Assessing age-related changes in primary osteocyte mechanosensitivity

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INTRODUCTION: Aging is associated with bone loss, which may be attributed to a dysregulation of mechanically driven bone homeostasis[1]. Osteocytes have been implicated in this process, as they are most abundant cell type found in bone and function as the primary mechanosensors of the bone microenvironment[1,2]. Recent studies have shown that ultrastructural changes in bone tissue occur with age[2]. More specifically, these studies have demonstrated an age-related decrease in osteocyte lacunae size, quantity of integrins that pin osteocyte dendrites to the canalicular wall, and osteocytic pericellular matrix production[2]. All these changes in bone tissue impair lacunocanalicular fluid dynamics, thereby compromising osteocytes' ability to sense oscillatory shear stress exerted on them during physical activity. However, alterations in aging osteocytes regarding their mechanosensitivity at the cellular level are not well understood, as osteocytes are difficult to isolate due to their terminal status and location within bone tissue. The aim of this study is to determine whether age-related changes in primary osteocyte mechanosensitivity exist at the cellular level. We hypothesize that primary osteocyte mechanosensitivity is indeed age-dependent, and that changes in mechanosensing can be observed through an attenuated response to loading via oscillatory fluid flow (OFF), regulation of bone effector cells, and downregulation of key mechanotransduction markers.
METHODS: Animal experiments followed guidelines approved by the Sunnybrook Research Institute. The long bones of young (yOCY, 2-3 months) and aged (aOCY, 12-20 months) adult C57BL/6 mice were removed from freshly sacrificed specimens. Both male and female mice are used for this study, bone samples from two mice of the same age and sex were pooled together during extractions. The samples underwent enzymatic digestions in LiberaseTM. Immunofluorescence staining of osteocyte-specific marker E11/podoplanin was performed to confirm the osteocyte population[3]. The MLO-Y4 osteocyte-like cell line was used as a reference group to compare with primary osteocytes. Static controls for mechanical loading experiments were included to compare results with oscillatory fluid flow (OFF)-stimulated groups. To assess response to mechanical loading, osteocytes were seeded into ibidi µ-slide channels and subjected to OFF at 2 Pa with a frequency of 1 Hz. The real-time calcium response to flow was determined by staining osteocytes with FURA-2 AM and recording the ratio of bound to unbound calcium ions to the indicator. Dendrite morphology studies were performed by imaging osteocytes before they were subjected to OFF for 2 hours. Osteocytes were imaged before applying OFF and 24 hours post-loading to determine dendrite elongation due to mechanical loading. Adenosine triphosphate (ATP) synthesis during OFF (1 Pa, 1 Hz) was quantified by collecting media and cell lysates, then measuring the luciferin-luciferase reaction. Regulation on osteoclast formation was assessed by treating differentiating RAW264.7 macrophages with osteocyte conditioned media from static or OFF-stimulated groups, and quantifying osteoclasts via tartrate-resistant acid phosphatase (TRAP) staining. To assess downstream effects of osteocyte regulation, a double conditioned media study was performed with MDA-MB-231 breast cancer cells. Osteoclast conditioned medium was collected to measure breast cancer cell migration through Transwell inserts. Statistical analyses were performed using paired, two-tailed t-tests, or a one- or two-way ANOVA followed by a post-hoc Tukey test with significance taken at $\alpha = 0.05$.
RESULTS: Based on E11 staining, the extracted cells from long bones yielded a 96-97% osteocyte population (3 different samples from 2-month-old male mice) (Fig 1A, B). Real-time calcium imaging results across four experiments consistently indicate a slight yet non-significant decrease in the % response to mechanical loading in aOCY (18-month-old female mice) compared to yOCY (2-month-old female) (Fig 1C) but did not exhibit a difference in the mean magnitude of the response (Fig 1D). Of interest, dendrite morphology studies indicate a reduction in dendrite elongation after OFF in aOCY (16-month-old male) versus their yOCY counterparts (2-month-old male) (Fig 2A, *p<0.05). Moreover, aOCYs had a lower number of dendrites per cell compared to yOCYs (Fig 2B, ***p<0.001). When subjected to OFF, aOCY (18-month-old female mice) produced lower quantities of ATP per cell compared to yOCY (2-month-old female) groups (Fig 2C, **p<0.01, ns = not significant). Conditioned media studies also revealed that while total osteoclast formation was significantly attenuated by mechanical loading in MLO-Y4 and yOCY groups, this effect was not observed in aOCY groups (Fig. 2A, B, ****p<0.0001, ns = not significant). While mechanical loading significantly reduces MDA-MB-231 migration by 63.0% and 60.4% in MLO-Y4 cells and yOCY respectively, loading of aOCY reduced MDA-MB-231 migration by 31.2% in comparison (Fig 3D, *p<0.05. **p<0.01).
DISCUSSION: A reliable method for our research group to extract primary murine osteocytes for in vitro mechanobiology experiments was developed. In general, aOCY groups exhibited a blunted response to mechanical loading compared to their yOCY counterparts. aOCYs produced less ATP per cell and % elongation of dendrites under OFF relative to yOCY groups. aOCYs exhibited reduced regulation of osteoclast formation when subjected to OFF, which consequently resulted in a lower reduction in MDA-MB-231 migration. To correlate findings from the in vitro functional assays to expression of related key osteocyte mechanotransduction markers, further studies will include RNA sequencing of osteocytes under static or OFF (mechanical loading) conditions.
SIGNIFICANCE/CLINICAL RELEVANCE: The consistent extraction of primary osteocytes allows for robust studies on osteocyte mechanobiology by using a relevant cell model that retains osteocyte-specific markers and functions as observed *in vivo*. The findings from this study will provide a more comprehensive understanding of aging on osteocyte mechanosensitivity at the cellular level and could further elucidate mechanisms by which osteocytes drive age-associated bone loss. Understanding these mechanisms could allow for the development of therapies to improve bone health in aging individuals.
REFERENCES: [1] Jilka, Curr Osteoporos Rep, 2016 [2] Hemmatian, Curr Osteoporos Rep, 2017 [3] Stern, Methods Mol Biol, 2015

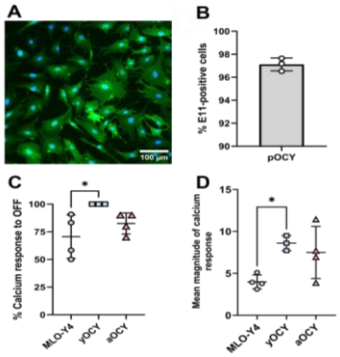

Figure 1. (A) Immunofluorescent staining of primary osteocyte cultures for E11/podoplanin (green) and DAPI (blue). (B) Percentage of E11 positive cells in n=3 samples. (C) Representative percent calcium response to OFF. (D) Representative mean magnitude of the calcium response to OFF

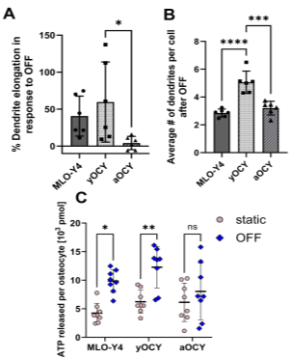

Figure 2. (A) Percent dendrite elongation after OFF (B) Number of osteocyte dendrites after OFF (C) Normalized ATP production from osteocytes.

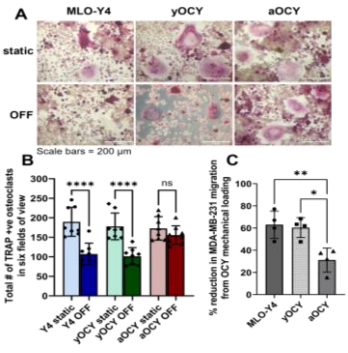

Figure 3. (A) Representative TRAP staining images of osteoclasts treated with osteocyte conditioned media (B) Total number of osteoclasts (C) Percent reduction in MDA breast cancer cell migration towards osteoclast conditioned media.