TRPV4-mediated mechanotransduction regulates the metabolic response of chondrocytes to dynamic loading

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Mechanical loading of joints plays a critical role in maintaining the health and function of articular cartilage. The mechanism(s) of chondrocyte mechanotransduction are not fully understood, but could provide important insights into new physical or pharmacologic therapies for joint diseases. Transient receptor potential vanilloid 4 (TRPV4), a Ca^{2+}-permeable osmomechano-TRP channel, is highly expressed in articular chondrocytes, and loss of TRPV4 function is associated with joint arthropathy and osteoarthritis. The goal of this study was to examine the hypothesis that TRPV4 transduces dynamic compressive loading in articular chondrocytes. We first confirmed the presence of physically induced, TRPV4-dependent intracellular Ca^{2+} signaling in agarose-embedded chondrocytes, and then used this model system to study the role of TRPV4 in regulating the response of chondrocytes to dynamic compression. Inhibition of TRPV4 during dynamic loading prevented acute, mechanically mediated regulation of proanabolic and anticatabolic genes, and furthermore, blocked the loading-induced enhancement of matrix accumulation and mechanical properties. Furthermore, chemical activation of TRPV4 by the agonist GSK1016790A in the absence of mechanical loading similarly enhanced anabolic and suppressed catabolic gene expression, and potently increased matrix biosynthesis and construct mechanical properties. These findings support the hypothesis that TRPV4-mediated Ca^{2+} signaling plays a central role in the transduction of mechanical signals to support cartilage extracellular matrix maintenance and joint health. Moreover, these insights raise the possibility of therapeutically targeting TRPV4-mediated mechanotransduction for the treatment of diseases such as osteoarthritis, as well as to enhance matrix formation and functional properties of tissue-engineered cartilage as an alternative to bioreactor-based mechanical stimulation.

mechanobiology | ion channel | calcium signaling | TGF-beta | tissue engineering

Articular cartilage is the dense connective tissue that lines the surfaces of diarthrodial joints and provides a low-friction surface for joint loading and articulation. The extracellular matrix (ECM) of articular cartilage is primarily comprised of proteoglycans and type II collagen, in addition to a sparse population of chondrocytes responsible for synthesizing and maintaining this tissue. The mechanical environment of articular cartilage plays an important role in regulating the development and maintenance of the tissue. For example, dynamic compressive loading of cartilage supports ECM biosynthesis (1), whereas abnormal loading, such as disuse, static loading, or altered joint biomechanics, can disrupt ECM homeostasis (2, 3) and lead to osteoarthritis (OA) (4), a degenerative joint disease characterized by an imbalance of chondrocyte anabolic and catabolic activities. Most of the hypotheses on the etiology of OA involve biomechanical loading as a factor (4, 5). As such, understanding chondrocyte mechanotransduction, i.e., how chondrocytes sense and respond to their physical environment, is vital to understanding how OA develops and progresses, and may lead to new treatments for this disease.

Chondrocyte mechanotransduction appears to involve the integration and transduction of multiple biophysical signals that arise from joint loading, including direct matrix, cellular, and nuclear strain, hydrostatic pressurization, fluid shear, and changes in tissue osmolarity (6). Ion channels, integrin signaling, and the primary cilia have all been implicated in transducing the external biophysical environment of chondrocytes into electrical and/or chemical intracellular signaling (7–9). Specifically, intracellular Ca^{2+} signaling has emerged as a common regulatory mechanism for controlling gene and protein expression (10–12).

The transient receptor potential vanilloid 4 (TRPV4) channel is a multimodally activated, Ca^{2+}-preferred membrane ion channel widely implicated in transducing external environmental cues into specific metabolic responses via the generation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients (13–15). Human TRPV4 mutations that alter channel function are known to disrupt normal skeletal development and joint health (14, 16–18), and similarly, targeted deletion of TRPV4 in mice leads to loss of chondrocyte osmotransduction and subsequently, severe joint degeneration (19). TRPV4-mediated Ca^{2+} signaling has also been shown to enhance chondrogenic gene expression in chondroprogenitor cell lines (20), as well as increase matrix synthesis in chondrocyte-based self-assembled constructs (21). However, the precise role of TRPV4 in transducing and regulating chondrocyte metabolic activity in response to mechanical loading is unclear.

Significance

Physiologic joint loading plays a critical role in the maintenance of articular cartilage structure and function, whereas abnormal loading can lead to pathologic changes in joint tissues. However, the mechanisms by which mechanical loading is transduced into intracellular signals that regulate chondrocyte homeostasis are not fully understood. In this study, we show that the mechanosensitive cation channel transient receptor potential vanilloid 4 (TRPV4) plays a critical role in the physiological link between mechanical loading and chondrocyte function. Specifically, TRPV4 acts a transducer of mechanical loading to regulate cartilage extracellular matrix biosynthesis. A better understanding of the mechanisms involved in chondrocyte mechanotransduction could enable the development of novel therapies for joint diseases such as osteoarthritis.

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The goal of this study was to examine the hypothesis that TRPV4 transduces dynamic compressive loading into signals that regulate cartilage homeostasis. We first confirmed the presence of TRPV4 channels in chondrocyte-laden agarose constructs that produced $[Ca^{2+}]_i$ transients in response to hypoosmotic swelling and TRPV4 agonist GSK1016790A (GSK101). The TRPV4 antagonist GSK205 was used to examine the role of this channel in regulating the response of chondrocytes to mechanical loading, whereas the GSK101 and osmotic loading were used to evaluate the effects of TRPV4 activation in the absence of mechanical loading.

Results

TRPV4 Channel Function in Agarose-Embedded Chondrocytes. Immunofluorescence staining for TRPV4 revealed the presence of TRPV4 in the cellular membrane of chondrocytes 3 d after casting in agarose (Fig. 1*A*). Using fluorescence ratio imaging, Ca^{2+} concentrations in individual agarose-embedded chondrocytes were measured in response to increases or decreases in osmolarity and TRPV4 activation. A significant increase in Ca^{2+} signaling was observed with both hypoosmotic treatments (400→200 mOsm and 600→400 mOsm, $P < 0.001$ and $P < 0.01$, respectively), as well as GSK101 treatment ($P < 0.001$), compared with their respective isoosmotic controls (Fig. 1 *B* and *C*), whereas the percentage of cells exhibiting a $[Ca^{2+}]_i$ signal was the same between the two isoosmotic controls (400→400 mOsm and 600→600 mOsm) (Fig. 1*C*). GSK101 produced higher percent cell signaling than 600→400 mOsm ($P = 0.04$), but not the 400→200 mOsm group ($P = 0.58$); the 400→200 mOsm group was also not different from the 600→400 mOsm group ($P = 0.15$). While both hypoosmotic and GSK101 treatments caused more cells to signal, GSK101 treatment generated more $[Ca^{2+}]_i$ transients in signaling cells than the two hypoosmotic treatments (Fig. 1*D*, $P < 0.05$). Preincubation with the TRPV4 inhibitor GSK205 inhibited the effect of hypotonic loading (600→400 mOsm and 400→200 mOsm), as well as that of GSK101 treatment, and returned the percent of cells signaling back to control levels (400→200 mOsm+GSK205 vs. 400→400 mOsm: $P = 0.08$; 600→400 mOsm+GSK205 vs. 600→600 mOsm: $P = 0.49$; GSK101+GSK205 vs. 400→400 mOsm: $P = 0.29$). Neither hyperosmotic loading condition tested (200→400 mOsm, 400→600 mOsm) significantly affected the percentage of cells signaling compared with the 400→400 mOsm control, indicating that TRPV4 activation is specific to hypoosmotic loading, and that signaling occurs in response to relative, and not absolute, changes in external osmolarity.

TRPV4 Inhibition During Dynamic Loading Inhibits Mechanically Regulated Gene Expression. Chondrocyte-laden constructs were cultured for 2 wk before loading to allow a neopericellular matrix to form (Fig. S1 *A* and *B*), which is thought to be an important structure for the transduction of mechanical signals (5, 22, 23). Constructs were dynamically loaded for 3 h, both in the presence and absence of GSK205, following which the media was changed and constructs collected for RNA extraction 24 and 72 h later. Transient GSK205 exposure alone had no effect on gene expression levels (Fig. 2*A*). *ACAN* gene expression was not significantly affected by loading (24 h: $P = 0.34$; 72 h: $P = 0.25$), nor was *COL2α1* (24 h: $P = 0.84$; 72 h: $P = 0.60$). However, 72 h later Loading+GSK205 caused a decrease in *COL2α1* ($P < 0.01$) and an increase in *NOS2* ($P = 0.02$) gene expression compared with control, an effect that was not present with loading alone. In addition, *ADAMTS5* expression was decreased with loading 72 h after loading ($P < 0.001$) and this effect was partially attenuated by GSK205 (loaded vs. loaded + GSK205: $P < 0.01$). Loading and GSK205 treatment had no effect on *SOX9* gene expression; however, *TGF-β3* expression was significantly increased at 24 h ($P < 0.01$) with loading and this effect was fully blocked by GSK205 (control vs. loaded + GSK205: $P = 0.78$). This effect was also transient, as *TGF-β3* expression in the loaded group returned to control levels by 72 h. The anabolic and anti-inflammatory effect of mechanical loading on chondrocyte gene expression was also dependent on the 2-wk preculture period; when chondrocyte-laden constructs were loaded on day 0 (3 d after casting), *ACAN* and *COL2α1* gene expression was suppressed and inflammatory genes *ADAMTS5* and *NOS2* were highly up-regulated (Fig. S1).

TRPV4 Inhibition During Dynamic Loading Inhibits Mechanically Regulated Enhancement of Matrix Accumulation and Functional Properties. To determine if the enhancement of construct biochemical and functional properties in response to dynamic loading was also TRPV4-dependent, precultured constructs underwent daily dynamic compressive loading for 4 wk (3 h/d, 5 d/wk), in the

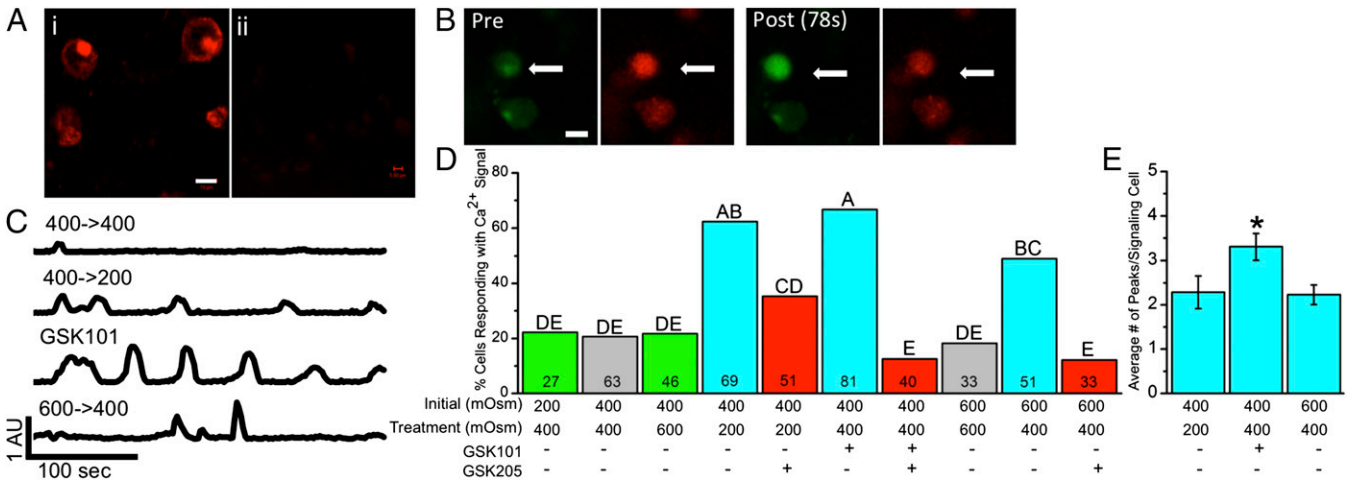

Fig. 1. TRPV4 function in agarose-embedded chondrocytes. (*A*) Positive labeling of TRPV4 in agarose-embedded chondrocytes (i) and no labeling in the no primary control (ii); scale bar, 10 μm. (*B*) Confocal images of a chondrocyte signaling in response to GSK101 (arrow), indicated by the increased ratio of green: red fluorescence post-GSK101 incubation; scale bar, 15 μm. (*C*) Representative Ca^{2+} traces for conditions that demonstrate significant $[Ca^{2+}]_i$ signaling compared with the isoosmotic control. (*D*) Percentage of cells responding to the below osmotic and chemical conditions. Data not sharing a common superscript letter indicate a significant difference ($P < 0.05$). Bars do not have error bars because the percent responding metric does not have an error associated with it. Numbers inside the bars are the total of number of cells in each group. (*E*) Average number of peaks elicited by a signaling cell during the imaging period. *$P < 0.05$ vs. other groups, $n = 5$–6, mean ± SEM.

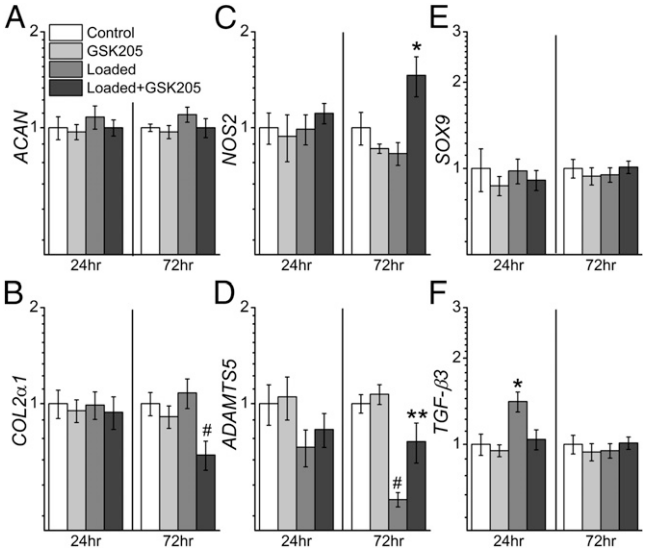

Fig. 2. TRPV4-dependent gene expression in 2-wk precultured chondrocyte-laden agarose constructs 24 and 72 h after mechanical loading. No change in (*A*) *ACAN* or (*E*) *SOX9* gene expression was observed. Mechanical loading enhanced *TGF-β3* (*F*) and suppressed *ADAMTS5* gene expression (*D*) in a TRPV4-dependent manner. Inhibition of TRPV4 during mechanical loading also enhanced *NOS2* (*C*) and suppressed *COL2α1* (*B*) gene expression. *greater than all other groups ($P < 0.05$); #smaller than all other groups ($P < 0.05$); **less than control and GSK205, greater than Loaded+GSK205 ($P < 0.05$), $n = 4$–6, mean ± SEM.

presence or absence of GSK205. Daily transient exposure to GSK205 had no effect on construct wet weight, biochemical content, or functional properties after 4 wk of culture (Fig. 3*A*). Dynamic loading significantly increased matrix accumulation as evidenced by the wet weight of the constructs ($P < 0.001$). Furthermore, inhibition of TRPV4 with GSK205 attenuated this response (Fig. 3*A*). Loaded disks also exhibited an increase in both total sulfated glycosaminoglycan (s-GAG) content ($P = 0.01$) and total collagen content ($P < 0.01$), whereas the presence of GSK205 prevented these increases (Fig. 3 *C* and *D*). Loaded disks also exhibited an increased dynamic modulus over free-swelling controls ($P = 0.02$), whereas loaded disks exposed to GSK205 had a significantly lower dynamic modulus than all other groups (Fig. 3*F*). In addition, although dynamic loading did not significantly affect the equilibrium Young's modulus of the constructs, the addition of GSK205 during loading significantly decreased this property (Fig. 3*E*, $P < 0.001$).

Direct Activation of TRPV4 Potently Drives Gene Expression, Biochemical, and Functional Changes Analogous To Dynamic Loading. To measure the direct effects of TRPV4 channel activation on chondrocyte gene expression, chondrocyte-laden constructs were transiently exposed to TRPV4 agonist GSK1016790A (GSK101). Similar to the effects of dynamic loading, *NOS2* ($P < 0.05$) and *ADAMTS5* ($P < 0.01$) gene expression were reduced 72 h after treatment with GSK101 (Fig. 4). *TGF-β3* gene expression was elevated 24-h posttreatment ($P < 0.001$), whereas GSK101 had no effect on *ACAN* (24 h: $P = 0.61$; 72 h: $P = 0.18$) and *SOX9* gene expression (24 h: $P = 0.50$; 72 h: $P = 0.94$). *COL2α1* (72 h) gene expression was also enhanced with GSK101 treatment ($P = 0.001$).

In addition to direct activation of TRPV4 using GSK101, we also examined the effects of osmotic loading as an alternative approach for activating TRPV4 and enhancing ECM production. We compared four different osmotic loading regimes (see *SI Materials and Methods* for details) and found that after 2 wk, both a +200-mOsm and −200-mOsm quasistatic stimulus enhanced total s-GAG accumulation (Fig. S2). We next compared this directly to 4 wk of daily GSK101 treatment, where we found GSK101 to be a more potent stimulus of matrix accumulation and functional property enhancement (Fig. 5). At both day 14 and day 28, daily GSK101 treatment caused an increase in wet weight and DNA per disk, as well as total s-GAG and collagen accumulation. When normalized to DNA content, s-GAG/DNA increased significantly with GSK101 treatment ($P = 0.001$), as did collagen–DNA ($P = 0.05$). Histologic staining for s-GAG and type II collagen further indicated an enhanced accumulation of both ECM components with GSK101. Agonist treatment also enhanced both the Young's ($P = 0.02$) and dynamic modulus ($P < 0.001$).

Discussion

Our results confirm the hypothesis that TRPV4 channel activation plays a critical role in the mechanoregulation of chondrocyte physiology and matrix metabolism in response to dynamic compressive loading. This study revealed a TRPV4-dependent response of chondrocytes to mechanical stimulation, involving transcriptional induction of anabolic growth factor gene expression and inhibition of proinflammatory mediators. This response was functionally validated in long-term culture, where inhibition of TRPV4 prevented mechanically induced chondrocyte ECM biosynthesis and matrix accumulation. The role of TRPV4 channel activation on chondrocyte physiology was further confirmed using direct chemical activation of TRPV4, which produced a similarly potent anabolic response as mechanical loading.

A compelling aspect of these findings is that they provide evidence for a specific transduction mechanism for an important but incompletely understood response of chondrocytes to mechanical loading that has been observed in a large number of previous studies. For example, compressive dynamic loading typically does not confer an anabolic response in chondrocyte-laden constructs for at least 2 wk (24), whereas the postponement of compressive loading for this same period is explicitly beneficial (25, 26). Moreover, whereas compressive loading of intact cartilage explants can stimulate proteoglycan synthesis immediately (1, 27), the response of chondrocytes embedded in agarose is enhanced with additional weeks of preculture (28). Cell–matrix interactions in the pericellular region are believed to play a critical role in transducing mechanical signals (29–31). Several studies

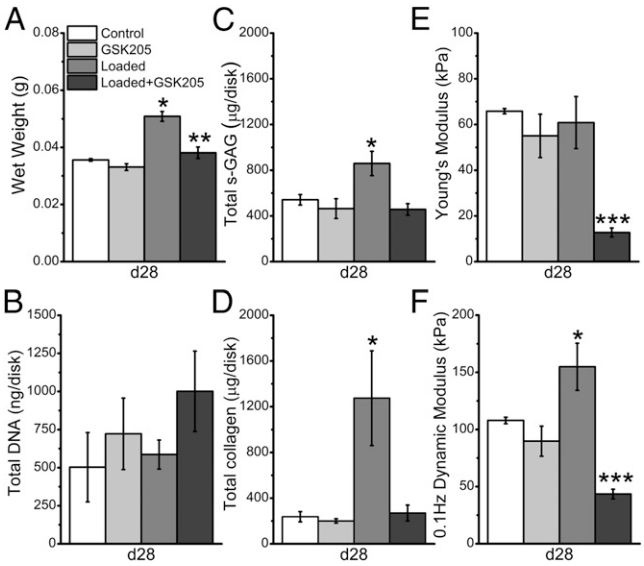

Fig. 3. TRPV4 antagonist GSK205 blocks the biosynthetic response of chondrocyte-laden constructs to dynamic mechanical loading. (*A*) Wet weight, (*B*) DNA content, (*C*) total s-GAG, (*D*) total collagen, (*E*) Young's modulus, and (*F*) dynamic modulus of constructs following 4 wk of mechanical loading. *greater than all other groups ($P < 0.05$); **greater than GSK205 ($P < 0.05$); ***smaller than all other groups ($P < 0.05$), $n = 3$–4, mean ± SEM.

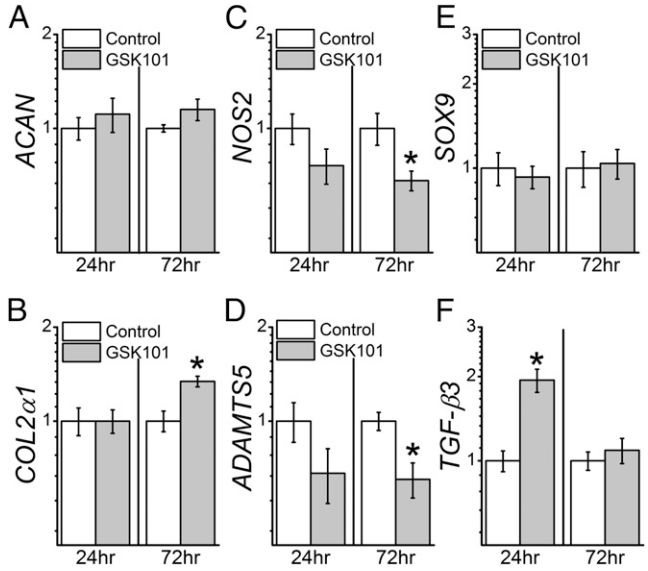

Fig. 4. TRPV4-dependent gene expression in 2-wk precultured chondrocyte-laden agarose constructs 24 and 72 h following TRPV4 agonist GSK101 treatment. GSK101 enhanced (*B*) *COL2α1* and (*F*) *TGF-β3* and decreased (*C*) *NOS2* (*D*) and *ADAMTS5* gene expression, with no effect on (*A*) *ACAN* and (*E*) *SOX9* expression. *greater than control ($P < 0.05$), $n = 4$–6, mean ± SEM.

have highlighted the association of preculture with accumulation of a proteoglycan-rich pericellular matrix (22, 23, 26, 32), which can contribute to strain shielding of the chondrocytes (22, 23), as well as the conversion of mechanical loading to changes in interstitial osmolarity (33, 34). The results of the present study not only reproduced this dependence of the anabolic effect of mechanical loading on preculture, but also suggest specific mechanisms involving the conversion of mechanical to osmotic stress, as well as the transduction of osmotic stress to an intracellular response via TRPV4 channel activation.

Ca^{2+} signaling elicited by hypoosmotic stress or GSK101 in our model system of chondrocyte-laden agarose constructs was attenuated back to control levels by GSK205, confirming that these responses were transduced via TRPV4. $[Ca^{2+}]_i$ transients were elicited by hypoosmotic challenge independent of base osmolarity, whereas isoosmotic and hyperosmotic loading had no effect on $[Ca^{2+}]_i$ signaling. These findings suggest that osmotically mediated activation of TRPV4 in chondrocytes is in response to relative, and not absolute, hypoosmotic stress. Thus, although cells may not signal in response to the hyperosmotic conditions of compression, they may be activated by relative hypoosmolarity when compression is removed. In addition, GSK101 elicited significantly more $[Ca^{2+}]_i$ transients during the imaging period than the hypoosmotic loading conditions (600→400 and 400→200mOsm), an indication that pharmacologic targeting of TRPV4 may provide a more potent method of activating TRPV4 than a single hypoosmotic stimulus of physiologic magnitude.

$[Ca^{2+}]_i$ signaling is believed to be one of the earliest events in the response of chondrocytes to mechanical stimulation (35–37). Previous studies have observed $[Ca^{2+}]_i$ signaling in response to mechanical loading (38, 39); interestingly, these studies detected $[Ca^{2+}]_i$ signaling in chondrocyte-laden constructs without substantial preculture (<72 h), although chondrocytes synthesize small amounts of pericellular proteoglycans even within 2 d of culture in agarose (29) that could contribute to mechanically induced $[Ca^{2+}]_i$ signaling. Thus, the effects of loading with and without preculture, observed in this study as well as in previous studies, may be due to differences in the characteristics of the $[Ca^{2+}]_i$ signal with preculture, or perhaps other environmental and cellular factors that change over time (24, 26, 28, 40). Although chondrocyte $[Ca^{2+}]_i$ signaling exhibits a high sensitivity to variations in osmolarity or volume (41–44), there is little or no sensitivity to physiologic levels of other physical stimuli, such as direct membrane stretch, suggesting that membrane connections to the pericellular matrix and/or intracellular cytoskeleton are important for the generation and transduction of $[Ca^{2+}]_i$ transients. TRPV4 has known physical interactions with both transmembrane proteins (integrins) (45, 46) and cytoskeletal elements (microtubules, actin, tubulin) (47, 48), and can transduce a diverse set of environmental signals (49). However, the complete

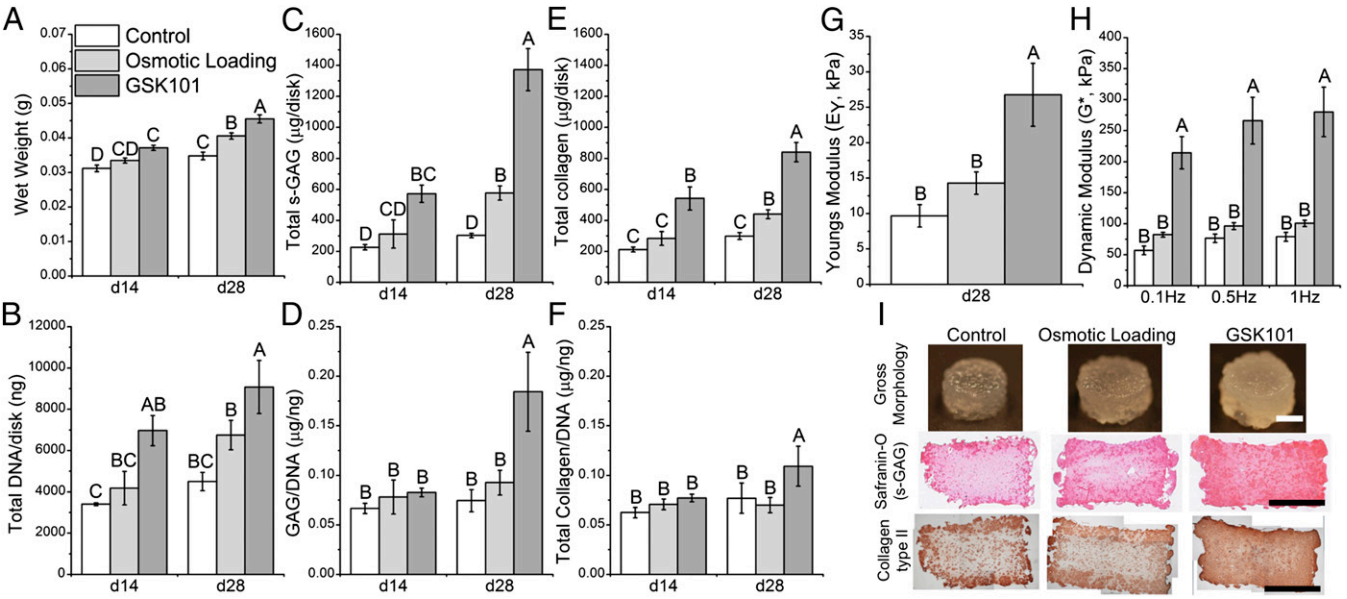

Fig. 5. TRPV4 activation via daily osmotic loading or GSK101 enhances extracellular matrix accumulation. (*A*) Wet weight, (*B*) DNA content, (*C*) total s-GAG, (*D*) s-GAG/DNA, (*E*) total collagen, (*F*) total collagen–DNA, (*G*) Young's modulus, and (*H*) dynamic modulus of constructs following 2 and 4 wk of stimulation. (*I*) Gross morphology, Safranin-O, and collagen type II immunostaining after 4 wk of culture; scale bar, 2 mm. Data not sharing a common superscript letter indicate a significant difference ($P < 0.05$), $n = 6$–8, mean ± SEM.

mechanism of TRPV4 channel activation during chondrocyte loading remains to be determined.

We observed transcriptional control of *ADAMTS5*, *NOS2*, *COL2α1*, and *TGF-β3* in response to mechanical loading or GSK101-mediated TRPV4 channel activation. Furthermore, inhibition of TRPV4 during loading prevented mechanoregulation of these genes. *ADAMTS5* is a previously identified mechanoresponsive gene in loaded chondrocyte constructs (26) and has also been implicated in mechanically induced models of OA (50). Interestingly, disruption of primary cilia, a structure also required for TRPV4-mediated transduction of osmotic swelling (8), has been shown to increase *ADAMTS5* gene expression (51). Mechanical loading of chondrocytes in agarose can reduce nitric oxide production (52), and our results suggest that mechanical activation of TRPV4 contributes to the suppression of *NOS2* expression. GSK101 enhanced *COL2α1* expression, and although *COL2α1* did not increase with loading, inhibiting TRPV4 during loading did reduce its expression. Thus, TRPV4-mediated Ca^{2+} appears to support ECM gene expression, particularly under dynamic mechanical loading. TRPV4-mediated enhancement of *COL2α1* gene expression has also been observed in the ATDC5 cell line (20). However, unlike the response of ATDC5 chondroprogenitors, we did not concurrently observe transcriptional enhancement of chondrocyte *SOX9* with TRPV4 activation, suggesting that fully differentiated articular chondrocytes use an alternate mechanism for *COL2α1* enhancement. Transforming growth factor (TGF) signaling plays an important role in cartilage development and homeostasis (53), as well as the response of other cell types to mechanical stimulation (54, 55). The mechanoresponsiveness of chondrocyte *TGF-β3* gene expression that preceded the regulation of the other genes measured in this study, along with its dependence on TRPV4 activity to mediate this response, represents an intriguing potential mechanism for the biophysical to biochemical transduction of mechanical loading in articular cartilage.

Inhibition of TRPV4 with GSK205 blocked the compositional and functional enhancement of mechanically loaded chondrocyte-laden constructs, further supporting the role of TRPV4-mediated mechanotransduction in regulating chondrocyte matrix metabolism. Functional testing of the mechanically loaded constructs revealed that the mechanically loaded disks with GSK205 had functional properties below that of the unloaded controls. Blocking TRPV4 during loading may have allowed for mechanical fatigue of the agarose in the setting of inhibited ECM production (e.g., a decrease in *COL2α1* expression) or perhaps increased catabolic or disrupted matrix organization processes (e.g., an increase in *NOS2 and ADAMTS5* expression). GSK101 treatment also produced increases in construct matrix accumulation and functional properties, which again could be due to increased production and/or suppressed catabolism and matrix organization. Future transcriptome-wide analyses (56, 57) may be useful in identifying the pathways that drive these loading-induced changes.

Activation of TRPV4 with GSK101 potently enhanced matrix accumulation and functional properties of the chondrocyte-agarose constructs, even in the absence of a preculture period. In addition, matrix deposition in GSK101-treated constructs appeared more uniform throughout the construct depth than in the control and osmotically treated groups (Fig. 5*I*). In this regard, the use of a small-molecule chemical agonist to accelerate tissue formation may have significant advantages over the use of growth factors or direct mechanical stimulation in a bioreactor, particularly for large anatomically shaped tissue grafts (58), where diffusion limitations or an inhomogeneous mechanical environment may lead to nonuniform tissue deposition. Determining whether TRPV4 targeting can also enhance the chondrogenesis and maturation of human stem-cell-based tissue engineered cartilage is an important next step under investigation.

In summary, the findings from this study demonstrate that TRPV4 activation is a key mechanism of mechanical signal transduction in articular chondrocytes. This finding further highlights the ability of mechanotransduction pathways to have profound effects on cellular physiology and function, as well as the ability of ion channel modulators to control these functions. Further understanding of this process may provide insights into the development of new therapies for cartilage repair or OA, as well as other diseases related to aberrant mechanotransduction (59).

Materials and Methods

Cell Culture. All cell culture was performed in standard feed media (DMEM-High Glucose, 10% FBS, 0.1 mM nonessential amino acids, 15 mM Hepes, 40 μg/mL L-proline, 1× antibiotics–antimycotics, and fresh 50 μg/mL vitamin C) unless otherwise noted, at 37 °C and 5% CO_2. Media osmolarity was measured and adjusted as detailed in *SI Materials and Methods*. To modulate TRPV4 channel activity, media was supplemented with 10 μM GSK205, a TRPV4 selective antagonist (8), or 1 nM GSK101 (Sigma-Aldrich), a TRPV4 selective agonist, with controls receiving the same amount of vehicle (0.1–0.2% DMSO) as the experimental groups. To limit exposure to these TRPV4 modulators, every treatment with GSK205 and GSK101 was followed by a wash in fresh feed media, aspiration, and a new feed of fresh media.

Cell Isolation and Construct Formation. Full-thickness porcine articular chondrocytes were enzymatically isolated from the femurs and ulnas of skeletally immature pigs (~30kg) as described previously (8) and frozen in freezing media (10% DMEM-High Glucose, 10% DMSO, 80% FBS). Thawed and pooled cell suspensions from at least five donors were washed once with PBS, suspended in feed media, and mixed 1:1 with 4% molten type VII agarose (Sigma-Aldrich) to form chondrocyte-laden disks (2% agarose, 4 ⌀ × 2.25 mm, 10–20 M cells/mL). All constructs were allowed 3 d to equilibrate before the start of each study (day 0). Media was changed for five consecutive days a week, except for the constructs used for Ca^{2+} imaging and during preculture periods, in which media was changed every 2–3 d.

Calcium Imaging. $[Ca^{+2}]_i$ was measured in the chondrocyte-laden agarose constructs within the first week of culture as described in *SI Materials and Methods*.

Custom Loading Bioreactor. Dynamic compressive loading was performed as previously described (60). Briefly, a closed-loop displacement controlled bioreactor with 24 individual polyacetal pistons connected to a linear stage driven by a stepper motor was used to deliver a 10% peak-to-peak sinusoidal strain (7% offset) at 1 Hz for 3 h/d. Unloaded control constructs were handled and cultured alongside loaded constructs.

Real-Time PCR. At day 0 and day 14, constructs intended for gene expression analysis were changed to their experimental media conditions and incubated statically or mechanically loaded for 3 h. After 24 or 72 h, whole constructs were stabilized in RNALater (Qiagen) and processed for quantitative real-time PCR analysis as described in *SI Materials and Methods*, using custom-designed primers (Table S1).

Biochemical Assays. Detailed methods can be found in *SI Materials and Methods*.

Histology and Immunohistochemistry. Detailed methods can be found in *SI Materials and Methods*.

Mechanical Testing. Detailed methods can be found in *SI Materials and Methods*.

Statistical Analysis. Data were analyzed using multiple-factor ANOVA, followed by a Fisher's post hoc test when appropriate. Nominal data were compared between treatment groups using the χ^2-test.

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