

Transient Receptor Potential Vanilloid 4 (TRPV4) knockdown decreases extracellular matrix synthesis via autophagy suppression in rat intervertebral disc

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INTRODUCTION: The intervertebral disc is the largest avascular, low nutrient organ in the human body¹. Autophagy is an important cell survival mechanism by self-digestion and recycling damaged components under stress conditions, primarily nutrient deprivation². Biologically, disc cells and their extracellular matrix are stimulated by physiological range of mechanical loading, and abnormal loading can result in disc degeneration. Therefore, the mechanobiological mechanisms that govern intradiscal homeostasis need to be clarified to understand the process of disc degeneration and to develop a therapeutic strategy for disc degenerative diseases³. One possible mechanosensitive regulator in disc homeostasis is Transient Receptor Potential Vanilloid 4 (TRPV4). The TRPV4 has been also reported to be activated under a physiological mechanical stimulation in disc nucleus pulposus (NP) cells *in vitro*⁴. We hypothesized that TRPV4 is involved in the maintenance of intradiscal autophagy. Our objective is to elucidate the role of TRPV4 in rat intervertebral disc autophagy and extracellular matrix metabolism through loss-of-function study with the RNA interference (RNAi) technique.

METHODS: *In-vitro* study: (1) Disc NP cells harvested from 12-week-old male Sprague-Dawley (SD) rats were used. Small interfering RNA (siRNA) was applied to knockdown TRPV4 by the reverse transfection method. Three different TRPV4-siRNA sequences were used to exclude the off-target effect. Cells after transfection were cultured in DMEM with or without 10% FBS for 24 h to simulate nutrient deprivation. Expression of AMPK, mTOR, p70/S6K, LC3-II, and a substrate p62/SQSTM1 as well as TRPV4 was measured by Western blotting (WB). (2) Next, cells after the transfection were cultured in serum-free DMEM with 10-ng/ml interleukin-1 beta (IL-1 β) for 24 h. Autophagy markers, extracellular matrix (COL2a1 and aggrecan), catabolic matrix metalloproteinases (MMPs), and anti-catabolic tissue inhibitor of metalloproteinase (TIMPs) were assessed by WB. The α -tubulin was used as a loading control. The intensities of the bands were quantified using ImageJ software. (3) Cell viability was assessed by the Cell Counting Kit-8 (CCK-8). (4) Apoptosis and senescence levels were determined by WB for PARP, Caspase-9 and p21/CIP1, p16/INK4A, and p53 expression. Apoptosis was also assessed by TUNEL staining. The *P*-values of < 0.05 were regarded as statistically significant.

***In-vivo* study:** Thirty-six 12-week-old male SD rats were used, and TRPV4 and control siRNAs were injected into respective discs using a 33-G needle. (5) To confirm *in-vivo* transfection, WB for TRPV4 and α -tubulin was conducted in rat disc NP-tissue protein extracts 2 and 28 d after injection. (6) A rat tail model of disc degeneration induced by temporary static compression was designed⁵. Axial force at 1.3 MPa was applied for 24 h and subsequently released. Radiographic and histological (Safranin-O) degeneration was assessed at 0, 7, 28, and 56 d after compression. (7) Extracellular matrix metabolism (COL2a1 and Aggrecan) and the notochordal marker (Brachyury) were assessed by immunofluorescence at 0, 28, and 56 d after compression.

RESULTS: *In-vitro* study: (1) In rat disc NP cells, TRPV4 expression significantly decreased by TRPV4 RNAi (70% or more). The LC3-II decreased and p70/S6K and p62/SQSTM1 increased, indicating autophagy suppression (Fig. 1). In addition, AMPK decreased and mTOR increased, suggesting a possible pathway between TRPV4 and autophagy. (2) Pro-inflammatory IL-1 β stimulation with TRPV4 RNAi further decreased AMPK, LC3-II, COL2a1, Aggrecan, and TIMPs and increased mTOR, p70/S6K, p62/SQSTM1, and MMPs, indicating enhancement of the effect of TRPV4 knockdown (Fig. 2). (3) Furthermore, in serum-free DMEM, CCK-8 assay exhibited decreased cell viability by TRPV4 RNAi (*P*<0.05). (4) Higher proportion of TUNEL positive cells was shown under IL-1 β stimulation with TRPV4 RNAi relative to the other conditions (*P*<0.05). In WB, IL-1 β stimulation with TRPV4 RNAi displayed increased expression in Caspase-9, p21/CIP1, p16/INK4A, and p53 and decreased expression in PARP (*P*<0.05).

***In-vivo* study:** (5) WB displayed sustained decreases in TRPV4 protein expression 2, 28, and 56 d after injection (*P*<0.05). (6) In the loaded, TRPV4 siRNA-injected discs, radiographic disc height significantly decreased compared to the other conditions at 28 and 56 d after compression (*P*<0.05) (Fig. 3). Safranin-O staining also showed advanced degenerative findings in the loaded, TRPV4 siRNA-injected discs relative to the other conditions at 28 and 56 d after compression (*P*<0.05). (7) Immunofluorescence showed that signals of COL2a1, Aggrecan, and Brachyury were significantly decreased in the loaded, TRPV4 siRNA-injected discs at 28 and 56 d after compression (*P*<0.05).

DISCUSSION: *In vitro*, the TRPV4 knockdown suppressed autophagy with AMPK inhibition in rat disc NP cells and developed further suppression of autophagy under pro-inflammatory IL-1 β stimulation. *In vivo*, intradiscal injection of TRPV4 siRNA was kept effective for 56 d, resulting in radiographic disc degeneration. This loss-of-function study demonstrated the involvement of TRPV4 in extracellular matrix metabolism of rat disc NP cells via autophagy. The TRPV4 could be a therapeutic target for intervertebral disc diseases via modulating autophagy.

SIGNIFICANCE: TRPV4 is a potential new molecular therapeutic target for degenerative, inflammatory disc diseases.

REFERENCES:1. Urban JP et al. *Arthritis Res Ther*, 2003. 2. Mizushima N et al. *Nature*, 2008. 3. Takeoka Y et al. *Int J Mol Sci*, 2021. 4. Takeoka Y et al. *Spine*, 2023. 5. Hirata H et al. *J Orthop Res*, 2014.

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FIGURES:

Figure 1

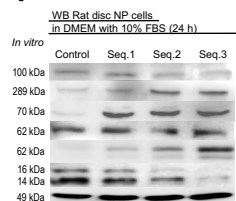


Figure 2

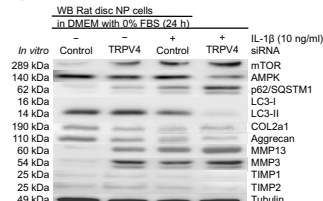


Figure 3

