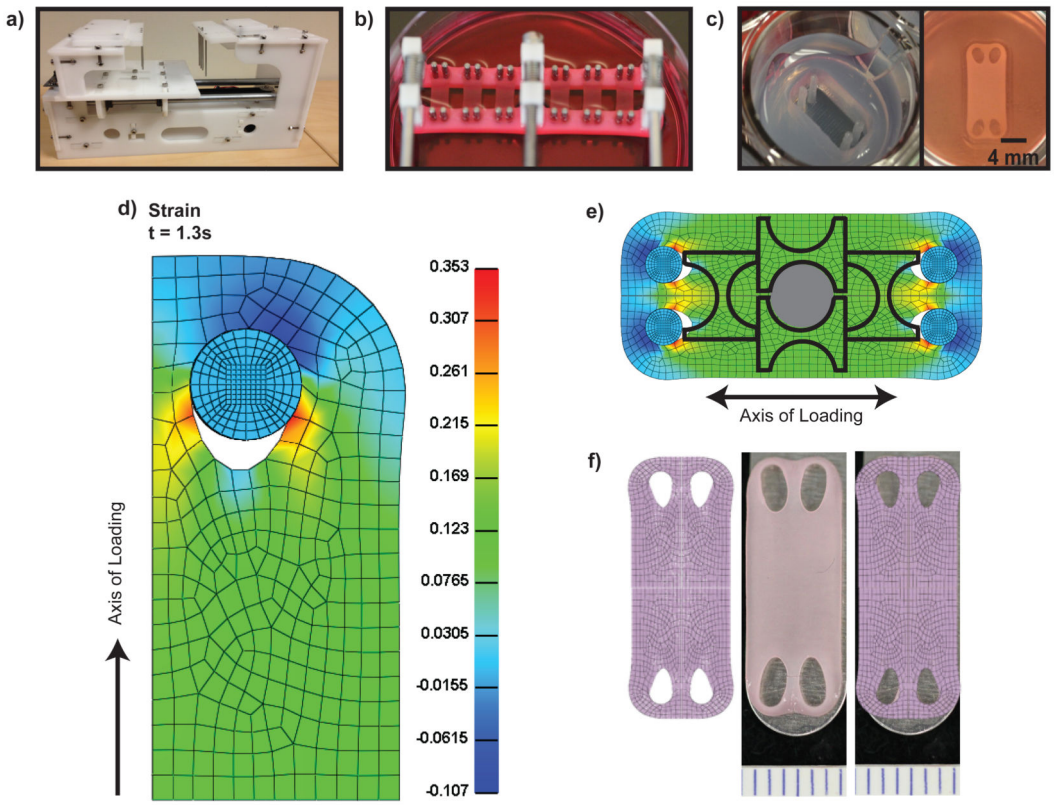

Figure 1. Large construct generation and uniform strain validation

Modeling of stress and strain distribution during tension stimulation enabled the rational design of constructs displaying uniformity through the central portion. An agarose mold was created to generate rectangular self-assembled constructs (a). A custom tensile loading device was created for Intermittent Tension Stimulation (InTenS) (b). A custom tensile loading device was also designed and fabricated for Continuous Tension Stimulation (CoTenS) of neocartilage (c). Tension stimulation was applied along the long axis. Modeling of stress and strain distribution during tension stimulation predicts uniform distribution through the center of the neocartilage using a biphasic model (d). From the finite element (FE) model, uniform areas of strain were selected to portion compressive samples (hatched circle) and dumbbell-shaped parallel and perpendicular samples for tensile testing (outline) (e). The FE model was validated by overlaying the model with the actual deformed neocartilage (f).

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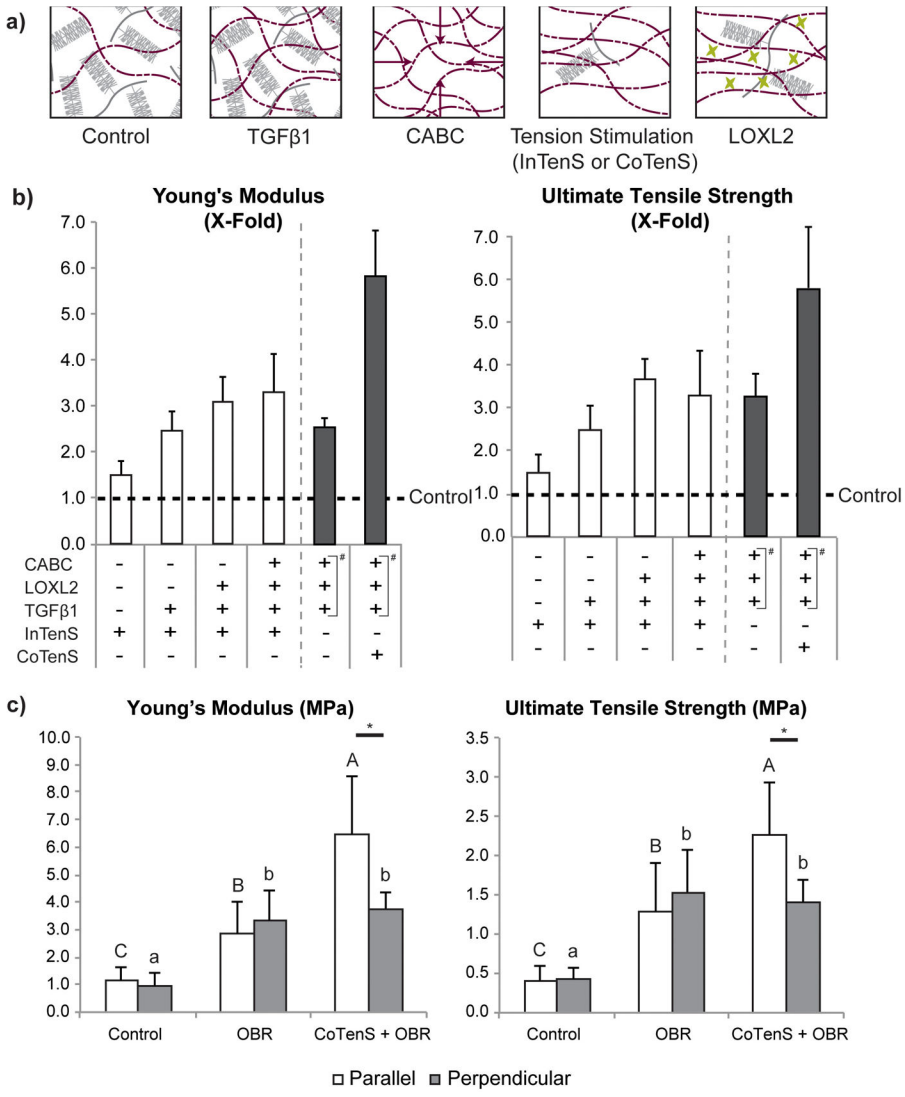

Figure 2. Tissue engineering of neocartilage with enhanced tensile properties

In combination with tension stimulation, transforming growth factor β1 (TGFβ1), chondroitinase-ABC (CABC), and lysyl oxidase-like protein 2 (LOXL2) were applied to achieve neocartilage with tensile properties that are on par with native tissue. The sequential application of these agents alters the extracellular matrix (a): TGFβ1 enhances collagen production; CABC temporarily causes loss of glycosaminoglycans (GAGs), allowing collagen fibers to be in closer proximity; tension stimulation aligns the collagen fiber network that is unrestricted by bulky GAGs; and LOXL2 crosslinks the aligned collagen matrix. A series of experiments investigated the step-wise addition of these agents to InTenS (b, white bars) and found increases in both Young's modulus and ultimate tensile strength (UTS). To quantitatively compare the data across these multiple experiments, Young's modulus and UTS values of treated groups were normalized to those of untreated control in each experiment. Fold-changes with respect to untreated control (dotted line) are presented. The absolute tensile properties are presented in Supplemental Table 1A and 1B. The combination of InTenS, TGFβ1, CABC, and LOXL2, achieved a Young's modulus 3.3-times

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control values. CoTenS elicited further dramatic increases in Young's modulus and UTS—both 5.8-times control values; these increases were found to be due to CoTenS. This is because the optimized bioactive regimen (OBR), consisting of TGFβ1, CABC, and LOXL2 (denoted in panel b by #), reached a Young's modulus and UTS only 2.5- and 3.3-times control values, respectively (b, dark bars). Moreover, CoTenS + OBR elicited anisotropy in stimulated neocartilage, with the parallel direction stiffer and stronger than the perpendicular direction (c). Neither the OBR regimen nor the no-treatment group was able to induce anisotropy. Two one-way ANOVAs, followed by a Tukey's *post hoc* test, were performed to statistically assess the results. Groups not connected by the same letter are statistically significant. Student's t-test was performed to compare tensile properties of parallel and perpendicular directions, $*p<0.05$. Data are represented as mean±SD.

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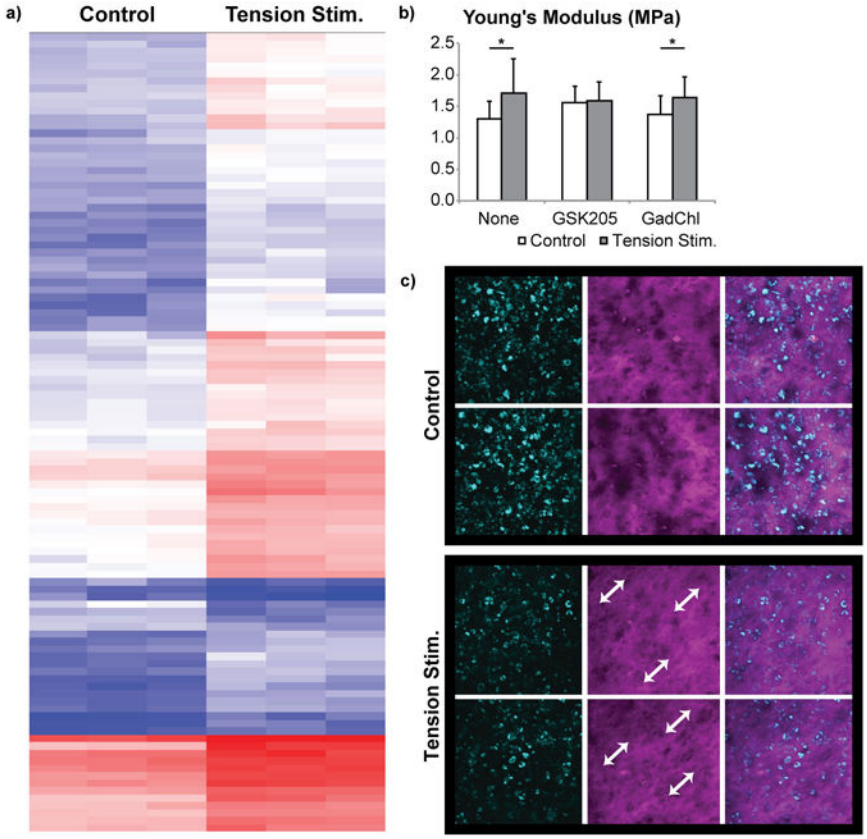

Figure 3. Under tension stimulation, the TRPV4 ion channel is implicated to initiate matrix remodeling

To investigate the effects of tension stimulation on increased tensile properties, microarray analysis and selective inhibitors were used to assess cell-based responses, while tissue-level responses (i.e., matrix alignment) were visualized using second harmonic generation (SHG) and quantified via mechanical analysis (see Figure 2). Microarray data (a) revealed significant differences in gene expression in the tension-treated group (red/blue indicate higher/lower amount of signal). Functional response (b) to tension stimulation is dependent on mechanosensitive TRPV4 channel activity, but, counter-intuitively, independent of stretch-activated channel (SAC) function. Student's t-test was used to assess statistical differences, $*p<0.05$. Data are represented as mean±SD. GSK205 and gadolinium chloride (GadChl) are inhibitors of the TRPV4 channel and SAC, respectively. SHG imaging (c) at 4 weeks revealed increased alignment of collagen fibers with tension stimulation, as shown with the white arrows.

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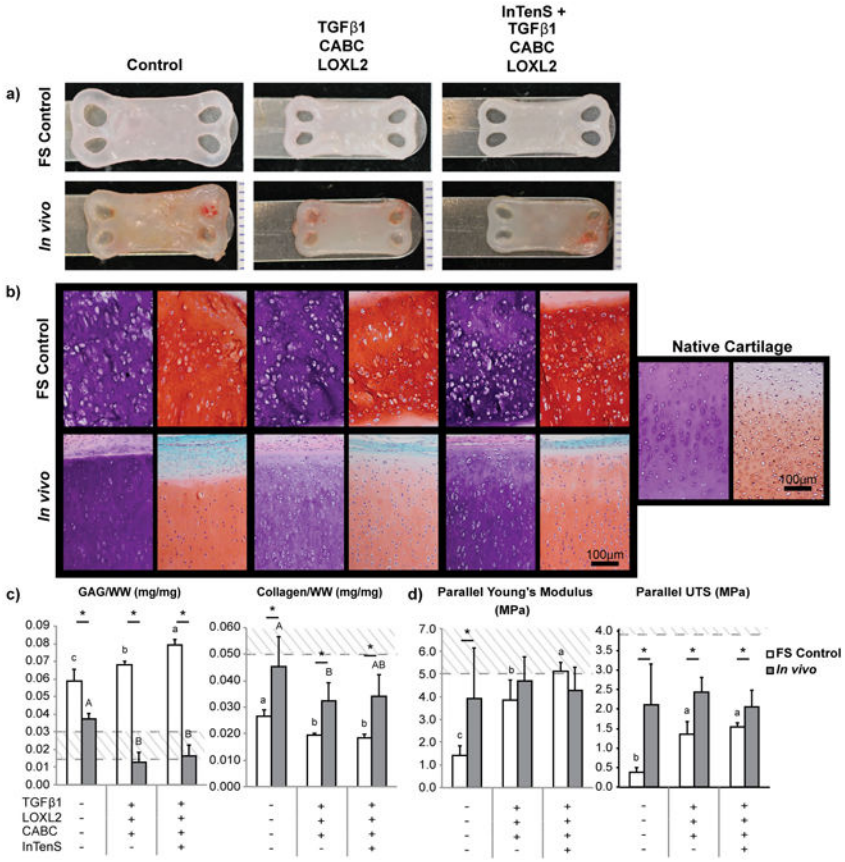

Figure 4. The *in vivo* environment results in neocartilage with morphological structure reminiscent of native articular cartilage

Implanted neocartilage constructs retained their shape after excision and are smaller compared to free-swelling (FS) culture conditions (a). Formalin-fixed, 5μm tissue sections were stained with H&E and Safranin-O/Fast Green (b) to visualize matrix and glycosaminoglycan (GAG) distribution, respectively. Staining in the GAG-rich FS neocartilage constructs was uniformly distributed. Implanted neocartilage attained a columnar morphology and matrix distribution reminiscent of native articular cartilage. Flatter cells can be identified toward the surface of the tissue, whereas elongated cells were distributed toward the center. *In vivo* conditions resulted in neocartilage with biochemical properties (c) closer to native tissue values, as indicated by the dotted lines, reducing GAG and increasing collagen content as compared to FS culture. InTenS-treated neocartilage in FS culture maintained a significantly higher Young's modulus than neocartilage treated with bioactive agents alone, which was greater than untreated controls (d). Further, tensile properties were maintained (for Young's modulus) or increased (for UTS) in the *in vivo* environment. Two one-way ANOVAs, followed by a Tukey's *post hoc* test, were performed to statistically assess the results. Groups not connected by the same letter are statistically significant. Student's t-test was also performed to compare FS and *in vivo* explanted groups, $*p<0.05$. Data are represented as mean±SD.

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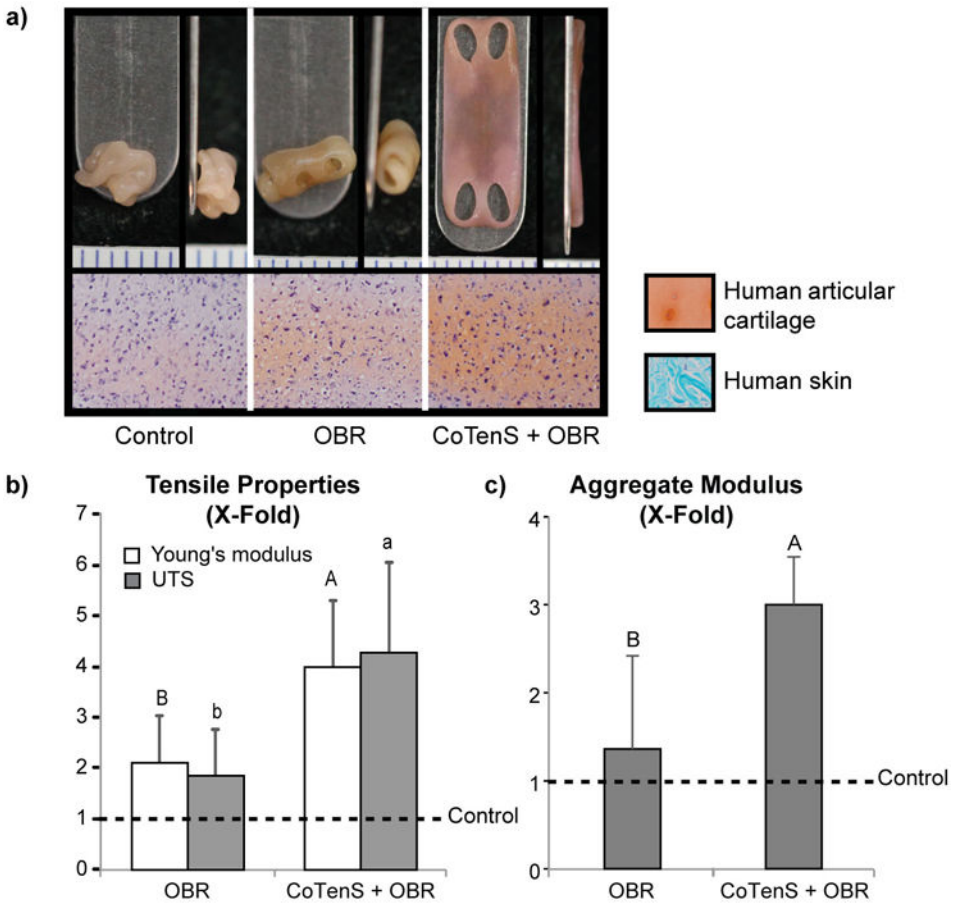

Figure 5. Translation of tension stimulation to human neocartilage

CoTenS was necessary in achieving flat and robust human neocartilage; without tension, neocartilage constructs folded and wrinkled non-uniformly (a, top). Tension stimulation also increased GAG content in the neocartilage, as compared to untreated constructs or those treated with an optimized bioactive regimen (OBR) (a, bottom). In addition, tension stimulation significantly increased the mechanical properties of human neocartilage. Young's modulus and UTS of treated groups were normalized to those of untreated control, and fold-changes with respect to untreated control (dotted line) are presented (b). The absolute tensile properties are presented in Supplemental Table 4. CoTenS + OBR enhanced the tensile modulus and strength to 4.0-times and 4.3-times, respectively, of control values, significantly greater than values achieved by OBR alone. Similarly, the compressive stiffness, as indicated by the aggregate modulus, significantly increased 3.0-times over control as a result of CoTenS + OBR treatment (c). Two one-way ANOVAs, followed by a Tukey's *post hoc* test, were used to statistically assess the results. Groups not connected by the same letter are statistically significant. Data are represented as mean±SD.