Hyaluronan Synthesis and Degradation Entail Limb Synovial Joint Cavitation

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INTRODUCTION: Synovial joints are essential for body movement, but mechanisms that create the joint cavity, permitting unhindered joint function within the joint interzone during joint morphogenesis, remain unclear [1]. We showed that fibroblast-like interzone cells produce a robust matrix (pericellular coat, mostly HA). Hyaluronan synthases (HA synthesizer) 1 (*HAS1*) and 2 (*HAS2*) are strongly expressed in the joint interzone. Microcavities appear within the pericellular spaces between interzone cells, merge, and coalesce into a single cavity. The fluid-filled microcavities contain sugars and proteins, and these molecular components significantly decrease with cavitation that accrues water by an osmoregulatory process [2]. We demonstrated that hyaluronidase (HAase) activity is significantly higher in the interzone compared to other joint tissues, and its expression is greater before cavitation (< E15.5) than after (> E16.5) [3]. We also revealed that transmembrane protein 2 (*TMEM2*, cell surface hyaluronidase) is strongly expressed in the interzone before cavitation than after. Joint-specific ablation of *HAS2* delays cavitation, and *TMEM2* expression is also temporally reduced. Further, interzone tissue samples show HA of varying sizes, including low molecular weight HA (LMW-HA, HA fragments) [4]. These data suggest that joint cavitation involves the synthesis and degradation of HA, assisting microcavity formation, merge, and coalescence. However, it is unclear which hyaluronidases are involved in joint formation and cavitation and what their possible roles are.

METHODS: All animal procedures were approved by IACUC. To first determine the changes in HA sizes as an indicator of HA degradation as a part of the cavitation process, we performed gel electrophoresis. Polysaccharides were extracted using the CD-1 mouse embryo hindlimbs at E14.5 and E15.5 from distinct locations: (a) bone (diaphysis), (b) cartilage including interzone, (c) muscle, and (d) skin. To quantify HA sizes and their changes, we developed a standard curve. We performed gel electrophoresis using lyophilized HAs with different sizes (HMW-, MMW (medium)-, LMW-HA, and a mixture (1:1:1 ratio)) at varying concentrations (1, 0.5, 0.25, and 0.125 mg/mL) and then quantified the intensity to generate a standard curve (N=5/group). To identify specific HAase activities that indicate the HA degradation process, we performed ELISA assays using CD-1 mouse embryonic tissues at E14.5 from the same locations described above. *TMEM2*, *HYAL1* (a lysosomal enzyme responsible for the intracellular degradation of HA), and *HYAL2* (a cell surface HAase) activities were determined (n=4/group). HAase activity (ng/mL) was normalized to a total protein by BCA assay (ug/mL) (N=4/group). To determine changes in water content and HA sizes, infrared spectral imaging was performed [6]. To determine possible regulators in the interzone before cavitation, mass spectrometry was performed using CD-1 mouse embryos at E14.5 and E18.5 from distinct locations: (a) bone, (b) cartilage, and (c) skin. Significance was determined by one-way ANOVA with Tukey's post hoc ($p<0.05$).

RESULTS: Gel electrophoresis of lyophilized HA showed a strong band at the respective HA sizes (HMW, MMW, and LMW-HA). With a mixture, the band range showed varying HA sizes, mimicking tissue samples **(Fig. 1A)**. With five replicates at different concentrations, a new method was developed to quantify HA sizes and their changes in tissue samples **(Fig. 1B)**. Gel electrophoresis showed that muscle (M) and skin (S) tissues contained polysaccharides of varying sizes but had a strong band in HMW-HA at E14.5. HMW-HA in muscle and bone remained at E15.5 **(Fig. 1C)**. Bone (B) and interzone (cartilage, C) tissue samples also showed HA of varying sizes, including HMW-HA at E14.5, but LMW-HA was dominant at E15.5. With quantification using the standard curve, HMW- and MMW-HA were notably decreased. Concurrently, LMW-HA increased, indicating HA breakdown to smaller HA fragments was progressive before cavitation **(Fig. 1D)**. ELISA assay showed that TMEM2 was significantly greater in bone (proliferating cartilage) and cartilage (joint interzone) samples than in muscle and skin at E14.5 ($p<0.05$) **(Fig. 2A)**. Similarly, *HYAL1* was also significantly greater in bone and cartilage samples than in muscle and skin ($p<0.05$) **(Fig. 2B)**. While overall expression levels were higher in all tissue samples compared to *TMEM2* and *HYAL1*, *HYAL2* expression level was not significantly different **(Fig. 2C)**. Mass spec data showed that the characteristics of top 50 proteins (E14.5/E18.5 ratio) were related to metabolism process (biological process), membrane (cellular component), and catalytic activity (molecular function).

DISCUSSION: Our data reveal new insights into mechanisms of synovial joint cavitation regarding HA metabolism. We have established a new method to quantify varying HA sizes and their changes in tissue samples. Polysaccharides are rapidly reduced in cartilage (joint interzone) before cavitation, particularly in HMW- and MMW-HA, indicating that HAase activity is notable, which generates HA fragments and alters cell signaling pathways. A rapid HA degradation in bone tissues may be due to the process of endochondral ossification. Similarly, *TMEM2* and *HYAL1* activities show similar patterns, suggesting that their distinct roles are well coordinated. *TMEM2* is a cell surface HAase that cleaves HMW-HA into intermediate-sized HA extracellularly, promoting cell migration, while *HYAL1* is a lysosomal (intracellular) HAase that degrades smaller HA fragments. *HYAL2* is also a cell surface HAase and has similar roles to *TMEM2*, and is also known to localize, in part, in the lysosome [5]. Their similar and distinct roles in HA will need to be further investigated. To further understand the distinct and collaborative roles of *TMEM2* and *HYAL2* in initiating HA cleavage during the cavitation process, conditional deletion of *TMEM2* using *GDF5-Cre* (*Tmem2$^{f/f;}$ GDF5-Cre*) is being performed.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates activities of *TMEM2*, *HYAL1*, and *HYAL2* in synovial joint cavitation, addressing their possible roles and develops a new method to quantify HA sizes. Understanding the roles of HAS (*HAS1* and *HAS2*) and hyaluronidases (*TMEM2*, *HYAL1*, and *HYAL2*) regulating HA metabolism will provide novel insights to treat joint conditions such as osteoarthritis.

REFERENCES: [1] Archer+1999, [2] Koyama+2025, [3] Kim+2022, [4] Kim+2023, [5] Hsu+2016, [6] Chen+2019.

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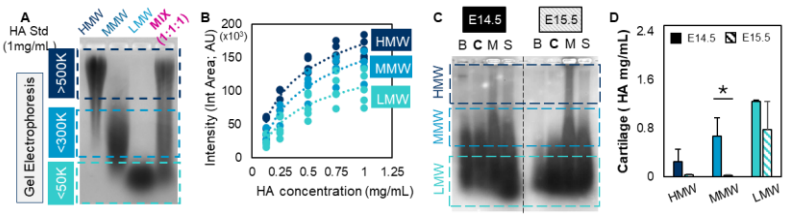

Fig 1. HA Quantification. (A) HA standard (HMW, MMW, LMW, and mixed) **(B)** HA standard curve. **(C)** HA gel electrophoresis (B =bone, C = cartilage (interzone), M = muscle, S = skin) E14.5 (left) and E15.5 (right), **(D)** Quantification of HA extracted from cartilage (interzone) (n=4/group) (E14.5 = solid, E15.5 (dashed box) (*p<0.05)

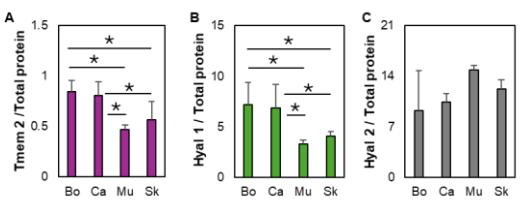

Fig 2. HAase activities in joint cavitation by ELISA. (A) Tmem2, **(B)** Hyal 1, **(C)** Hyal 2 (B =bone, C = cartilage, M = muscle, S = skin), (E14.5, N = 4/group, ELISA (ng/mL) / Total protein (BCA assay); *p<0.05)