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Transient receptor potential vanilloid 4 as a regulator of induced pluripotent stem cell chondrogenesis

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Abstract

Transient receptor potential vanilloid 4 (TRPV4) is a polymodal calcium-permeable cation channel that is highly expressed in cartilage and is sensitive to a variety of extracellular stimuli. The expression of this channel has been associated with the process of chondrogenesis in adult stem cells as well as several cell lines. Here, we used a chondrogenic reporter (*Col2a1-GFP*) in murine induced pluripotent stem cells (iPSCs) to examine the hypothesis that TRPV4 serves as both a marker and a regulator of chondrogenesis. Over 21 days of chondrogenesis, iPSCs showed significant increases in *Trpv4* expression along with the standard chondrogenic gene markers *Sox9*, *Acan*, and *Col2a1*, particularly in the green fluorescent protein positive (GFP+) chondroprogenitor subpopulation. Increased gene expression for *Trpv4* was also reflected by the presence of TRPV4 protein and functional Ca²⁺ signaling. Daily activation of TRPV4 using the specific agonist GSK1016790A resulted in significant increases in cartilaginous matrix production. An improved understanding of the role of TRPV4 in chondrogenesis may provide new insights into the development of new therapeutic approaches for diseases of cartilage, such as osteoarthritis, or channelopathies and hereditary disorders that affect cartilage during

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AUTHOR CONTRIBUTIONS

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Wolfgang Liedtke and Farshid Guilak contributed equally to this study.

CONFLICT OF INTEREST

V.P. Willard and F. Guilak are employees and shareholders of Cytex Therapeutics, Inc. The other authors declared no potential conflicts of interest.

development. Harnessing the role of TRPV4 in chondrogenesis may also provide a novel approach for accelerating stem cell differentiation in functional tissue engineering of cartilage replacements for joint repair.

Keywords

adipose stem cell; hypertrophy; mesenchymal stem cell; tissue engineering; TRP channel

1 | INTRODUCTION

Transient receptor potential vanilloid 4 (TRPV4) is a polymodal calcium-permeable cation channel that is sensitive to a variety of extracellular stimuli and can serve as an integrator of physical and chemical signals in the cellular microenvironment. First discovered in 2000,^{1,2} TRPV4 was initially found to serve as a transducer of osmotic signals.³⁻⁵ It has since been demonstrated to function in signaling related to pain and inflammation.⁶⁻⁸ Subsequently, TRPV4 has been found to be expressed and functionally relevant in several musculoskeletal tissues, including cartilage,⁹⁻¹¹ bone,^{12,13} intervertebral disc,¹⁴ and synovium,^{15,16} where it plays highly complex and diverse roles in different contexts.

In particular, TRPV4 is highly expressed in cartilaginous tissues and is essential in skeletal development as well as joint homeostasis. For example, gain-of-function mutations in TRPV4, termed “channelopathies,” can result in skeletal dysplasias that range from mild deformities to neonatal lethality.¹⁷⁻²¹ In chondrocytes, TRPV4 helps to maintain cartilage homeostasis by serving as a mechanosensor, secondary to osmotic changes in the cartilage matrix caused by mechanical loading.¹¹ In this respect, TRPV4 has been shown to regulate the anabolic response of chondrocytes to compressive loading.²² The global loss-of-function of TRPV4 (eg, *Trpv4*^{-/-} mice) results in premature cartilage loss secondary to bone remodeling,⁹ whereas cartilage-specific deletion of *Trpv4* appears to protect against age-related osteoarthritis,²³ revealing the diverse function of this ion channel in different tissues.²⁴

The effects of TRPV4 on skeletal development appear to involve the regulation of stem and progenitor cell differentiation. In ATDC5 and C3H10T1/2 cell lines undergoing chondrogenesis, the expression pattern of TRPV4 was found to be similar to those of chondrogenic marker genes, such as type II collagen (*Col2a1*) and aggrecan (*Acan*).¹⁰ A similar pattern of TRPV4 expression has also been observed in human induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), and adipose stem cells (ASCs) undergoing chondrogenesis.²⁵⁻²⁸ Furthermore, ASCs isolated from *Trpv4*^{-/-} mice exhibit reduced chondrogenic differentiation potential and enhanced osteogenic potential compared to ASCs from *Trpv4*^{+/+} mice.²⁹ Interestingly, the chemical activation of TRPV4 induced *Sox9*-dependent reporter activity,¹⁰ and iPSCs derived from patients with a lethal skeletal dysplasia caused by TRPV4 mutation show significantly decreased chondrogenesis and expression of *COL2A1*, *SOX9*, *ACAN*, type X collagen (*COL10A1*), and runt-related protein 2 (*RUNX2*).³⁰ Taken together, these findings suggest that TRPV4 is not only a

marker of differentiation, but could serve as a functional role in chondrogenesis, upstream of other chondrogenic genes such as *SOX9*.

Here, we examined the hypothesis that TRPV4 serves both as a marker and a regulator of chondrogenesis in iPSCs. Due to their virtually unlimited expansion and differentiation potentials, iPSCs can provide an excellent model system for studying cartilage development or disease modeling in vitro using genetically defined populations of cells.³¹ In this regard, iPSCs may provide an adaptable system to study the functional role of this channel under physiologic conditions as well as with TRPV4 channelopathies. However, even using clonal cell lines, iPSCs tend to exhibit heterogeneous differentiation.²⁸ In this study, we used a *Col2a1-GFP* reporter in murine iPSCs³² to determine if TRPV4 is specifically expressed as a marker of the chondrogenic subpopulation sorted from differentiated iPSCs, and to determine if TRPV4 serves not only as a marker but also plays a functional role in regulating chondrogenesis in iPSC-derived chondrocytes. Understanding the role of TRPV4 in chondrogenesis will improve our understanding of cartilage development and hopefully lead to new methods to accelerate stem cell differentiation for applications in cartilage tissue engineering.

2 | MATERIALS AND METHODS

2.1 | iPSCs culture and differentiation

iPSCs were derived, cultured, and differentiated toward the chondrogenic lineage as previously described.³¹⁻³³ Briefly, tail fibroblasts from adult C57BL/6 mice were reprogrammed to pluripotency with a single doxycycline-inducible lentiviral vector that expressed mouse cDNAs for Oct4 (Pou5f1), Sox2, Klf4, and c-Myc.³⁴ Undifferentiated iPSCs were maintained on mitomycin C-treated mouse embryonic feeder (MEFs, MilliporeSigma, Burlington, Massachusetts) in iPSC media containing Dulbecco's modified Eagle medium-high glucose (DMEM-HG, Gibco, Waltham, Massachusetts), 20% lot-selected fetal bovine serum (FBS) (Atlanta Biologicals, Minneapolis, Massachusetts), 100 nM Modified Eagle Medium (MEM, Gibco), nonessential amino acids (NEAA, Gibco), 55 μ M β -mercaptoethanol (2-me, Gibco), 25 ng/mL gentamicin (Gibco), and 1000 U/mL mouse leukemia inhibitory factor (LIF, MilliporeSigma). Nucleofection (Amaxa, Lonza, Basel, Switzerland) of a construct with the *Col2a1* promoter driving green fluorescent protein (GFP)³⁵ was used to generate iPSC clones with stable integration of the reporter. Chondrogenic differentiation was carried out in high density micromass culture for 15 days while cultured in serum-free chondrogenic medium containing DMEM-HG, NEAA, 2-me, insulin-transferrin-selenous acid (ITS+ premix, BD, Franklin Lakes, New Jersey), penicillin-streptomycin, 50 μ g/mL L-ascorbic acid 2-phosphate, and 40 μ g/mL L-proline. For days 3 to 5 only, 50 ng/mL murine BMP-4 (R&D Systems, Minneapolis, Minnesota) and 100 nM dexamethasone (Sigma, St. Louis, Missouri) were added to the medium. At 0, 7, 14, and 21 days, cells within the micromasses were lysed, total RNA was isolated (Norgen Biotek), and cDNA was generated using superscript VILO (Life Technologies, Carlsbad, California). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green (Applied Biosystems, Waltham, Massachusetts) and gene-specific primers for *Nanog*, *Sox2*, *Vim*, *Sox9*, *Acan*, *Col2a1*, and *Trpv4*, provided in Table

1. Fold changes in gene expression were calculated with the 2^{-Ct} method using 18S rRNA as a house-keeping gene. At each time point, additional micromasses were lysed with RIPA buffer containing protease and phosphatase inhibitors (Sigma). Thirty μg of total protein per sample was used for Western blots against SOX2 (#14962), TRPV4 (#65893), and β -actin (#8457) (all antibodies from Cell Signaling Technology, Danvers, Massachusetts). Finally, micromasses at each time point were fixed with 4% paraformaldehyde and stained for sulfated glycosaminoglycan (sGAG) with Alcian blue as an indicator of chondrogenesis. To ensure reproducibility of the results, the full differentiation process from pluripotent cells to micromasses, GFP sorting, and subsequent culture (as described below) was repeated three times. The total sample sizes for each assay (indicated in the figure legends) include biological replicates collected over the course of three separate differentiation experiments.

2.2 | *Trpv4* expression in sorted chondrogenitor subpopulations of iPSCs

To determine the specificity of *Trpv4* expression to the chondrogenic subpopulation of iPSCs, a *Col2a1-GFP* reporter integrated in the cells was used to sort the chondrogenic cell population (GFP+) from the nonchondrogenic cells (GFP-) as described previously.³² At the end of the 15-day culture period, micromasses were digested and single cells were sorted on the basis of positive GFP expression using flow cytometry. GFP+ cells were expanded for two passages in chondrogenic medium with the addition of 10% FBS and 4 ng/mL basic fibroblast growth factor (Roche). After sorting, cells were lysed for RNA isolation and subsequent qRT-PCR to measure expression of *Nanog*, *Sox9*, *Acan*, *Col2a1*, and *Trpv4* as described above, or seeded on gelatin-coated coverslips for 48 hours for TRPV4 immunocytochemistry. Cells were fixed with 4% paraformaldehyde, permeabilized, and labeled with Hoechst 33342 and an anti-TRPV4 antibody (#ACC-034, Alomone Labs, Jerusalem, Israel). Undifferentiated iPSCs and knee articular chondrocytes (enzymatically isolated from the femoral condyles of 12-day old C57BL/6 mice) served as controls.

2.3 | Ca^{2+} signaling and functional activity of TRPV4

To determine the expression and functional activity of TRPV4, samples were collected and sorted into GFP+ and GFP- groups. Cells were expanded for 48 hours, and TRPV4-mediated Ca^{2+} signaling was measured in the sorted cell populations using confocal calcium ratio imaging or a fluorometric imaging plate reader. For confocal imaging, cells were seeded onto gelatin-coated coverslips at 10 k cells/cm². Cells were loaded with Ca^{2+} sensitive dyes, Fluo4-AM and Fura Red (ThermoFisher Scientific, Waltham, Massachusetts),³¹ and imaged using a laser scanning confocal microscope (LSM 510, Zeiss). After measuring dose-dependent response to 0, 0.1, 1, 10, or 100 nM GSK1016790A (GSK101), a TRPV4-specific agonist, cells were stimulated with 0, 1, or 10 nM GSK101 to induce Ca^{2+} signaling. Undifferentiated iPSCs and primary articular chondrocytes from 12-day-old mice served as controls. To determine the mechanisms of Ca^{2+} , cells were seeded into gelatin-coated 96-well plates at 10 k cells/cm² and imaged using a fluorometric imaging plate reader (FLIPR, Molecular Devices). To determine the source of Ca^{2+} flux, cells were treated with 10 nM GSK101 with or without 10 μM GSK205 (TRPV4-specific inhibitor), 3 μM thapsigargin (inhibitor of intracellular Ca^{2+} release; MilliporeSigma), or in Ca^{2+} -free medium containing 10 mM EGTA (MilliporeSigma).^{11,36}

2.4 | Effects of dynamic TRPV4 activation on chondrogenesis and matrix formation

To determine if dynamic TRPV4 activation could stimulate chondrogenesis and cartilage matrix accumulation, GFP⁻ and GFP⁺ populations were sorted based on the *Col2a1-GFP* reporter. The cells were subsequently passaged twice and then reformed in micromass culture. For 7 days, micromasses were cultured in chondrogenic medium containing 10 ng/mL transforming growth factor beta 3 (TGF- β 3) and 10 ng/mL bone morphogenetic protein 6 (BMP-6) (R&D Systems) to induce chondrogenesis. These growth factors were then removed, and the micromasses were cultured for 14 more days with daily treatment of either 10 nM GSK101, or 10 nM GSK101 + 10 μ M GSK205³⁶ for 1 hour per day. After the 1-hour treatment, micromasses were washed with warm phosphate buffered saline and fed fresh culture medium. On day 21, micromasses were digested in papain (MilliporeSigma) overnight and then analyzed using the PicoGreen assay (Life Technologies) to measure double-stranded DNA, the hydroxyproline assay for measuring total collagen content, and the dimethylmethylene blue assay for measuring the total sulfated glycosaminoglycan content of tissues.^{31,32} To determine the chondrogenic response to GSK101 treatment at the transcriptional level, cells within micromasses were lysed for RNA isolation and subsequent qRT-PCR as described previously, using the gene-specific primers for *Sox9*, *Acan*, and *Col2a1* from Table 1. Additional micromasses were fixed with 4% paraformaldehyde, paraffin embedded, and cross-sections were cut at 8- μ m thickness. The resulting slides were stained with Safranin-O/fast green/hematoxylin. Immunohistochemistry for collagens type I (#72026, Cell Signaling Technology) and II (#II-II6B3, Developmental Studies Hybridoma Bank, Iowa City, Iowa) was performed as described previously.³² Tibial plateaus from adult C57BL/6 mice served as positive controls for osteochondral staining.

2.5 | Statistical analysis

Statistical analysis was performed using a one-way analysis of variance with a Tukey's HSD post hoc test where applicable ($\alpha = .05$). Chi-square analysis was used for confocal calcium ratio imaging to determine differences in the percentage of cells responding to GSK101 ($\alpha = .05$).

3 | RESULTS

3.1 | *Trpv4* expression increases during chondrogenesis of iPSCs

Over 21 days of micromass culture, iPSCs underwent chondrogenic differentiation as exhibited by increased Alcian blue staining (Figure 1A). Quantitative RT-PCR showed significant decreases in expression for the pluripotency genes *Nanog* and *Sox2* during this process, while the mesenchymal marker *Vim* and chondrogenic markers *Sox9*, *Acan*, and *Col2a1* were increased by approximately 20-, 100-, 90-, and 1600-fold, respectively (Figure 1B). Concomitantly, *Trpv4* expression increased by over 50-fold during chondrogenesis. Western blot analysis of SOX2 and TRPV4 confirmed that these changes in gene expression resulted in accompanying changes in protein production during micromass differentiation (Figure 1C).

3.2 | *Trpv4* expression is specific to the chondrogenic cell population identified by a *Col2a1-GFP* reporter

Following 15 days of chondrogenic culture in micromass, cells were enzymatically isolated and sorted into GFP⁺ and GFP⁻ populations based on a *Col2a1-GFP* reporter. Expression for the pluripotency gene *Nanog* decreased significantly for both sorted populations and primary chondrocytes, as compared to undifferentiated iPSCs (Figure 2). GFP⁺ cells showed significantly increased *Sox9*, *Acan*, *Col2a1*, and *Trpv4* expression as compared to GFP⁻ cells or undifferentiated iPSCs. GFP⁺ cells also showed higher *Sox9* and *Trpv4* expression, but lower *Acan* and *Col2a1* expression relative to primary chondrocytes. Immunocytochemistry labeling for TRPV4 protein confirmed that expression was specific to the GFP⁺ population (Figure 2).

3.3 | GFP⁺ chondroprogenitor cells exhibit TRPV4-mediated Ca²⁺ signaling

Ca²⁺ measurements indicated that GFP⁺ cells had a higher level of basal signaling compared to the GFP⁻ cells or undifferentiated iPSCs (Figure 3A). GFP⁺ chondroprogenitor cells and primary chondrocytes showed significantly higher TRPV4-mediated Ca²⁺ signaling with GSK101 treatment as compared to iPSCs or GFP⁻ cells using either confocal fluorescence or FLIPR assays (Figure 3). The GFP⁺ population also increased Ca²⁺ signaling in response to a low dose of GSK101, while the GFP⁻ and iPSC groups only increased signaling in response to a high dose of GSK101 (Figure 3B,C). Calcium signaling showed a clearly dose-dependent response to GSK101, statistically significant at 10 nM GSK101 (Figure 3D). TRPV4-mediated Ca²⁺ signaling was inhibited by the TRPV4-specific inhibitor GSK205 and by removal of Ca²⁺ from the extracellular medium, indicating TRPV4-mediated Ca²⁺ influx was necessary for the observed signaling response (Figure 3E). Thapsigargin treatment did not result in a statistically significant inhibition of Ca²⁺ signaling.

3.4 | Transient activation of TRPV4 enhances cell proliferation and chondrogenesis in iPSC-derived chondroprogenitors

GFP⁺ and GFP⁻ cells were formed into micromasses and were treated with GSK101 or GSK101+GSK205 for 1 hour per day, in the absence of chondrogenic growth factors. In control conditions, micromasses generated from GFP⁺ cells contained higher sGAG/DNA ratio than micromasses generated from GFP⁻ cells, consistent with previous findings.³² Activation of TRPV4 with GSK101 significantly increased DNA content as well as sGAG/DNA ratio compared to control conditions or treatment with GSK101 and GSK205, notably only in micromasses containing GFP⁺ cells (Figure 4B). Total collagen/DNA was not affected, underscoring the specificity of the observed response as well as the general metabolic health of the GFP⁻ cells. qRT-PCR revealed similar results at the transcriptional level, with GFP⁺ cells displaying significantly greater *Sox9* and *Acan* expression in response to GSK101 treatment compared to controls conditions, consistent with the observed increase in sGAG production (Figure 4C). These changes were not observed in the GFP⁻ cells. Expression of *Col2a1* was not significantly altered in either cell population in response to TRPV4 activation, consistent with no observed changes in total collagen production. Histologically, significant increases in tissue accumulation and Safranin-O staining were observed only in GFP⁺ micromasses treated with GSK101 (Figure

5). The stimulatory effects of GSK101 on the GFP+ cells were inhibited by the TRPV4 antagonist, GSK205. Across the treatment groups, micromasses formed from GFP+ cells stained strongly for collagen II with minimal collagen I signal, indicative of a chondrogenic phenotype (Figure 5). In contrast, GFP- micromasses stained strongly for collagen I with minimal collagen II staining.

4 | DISCUSSION

Our findings indicate that TRPV4 serves as critical regulator of chondrogenesis in iPSCs. TRPV4 expression increased over time in micromass-differentiated iPSCs, paralleling the expression of chondrogenic markers such as *Sox9*, *Acan*, and *Col2a1*. Importantly, this expression was specific to the chondrogenic cell population (GFP+) and absent in the nonchondrogenic population (GFP-). Furthermore, gene expression of *Trpv4* led to functional expression of TRPV4 protein as well as the formation of functional ion channels, as verified by fluorescence imaging of Ca²⁺ signaling. Importantly, we found that TRPV4 serves not just as a marker of chondrogenesis, but also as a regulator of chondrogenesis and cartilage extracellular matrix synthesis. In the purified GFP+ chondrogenic cell population, daily activation of TRPV4 increased cell number and matrix production, mirroring the response of primary chondrocytes.²²

TRPV4 gene and protein expression increased significantly and monotonically in iPSC micromasses as they underwent chondrogenesis. This finding is consistent with our previous observations in human stem cells, such as bone marrow-derived MSCs and ASCs, which exhibit increases of several orders of magnitude in *Trpv4* expression during chondrogenesis.²⁵⁻²⁸ During development, *Trpv4* is one of the earliest expressed genes in cartilage, appearing during mesenchymal condensations at 12.5 days post coitum.³⁷ These findings suggest that in a wide range of stem/progenitor cells, TRPV4 serves as a marker of chondrogenesis, with expression patterns similar to classical chondrogenic marker genes such as *Sox9*, *Col2a1*, and *Acan*, providing an additional indicator of chondrogenic differentiation.

The purification of chondrogenic progenitors using a *Col2a1-GFP* reporter revealed that in the heterogeneous population of cells within a differentiating micromass, the gene and protein expression of TRPV4 as well as functional activity of TRPV4 was selectively localized to the GFP+ population. Furthermore, the GFP+ cells showed significantly higher sensitivity to the TRPV4 agonist, GSK101, as verified by single-cell and FLIPR-based imaging of Ca²⁺ signaling. This TRPV4-mediated Ca²⁺ signaling required influx of extracellular Ca²⁺, as the response was inhibited by GSK205 or removal of Ca²⁺ from the medium, but not by inhibition of intracellular Ca²⁺ release by thapsigargin. While using the *Col2a1-GFP* reporter provided a robust method for purifying the chondrogenic cell population, it also prevented us from validating these results using iPSC lines of different genetic backgrounds. We have recently published work describing the purification of highly chondrogenic cells from differentiated hiPSC lines with or without the use of a genetic reporter,²⁸ allowing future studies to further validate these findings in human cells.

Not only was *Trpv4* expression and function associated with chondrogenesis, we also observed that activation of TRPV4 signaling in GFP+ cells resulted in increased expression of chondrogenic marker genes as well as enhanced chondrogenic matrix production as observed by increased cell number and sGAG/DNA ratio. The increase in biosynthetic activity induced by the GSK101 TRPV4-activator was specific to TRPV4, as it was inhibited by GSK205, a TRPV4-selective antagonist. Increases in chondrogenic marker expression, matrix production, and cell proliferation were not observed in the GFP- (nonchondrogenic) cells after GSK101 stimulus, possibly due to the distinct Ca²⁺ signaling response of these cells, or due to this heterogeneous cell population having already differentiated into other cell lineages. The enhanced biosynthesis of GFP+ cells is consistent with previous studies showing that stimulation of TRPV4 with mechanical, osmotic, or chemical activators results in a strong anabolic response in primary chondrocytes, characterized by significant increases in extracellular matrix synthesis and accumulation.^{22,38,39} It is important to note that while mechanical factors can enhance chondrogenesis,⁴⁰ the ability to accelerate cartilage tissue formation using a small molecule chemical agonist may have significant advantages over the use of growth factors or direct mechanical stimulation (eg, in a bioreactor), particularly for large, inhomogeneous, or anatomically shaped tissue grafts.^{41,42}

The specific mechanisms involved in TRPV4 enhancement of chondrogenesis remain to be determined. Previous studies suggest that activation of TRPV4 drives expression of *Sox9* via Ca²⁺/calmodulin signaling,¹⁰ which is known to play a role in cartilage homeostasis and interact with the TGF- β pathway.^{43,44} TGF- β and TRPV4 signaling pathways demonstrate synergistic transcriptomic profiles, including targets of SMAD3, JUN, and SP1, while also displaying Ca²⁺/calmodulin-dependence.⁴⁴ Together with our results, these findings depict TRPV4 as an active member in chondrogenic development, where it directly drives chondrogenic gene expression, including *Sox9*, by Ca²⁺/calmodulin-transduced interaction with the TGF- β pathway.^{10,14} It is also of interest to note that activation of TRPV4 by GSK101 appears to require the presence of extracellular matrix molecules,²² particularly hyaluronic acid,⁴⁵ which may be associated with the increased matrix production observed in GFP+ cells.

5 | CONCLUSION

In summary, our findings indicate that TRPV4 serves both as a marker and a regulator of iPSC chondrogenesis. Within a heterogeneous population of cells in a micromass, the enhancement of chondrogenesis appears to occur specifically in the GFP+, chondroprogenitor cell population. An improved understanding of the role of TRPV4 in chondrogenesis promises to provide new insights into the development of new therapeutic approaches for diseases of cartilage such as osteoarthritis or hereditary disorders that affect cartilage during development.^{46,47} The role of TRPV4 signaling in iPSC-derived chondrocytes mimics that seen in native articular chondrocytes; thus, iPSC chondrogenesis could provide a model system for studying the role of TRPV4 and TRPV4 channelopathies in cartilage development and pathogenesis. Controlled modulation of activity of TRPV4 evolves as a promising novel approach for optimizing stem cell differentiation in functional tissue engineering of cartilage replacements for joint repair.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Significance statement

Transient receptor potential vanilloid 4 (TRPV4) is an osmo- and mechanosensitive cation channel that is highly expressed in chondrocytes. This study examined the hypothesis that TRPV4 serves as a marker as well as a regulator of chondrogenesis in induced pluripotent stem cells. During the process of chondrogenesis, *Trpv4* expression mirrored the expression of classic chondrogenic markers (e.g., *Sox9*, *Acan*, and *Col2a1*). Furthermore, intermittent daily activation of TRPV4 significantly increased cartilaginous matrix production. An improved understanding of the role of TRPV4 in chondrogenesis may provide new insights into the development of new therapeutic approaches for diseases that affect cartilage.

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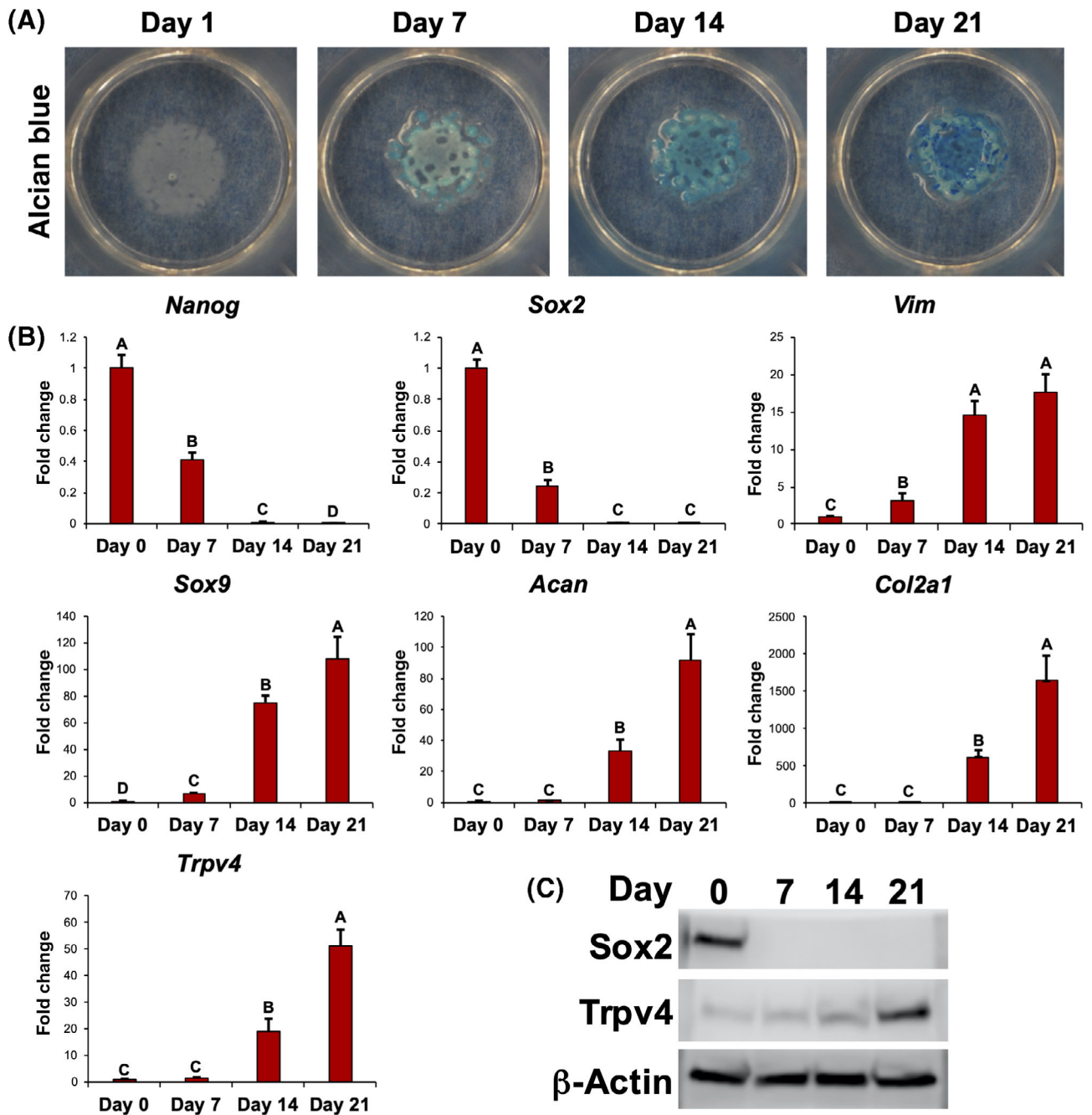


FIGURE 1.

Trpv4 expression during chondrogenesis of induced pluripotent stem cells (iPSCs). A, Alcian blue staining of iPSCs undergoing chondrogenic differentiation over 21 days. B, Gene expression for pluripotency genes *Nanog* and *Sox2*, mesenchymal marker *Vim*, and chondrogenic markers *Sox9*, *Acan*, and *Col2a1*, and *Trpv4*. Mean \pm SEM, $n = 5$ per group, groups not sharing the same letter are statistically different from one another (analysis of variance with Tukey's post hoc, $P < .05$). C, Western blots for SOX2 and TRPV4, as well as loading control β -actin

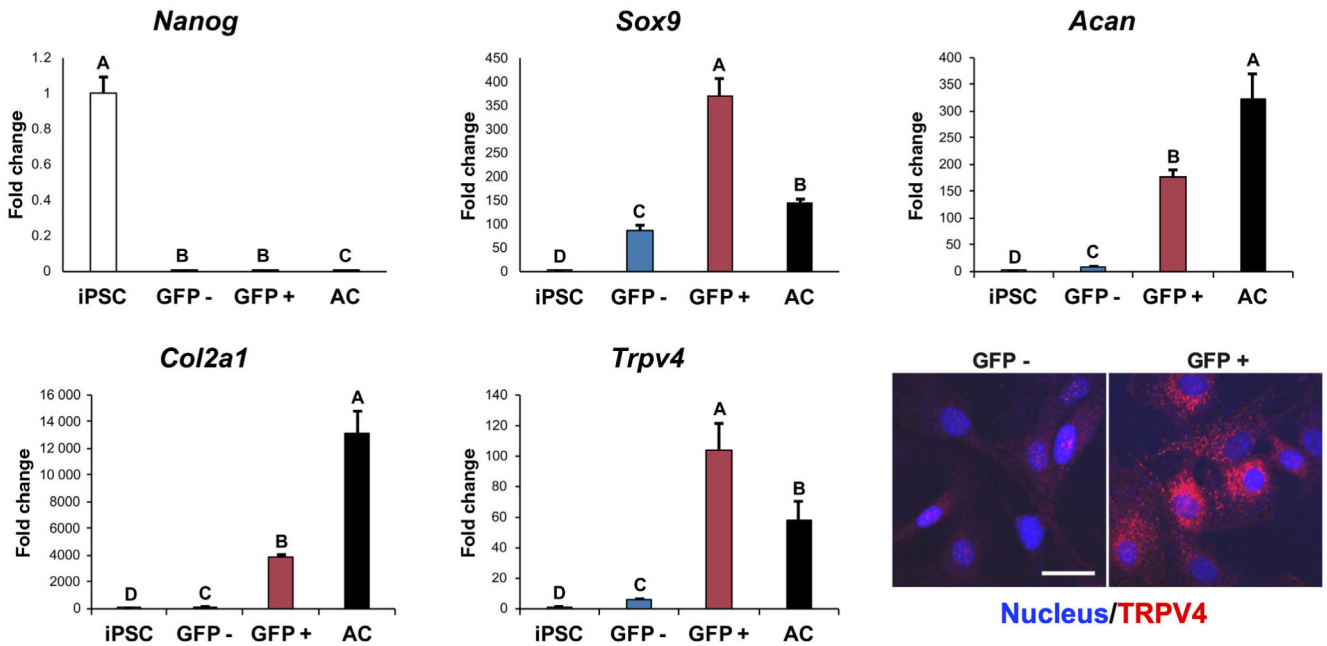
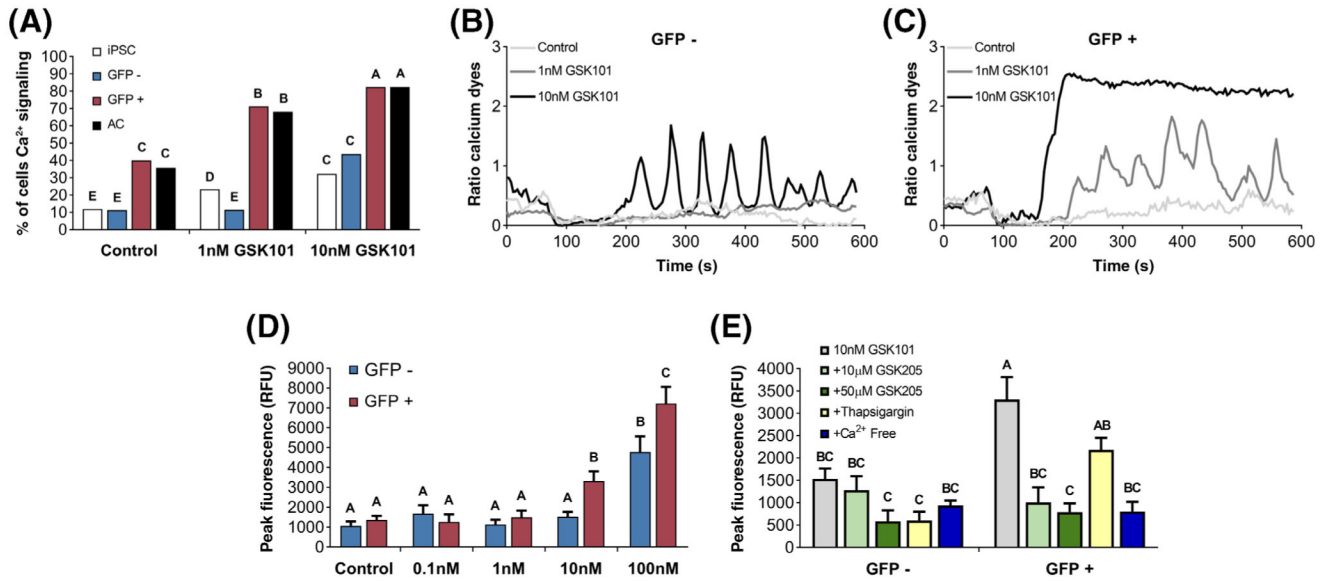
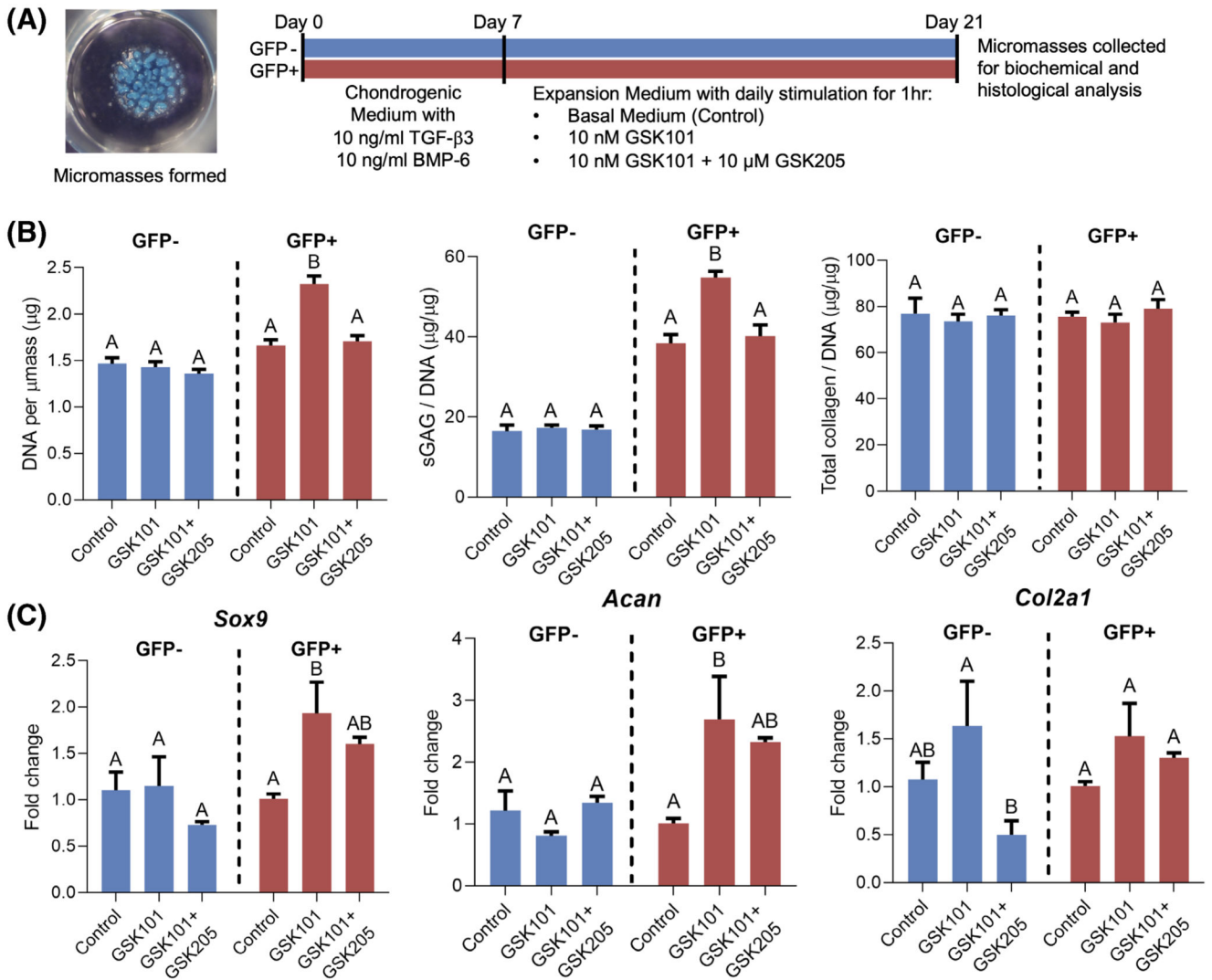


FIGURE 2.

Trpv4 expression within chondrogenic cell population identified by *Col2a1-GFP* reporter. Induced pluripotent stem cells (iPSCs) underwent chondrogenesis for 15 days and were sorted into GFP+ and GFP- populations based on a *Col2a1-GFP* reporter before quantitative reverse transcription polymerase chain reaction (qRT-PCR) gene expression analysis for pluripotency gene *Nanog*, chondrogenic markers *Sox9*, *Acan*, and *Col2a1*, and *Trpv4*. Controls: undifferentiated iPSCs and isolated primary murine articular chondrocytes (AC). Mean \pm SEM, $n = 5$, groups not sharing the same letter are statistically different from one another (analysis of variance with Tukey's post hoc, $P < .05$). Bottom right: GFP+ cells also showed positive immunolabeling for TRPV4 protein, while GFP- cells had little to no staining (nucleus stained with Hoechst 33342)

**FIGURE 3.**

TRPV4-mediated Ca²⁺ signaling. A, GFP-sorted induced pluripotent stem cell (iPSC)-derived chondroprogenitor cell response to TRPV4-mediated Ca²⁺ signaling with GSK1016790A (GSK101) treatment measured by confocal cell traces, as compared to undifferentiated iPSCs or primary murine articular chondrocytes (AC) (N = 3 experiments, n = 78-161 cell traces per group; groups not sharing the same letter are statistically different from one another by Chi-square test, $P < .05$). Representative Ca²⁺ traces in response to increasing concentrations of GSK101 in (B) GFP- and (C) GFP+ chondroprogenitor cells. D, Fluorometric imaging plate reader (FLIPR)-based assay dose-response of GFP- and GFP+ populations in response to GSK101 (n = 8). E, TRPV4-mediated Ca²⁺ signaling response to TRPV4-specific inhibitor GSK205, thapsigargin, and removal of Ca²⁺ from the extracellular medium (Ca²⁺ Free) as measured by FLIPR (n = 5). Mean \pm SEM, groups not sharing the same letter are statistically different from one another by analysis of variance with Tukey's post hoc, $P < .05$

**FIGURE 4.**

Cell proliferation, sulfated glycosaminoglycan (sGAG) production, and chondrogenic gene expression in induced pluripotent stem cell (iPSC)-derived chondroprogenitors after transient activation of TRPV4. A, GFP⁺ and GFP⁻ cells were formed into micromasses and underwent chondrogenesis for 7 days before being treated with GSK1016790A (GSK101) or GSK101+GSK205 for 1 hour per day, in the absence of chondrogenic growth factors. B, Day 21 biochemical analysis for DNA, sGAG, and collagen content showed that activation of TRPV4 with GSK101 increased DNA content, sGAG/DNA ratio in GFP⁺ groups only. Total collagen/DNA was not affected. C, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) at day 21 showed significantly increased *Sox9* and *Acan* expression in GFP⁺ cells in response to GSK101 treatment compared to controls conditions. *Col2a1* expression was not significantly altered in either cell population in response to TRPV4 activation. Mean \pm SEM, n = 5-6 for qPCR, n = 5 for biochemical measurements, groups not sharing the same letter are statistically different from one another (analysis of variance with Tukey's post hoc, $P < .05$)

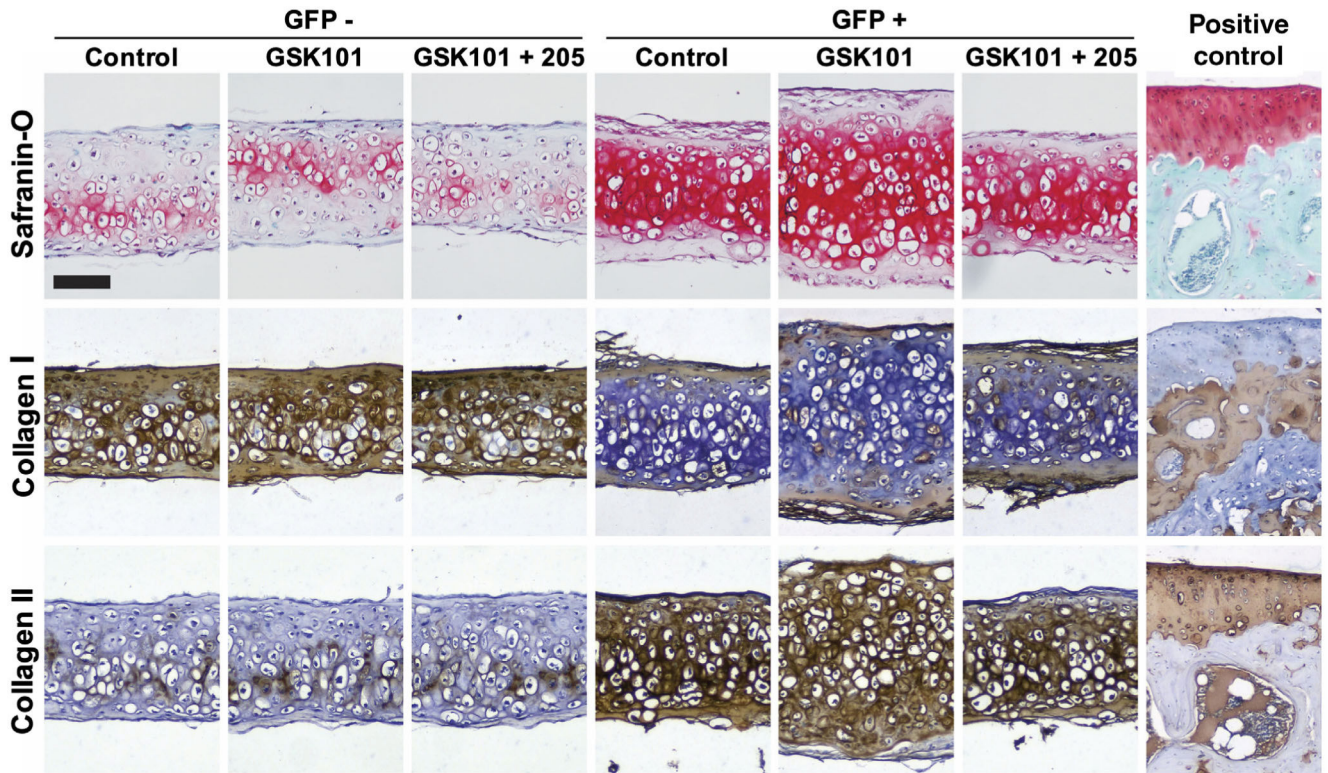


FIGURE 5.

Tissue accumulation, Safranin-O staining, and collagen types I and II immunohistochemistry (IHC) in induced pluripotent stem cell (iPSC)-derived chondroprogenitors after transient activation of TRPV4. Increases in tissue thickness and Safranin-O labeling were observed in GSK101-treated GFP+ cells at day 21, and this effect was inhibited by the TRPV4 antagonist, GSK205. IHC revealed increased collagen type II staining in GFP+ micromasses, while GFP- micromasses displayed increased collagen type I staining. Positive controls were performed on the mouse tibial plateau. Scale bar = 100 μ m

TABLE 1

Primers used for gene expression assays

Target	Forward primer	Reverse primer
r188	5'-CGGCTACCAATCCAAAGGAA-3'	5'-GGGCCTCGAAAGAGTCCTGT-3'
Nanog	5'-GCACATCAAGGACAGGTTTCA-3'	5'-CGCTTGCACATTCATCCTTTTG-3'
Sox2	5'-AAGGCCCTTCATGGTATGGTC-3'	5'-CTCGGTCTCGGACAAAAAGTT-3'
Vim	5'-CAAAACGAGTACCCTGGAGACAG-3'	5'-TAGCAGCTTCAAGGGCAAAAA-3'
Sox9	5'-GAAAGTCGGTGAAGAACGGAC-3'	5'-CAGCCCTTGAAGATAGCAT-3'
Acan	5'-GCATGAGAGAGCGGAATGGA-3'	5'-CTGATCTCGTAGCGGATCTTTCTTCT-3'
Col2a1	5'-TCCAGATGACTTTCCCTCCGCTCA-3'	5'-AGGTAGGGCGATGCTGTTCTTACA-3'
Trpv4	5'-TCTTCAACCCTCACCCGCTACT-3'	5'-TCCACTGTGGTCCCGGTAAG-3'