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Removing or truncating connexin 43 in murine osteocytes alters cortical geometry, nanoscale morphology, and tissue mechanics in the tibia

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ABSTRACT

Gap junctions are formed from ubiquitously expressed proteins called connexins that allow the transfer of small signaling molecules between adjacent cells. Gap junctions are especially important for signaling between osteocytes and other bone cell types. The most abundant type of connexin in bone is connexin 43 (Cx43). The C-terminal domain of Cx43 is thought to be an important modulator of gap junction function but the role that this domain plays in regulating tissue-level mechanics is largely unknown. We hypothesized that the lack of the C-terminal domain of Cx43 would cause morphological and compositional changes as well as differences in how bone responds to reference point indentation (RPI) and fracture toughness testing. The effects of the C-terminal domain of Cx43 in osteocytes and other cell types were assessed in a murine model (C57BL/6 background). Mice with endogenous Cx43 in their osteocytes removed via a Cre-loxP system were crossed with knock-in mice which expressed Cx43 that lacked the C-terminal domain in all cell types due to the insertion of a truncated allele to produce the four groups used in the study. The main effect of removing the C-terminal domain from osteocytic Cx43 increased cortical mineral crystallinity ($p = 0.036$) and decreased fracture toughness ($p = 0.017$). The main effect of the presence of the C-terminal domain in other cell types increased trabecular thickness ($p < 0.001$), cortical thickness ($p = 0.008$), and average RPI unloading slope ($p = 0.004$). Collagen morphology was altered when either osteocytes lacked Cx43 ($p = 0.008$) or some truncated Cx43 was expressed in all cell types ($p < 0.001$) compared to controls but not when only the truncated form of Cx43 was expressed in osteocytes ($p = 0.641$). In conclusion, the presence of the C-terminal domain of Cx43 in osteocytes and other cell types is important to maintain normal structure and mechanical integrity of bone.

1. Introduction

Gap junctions are ubiquitously expressed protein channels that allow for rapid signaling between adjacent cells by linking the cells' cytoplasm and permitting ions and small molecules (<1 kDa) to be transported between the cells [1]. The most common gap junction channel in bone is formed from connexin 43 (Cx43) wherein six Cx43 molecules assemble into a hemichannel in each cell and the two hemichannels dock to complete the gap junction [2]. In bone gap junctions are important for communication within the osteocyte network and between osteocytes and other cell types on the surface of bone to coordinate structural changes and respond to mechanical loading/unloading [3–6]. The clinical importance of proper Cx43 gap junctions function is apparent in oculodentoligcal dysplasia (ODDD) which is characterized by craniofacial and skeletal abnormalities caused by mutations in *GJA1* that result in amino acid changes at critical highly conserved locations in Cx43's structure [7].

Cx43's structure consists of an intracellular amino terminus, four transmembrane loops connected by two extracellular loops and one intracellular loop, and an intracellular carboxyl terminus [1]. The N-terminal domain is small compared to the C-terminal domain and modulates the voltage-gated behavior of the channel whereas the larger C-terminal domain serves as a scaffold for downstream signaling molecules to associate with the channel and regulates channel closing [8–10]. Due to both channel dependent and independent functions of the C-terminal domain [11,12], it has been the target of recent research. However, the role the C-terminal domain plays in regulating the quality and mechanical integrity of bone tissue is a largely uninvestigated area despite evidence that tissue-level mechanical properties are altered at

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the macroscale with the removal of Cx43 [13,14]. Additionally, while studies have investigated conditional knock outs of Cx43 in either osteoblasts and osteocytes or only osteocytes [4,6,14], the role the C-terminal domain plays in determining tissue quality was not directly investigated and was limited to its modulation of parathyroid hormone treatment [13].

The current study sought to address the importance of the C-terminal domain of Cx43 within osteocytes and other cell types in determining the microscale mechanics of bone by identifying changes in tissue composition that could alter the bone's mechanical integrity, e.g. collagen morphology. To assess the role of C-terminal domain, genetically modified mice were used that either express a truncated form of Cx43 without the C-terminal domain or lack endogenous Cx43 in their osteocytes [6,15]. Changes to the nanoscale morphology of collagen due to the altered Cx43-mediated signaling were investigated for the first time. We hypothesized that the lack of the C-terminal domain of Cx43 would alter collagen morphology, tissue composition, and mechanical properties as measured by reference point indentation and fracture toughness testing in a murine model.

2. Materials and methods

2.1. Animals

Knock-in mice containing one allele coding for endogenous Cx43 and a mutant allele that has the C-terminal end of the Cx43 truncated at amino acid 258 (Cx43K258stop) [15] were crossed with mice having two loxP-flanked (floxed) endogenous Cx43 encoding alleles (fl/fl) [16] to generate mice with one truncated Cx43 allele and one floxed allele (ΔCT/fl). ΔCT/fl mice were bred with mice expressing Cre recombinase under the control of an 8 kb fragment of murine dentin matrix protein 1 promoter (DMP1-8kb-Cre) with both alleles floxed (fl/fl:Cre) which selectively removes Cx43 from osteocytes [6]. This cross resulted in mice able to express endogenous Cx43 in all cell types (fl/fl), mice lacking Cx43 in osteocytes but able to express endogenous Cx43 in all other cell types (fl/fl:Cre), mice able to express both endogenous and truncated Cx43 in all cell types (ΔCT/fl), and mice able to express only truncated Cx43 in their osteocytes but able to express both endogenous and truncated Cx43 in all other cell types (ΔCT/fl:Cre). All mice were developed on a C57BL/6 background, given ad libitum access to food and water, and kept in a 12 h light/dark cycle environment. After sacrifice via cervical dislocation while under isoflurane anesthesia, left and right tibiae were harvested from four month old female mice, cleaned of soft tissue, snap frozen in liquid nitrogen, and stored at −80 °C. All work was performed with prior IACUC approval from the Indiana University School of Medicine.

2.2. Raman spectroscopy

Samples were submerged in a PBS bath and allowed to thaw for 30 min. Once thawed, the periosteum was stripped from the medial surface of the right tibiae and PBS was removed from the bath until only the medial surface was exposed (n = 6 to 12 per group). A LabRAM HR 800 Raman Spectrometer (HORIBA Jobin Yvon, Edison, NJ) with a 660 nm laser focused on the exposed surface (spot size of ~10 μm) through an integrated BX41 microscope (Olympus, Tokyo, Japan) with a 50X objective (NA = 0.75) was used to record Raman spectra. Five locations were measured between the medial malleolus and the tibia-fibula junction (TFJ) 1 to 2 mm apart along the native medial surface of the bone while in the PBS bath. Five 20 s acquisitions were averaged at each location between 700 cm^{-1} and 1800 cm^{-1} and a 5 point linear baseline correction was applied in LabSpec 5 (HORIBA Jobin Yvon). The areas of the PO_4^{3-} $\nu1$, CO_3^{2-} $\nu1$, and Amide III bands and the full width at half maximum (FWHM) of a Gaussian fit of the PO_4^{3-} $\nu1$ peak were calculated using OriginPro 8.6 (OriginLab, Northampton, MA) at each location as previously described [17]. Type B carbonate substitution, crystallinity/maturity, and relative matrix mineralization were defined as the band area ratio of CO_3^{2-} $\nu1/PO_4^{3-}$ $\nu1$, 1/FWHM of PO_4^{3-} $\nu1$, and band area ratio of PO_4^{3-} $\nu1$ /Amide III, respectively. Following imaging, samples were wrapped in PBS soaked gauze and stored at −20 °C.

2.3. Reference point indentation (RPI)

RPI was performed using a BioDent Hfc microindenter (Active Life Scientific, Santa Barbara, CA) in the same region and along the same surface as Raman spectroscopy locations on the right tibiae (n = 6–12 per group). Beginning just proximal to the medial malleolus, 4 to 5 locations 1 to 1.5 mm apart were indented using Bone Probe 3 (BP3) along the medial surface of the bone while submerged in a PBS bath and secured in the manufacturer provided stage. Ten cycles of a 2 N indentation force were applied at a frequency of 2 Hz. A custom MATLAB (MathWorks, Natick, Massachusetts) program was used to calculate the 1st cycle indentation distance (ID 1st), 1st cycle energy dissipation (ED 1st), 1st cycle unloading slope (US 1st), 1st cycle creep indentation distance (CID 1st), indentation distance increase (IDI), total indentation distance (TID), total energy dissipation (ED Tot), average creep indentation distance (CID Avg), average energy dissipation from cycles 3 to 10 (ED Avg), and average unloading slope (US Avg) [18]. Following imaging, samples were wrapped in PBS soaked gauze and stored at −20 °C.

2.4. Atomic force microscopy

After Raman and RPI, a 6 mm section between the TFJ and malleoli was removed using a low speed sectioning saw, mounted lateral-side up to a steel disk with cyanoacrylate glue, and polished using a 3 μm diamond suspension (n = 4 per group). Each sample was treated for 15 min with 0.5 M EDTA at a pH of 8.0 followed by sonication for 5 min in ultrapure water. This process was repeated 3 times. Samples were imaged using a BioScope Catalyst AFM in peak force tapping mode (Bruker, Santa Barbara, CA). A 3.5 μm × 3.5 μm image was acquired from 4 to 5 locations spaced ~1 mm apart along the polished surface of the sample using a silicon cantilever with a silicon probe (tip radius ~8 nm). From each error image, 2D Fast Fourier Transforms (2D FFTs) were performed on 10 to 15 fibrils (50 to 60 fibrils per bone) to obtain an individual fibril's D-spacing from the first harmonic peak of the power spectrum as previously described [17,19,20].

2.5. Micro-computed Tomography (μCT)

Left tibiae were thawed and hydrated for 30 min in PBS. Samples were wrapped in Parafilm M (Bemis, Oshkosh, WI) to maintain hydration and were scanned in air with the long axis of the bone vertical using a Skyscan 1172 system (Bruker microCT, Kontich, Belgium; n = 6 to 9 per group). Scans were performed using a 12.5 μm voxel size with a source voltage of 59 kV and current of 167 μA through a 0.5 mm Al filter. NRecon (Bruker microCT) was used to reconstruct voxels with attenuation coefficients ranging from 0 to 0.11, apply a beam hardening correction of 40%, and apply a ring artifact correction of 5. Mineral density was calculated using daily scans of manufacturer supplied hydroxyapatite (HA) phantoms of 0.25 g/cm^3 and 0.75 g/cm^3. Reconstructed scans were rotated using Dataviewer (Bruker microCT) to ensure precise vertical alignment. The metaphyseal region of interest extended from the distal end of the proximal growth plate to 10% of the distance between the growth plate and the TFJ. A custom MATLAB script segmented the cortical shell from trabecular region of interest which followed the contour of the cortical shell in each slice. CTAn (Bruker microCT) was used to compute bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), structure model index (SMI), connectivity density (Conn.Dn), and bone mineral density (BMD). The cortical standard site was defined as a 7 slice region centered on the slice that was 80% the distance between the growth plate and the TFJ from the growth