Acta Biomaterialia 9 (2013) 5554–5561

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

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

Sriram V. Eleswarapu [a,b,c], Kyriacos A. Athanasiou [a,b,*]

[a] Department of Biomedical Engineering, University of California, Davis, CA 95616, USA
[b] Department of Bioengineering, Rice University, Houston, TX, USA
[c] Medical Scientist Training Program, Baylor College of Medicine, Houston, TX, USA

ARTICLE INFO

Article history:
Received 11 August 2012
Received in revised form 19 October 2012
Accepted 26 October 2012
Available online 2 November 2012

Keywords:
TRPV4
Cartilage tissue engineering
Self-assembly
Biomechanics

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 the 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: (i) to determine whether TRPV4 activation would enhance self-assembled constructs; (ii) to identify an optimal treatment time window for TRPV4 activation; and (iii) to compare TRPV4 activation which Na^+/K^+ pump inhibition, which has previously been shown to improve the construct tensile properties. This study employed a two phase approach. In Phase I self-assembled constructs were grown for 4 weeks and subjected to treatment with the TRPV4 agonist 4α-phorbol-12,13-didecanoate (4α-PDD) during three treatment time windows: $t = 6–10$, $t = 10–14$, and $t = 14–18$ days. Treatment for $t = 10–14$ 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 4α-PDD and Na^+/K^+ pump inhibition using ouabain. Treatment with 4α-PDD produced improvements on a par with ouabain (91–107% 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.

1. 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 biological 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 prefabricated, 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 physiological loading conditions experienced by native cartilage in the intact

* Corresponding author at: Department of Biomedical Engineering, University of California, Davis, CA 95616, USA. Tel.: +1 530 754 6645; fax: +1 530 754 5739.
E-mail address: athanasiou@ucdavis.edu (K.A. Athanasiou).

1742-7061/$ - see front matter © 2012 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2012.10.031

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 Na^+/K^+ pump [16], an ATPase that pumps ions against a concentration gradient to maintain a higher intracellular concentration of K^+ than Na^+. Thus, by inhibiting the Na^+/K^+ pump hydrostatic pressure produces increased levels of intracellular Na^+. A recent study from our group [24] showed that selective inhibition of the Na^+/K^+ pump using 20 μM 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 to 550 mOsm [20], has been shown to produce transient increases in intracellular 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 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: (i) to determine whether TRPV4 activation would enhance the biochemical and biomechanical properties of self-assembled constructs; (ii) to identify an optimal treatment time window for TRPV4 activation; and (iii) to compare TRPV4 activation with Na^+/K^+ pump inhibition. This study employed a two phase approach. In Phase I constructs were self-assembled from bovine chondrocytes and subjected to treatment with the TRPV4 agonist 4α-phorbol-12,13-didecanoate (4α-PDD) during three treatment time windows: $t = 6–10$, $t = 10–14$, 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 4α-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 4α-PDD and Na^+/K^+ pump inhibition using ouabain. We also examined a combination of 4α-PDD and ouabain. It was hypothesized that: (i) TRPV4 activation would improve construct properties; (ii) an optimal treatment time window exists in which the constructs undergo greatest improvement; and (iii) activation of TRPV4 would produce results comparable with those observed with inhibition of the Na^+/K^+ pump. Assessments included gross morphology, biochemical analysis for GAG and collagen, and biomechanical testing.

2. Materials and methods

2.1. 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 components Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg ml^{-1} 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 μg ml^{-1} ascorbate-2-phosphate, 40 μg ml^{-1} L-proline, and 100 μg ml^{-1} sodium pyruvate (Fisher Scientific). Chondrogenic medium contains 151 mM Na^+, 5.2 mM K^+, and 1.7 mM Ca^{2+}. Medium osmolarity was assessed using a VAPRO 5520 vapor pressure osmometer (Wescor) and was determined to be ~347 mOsm.

2.2. Chondrocyte isolation

Cartilage was harvested from the distal femurs and patello-femoral grooves of 1-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 °C in DMEM containing 20% fetal bovine serum and 10% dimethyl sulfoxide.

2.3. 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.

2.4. 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 μl of chondrogenic medium was deposited into each preformed cylindrical agarose well, followed by the addition of 400 μl of chondrogenic medium 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 released and transferred to 48-well plates unrestricted by circumferential confinement. Constructs received 500 μl of medium change every 24 h and remained in culture until $t = 28$ days. All culture was performed at 37 °C and 10% CO_2, simulating hypoxic conditions that have produced consistent biosynthetic results previously with self-assembled cartilage constructs in our laboratory [24].

2.5. 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, 4α-PDD (Enzo Life Sciences), during three treatment windows: t = 6–10, t = 10–14, and t = 14–18 days. During treatment constructs were cultured in Petri dishes for 1 h with ~4 ml of chondrogenic medium containing 10 μM 4α-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 4α-PDD before the constructs were returned to their wells. Treatment occurred at the same time every day over the course of 5 days.

2.6. Phase II. TRPV4 activation versus Na^+/K^+ pump inhibition

A previous study from our group showed that inhibition of the Na^+/K^+ 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^+/K^+ 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 μM 4α-PDD during t = 10–14 days (see Section 3 for details). The regimen for Na^+/K^+ pump inhibition was selected from previous work done by our group [24]: 20 μM ouabain during t = 10–14 days. During t = 10–14 days constructs were cultured in Petri dishes for 1 h with ~4 ml of chondrogenic medium containing either 10 μM 4α-PDD, 20 μM 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.

2.7. Gross morphology and specimen portioning

At t = 28 days the constructs were removed from culture. Photographs were taken and the dimensions were measured from photographs using ImageJ software (National Institutes of Health). Wet weights (WW) were recorded, and the constructs were portioned for analysis. A 3 mm diameter punch was taken from the construct center for indentation testing. The remaining outer ring was divided into two semilunar portions, one for biochemical analysis and one for tensile testing.

2.8. Biochemical analysis

Biochemical samples were weighed wet, frozen, and lyophilized. Samples were digested with 125 μg ml^{-1} papain (Sigma) for 18 h at 65 °C. Total DNA content was assessed with a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and the cell number estimated assuming 7.8 pg DNA per cell. Sulfated glycosaminoglycan (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 in making comparisons.

2.9. 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 gage 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 gage length, and these tabs were gripped during testing (for more details, including photographs of the process, see Natoli et al. [24]). A uniaxial materials testing system (Instron Model 5565) was employed to determine the tensile properties. Tensile tests were performed until failure at a strain rate of 1% of the gage length per second. Force–displacement curves were generated, and stress–strain curves were calculated by normalizing to the specimen dimensions. The 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.

2.10. 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 phosphate-buffered saline. 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].

2.11. Statistical analysis

All quantitative biochemical and biomechanical assessments were made using n = 6–8, i.e. each group for every quantitative test had a minimum of 6 samples and a maximum of 8 samples. Data are presented as means ± standard deviations. Single factor ANOVA was employed in each phase of the study to assess 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).

3. Results

3.1. 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 4α-PDD, an agonist of the TRPV4 channel. We examined the use of 10 μM 4α-PDD during three different treatment time windows: t = 6–10, t = 10–14, 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 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 4α-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 with the control. There were no differences observed in GAG content across treatment time windows.

Table 1
Diameter, thickness, and wet weight (WW) of the tissue engineered constructs.

Group	Diameter (mm)	Thickness (mm)	WW (mg)
Phase I (10 μM 4α-PDD)			
Control	6.29 ± 0.17	0.62 ± 0.05	36.7 ± 3.1
Treatment on days 6–10	6.27 ± 0.16	0.63 ± 0.05	37.2 ± 2.8
Treatment on days 10–14	6.31 ± 0.18	0.63 ± 0.05	36.2 ± 2.4
Treatment on days 14–18	6.26 ± 0.16	0.62 ± 0.06	37.2 ± 2.8
Phase II			
Control	6.35 ± 0.06[a]	0.68 ± 0.10[a]	38.3 ± 1.5[a]
10 μM 4α-PDD	6.30 ± 0.13[a]	0.63 ± 0.10[a]	39.6 ± 2.3[a]
20 μM ouabain	5.29 ± 0.04[b]	0.35 ± 0.08[b]	15.2 ± 0.6[b]
Combination	5.45 ± 0.18[b]	0.45 ± 0.08[b]	16.3 ± 1.3[b]

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 4α-PDD and ouabain had significantly reduced diameters (0.83× and 0.86× control, respectively), thicknesses (0.51× and 0.67× control), and WW (0.40× and 0.43× control). Data are presented as means ± standard deviations. Lower case letters denote significant differences within a column; groups not connected by the same letter are considered significantly different ($P < 0.05$).

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 Fig. 1. Young's moduli for the control, treatment on days 6–10, treatment on days 10–14, and treatment on days 14–18 were 269 ± 73, 328 ± 80, 681 ± 224, and 464 ± 69 kPa, respectively. Constructs treated with 4α-PDD during days 10–14 had the highest Young's moduli (153% increase over control). UTS values for the control, treatment on days 6–10, treatment on days 10–14, and treatment on days 14–18 were 112 ± 43, 138 ± 27, 261 ± 94, and 182 ± 46 kPa, respectively. Constructs treated with 4α-PDD during days 10–14 had the highest UTS (133% increase over control). With respect to compressive stiffness, the aggregate moduli for the control, treatment on days 6–10, treatment on days 10–14, and treatment on days 14–18 were 75 ± 19, 72 ± 21, 82 ± 20, and 76 ± 25 kPa, respectively. No differences were found in aggregate moduli across groups.

Altogether, treatment with 4α-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.

3.2. Phase II. TRPV4 activation versus Na^+/K^+ pump inhibition

In Phase II we compared the effects of TRPV4 activation with the effects of Na^+/K^+ 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 μM 4α-PDD, 20 μM 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 the constructs treated with ouabain or with the combination of 4α-PDD and ouabain were visibly smaller than the control constructs or constructs treated with 4α-PDD alone (Fig. 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 WW values (60% and 57% decreases).

Construct biochemical data for Phase II are provided in Table 2. Treatment with 4α-PDD, ouabain, or a combination resulted in significant increases in collagen (% WW) compared with the control (increases of 80%, 118%, and 93%, respectively), but no differences between each other. Collagen production per cell was greatest in constructs treated with 4α-PDD (85% increase over control), with no differences among control, ouabain, or the combination 4α-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 4α-PDD and ouabain (57% decrease from control). No differences were observed in cell number across groups.

Biomechanical properties were again assessed by uniaxial tensile and creep indentation testing. Tensile stiffness and strength data for Phase II are shown in Fig. 3. Young's moduli for the control, treatment with 4α-PDD, treatment with ouabain, and combined treatment were 282 ± 105, 538 ± 133, 572 ± 136, and 583 ± 121 kPa, respectively. Treatment with 4α-PDD, ouabain, and their combination resulted in significant increases in Young's moduli compared with the control (91%, 103%, and 107% increases, respectively), but no differences between each other. UTS values for the control, treatment with 4α-PDD, treatment with ouabain, and combined treatment were 106 ± 29, 203 ± 64, 256 ± 89, and 251 ± 61 kPa, respectively. Treatment with ouabain or with the combination of 4α-PDD and ouabain produced the greatest improvements in construct UTS (141% and 136% increases over control, respectively), followed by treatment with 4α-PDD (91% increase over control, but not statistically significant). In terms of compressive properties, aggregate moduli for control, treatment with 4α-PDD, treatment with ouabain, and combined treatment were 67 ± 14, 73 ± 18, 67 ± 19, and 74 ± 23 kPa, respectively. No differences were found in aggregate moduli across groups.

Table 2
Biochemical content of tissue engineered constructs.

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

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

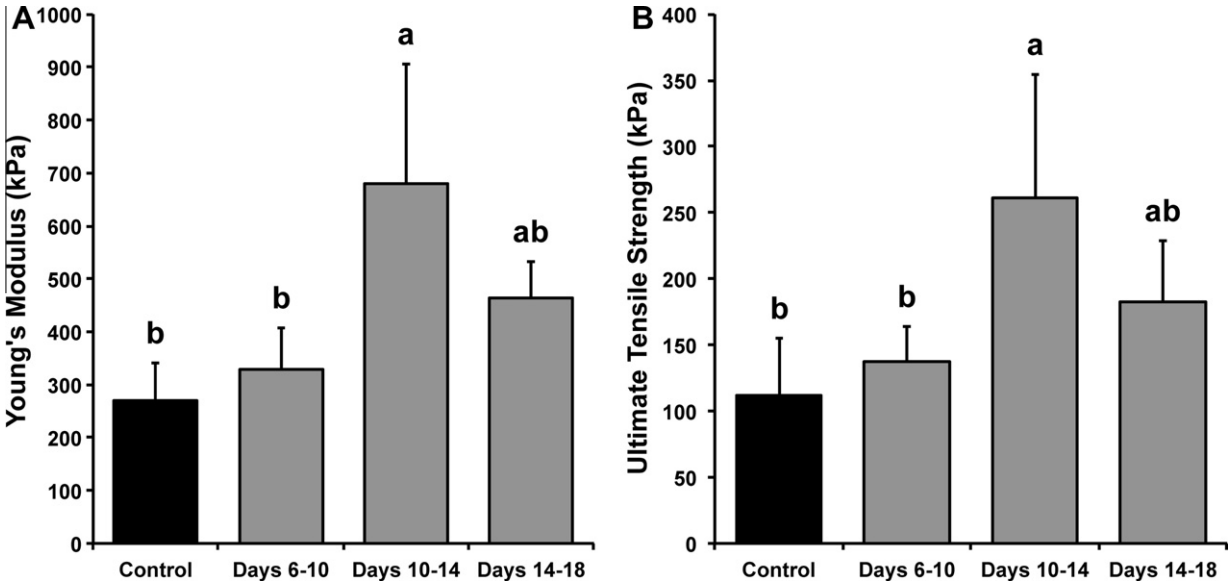

Fig. 1. Phase I tensile properties of tissue engineered constructs. (A) Tensile stiffness for all groups. Constructs treated with 4α-PDD during days 10–14 had the highest Young's moduli (153% greater than control). (B) Tensile strength for all groups. Constructs treated with 4α-PDD during days 10–14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

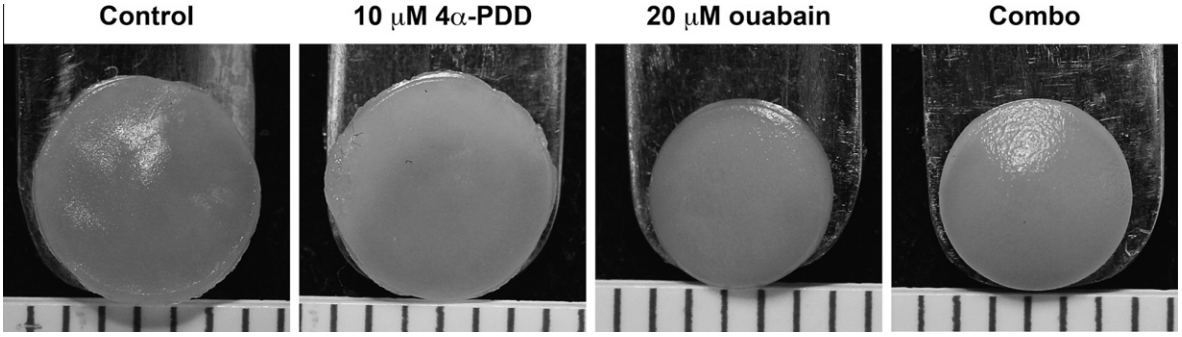

Fig. 2. Phase II gross morphology of tissue engineered constructs at 4 weeks. From left to right, representative photographs of constructs from the control group treated with 10 μM 4α-PDD, treated with 20 μM ouabain, and treated with both with 10 μM 4α-PDD and 20 μM ouabain. Constructs treated with ouabain or with both 4α-PDD and ouabain were visibly smaller than control constructs or constructs treated with 4α-PDD alone. Scale markings are spaced 1 mm apart.

3.3. 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 Eq. (1), FI equally weights the compressive stiffness (E^C), tensile stiffness (E^T), GAG content (G), and collagen content (C). The subscripts 'nat' and 'sac' represent values for native and self-assembled cartilage, respectively.

$$\mathrm{FI} = \frac{1}{4}\left(\left(1 - \frac{(G_{nat} - G_{sac})}{G_{nat}}\right) + \left(1 - \frac{(C_{nat} - C_{sac})}{C_{nat}}\right) + \left(1 - \frac{\left(E^T_{nat} - E^T_{sac}\right)}{E^T_{nat}}\right) + \left(1 - \frac{\left(E^C_{nat} - E^C_{sac}\right)}{E^C_{nat}}\right)\right) \quad (1)$$

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 4α-PDD, treatment with ouabain, and combined treatment, respectively.

4. 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: (i) TRPV4 activation resulted in significant improvements in construct biochemical and biomechanical properties; (ii) culture days 10–14 were identified as the optimal treatment time window to produce the greatest improvements in constructs; and (iii) activation of TRPV4, a Ca^{2+}-permeable channel, produced results comparable with Na^+/K^+ 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

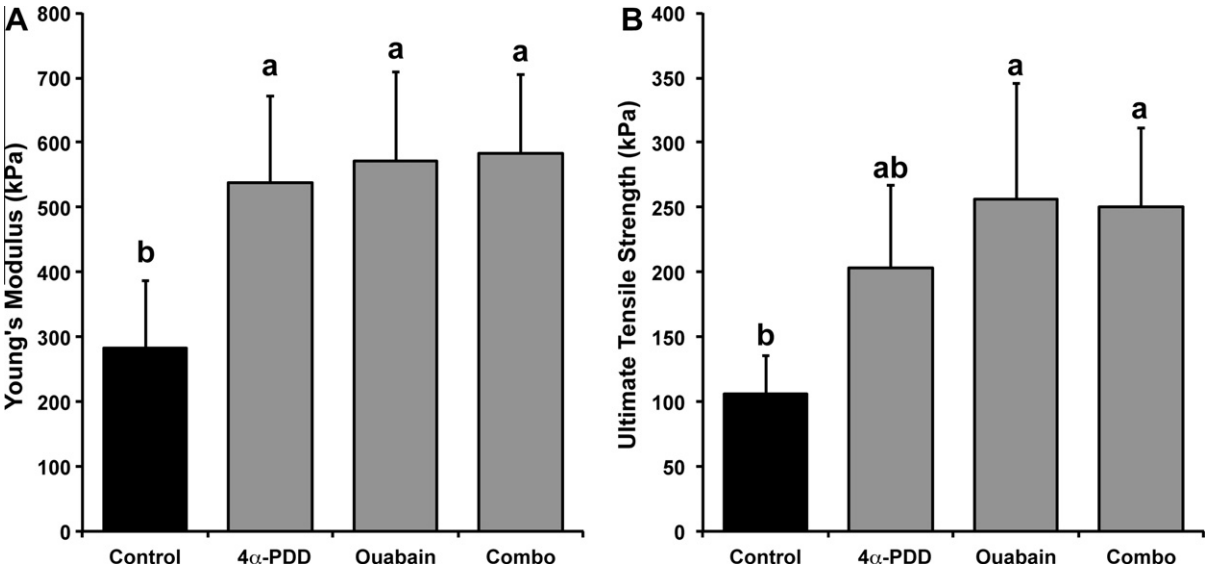

Fig. 3. Phase II tensile properties of self-assembled cartilage constructs. (A) Tensile stiffness for all groups. Treatment with 4α-PDD, ouabain, and 4α-PDD and ouabain in combination resulted in significant increases in the Young's moduli compared with the control (91%, 103%, and 107% increases, respectively), but no differences between each other. (B) Tensile strength for all groups. Treatment with ouabain or the combination of 4α-PDD and ouabain improved construct UTS (141% and 136% increases over the control, respectively), followed by treatment with 4α-PDD (91% increase over control, but not statistically significant). Data are presented as means ± standard deviations. Lower case letters denote significant differences; groups not connected by the same letter are considered significantly different ($P < 0.05$).

culture days 10–14. Compared with the control, treatment with the TRPV4 channel agonist 4α-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 throughout the culture period. This rapid synthesis of GAG with no new collagen secretion contributes to pre-stress within the nascent ECM, thereby compromising the tensile properties of the engineered tissue [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 4α-PDD was compared with Na^+/K^+ pump inhibition using ouabain. A previous study by our group showed that inhibition of the Na^+/K^+ pump using 20 μM 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 μM 4α-PDD produced improvements in construct tensile properties that were comparable with 20 μM ouabain, with no added benefit when the two stimuli were combined. Specifically, application of either 4α-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 the 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^+/K^+ 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^{2+} ionophore, did not outperform individual application of 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 4α-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 4α-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 $Na^+/K^+/2Cl^-$ co-transporter to increase the tonicity of the intracellular compartment, thereby encouraging the cell to return to its initial state [20,44]. Similarly, the Na^+/K^+ pump is an ATPase that pumps ions against a concentration gradient to keep intracellular K^+ higher than Na^+; inhibition of this pump, as with ouabain in the present study, leads to increased intracellular Na^+ [24]. Other channels, such as the $Na^+/$

H^+ transporter and Ca^{2+} 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 demonstrated 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 involving 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^{2+}-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 the net improvements were on a par with those produced by inhibiting the Na^+/K^+ 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. examining 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).

Acknowledgements

This work was funded by Grants NIAMS R01AR053286 and NIGMS T32GM007730 from the National Institutes of Health. S.V.E. and K.A.A. conceived and designed the experiments, S.V.E. acquired the data, S.V.E. and K.A.A. reviewed the data, and S.V.E. and K.A.A. wrote and edited the paper.

References

[1] Buckwalter JA. Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther 1998;28:192–202.
[2] Darling EM, Athanasiou KA. Biomechanical strategies for articular cartilage regeneration. Ann Biomed Eng 2003;31:1114–24.
[3] Hu JC, Athanasiou KA. A self-assembling process in articular cartilage tissue engineering. Tissue Eng 2006;12:969–79.
[4] Ofek G, Revell CM, Hu JC, Allison DD, Grande-Allen KJ, Athanasiou KA. Matrix development in self-assembly of articular cartilage. PloS One 2008;3:e2795.
[5] Chowdhury TT, Knight MM. Purinergic pathway suppresses the release of NO and stimulates proteoglycan synthesis in chondrocyte/agarose constructs subjected to dynamic compression. J Cell Physiol 2006;209:845–53.
[6] Mouw JK, Imler SM, Levenston ME. Ion-channel regulation of chondrocyte matrix synthesis in 3D culture under static and dynamic compression. Biomech Model Mechanobiol 2007;6:33–41.
[7] Hunter CJ, Mouw JK, Levenston ME. Dynamic compression of chondrocyte-seeded fibrin gels: effects on matrix accumulation and mechanical stiffness. Osteoarthritis Cartilage 2004;12:117–30.
[8] Likhitpanichkul M, Guo XE, Mow VC. The effect of matrix tension-compression nonlinearity and fixed negative charges on chondrocyte responses in cartilage. Mol Cell Biomech 2005;2:191–204.
[9] Lima EG, Bian L, Ng KW, Mauck RL, Byers BA, Tuan RS, et al. The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. Osteoarthritis Cartilage 2007;15:1025–33.
[10] Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng 2003;9:597–611.
[11] Edlich M, Yellowley CE, Jacobs CR, Donahue HJ. Cycle number and waveform of fluid flow affect bovine articular chondrocytes. Biorheology 2004;41:315–22.
[12] Edlich M, Yellowley CE, Jacobs CR, Donahue HJ. Oscillating fluid flow regulates cytosolic calcium concentration in bovine articular chondrocytes. J Biomech 2001;34:59–65.
[13] Yellowley CE, Jacobs CR, Donahue HJ. Mechanisms contributing to fluid-flow-induced Ca^{2+} mobilization in articular chondrocytes. J Cell Physiol 1999;180:402–8.
[14] Wilkins RJ, Browning JA, Urban JP. Chondrocyte regulation by mechanical load. Biorheology 2000;37:67–74.
[15] Urban JP, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. J Cell Physiol 1993;154:262–70.
[16] Hall AC. Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. J Cell Physiol 1999;178:197–204.
[17] Elder BD, Athanasiou KA. Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. Tissue Eng B Rev 2009;15:43–53.
[18] Elder SH, Sanders SW, McCulley WR, Marr ML, Shim JW, Hasty KA. Chondrocyte response to cyclic hydrostatic pressure in alginate versus pellet culture. J Orthop Res 2006;24:740–7.
[19] Smith RL, Lin J, Trindade MC, Shida J, Kajiyama G, Vu T, et al. Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. J Rehabil Res Dev 2000;37:153–61.
[20] Erickson GR, Alexopoulos LG, Guilak F. Hyper-osmotic stress induces volume change and calcium transients in chondrocytes by transmembrane, phospholipid, and G-protein pathways. J Biomech 2001;34:1527–35.
[21] Erickson GR, Northrup DL, Guilak F. Hypo-osmotic stress induces calcium-dependent actin reorganization in articular chondrocytes. Osteoarthritis Cartilage 2003;11:187–97.
[22] Sanchez JC, Danks TA, Wilkins RJ. Mechanisms involved in the increase in intracellular calcium following hypotonic shock in bovine articular chondrocytes. Gen Physiol Biophys 2003;22:487–500.
[23] Sanchez JC, Wilkins RJ. Changes in intracellular calcium concentration in response to hypertonicity in bovine articular chondrocytes. Comp Biochem Physiol A Mol Integr Physiol 2004;137:173–82.
[24] Natoli RM, Skaalure S, Bijlani S, Chen KX, Hu J, Athanasiou KA. Intracellular Na(+) and Ca(2+) modulation increases the tensile properties of developing engineered articular cartilage. Arthritis Rheum 2010;62:1097–107.
[25] Elder BD, Athanasiou KA. Effects of temporal hydrostatic pressure on tissue-engineered bovine articular cartilage constructs. Tissue Eng A 2009;15:1151–8.
[26] Gu WY, Lai WM, Mow VC. A mixture theory for charged-hydrated soft tissues containing multi-electrolytes: passive transport and swelling behaviors. J Biomech Eng 1998;120:169–80.
[27] Yellowley CE, Hancox JC, Donahue HJ. Effects of cell swelling on intracellular calcium and membrane currents in bovine articular chondrocytes. J Cell Biochem 2002;86:290–301.
[28] Palmer GD, Chao PH, Raia F, Mauck RL, Valhmu WB, Hung CT. Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading. Osteoarthritis Cartilage 2001;9:761–70.
[29] Tew SR, Peffers MJ, McKay TR, Lowe ET, Khan WS, Hardingham TE, et al. Hyperosmolarity regulates SOX9 mRNA posttranscriptionally in human articular chondrocytes. Am J Physiol Cell Physiol 2009;297:C898–906.
[30] Phan MN, Leddy HA, Votta BJ, Kumar S, Levy DS, Lipshutz DB, et al. Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes. Arthritis Rheum 2009;60:3028–37.
[31] Guilak F, Leddy HA, Liedtke W. Transient receptor potential vanilloid 4: the sixth sense of the musculoskeletal system? Ann NY Acad Sci 2010;1192:404–9.
[32] Hung CT. Transient receptor potential vanilloid 4 channel as an important modulator of chondrocyte mechanotransduction of osmotic loading. Arthritis Rheum 2010;62:2850–1.
[33] Eleswarapu SV, Chen JA, Athanasiou KA. Temporal assessment of ribose treatment on self-assembled articular cartilage constructs. Biochem Biophys Res Commun 2011;414:431–6.
[34] Elder BD, Athanasiou KA. Effects of confinement on the mechanical properties of self-assembled articular cartilage constructs in the direction orthogonal to the confinement surface. J Orthop Res 2008;26:238–46.
[35] Woessner Jr JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440–7.
[36] Athanasiou KA, Agarwal A, Muffoletto A, Dzida FJ, Constantinides G, Clem M. Biomechanical properties of hip cartilage in experimental animal models. Clin Orthop Relat Res 1995;316:254–66.
[37] Elder BD, Athanasiou KA. Systematic assessment of growth factor treatment on biochemical and biomechanical properties of engineered articular cartilage constructs. Osteoarthritis Cartilage 2009;17:114–23.
[38] Responte DJ, Arzi B, Natoli RM, Hu JC, Athanasiou KA. Mechanisms underlying the synergistic enhancement of self-assembled neocartilage treated with chondroitinase-ABC and TGF-beta1. Biomaterials 2012;33:3187–94.
[39] Natoli R, Revell CM, Athanasiou K. Chondroitinase ABC treatment results in increased tensile properties of self-assembled tissue engineered articular cartilage. Tissue Eng A 2009;15:3119–28.
[40] Schwartz MH, Leo PH, Lewis JL. A microstructural model for the elastic response of articular cartilage. J Biomech 1994;27:865–73.

[41] Natoli RM, Responte DJ, Lu BY, Athanasiou KA. Effects of multiple chondroitinase ABC applications on tissue engineered articular cartilage. J Orthop Res 2009. http://dx.doi.org/10.1002/jor.20821.
[42] Asanbaeva A, Masuda K, Thonar EJ, Klisch SM, Sah RL. Mechanisms of cartilage growth: modulation of balance between proteoglycan and collagen in vitro using chondroitinase ABC. Arthritis Rheum 2007;56:188–98.
[43] Huey DJ, Athanasiou KA. Maturational growth of self-assembled, functional menisci as a result of TGF-beta1 and enzymatic chondroitinase-ABC stimulation. Biomaterials 2011;32:2052–8.
[44] Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. Exp Physiol 1996;81:535–45.
[45] Muramatsu S, Wakabayashi M, Ohno T, Amano K, Ooishi R, Sugahara T, et al. Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation. J Biol Chem 2007;282:32158–67.
[46] Clark AL, Votta BJ, Kumar S, Liedtke W, Guilak F. Chondroprotective role of the osmotically sensitive ion channel transient receptor potential vanilloid 4: age- and sex-dependent progression of osteoarthritis in Trpv4-deficient mice. Arthritis Rheum 2010;62:2973–83.