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CONTRACTING ORGANIZATION: Massachusetts General Hospital

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project is to determine the roles of Piezo mechano-sensing channels in osteoarthritis (OA) and pain associated with OA using mouse genetic models. Two major aims are proposed.1) assessment of the effect of cartilage-specific loss of Piezo1 and Piezo2 in OA progression in surgically created OA in mice, and 2) assessment of OA-associated pain in Piezo2 haplo-insufficient mice.
The scope of this project is generation of genetic mouse models and analysis of OA for Aim 1 (neuron specific Piezo KO) and Aim 2 (cartilage-specific Piezo KO).
Major findings: The Aim2 to assess OA progression in mice with joint-specific Piezo conditional KO [cKO, *Gdf5-Cre:Piezo1(fl/fl):Piezo2(fl/fl*)] was completed. Piezo 1/2 deletion in developing joints does not disturb joint development based on the histological finidngs at 3 and 6 months of age. Therefore, Piezo channels are not essential for joint cartilage development. Induction of OA via destabilization of medial meniscus (DMM) surgery caused OA in both Piezo cKO and control mice. The cohort of Piezo cKO included a greater number of mice that showed lower grade OA compared with the control cohort, although it does not reach the statistical significance. This result suggests that Piezo genetic deletion might have modest beneficial effects on OA progression. In an in vitro system using fluid flow shear stress (FFSS), we assessed expression of mechanical stress-induced OA-associated genes in control and Piezo cKO articular chondrocytes. Both control and Piezo cKO chondrocytes showed similar upregulation of OA-associated genes, suggesting that different mechanotransducer other than Piezo 1/2 channels dominantly mediate FFSS. The Aim 1. we have generated mice with neuron-specific Piezo knockdown (Piezo1 homozygous, Piezo 2 heterozygous). They showed no overt neurological deficits. Unfortunately, when we performed DMM surgery on mice. After one measurement, the incapacitance meter started malfunctioning and the device was not repaired in a timely manner to perform measurements for the mice prepared for the experiments.

The significance of these findings indicate that 1) Piezo1 and 2 are not required for normal articular and growth plate chondrocytes *in vivo* (at least up to 6 months) and suggest that 2) Piezo 1 and 2 might modestly contribute to OA progression and that different mechanotransducers other than Piezo1 and 2 dominantly mediate mechanical stress *in vitro*. 3) These in vivo results are not fully compatible to the suggested role of Piezo channels in articular chondrocytes based on in vitro study (Lee W et al 2014 PNAS, ;111(47):E5114-22). These findings were published in *Osteoarthritis and Cartilage* as well as in BioRxiv.

15. SUBJECT TERMS
Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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Standard Form 298 (Rev. 8-98)

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Cumulative mechanical stress is a major mechanism of Osteoarthritis (OA) development. Our central hypothesis is that suppressing mechanosensing channels inhibits progression of OA. This project aims to specifically determine the role of Piezo mechano-sensing channels that were recently shown to play critical roles in bone development and pain sensing in OA. In this project, OA is surgically induced in mice with cartilage-specific or neuron-specific Piezo channel deletion to assess OA and OA-associated pain. The purpose is to provide scientific basis whether Piezo channels can be therapeutic targets for OA.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Osteoarthritis, mouse, genetic models, mechano-sensing ion channel, Piezo1, Piezo2, pain, joint, synovial.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The following aims and tasks are proposed in SOW during the entire research period:

Major Task 1 (Aim1) : Establish animal model and system validation (neuron-specific Piezo2 and Piezo1 ablation)

Major Task 2 (Aim1) : Evaluation of Piezo deletion effects on OA (neuron-specific Piezo2 and Piezo1 ablation).

Major Task 3 (Aim2) : Establish animal model and system validation (cartilage-specific Piezo deletion)

Major Task 4 (Aim2) : Evaluation of Piezo deletion effects on OA (cartilage-specific Piezo deletion).

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1 and 2 (Aim1) : Establish animal model and system validation (neuron-specific Piezo2 and Piezo1 ablation)

1) Major activities: We established mouse colonies, performed DMM surgery, subjected them to incapacitance test.

2) Specific objectives: Subtask 1) To generate sensory nerve specific Piezo2 (heterozygous) with Piezo1 (homozygous) deletion in mice. Subtask 2) confirmation of gene deletion and the absence of an overt basal phenotype. Subtask 3) confirmation of the absence of basal cartilage phenotype.

3) Significant results: We generated tamoxifen-inducible neuron-specific Piezo2 heterozygous, Piezo1 homozygous deletion (cKO) and confirmed that these mice do not have an abnormal basal phenotype. We performed DMM surgery at 3 months on a rolling basis. At one month after the DMM surgery, we performed static wight-bearing analysis on two cKO mice. Although this preliminary measurement suggested a pain reduction in cKO mice, this study was not completed because of malfunction of the incapacitance meter. The manufacturer was not able to repair the device in a timely manner to conduct the planned experiments.

4) Other achievements: Nothing to report.

Major Task 3 and 4 (Aim2) : Evaluation of Piezo deletion effects on OA (cartilage-specific Piezo2 and Piezo1 ablation)

1) Major activities: We generated Piezo1/2 KO mice, induced OA via DMM surgery at 3 months of age. Pain is analyzed by the incapacitance test, and histology is analyzed at 6 months of age (3 months after OA induction). We harvested samples and evaluated OA scores.

2) Specific objectives: Subtask 1) To evaluate pain in cartilage-specific Piezo KO mice with OA. Subtask 2) To evaluate the cartilage phenotype and pain in cartilage specific Piezo KO mice with OA.

3) Significant results: For the pain assessment performed by the incapacitance meter test that measures left limb/right limb weight bearing balance, we did not observed beneficial effects of Piezo1/2 deletion in cartilage.
With regard to DMM-induced OA, both control and Piezo cKO mice showed moderate to severe OA upon DMM surgery. Piezo1/2 KO might show less severe OA damage. In vitro experiments using primary chondrocytes missing Piezo1/2 showed normal response to fluid flow shear stress, suggesting mechanosensing systems other than Piezo1/2 mediate mechanotransduction. **These results were published (Young C and Kobayashi T. *Osteoarthritis Cartilage* 2023 (6):775-779)**

4) Other achievements: Nothing to report.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project provided learning opportunities for two US college graduates (BS) and a Chinese MD, PhD student and a Russian MD, PhD student. This project and its progress are also presented at departmental meetings.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Major findings were published as a freely-accessible pre-print (bioRxiv 2022.10.07.511314 as well as as a peer-reviewed article (Osteoarthritis and Cartilage 2023 ***6*** 775-779).

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Mechanical stress is a major mechanism for OA. However, critical mechanotransducers that mediate this process are not known. Among several different mechanosensing systems, Piezo channels have been ones of the top candidates as such critical mechanotransducers because they are activated by high-level, injurious mechanical load, and that in vitro and ex vivo data have suggested that Piezo inhibition has chondroprotective effects. Until this present study, direct in vivo data regarding the effect of Piezo inhibition on OA have not been available. This study addressed this question. The results revealed limited roles of Piezo channels in OA and suggested existence of other mechanosensing systems that play independent or overlapping role in OA.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not Applicable

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Limited roles of Piezo mechanosensing channels in articular cartilage development and osteoarthritis progression.
Young C, Kobayashi T.*Osteoarthritis Cartilage* (IF: 6.58; Q1). 2023 Jun;31(6):775-779. doi: 10.1016/j.joca.2023.01.576. Epub 2023 Feb 17.PMID: 36805475

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Preprint:

Limited roles of Piezo mechanosensing channels in articular cartilage development and osteoarthritis progression
Cameron Young, Tatsuya Kobayashi
bioRxiv 2022.10.07.511314; doi: https://doi.org/10.1101/2022.10.07.511314

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

https://www.biorxiv.org/search/young%252Bcameron. :Preprint site.

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Tatsuya Kobayashi*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-4264-5117
Nearest person month worked:	*4*
Contribution to Project:	*Mouse management and analysis*
Funding Support:	*NIH and current project*

Name:	*Cameron Young*
Project Role:	*Research technician*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	*Mouse management, surgery, data analysis*
Funding Support:	*NIH and current project*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:

Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

Nothing to Report

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Publication in OAC attached

Osteoarthritis and Cartilage 31 (2023) 775–779

Osteoarthritis and Cartilage

Brief Report

Limited roles of Piezo mechanosensing channels in articular cartilage development and osteoarthritis progression

C. Young †, T. Kobayashi † ‡ *

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SUMMARY

Objective: To investigate the role of Piezo1 and Piezo2 in surgically induced osteoarthritis (OA) in mice.
Design: Male conditional knockout (cKO) mice missing *Piezo1* and *Piezo2* in the joint using *Gdf5-Cre* transgenic mice were induced with post-traumatic OA by destabilization of the medial meniscus (DMM) of the right knee joint at 12 weeks of age. The severity of OA was histologically assessed at 24 weeks of age. OA-associated pain was evaluated by static weight bearing analysis. Additionally, articular chondrocytes isolated from cKO mice were exposed to fluid flow shear stress (FFSS) to evaluate the expression of OA-associated genes.
Results: Mice with conditional deletion of *Piezo1* and *Piezo2* showed normal joint development with no overt histological changes in the knee joint at 12 weeks and 24 weeks. DMM surgery induced moderate to severe OA in both control and cKO mice (median OARSI score: control, 4.67; cKO, 4.23, $P = 0.3082$), although a few cKO mice showed milder OA. Pain assessment by static weight-bearing analysis suggested Piezo ablation in the joint has no beneficial effects on pain. FFSS increased the expression of OA-related genes both in control and cKO mice to similar extents.
Conclusion: Piezo1 and Piezo2 are not essential for normal joint development. Genetic ablation of Piezo channels did not confer evident protective effects on OA progression in mice. *In vitro* data suggests that different mechanotransducers other than Piezo channels mediate FFSS in mechanical stress-induced gene expression.

Introduction

Osteoarthritis (OA), a common disease characterized by chronic pain and degeneration of joints, widely affects people worldwide with increasing prevalence. OA is strongly associated with aging and injury, and cumulative and/or excessive mechanical stress plays a central role in the pathogenesis of OA. In addition to direct physical damage to the cartilage, many factors, such as inflammation and cellular senescence, are known to contribute to development and progression of OA. Cells in the joint affected by OA respond to mechanical stress and secrete bioactive molecules that could further progress OA. Mechanical stress is sensed by the cell through multiple mechanisms such as primary cilium, integrins, and mechanosensing ion channels[1]. Among diverse groups of ion channels expressed in chondrocytes, two groups of mechanosensitive calcium ion channels, transient receptor potential (TRP) channels, including TRP vanilloid-4 (Trpv4), and Piezo-1 (Piezo1) and -2 (Piezo2), are particularly of interest as possible therapeutic targets for OA[2]. Trpv4 appears to regulate anabolic chondrocyte metabolism and Ca^{2+} levels in response to low strain, physiologic mechanical stress maintaining proper extracellular matrix for cartilage health[3], whereas the effect of its inhibition on OA is unclear. Conditional ablation of *Trpv4* reduces age-related OA-like changes, although it does not significantly alter the severity of OA induced by destabilization of the medial meniscus (DMM)[4]. Piezo channels are broadly expressed in different types of cells. Piezo1 is highly expressed in skeletal tissues, including cartilage, and is activated by high-level, injurious strain[5]. A study using isolated chondrocytes showed that Piezo1 and Piezo2 synergistically regulate Ca^{2+} influx in response to mechanical stress[6]. Furthermore, this study demonstrated that pharmacological Piezo inhibition reduced chondrocyte apoptosis in cartilage explants upon injurious mechanical loading. Inflammatory cytokines and matrix-degrading

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1063-4584/

enzymes, such as interleukins and matrix metalloproteinases, are well-known factors that accelerate OA[7,8]. Interleukin-1α enhances Piezo1 expression, which appears to further amplify the effect of mechanical stress to form a feed-forward loop to promote OA progression[9]. These studies suggest that Piezo ion channels play a significant role as a mediator of mechanical stress during OA development and progression. In this study, we have tested the hypothesis that the conditional ablation of Piezo channels in the joint confers protective effects in a mouse OA model.

Method

Mice

Floxed *Piezo1* and floxed *Piezo2* mice were purchased from the Jackson laboratory. *Gdf5-Cre* transgenic mice were previously described[10]. Mice were in a congenic strain derived from the C57/BL6 and FBV strains. Only male mice were subjected to the study. Doubly conditional homozygous knockout males (cKO, *Gdf5-Cre:Piezo1*$^{fl/fl}$*:Piezo2*$^{fl/fl}$) and male Cre-negative controls (*Piezo1*$^{fl/fl}$*:Piezo2*$^{fl/fl}$) were generated and were subjected to DMM surgery on a rolling basis. cKO mice and controls were not analyzed as pairs. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital and by the Animal Care and Use Review Office (ACURO) of the U.S. Army Medical Research and Development Command (USAMRDC).

DMM surgery

The DMM surgery was performed to induce OA according to an established protocol with minor modifications[11]. Briefly, under anesthesia, the meniscotibial ligament was cut through a 1 cm skin incision and a 0.3 cm incision of the right joint capsule. The joint capsule was then sutured with 7-0 Vicryl sutures, and the skin incision was closed using 9 mm wound clips. Wound clips were removed 14 days after the operation. The surgery was performed by a single surgeon in a consistent manner. A total of 11 mice for the control group and 12 mice for the cKO group were collected for histological analysis.

Mouse dissection and histological analysis

Three cKO and three control male mice were randomly allocated at each time point and sacrificed at 12 weeks or 24 weeks without surgery for baseline histological assessment, and mice that received DMM surgery were sacrificed at 24 weeks (12 weeks post-operation). Right hindlimbs were fixed in 10% Formaldehyde-PBS, decalcified, paraffin-processed, sectioned, and stained with Safranin-O or hematoxylin and eosin (H/E) according to a standard procedure. OA was scored using the Osteoarthritis Research Society International (OARSI) mouse OA scoring system[12]. Sagittal sections from three different cutting planes of the mid-tibial plateau per mouse were blindly evaluated by two inspectors. A single OARSI score per mouse was then calculated by averaging a total of 6 values. Due to the difficulty of obtaining comparable sections in the femoral condyle, only the medial tibial plateau was evaluated.

Articular chondrocyte isolation and fluid flow shear stress

Articular chondrocytes were obtained from 10-day-old mouse knee and hip joints from 3 cKO and 3 control mice from a single litter according to the method previously described[13]. Briefly, the tibial condyle or hip joint was manually dissected. The soft tissues and subchondral tissues were manually removed as much as possible. Cartilage pieces were digested overnight in a growth medium (DMEM containing 10% fetal bovine serum) and 0.2% collagenase II (Worthington) to disperse chondrocytes. Cells were passed through 0.40 μm nylon mesh, resuspended in the growth medium, plated in 48-well plates, and grown to confluence. For the experiment to test the effect of fluid flow shear stress (FFSS), cells from knee joints with same genotypes were combined, and split them into FFSS+ and FFSS− groups. FFSS was applied for 4 h by shaking the plate at 1,200 rpm on a mixing platform (Thermomixe R, Eppendorf).

qRT-PCR

Gene expression was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). RNA was isolated from cells using the Directzol RNA Mini-Prep Kit (Zymo Research) and converted to cDNA using the Verso cDNA synthesis kit (Thermo Scientific). qRT-qPCR was performed using the PerfeCTa SYBR Green Supermix (QuantaBio). The values were normalized to *Actb*. The relative expression to control groups was calculated. PCR primer sequences are as follows: *Actb*-L, 5′- GCACTGTGTTGGCATAGAGG -3′ and *Actb*-R, 5′- GTTCCGATGCCCTGAGGCTCTT -3′; *Piezo1*-L, 5′- GATTGGGCAGCGTATGAACT -3′ and *Piezo1*-R, 5′- GTACAGCAGGAACAGCGTGA -3′; *Piezo2*-L, 5′- CCTCGTGTTTGGGATTCACT -3′ and *Piezo2*-R, 5′- GTAGCCACAGCGGATTTGAT -3′; *Acan*-L, 5′- GAAGAGCCTCGAATCACCTG-3′ and *Acan*-R, 5′- ATCCTGGGCACATTATGGAA -3′; *Mmp13*-L, 5′- GCCATTTCATGCTTCCTGAT -3′ and *Mmp13*-R, 5′- TTTTGGGATGCTTAGGGTTG -3′; *Wnt11*-L, 5′–CAGGATCCCAAGCCAATAAA -3′ and *Wnt11*-R, 5′- GACAGGTAGCGGGTCTTGAG -3′; *Il6*-L, 5′- CACAAGTCCGGAGAGGAGAC -3′ and *IL6*-R, 5′- TCCACGATTTCCCAGAGAAC -3′.

Pain assessment and OA scoring

The OA-associated pain was assessed using an incapacitance meter (BioSeb, please refer to the company's web page for further information) at 4, 8 and 12 weeks after the DMM surgery. The weight distribution difference between the injured (right) and unaffected (left) hind limb was quantified to evaluate pain. Ten measurements for each mouse at each time point were performed. The cKO and control mice were compared as unpaired groups at each time point separately.

Statistical analysis

Sample numbers were empirically determined based on previously available data in similar experimental conditions. Statistical analysis was performed using the Graphpad Prism 9.4. General information about statistical methods can be found on the GraphPad website, and specific information is found in figure legends. Because of the malfunction of assay devices, some data points were not obtained. Analysis was performed with all available data points.

Results

To confirm the deletion of *Piezo1* and *Piezo2*, articular chondrocytes were isolated from the knee and hip joints of cKO and control mice. Relative expression of *Piezo1* and *Piezo2* was decreased by 50–75% in the hip and knee joint articular chondrocytes in cKO mice [Fig. 1(A)]. This partial reduction is likely due to contamination of non-articular chondrocytes including growth plate chondrocytes that are difficult to eliminate by manual dissection because the recombination of *Gdf5-Cre* that occurs in joint primordia during embryonic development is very efficient and specific to cells in the joint[10]. Sagittal sections of the knee at 12 weeks of age and 24 weeks of age without surgical intervention did

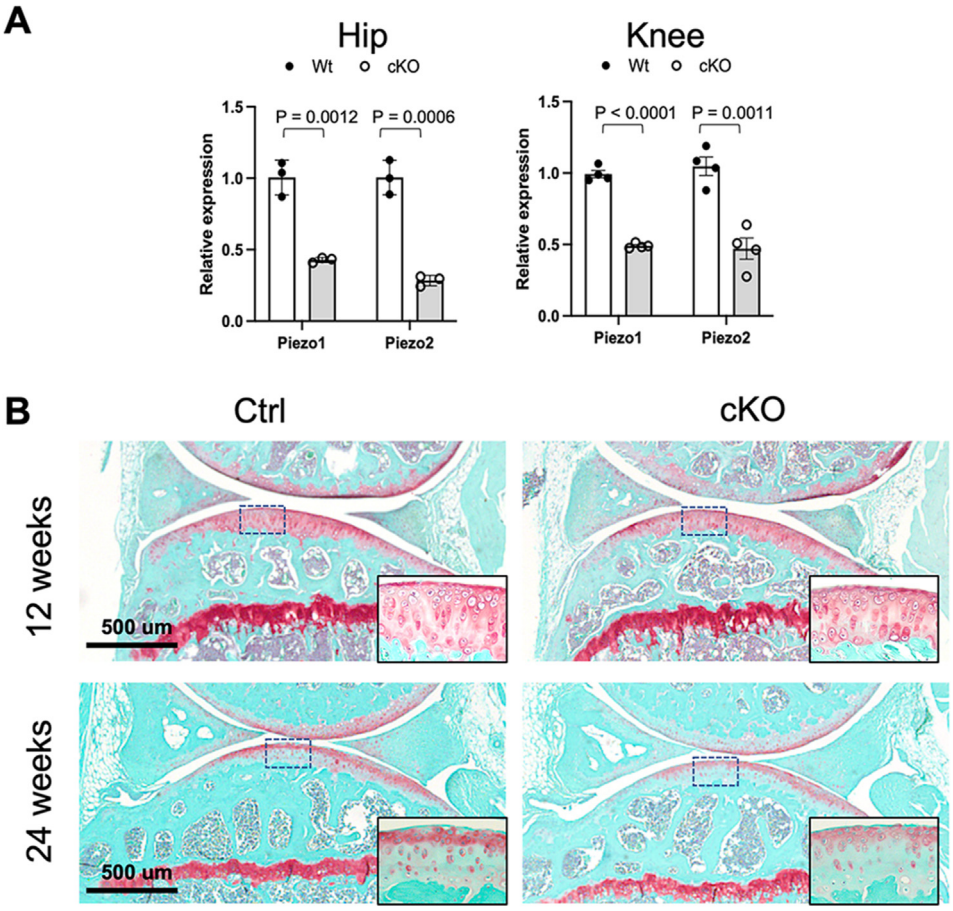

Fig. 1

Osteoarthritis and Cartilage

Knee joint morphology of *Piezo1* and *Piezo2* conditional knockout mice. (A) Relative expression of *Piezo1* and *Piezo2* in primary chondrocytes from knee and hip joints of *Piezo1* and *Piezo2* doubly conditional knockout (cKO, *Gdf5-Cre:Piezo1* $^{fl/fl}$*:Piezo2*$^{fl/fl}$) mice. Hip *Piezo1*, Est Diff −0.577, 95% CI −0.773 ~ −0.380; Hip *Piezo2*, Est Diff −0.721, 95% CI −0.922 ~ −0.519; Knee *Piezo1*, Est Diff −0.571, 95% CI −0.797 ~ −0.346; Knee *Piezo2*, Est Diff −0.559, 95% CI −0.959 ~ −0.159. $N = 3–4$. Two-tailed, two-sample *t*-test. (B) Safranin-O stained sections of the knee of mice with indicated genotypes and ages. Insets are magnified views of boxed areas. Three mice per group. No overt abnormalities are found in cKO mice.

not show overt abnormalities, suggesting that Piezo channels are not essential for normal articular cartilage development and maintenance [Fig. 1(B)].

To test whether genetic ablation of Piezo channels affects OA initiation and progression, we surgically induced OA in cKO mice. OA was evaluated 12 weeks after DMM surgery in the tibial plateau according to the OARSI scoring system[12]. Both cohorts developed moderate to severe OA, but a few cKO mice showed milder OA (control: median OARSI score 4.67, $n = 11$; cKO: median OARSI score 4.23, 95%, $n = 12$) [Fig. 2(A)]. Mice were also assessed for pain by the incapacitance meter test. We did not find significant differences between the control and cKO groups, suggesting that Piezo channel ablation in the joint does not appear to have beneficial effects on OA-pain [Fig. 2(B)].

To investigate the effect of Piezo ablation on the molecular response to mechanical stress, articular chondrocytes isolated from the hip joints of cKO and control mice were exposed to FFSS. The induction of several OA-related genes was assessed. FFSS reduced the expression of *Acan* and increased the expression of *Mmp13*, *Wnt11*, and *Il6* in both cKO and control cells [Fig. 2(C)], although the extent of gene expression change in cKO cells was slightly greater in *Il6* and smaller in *Wnt11* than in control cells.

Discussion

The critically important roles of Piezo mechanosensing channels in bone formation and homeostasis have been demonstrated[14]. However, except for a few studies suggesting possible chondroprotective effects of Piezo1 inhibition[6,15], the roles of Piezo channels in cartilage and OA have not been directly demonstrated *in vivo*. Since Piezo channels are activated by strains at injurious levels[5] and Piezo1 expression is regulated by inflammatory cytokine signaling[9], it is an attractive hypothesis that Piezo channels mediate injurious mechanical stress and thereby promote OA progression. In this study, we attempted rigorously test this hypothesis *in vivo* using a genetic model. Our data show that genetic

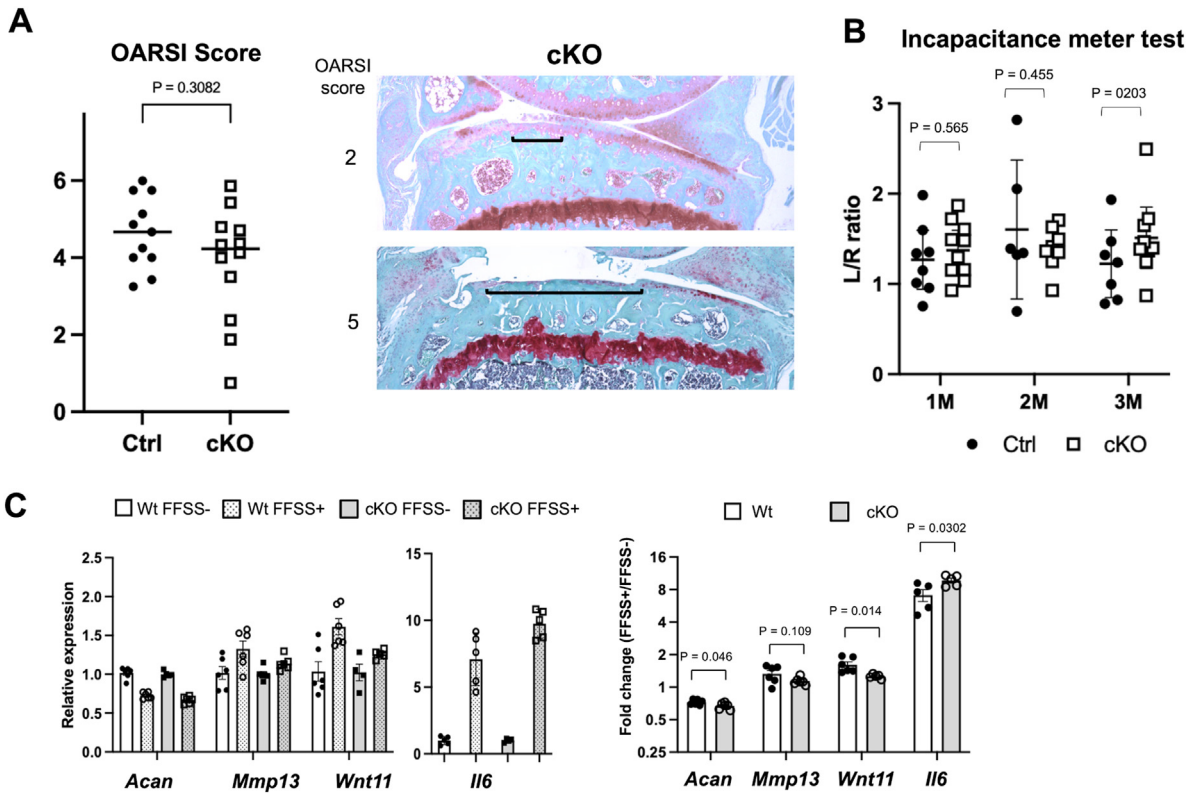

Fig. 2

Osteoarthritis and Cartilage

Effects of *Piezo1* and *Piezo2* ablation on OA progression. (A) OA was evaluated on the tibial plateau of *Piezo1* and *Piezo2* doubly conditional knockout (cKO, *Gdf5-Cre:Piezo1* $^{fl/fl}$*:Piezo2* $^{fl/fl}$) mice. *Left.* OARSI OA score. Both control and cKO mice develop moderate to severe OA 12 weeks after DMM. The cKO group contains a few samples with mild OA legions. $N = 11$ for control (Ctrl) and 12 for cKO. The median value is indicated. Estimated median difference (Hodges-Lehmann) − 0.555. Two-tailed Mann–Whitney *U* test. *Right.* Representative images of cKO knees with indicated OARSI scores. Brackets indicate the legion that reaches the calcified cartilage. (B) Pain assessment by the incapacitance meter test. The left-right asymmetry of hindlimb weight bearing (L/R ratio) was quantified at 1, 2, and 3 months after DMM surgery. The average of 10 measurements per mouse is plotted. The mean value and 95% CI are indicated by bars. Ctrl vs cKO, 1M, Est Diff 0.106, 95% CI −0.243 ~ −0.455; 2M, Est Diff −0.212, 95% CI −0.810 ~ −0.386; 3M, Est Diff 0.287, 95% CI −0.173 ~ 0.748. $N = 6–10$ mice per group. Two-tailed standard unpaired nested *t*-test (a version of nested ANOVA for a two-group comparison) was performed using all the data points. (C) Gene expression changes in primary articular chondrocytes after 4 h of FFSS stimulation. A representative result from multiple experiments is shown. FFSS altered gene expression similarly in both Ctrl and cKO cells. Relative expression of indicated genes are shown in the left panel. Fold change (FFSS+/FFSS−) differences between Wt and cKO groups are shown in the right panel, *Acan*, Est Diff −0.056, 95% CI −0.112 ~ −0.0011; *Mmp13*, Est Diff −0.188, 95% CI −0.426 ~ −0.0501; *Wnt11*, Est Diff −0.353, 95% CI −0.618 ~ −0.088; *Il6*, Est Diff −2.651, 95% CI 0.325 ~ 4.976. $N = 5–6$. The extent of gene expression changes (fold changes) upon FFSS treatment was compared between Wt and cKO chondrocytes. Two-tailed, two-sample *t-test*.

ablation of *Piezo1* and *Piezo2* has limited effects on cartilage development or OA progression.

Chondrocytes sense mechanical stress through multiple systems. During OA progression, molecules produced by cells in the joint in response to excessive and improper mechanical stimulation are considered to promote the progression of OA. Thus far, our *in vitro* data do not strongly support the role of Piezo channels mediating mechanical stress to the expression of OA-promoting molecules but rather suggest that different mechanosensing mechanisms other than Piezo channels dominantly mediate mechanical stress-induced gene regulation in chondrocytes. We did not find significant differences in the incapacitance meter test, which has been used to assess knee joint pain in rodent arthritis models. Due to the relatively high variability and the non-direct pain measurement of this method, the result needs a cautious interpretation, but the data consistently suggest that Piezo channel ablation has limited effects on OA outcome.

The absence of significant differences in this study is a major weakness, which could be caused by many theoretically possible reasons. These also include rather trivial issues in experimental consistency, gene ablation efficiency, experimental design, and assay sensibility; these negative data should be interpreted with caution. Additionally, we used only males in this study because of the sex difference in mouse OA development; it is unknown whether Piezo ablation has sex-dependent effects. However, based on the observation that the Piezo cKO cohort contained samples with milder OA,

the possibility remains that Piezo inhibition might delay OA progression, if not robustly inhibit it, which requires further investigation. In this study, we specifically focus on the role of Piezo channels in the joint tissue. From the point of view of translational research, the possibility that Piezo channels in cells outside of the joint may have a modulatory role in OA may be a future research agenda.

Author contributions

T.K. conceived the project. T.K. and C.Y. performed experiments and wrote the manuscript.

Conflict of interest

We have no conflicts of interest to disclose.

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