



Published in final edited form as:

*Acta Biomater.* 2013 March ; 9(3): 5554–5561. doi:10.1016/j.actbio.2012.10.031.

## TRPV4 channel activation improves the tensile properties of self-assembled articular cartilage constructs

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### Abstract

A persistent hurdle in the field of tissue regeneration is to produce tissues with biochemical and biomechanical properties robust enough to meet the aggressive physiological demands of the native milieu. In an effort to improve these properties, tissues grown *in vitro* are often subjected to mechanical stimuli that aim to recapitulate *in vivo* physiology. These mechanical stimuli are thought to produce downstream alterations in intracellular ion concentrations, which ultimately give rise to increased biosynthesis. There is mounting evidence that these perturbations in the cellular microenvironment are regulated by the Ca(2+)-permeable transient receptor potential vanilloid 4 (TRPV4) channel. In this study, we examined the effects of targeted TRPV4 activation on self-assembled articular cartilage constructs. The objectives of this study were 1) to determine whether TRPV4 activation would enhance self-assembled constructs, 2) to identify an optimal treatment time window for TRPV4 activation, and 3) to compare TRPV4 activation to Na(+)/K(+) pump inhibition, which has been shown previously to improve construct tensile properties. This study employed a two-phased approach. In Phase I, self-assembled constructs were grown for 4 weeks and subjected to treatment with the TRPV4 agonist 4alpha-phorbol-12,13-didecanoate (4alpha-PDD) during three treatment time windows:  $t=6-10$  days,  $t=10-14$  days, and  $t=14-18$  days. Treatment during  $t=10-14$  days produced an 88% increase in collagen and a 153% increase in tensile stiffness. This treatment window was carried forward to Phase II. In Phase II, we performed a head-to-head comparison between TRPV4 activation using 4alpha-PDD and Na(+)/K(+) pump inhibition using ouabain. Treatment with 4alpha-PDD produced improvements on par with ouabain (91% to 107% increases in tensile stiffness). The results of this study demonstrate the effectiveness of ion channel modulation as a strategy for improving engineered tissues. To our knowledge, this is the first study to examine TRPV4 channel activation in tissue engineering.

### Keywords

TRPV4; cartilage tissue engineering; self-assembly; biomechanics

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**Disclosures** There are no conflicts of interest to report.

**Author Contributions** SVE and KAA conceived and designed the experiments. SVE acquired the data. SVE and KAA reviewed the data. SVE and KAA wrote and edited the paper.

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## Introduction

Many tissue types lack an intrinsic physiological capacity to heal in response to injury or degeneration. Tissues that do recover after acute injury are often replaced with physiologically inferior replacement tissue. This poses a significant clinical burden, particularly in the field of cartilage regeneration. Injury to the cartilage found at the articulating surfaces of diarthrodial joints is irreversible and leads inescapably to pain and disability [1]. Tissue engineering aims to replace damaged articular cartilage by producing biologic replacements *in vitro* for eventual *in vivo* implantation. A persistent challenge in cartilage tissue engineering is to produce biomaterials with biochemical and biomechanical properties robust enough to meet the aggressive physiological demands of the native joint [2]. To address this challenge, our laboratory has developed a self-assembly process for engineering cartilage constructs [3]. Self-assembly involves seeding chondrocytes at high density into pre-fabricated, non-adherent, cylindrical molds. Cells condense into disc-shaped constructs and, over time, synthesize an extracellular matrix (ECM) rich in collagen and sulfated glycosaminoglycans (GAG), components that give the tissue its tensile and compressive integrity [4]. To date, however, native tissue functional properties remain elusive.

A variety of mechanical stimulation modalities have been examined for the potential to affect chondrocyte physiology. These stimuli have been informed by the dynamic physiologic loading conditions experienced by native cartilage in the intact joint. By studying the effects of these mechanical stimuli on chondrocyte physiology, targeted strategies for enhanced cartilage engineering may be devised. Example stimuli include dynamic compression [5-10], fluid shear [11-13], hydrostatic pressure [14-19], and osmotic stress [20-23]. Underlying these studies is the idea that changes in the macroscopic environment of the tissue can give rise to beneficial perturbations in the *in situ* cellular microenvironment. Dynamic changes at the cellular level manifest physiologically as transient alterations in intracellular ion concentrations. For example, hydrostatic pressure inhibits the action of the  $\text{Na}^+/\text{K}^+$  pump [16], an ATPase that pumps ions against a concentration gradient to maintain a higher intracellular concentration of  $\text{K}^+$  than  $\text{Na}^+$ . Thus, by inhibiting the  $\text{Na}^+/\text{K}^+$  pump, hydrostatic pressure produces increased levels of intracellular  $\text{Na}^+$ . A recent study from our group [24] showed that the selective inhibition of the  $\text{Na}^+/\text{K}^+$  pump using 20  $\mu\text{M}$  ouabain in self-assembled cartilage constructs was able to produce significant increases in collagen content and tensile properties, a result that recapitulated our group's previous success with hydrostatic pressure [25].

Another example of a dynamic tissue-level stimulus giving rise to changes at the cellular level is cyclic deformational loading. During joint motion, compressive loading of cartilage causes fluid expulsion, which can create a microenvironment of temporary hyper-osmotic stress or increased charge density for chondrocytes within the tissue [26]. This kind of hyper-osmotic stress – for example, from 310 mOsm to 550 mOsm [20] –has been shown to produce transient increases in intracellular  $\text{Ca}^{2+}$  [20, 21, 27], which can drive gene expression toward ECM biosynthesis [28, 29]. The precise mechanism underlying this osmoregulation in cartilage remains unclear. However, there is mounting evidence that the chondrocyte response to osmotic stress may be regulated by the transient receptor vanilloid 4 (TRPV4) channel [30, 31], a  $\text{Ca}^{2+}$ -permeable membrane protein found across many tissue types [32]. Although a handful of recent papers have examined the molecular and cellular physiology of the TRPV4 channel in chondrocytes, no study to date has selectively targeted the TRPV4 channel for use in a tissue engineering strategy.

Encouraged by results from the literature that suggest that the TRPV4 channel plays a vital role in chondrocyte physiology, we decided to examine the effects of TRPV4 activation on self-assembled articular cartilage constructs. The objectives of this study were 1) to determine whether TRPV4 activation would enhance the biochemical and biomechanical properties of self-assembled constructs, 2) to identify an optimal treatment time window for TRPV4 activation, and 3) to compare TRPV4 activation to Na<sup>+</sup>/K<sup>+</sup> pump inhibition. This study employed a two-phased approach. In Phase I, constructs were self-assembled from bovine chondrocytes and subjected to treatment with the TRPV4 agonist 4 $\alpha$ -phorbol-12,13-didecanoate (4 $\alpha$ -PDD) during three treatment time windows:  $t=6-10$  days,  $t=10-14$  days, and  $t=14-18$  days. These treatment periods were selected based on previous work that showed time-dependent differences in construct properties [25, 33]. Constructs were grown until  $t=28$  days, at which time they were evaluated morphologically, biochemically, and biomechanically. The optimal 4 $\alpha$ -PDD treatment time window was then carried forward to Phase II. In Phase II, we performed a head-to-head comparison between TRPV4 activation using 4  $\alpha$ -PDD and Na<sup>+</sup>/K<sup>+</sup> pump inhibition using ouabain; we also examined the combination of 4 $\alpha$ -PDD and ouabain. It was hypothesized that 1) TRPV4 activation would improve construct properties, 2) an optimal treatment time window exists for which constructs undergo greatest improvement, and 3) activation of TRPV4 would produce results comparable to those observed with inhibition of the Na<sup>+</sup>/K<sup>+</sup> pump. Assessments included gross morphology, biochemical analysis for GAG and collagen, and biomechanical testing.

## Materials and Methods

### Chondrogenic medium

This study employed a chemically defined medium termed “chondrogenic medium,” which has been used previously by our group [4, 24, 34] and contains the following components: DMEM with 4.5 mg/mL of glucose and L-glutamine (Invitrogen); 100 nM dexamethasone (Sigma); 0.1 mM non-essential amino acids (Invitrogen); 1% ITS+ (insulin, human transferrin, and selenous acid; BD Biosciences); 1% penicillin-streptomycin-fungizone (BioWhittaker); 50  $\mu$ g/mL ascorbate-2-phosphate; 40  $\mu$ g/mL L-proline; and 100  $\mu$ g/mL sodium pyruvate (Fisher Scientific). Chondrogenic medium contains 151 mM Na<sup>+</sup>, 5.2 mM K<sup>+</sup>, and 1.7 mM Ca<sup>2+</sup>. Medium osmolarity was assessed using a VAPRO 5520 vapor pressure osmometer (Wescor) and was determined to be ~347 mOsm.

### Chondrocyte isolation

Cartilage was harvested from the distal femurs and patellofemoral grooves of week-old male calves (Research 87, Inc.) shortly after slaughter, then digested in 0.2% collagenase type II (Worthington) for 24 h. To normalize variability among animals, each leg came from a different animal, and cells from 8 legs were pooled to create a mixture of chondrocytes. Separate harvests were conducted for each phase of this study. Cells were counted using a hemocytometer and then frozen at  $-80^{\circ}\text{C}$  in DMEM containing 20% FBS and 10% DMSO.

### Preparation of agarose wells for construct self-assembly

Cylindrical, non-adherent wells were prepared using a technique adapted from previous work [3, 4]. Briefly, a stainless steel mold consisting of 5 mm diameter cylindrical prongs was placed into sterile, molten 2% agarose in a 48-well plate. The agarose solidified at room temperature for 1 h, and the stainless steel mold was carefully removed. Two changes of chondrogenic medium were used to completely saturate the agarose well by the time of cell seeding.

## Self-assembly of cartilage constructs

Chondrocytes were thawed and counted within 5 days of being isolated and frozen. After thawing, cell viability was >90%. To create each construct, a suspension of 5.5 million cells in 100  $\mu$ L of chondrogenic media was deposited into each pre-formed cylindrical agarose well, followed by addition of 400  $\mu$ L chondrogenic media after 4 h. Cells settled and condensed into free-floating cylindrical disc-shaped constructs;  $t=1$  day was defined as 24 h after seeding. All constructs were cultured in the agarose wells until  $t=10$  days, at which point they were gently unconfined and transferred to 48-well plates unrestricted by circumferential confinement. Constructs received 500  $\mu$ L medium change every 24 h and remained in culture until  $t=28$  days. All culture was performed at 37°C and 10% CO<sub>2</sub>, simulating hypoxic conditions that have produced consistent biosynthetic results previously with self-assembled cartilage constructs in our laboratory [24].

### Phase I: Evaluation of treatment time windows for TRPV4 channel activation

In Phase I, we tested the hypothesis that TRPV4 channel activation can improve the biochemical and biomechanical properties of tissue engineered cartilage. We further sought to determine the optimal time window for performing this stimulation. Self-assembled constructs were treated with a TRPV4 channel agonist, 4 $\alpha$ -PDD (Enzo Life Sciences), during three treatment windows:  $t=6-10$  days,  $t=10-14$  days, and  $t=14-18$  days. During treatment, constructs were cultured in petri dishes for 1 h with ~4 mL chondrogenic medium containing 10  $\mu$ M 4 $\alpha$ -PDD. Control constructs were also moved to petri dishes containing chondrogenic medium during this time. Treatment was followed by a 30 min wash step in chondrogenic medium without 4 $\alpha$ -PDD before the constructs were returned to their wells. Treatment occurred at the same time every day over the course of 5 days.

### Phase II: TRPV4 activation versus Na<sup>+</sup>/K<sup>+</sup> pump inhibition

A previous study from our group showed that inhibition of the Na<sup>+</sup>/K<sup>+</sup> pump improved the tensile properties of tissue engineered cartilage [24]. In Phase II, we performed a head-to-head comparison between TRPV4 activation and Na<sup>+</sup>/K<sup>+</sup> pump inhibition. We further sought to determine the effects of the combination of these two stimuli. The regimen for TRPV4 activation was chosen from the most effective treatment time window determined in Phase I: 10  $\mu$ M 4 $\alpha$ -PDD during  $t=10-14$  days (see Results section for details). The regimen for Na<sup>+</sup>/K<sup>+</sup> pump inhibition was selected from previous work done by our group [24]: 20  $\mu$ M ouabain during  $t=10-14$  days. During  $t=10-14$  days, constructs were cultured in petri dishes for 1 h with ~4 mL chondrogenic medium containing either 10  $\mu$ M 4 $\alpha$ -PDD, 20  $\mu$ M ouabain (Sigma), or both agents. Control constructs were also moved to petri dishes containing chondrogenic medium during this time. Treatment was followed by a 30 min wash step in chondrogenic medium before the constructs were returned to their wells. Treatment occurred at the same time every day over the course of 5 days.

### Gross morphology and specimen portioning

At  $t=28$  days, constructs were removed from culture. Photographs were taken, and dimensions were measured from photographs using ImageJ software (National Institutes of Health). Wet weights (WW) were recorded, and constructs were portioned for analysis. A 3 mm diameter punch was taken from the construct's center for indentation testing. The remaining outer ring was divided into two semilunar portions, one for biochemical analysis and one for tensile testing.

### Biochemical analysis

Biochemical samples were weighed wet, frozen, and lyophilized. Samples were digested with 125  $\mu$ g/mL papain (Sigma) for 18 h at 65°C. Total DNA content was assessed with the

Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and cell number was estimated assuming 7.8 pg DNA per cell. Sulfated GAG content was quantified using the Blyscan Glycosaminoglycan Assay (Biocolor). Following hydrolysis with 4 N sodium hydroxide for 20 min at 110°C, total collagen content was quantified with a modified chloramine-T hydroxyproline assay [3, 35]. Sircol collagen standard (Biocolor) was used such that the standard curve reflected collagen amount, eliminating the need to convert hydroxyproline to collagen. Total collagen and sulfated GAG were normalized to WW and cell number for making comparisons.

### Tensile testing

Each semilunar specimen designated for tensile testing was cut into a dog-bone shape. To prepare the dog-bone shape, a 3 mm diameter punch was used to cut a semicircle from the curved, uncut edge of the semilunar specimen. Care was taken to ensure a 1 mm gauge length, which was confirmed with ImageJ software. Specimen thickness and width were also measured from photographs using ImageJ software. Specimens were then affixed with glue to paper tabs outside the gauge length, and these tabs were gripped during testing (for more details, including photographs of the process, please see reference [24]). A uniaxial materials testing system (Instron Model 5565) was employed to determine tensile properties. Tensile tests were performed until failure at a strain rate of 1% of the gauge length per second. Force-displacement curves were generated, and stress-strain curves were calculated by normalizing to specimen dimensions. Young's modulus, a measure of tensile stiffness, was determined by least squares fitting of the linear region of the stress-strain curve. The ultimate tensile strength (UTS) was determined as the maximum stress reached during a test.

### Creep indentation testing

A creep indentation apparatus was used to determine the compressive behavior of each construct [36]. Each 3 mm sample was affixed to a stainless steel surface and equilibrated for 20 min in PBS. A 0.7 g (0.007 N) mass was applied with a 0.8 mm diameter flat, porous indenter tip, and specimens crept until equilibrium. Specimen thickness was measured from photographs using ImageJ software. Aggregate modulus, a measure of compressive stiffness, was calculated using a semi-analytical, semi-numeric, linear biphasic model [36].

### Statistical analysis

All quantitative biochemical and biomechanical assessments were made using  $n=6-8$ ; that is, each group for every quantitative test had a minimum of 6 samples and a maximum of 8 samples. Data are presented as means  $\pm$  standard deviations. Single factor ANOVA was employed in each phase of the study to assess for differences among experimental groups. Statistical significance was defined as  $p<0.05$ . If significant differences were observed, a Tukey's HSD *post hoc* test was performed to determine specific differences among groups. All statistical analyses were performed using JMP 9.0.2 (SAS Institute).

## Results

### Phase I: Evaluation of treatment time windows for TRPV4 channel activation

In Phase I, we studied the effects of treating self-assembled articular cartilage constructs with 4 $\alpha$ -PDD, an agonist of the TRPV4 channel. We examined the use of 10  $\mu$ M 4 $\alpha$ -PDD during three different treatment time windows:  $t=6-10$  days,  $t=10-14$  days, and  $t=14-18$  days. Gross morphological measurements of all constructs at  $t=28$  days are presented in Table 1. No differences were found in construct diameter, thickness, or wet weight (WW) among groups.

Biochemical analyses were conducted to quantify construct cellularity, collagen content, and GAG content. Construct biochemical data are provided in Table 2. No differences were found in cell numbers across treatment times. Collagen/WW was highest in constructs treated with 4 $\alpha$ -PDD during days 10-14 (88% increase over control), followed by constructs treated during days 14-18 (40% increase over control). When normalized to cell number, these differences in collagen content were also seen, with approximately the same increases in magnitude compared to control. There were no differences observed in GAG content across treatment time windows.

Uniaxial tensile and creep indentation tests were performed to determine construct tensile and compressive properties. Tensile stiffness and strength data for Phase I are shown in Figure 1. Young's moduli for control, treatment on days 6-10, treatment on days 10-14, and treatment on days 14-18 were  $269 \pm 73$ ,  $328 \pm 80$ ,  $681 \pm 224$ , and  $464 \pm 69$  kPa, respectively. Constructs treated with 4 $\alpha$ -PDD during days 10-14 had the highest Young's moduli (153% increase over control). UTS values for control, treatment on days 6-10, treatment on days 10-14, and treatment on days 14-18 were  $112 \pm 43$ ,  $138 \pm 27$ ,  $261 \pm 94$ , and  $182 \pm 46$  kPa, respectively. Constructs treated with 4 $\alpha$ -PDD during days 10-14 had the highest UTS (133% increase over control). With respect to compressive stiffness, the aggregate moduli for control, treatment on days 6-10, treatment on days 10-14, and treatment on days 14-18 were  $75 \pm 19$ ,  $72 \pm 21$ ,  $82 \pm 20$ , and  $76 \pm 25$  kPa, respectively; no differences were found in aggregate moduli across groups.

Altogether, treatment with 4 $\alpha$ -PDD during days 10-14 provided the greatest increases in collagen content and tensile properties. Based on these results, this treatment regimen was carried forward into Phase II.

## Phase II: TRPV4 activation versus Na<sup>+</sup>/K<sup>+</sup> pump inhibition

In Phase II, we compared the effects of TRPV4 activation to the effects of Na<sup>+</sup>/K<sup>+</sup> pump inhibition, and we further studied whether the combination of these two stimuli would outperform their individual use. Self-assembled articular cartilage constructs were treated with either 10  $\mu$ M 4 $\alpha$ -PDD, 20  $\mu$ M ouabain, or a combination of the two during  $t=10-14$  days. Constructs were grown in culture to  $t=28$  days. At the end of culture, constructs treated with ouabain or with the combination of 4 $\alpha$ -PDD and ouabain were visibly smaller than control constructs or constructs treated with 4 $\alpha$ -PDD alone (Figure 2). Diameter, thickness, and WW values are provided in Table 1. Constructs treated with ouabain or with both agents had significantly smaller diameters (17% and 14% decreases from control, respectively), thicknesses (49% and 33% decreases), and wet weights (60% and 57% decreases).

Construct biochemical data for Phase II are provided in Table 2. Treatment with 4 $\alpha$ -PDD, ouabain, or their combination resulted in significant increases in collagen/WW compared to control (increases of 80%, 118%, and 93%, respectively), but no differences between each other. Collagen production per cell was greatest in constructs treated with 4 $\alpha$ -PDD (85% increase over control), with no differences among control, ouabain, or the combination 4 $\alpha$ -PDD and ouabain. GAG/WW was not different across groups, but GAG production per cell was significantly decreased in constructs treated with ouabain (60% decrease from control) and the combination of 4 $\alpha$ -PDD and ouabain (57% decrease from control). No differences were observed in cell number across groups.

Biomechanical properties were again assessed with uniaxial tensile and creep indentation testing. Tensile stiffness and strength data for Phase II are shown in Figure 3. Young's moduli for control, treatment with 4 $\alpha$ -PDD, treatment with ouabain, and combined treatment were  $282 \pm 105$ ,  $538 \pm 133$ ,  $572 \pm 136$ , and  $583 \pm 121$  kPa, respectively. Treatment with 4 $\alpha$ -PDD, ouabain, and their combination resulted in significant increases in

Young's moduli compared to control (91%, 103%, and 107% increases, respectively), but no differences between each other. UTS values for control, treatment with 4 $\alpha$ -PDD, treatment with ouabain, and combined treatment were  $106 \pm 29$ ,  $203 \pm 64$ ,  $256 \pm 89$ , and  $251 \pm 61$  kPa, respectively. Treatment with ouabain or with the combination of 4 $\alpha$ -PDD and ouabain produced the greatest improvements in construct UTS (141% and 136% increases over control, respectively), followed by treatment with 4 $\alpha$ -PDD (91% increase over control, but not statistically significant). In terms of compressive properties, aggregate moduli for control, treatment with 4 $\alpha$ -PDD, treatment with ouabain, and combined treatment were  $67 \pm 14$ ,  $73 \pm 18$ ,  $67 \pm 19$ , and  $74 \pm 23$  kPa, respectively; no differences were found in aggregate moduli across groups.

### Functionality index

A previously developed functionality index (FI) was used to quantify the similarity between self-assembled constructs and native tissue in Phase II. As shown in Equation 1, the FI equally weights the compressive stiffness ( $E^C$ ), tensile stiffness ( $E^T$ ), GAG content (G), and collagen content (C). The subscripts 'nac' and 'sac' represent values for native and self-assembled cartilage, respectively.

$$FI = \frac{1}{4} \left( \left( 1 - \frac{(G_{nat} - G_{sac})}{G_{nat}} \right) + \left( 1 - \frac{(C_{nat} - C_{sac})}{C_{nat}} \right) + \left( 1 - \frac{(E_{nat}^T - E_{sac}^T)}{E_{nat}^T} \right) + \left( 1 - \frac{(E_{nat}^C - E_{sac}^C)}{E_{nat}^C} \right) \right) \quad (\text{Eq. 1})$$

Native values previously determined by our laboratory for the purpose of FI calculation were 12.1 MPa, 0.2 MPa, 5.5%, and 15% for Young's modulus, aggregate modulus, GAG/WW, and collagen/WW, respectively [37, 38]. The FI yields a score of 1 when construct properties are equivalent to those of native tissue. FI values were 0.41, 0.63, 0.70, and 0.69 for control, treatment with 4 $\alpha$ -PDD, treatment with ouabain, and combined treatment, respectively.

### Discussion

This study employed a two-phased approach to evaluate the effects of TRPV4 channel activation on tissue engineered articular cartilage. Experimental results supported the hypotheses motivating this study: 1) TRPV4 activation resulted in significant improvements in construct biochemical and biomechanical properties; 2) culture days 10-14 were identified as the optimal treatment time window to produce the greatest improvements in constructs; and 3) activation of TRPV4, a Ca<sup>2+</sup>-permeable channel, produced results comparable to Na<sup>+</sup>/K<sup>+</sup> pump inhibition. To our knowledge, this is the first study to examine TRPV4 channel activation in tissue engineering. The results of this investigation demonstrate that direct chemical modulation of intracellular ion concentrations can be a powerful tool in tissue engineering.

In Phase I, it was found that the optimal time window for TRPV4 activation in self-assembled articular cartilage constructs is during culture days 10-14. Compared to control, treatment with the TRPV4 channel agonist 4 $\alpha$ -PDD during days 10-14 led to significant improvements in collagen content (88% increase), tensile stiffness (153% increase), and tensile strength (130% increase). However, constructs were not improved by treatment during days 6-10 or 14-18, thereby highlighting the importance of timing during *in vitro* tissue development. The beneficial effects of treatment during days 10-14 are corroborated by previous work showing that other stimuli also produce their maximal effects during this time window [24, 25]. To understand why this time period is so crucial, it is important to consider the developmental milestones of constructs during self-assembly, a process that has

been shown to resemble *in vivo* cartilage development [4]. During self-assembly, collagen production peaks between days 10-14 of culture, while GAG production continues indiscriminately. This rapid synthesis of GAG with no new collagen secretion contributes to pre-stress within the nascent ECM, thereby compromising the engineered tissue's tensile properties [39, 40]. Directly modulating this imbalance between GAG and collagen has been shown to improve the tensile properties of self-assembled constructs [39, 41]. Thus, during days 10-14, before collagen production tapers and GAG production ramps up, cells within the developing construct may be more susceptible to interventions that induce new collagen biosynthesis. Based on the results from Phase I, the optimal treatment time window of  $t=10-14$  days was carried forward to Phase II.

In Phase II, TRPV4 activation using 4 $\alpha$ -PDD was compared to Na<sup>+</sup>/K<sup>+</sup> pump inhibition using ouabain. A previous study from our group showed that inhibition of the Na<sup>+</sup>/K<sup>+</sup> pump using 20  $\mu$ M ouabain during days 10-14 of culture improved the collagen content and tensile properties of self-assembled articular cartilage constructs [24], results that were corroborated by the present study. It was found that 10  $\mu$ M 4 $\alpha$ -PDD produced improvements in construct tensile properties that were comparable to 20  $\mu$ M ouabain, with no added benefit when the two stimuli were combined. Specifically, application of either 4 $\alpha$ -PDD or ouabain led to an approximately 2-fold increase in tensile stiffness. Though each agent produced an identical net enhancement in tensile stiffness, it is clear from the differences in construct sizes and biochemical content that the precise physiological responses to these agents, and therefore the mechanisms underlying tensile improvements, vary considerably. Moreover, combined treatment resulted in the same changes in size and biochemical content observed for treatment with ouabain alone, implying that Na<sup>+</sup>/K<sup>+</sup> pump inhibition predominates over TRPV4 activation in producing effects at the cell and tissue levels. This observation is similar to a previous observation in which constructs treated with a combination of ouabain and ionomycin, a Ca<sup>2+</sup> ionophore, did not outperform individual application either agent [24].

Notably, treatment with ouabain significantly reduced GAG production on a per-cell basis. Lower GAG levels are associated with decreased size and wet weight in cartilage [42], and the subsequent reduction in size in ouabain-treated constructs led to an increase in the percentage of collagen per wet weight, even though the per-cell production of collagen did not change. These phenomena suggest that ouabain treatment promotes a maturational growth phenotype, in which the tissue maintains a uniform size during ECM synthesis and remodeling, rather than an expansive growth phenotype, in which the tissue experiences a volumetric increase in size during ECM deposition [39, 42, 43]. Unlike ouabain, treatment of constructs with 4 $\alpha$ -PDD resulted in increased collagen production per cell, with no change in GAG production per cell. This net increase in collagen deposition at a steady level of GAG production is likely responsible for the improved tensile stiffness of constructs treated with 4 $\alpha$ -PDD.

Further work is necessary to determine how ion channel modulators elicit changes in chondrocyte ECM synthesis. Ion channels are thought to be involved in the cellular response to dynamic compression [5-7], fluid shear [11-13], hydrostatic pressure [14-17], and osmotic stress [20-23]. Specifically, ion channels play a role in the chondrocyte response to changes in volume; for example, a decrease in cell volume activates the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter to increase the tonicity of the intracellular compartment, thereby encouraging the cell to return to its initial state [20, 44]. Similarly, the Na<sup>+</sup>/K<sup>+</sup> pump is an ATPase that pumps ions against a concentration gradient to keep intracellular K<sup>+</sup> higher than Na<sup>+</sup>; inhibition of this pump, as with ouabain in the present study, leads to increased intracellular Na<sup>+</sup> [24]. Other channels, such as the Na<sup>+</sup>/H<sup>+</sup> transporter and Ca<sup>2+</sup> permeable channels, are also involved in volume regulation, among other roles, in response to mechanical or osmotic loading. In



particular, the TRPV4 channel has been shown to play a central role in regulating the chondrocyte response to osmotic stress [30, 31], as well as in promoting chondrogenic differentiation [45]. TRPV4 may also be implicated in osmotic stress-related pathogenesis of osteoarthritis [46]. The present study demonstrates that TRPV4 activation in engineered cartilage constructs can produce observable, tissue-level changes. Because of the osmosensitivity of TRPV4, it will be important in the future to examine the combined effects of osmotic stress and TRPV4 modulation on tissue engineered cartilage. Future studies that involve confocal imaging of intact self-assembled constructs may provide a better understanding of the importance of TRPV4 during *in vitro* tissue development.

In summary, this study investigated whether activation of the Ca<sup>2+</sup>-permeable TRPV4 channel would alter the biochemical and biomechanical properties of tissue engineered articular cartilage. It was shown that TRPV4 activation improved construct tensile properties, that the effects of TRPV4 activation were time-dependent, and that net improvements were on par with those produced by inhibiting the Na<sup>+</sup>/K<sup>+</sup> pump. To our knowledge, this is the first study to examine TRPV4 channel activation in tissue engineering. The results of this study demonstrate the effectiveness of ion channel modulation as a strategy for improving the functional properties of engineered tissues. Further investigation of the role of TRPV4 in self-assembled constructs should be undertaken at both a mechanistic level (e.g., examine cell volume regulation and calcium transients *in situ*) and at a functional engineering level (e.g., assessment of different durations of TRPV4 activation, or combining TRPV4 activation or inhibition with hyper-osmotic or hypo-osmotic stimulation).

## Acknowledgments

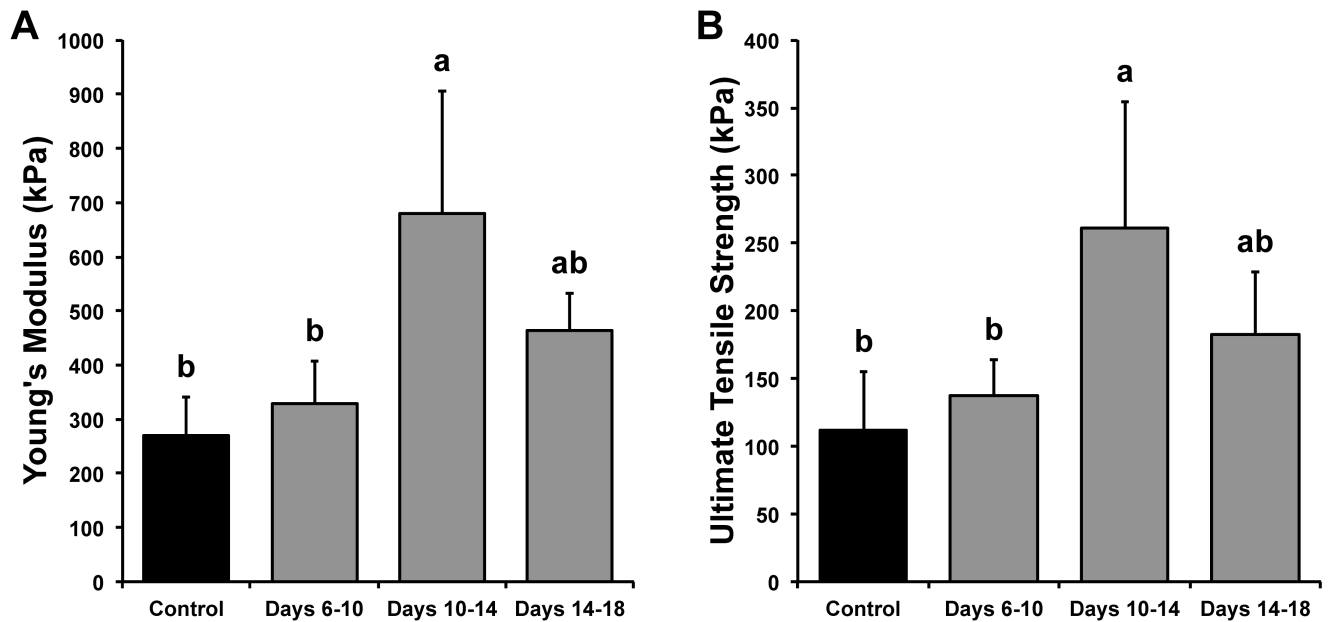
**Grants** This work was funded by the following grants from the National Institutes of Health: NIAMS R01AR053286 and NIGMS T32GM007730.

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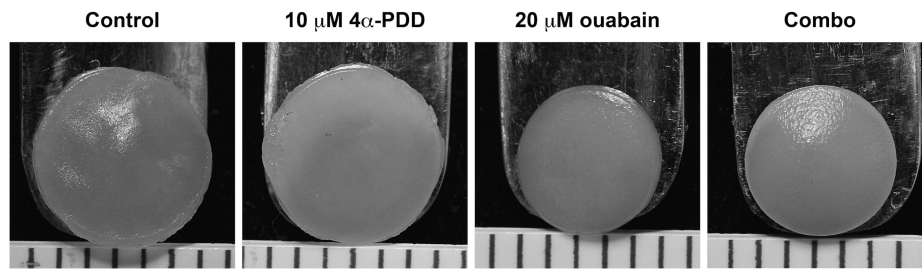
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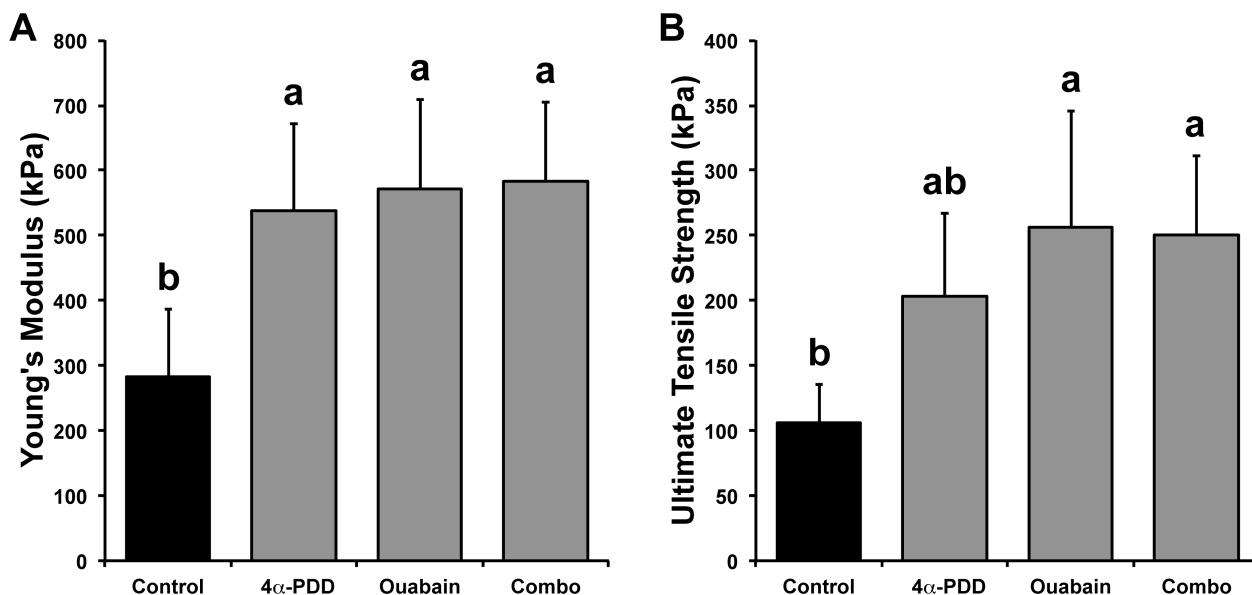


**Figure 1.** Phase I tensile properties of tissue engineered constructs. (A) Tensile stiffness for all groups. Constructs treated with 4 $\alpha$ -PDD during days 10-14 had the highest Young's moduli (153% greater than control). (B) Tensile strength for all groups. Constructs treated with 4 $\alpha$ -PDD during days 10-14 had the highest ultimate tensile strength (UTS) (133% greater than control). Data are presented as means + standard deviations. Lowercase letters denote significant differences; groups not connected by the same letter are considered significantly different ( $p < 0.05$ ).



**Figure 2.**

Phase II gross morphology of tissue engineered constructs at 4 weeks. From left to right: representative photographs of constructs from the control group, treated with 10  $\mu\text{M}$  4 $\alpha$ -PDD, treated with 20  $\mu\text{M}$  ouabain, and treated with both with 10  $\mu\text{M}$  4 $\alpha$ -PDD and 20  $\mu\text{M}$  ouabain. Constructs treated with ouabain or with both 4 $\alpha$ -PDD and ouabain were visibly smaller than control constructs or constructs treated with 4 $\alpha$ -PDD alone. Scale markings are spaced 1 mm apart.



**Figure 3.**

Phase II tensile properties of self-assembled cartilage constructs. (A) Tensile stiffness for all groups. Treatment with 4 $\alpha$ -PDD, ouabain, and their combination resulted in significant increases in Young's moduli compared to control (91%, 103%, and 107% increases, respectively), but no differences between each other. (B) Tensile strength for all groups. Treatment with ouabain or with the combination of 4 $\alpha$ -PDD and ouabain improved construct UTS (141% and 136% increases over control, respectively), followed by treatment with 4 $\alpha$ -PDD (91% increase over control, but not statistically significant). Data are presented as means + standard deviations. Lowercase letters denote significant differences; groups not connected by the same letter are considered significantly different ( $p < 0.05$ ).

**Table 1**

Diameter, thickness, and wet weight (WW) of tissue engineered constructs. In Phase I, no differences were found in construct diameter, thickness, or WW among groups. In Phase II, constructs treated with ouabain or with both 4 $\alpha$ -PDD and ouabain had significantly reduced diameters (0.83x and 0.86x control, respectively), thicknesses (0.51x and 0.67x control), and WW (0.40 and 0.43x control). Data are presented as means  $\pm$  standard deviations. Lowercase letters denote significant differences within a column; groups not connected by the same letter are considered significantly different ( $p < 0.05$ ).

Group	Diameter (mm)	Thickness (mm)	WW (mg)
<i>Phase I (10 <math>\mu</math>M 4<math>\alpha</math>-PDD)</i>			
Control	6.29 $\pm$ 0.17	0.62 $\pm$ 0.05	36.7 $\pm$ 3.1
Treatment on days 6-10	6.27 $\pm$ 0.16	0.63 $\pm$ 0.05	37.2 $\pm$ 2.8
Treatment on days 10-14	6.31 $\pm$ 0.18	0.63 $\pm$ 0.05	36.2 $\pm$ 2.4
Treatment on days 14-18	6.26 $\pm$ 0.16	0.62 $\pm$ 0.06	37.2 $\pm$ 2.8
<i>Phase II</i>			
Control	6.35 $\pm$ 0.06 <sup>a</sup>	0.68 $\pm$ 0.10 <sup>a</sup>	38.3 $\pm$ 1.5 <sup>a</sup>
10 $\mu$ M 4 $\alpha$ -PDD	6.30 $\pm$ 0.13 <sup>a</sup>	0.63 $\pm$ 0.10 <sup>a</sup>	39.6 $\pm$ 2.3 <sup>a</sup>
20 $\mu$ M ouabain	5.29 $\pm$ 0.04 <sup>b</sup>	0.35 $\pm$ 0.08 <sup>b</sup>	15.2 $\pm$ 0.6 <sup>b</sup>
Combo	5.45 $\pm$ 0.18 <sup>b</sup>	0.45 $\pm$ 0.08 <sup>b</sup>	16.3 $\pm$ 1.3 <sup>b</sup>

Table 2

Biochemical content of tissue engineered constructs. In Phase I, collagen/WW was highest in constructs treated with 4 $\alpha$ -PDD during days 10-14 (1.88x control), followed by constructs treated during days 14-18 (1.40x control). These differences in collagen content were upheld when normalized to cell number. There were no differences observed in GAG content in Phase I. In Phase II, treatment with 4 $\alpha$ -PDD, ouabain, or their combination resulted in significant increases in collagen/WW compared to control (1.80x, 2.18x, and 1.93x control, respectively), but no differences between each other. Collagen production per cell was greatest in constructs treated with 4 $\alpha$ -PDD (1.85x control). GAG production per cell was significantly decreased in constructs treated with ouabain (0.40x control) and the combination of 4 $\alpha$ -PDD and ouabain (0.43x control). No differences in cell number were observed in Phase I or Phase II. Data are presented as means  $\pm$  standard deviations. Lowercase letters denote significant differences within a column; groups not connected by the same letter are considered significantly different ( $p < 0.05$ ).

Group	Total cells ( $\times 10^6$ )	Collagen (% WW)	GAG (% WW)	Collagen ( $\mu\text{g}/10^6$ cells)	GAG ( $\mu\text{g}/10^6$ cells)
<i>Phase I (10 <math>\mu\text{M}</math> 4<math>\alpha</math>-PDD)</i>					
Control	5.64 $\pm$ 0.33	5.9 $\pm$ 0.7 <sup>c</sup>	3.6 $\pm$ 0.5	383 $\pm$ 60 <sup>c</sup>	232 $\pm$ 44
Treatment on days 6-10	5.73 $\pm$ 0.37	6.2 $\pm$ 1.1 <sup>bc</sup>	3.7 $\pm$ 0.4	401 $\pm$ 82 <sup>bc</sup>	245 $\pm$ 39
Treatment on days 10-14	5.71 $\pm$ 0.43	11.1 $\pm$ 2.3 <sup>a</sup>	3.4 $\pm$ 0.9	694 $\pm$ 97 <sup>a</sup>	212 $\pm$ 46
Treatment on days 14-18	5.67 $\pm$ 0.46	8.3 $\pm$ 1.1 <sup>b</sup>	3.7 $\pm$ 0.7	548 $\pm$ 123 <sup>ab</sup>	242 $\pm$ 62
<i>Phase II</i>					
Control	5.73 $\pm$ 0.24	5.5 $\pm$ 0.7 <sup>b</sup>	4.0 $\pm$ 0.8	363 $\pm$ 37 <sup>b</sup>	268 $\pm$ 50 <sup>a</sup>
10 $\mu\text{M}$ 4 $\alpha$ -PDD	5.79 $\pm$ 0.29	9.8 $\pm$ 1.8 <sup>a</sup>	4.5 $\pm$ 1.1	669 $\pm$ 127 <sup>a</sup>	305 $\pm$ 74 <sup>a</sup>
20 $\mu\text{M}$ ouabain	5.55 $\pm$ 0.20	11.9 $\pm$ 2.5 <sup>a</sup>	4.0 $\pm$ 1.1	325 $\pm$ 67 <sup>b</sup>	108 $\pm$ 28 <sup>b</sup>
Combo	5.71 $\pm$ 0.26	10.5 $\pm$ 2.7 <sup>a</sup>	4.1 $\pm$ 1.3	296 $\pm$ 63 <sup>b</sup>	116 $\pm$ 39 <sup>b</sup>