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The development of inter-strain variation in cortical and trabecular traits during growth of the mouse lumbar vertebral body

M. A. Ramcharan^{1,2,3}, M. E. Faillace², Z. Guengerich², V. A. Williams², and K. J. Jepsen³

¹Department of Biomedical Engineering, The City College of New York c/o CUNY Graduate Center, New York, NY, USA

²Leni and Peter W. May Department of Orthopaedics, Icahn School of Medicine at Mount Sinai, New York, NY, USA

³Department of Orthopaedic Surgery, University of Michigan, 109 Zina Pitcher Place, Room 2001, Ann Arbor, MI 48109-2200, USA

Abstract

Summary—How cortical and trabecular bone co-develop to establish a mechanically functional structure is not well understood. Comparing early postnatal differences in morphology of lumbar vertebral bodies for three inbred mouse strains identified coordinated changes within and between cortical and trabecular traits. These early coordinate changes defined the phenotypic differences among the inbred mouse strains.

Introduction—Age-related changes in cortical and trabecular traits have been well studied; however, very little is known about how these bone tissues co-develop from day 1 of postnatal growth to establish functional structures by adulthood. In this study, we aimed to establish how cortical and trabecular tissues within the lumbar vertebral body change during growth for three inbred mouse strains that express wide variation in adult bone structure and function.

Methods—Bone traits were quantified for lumbar vertebral bodies of female A/J, C57BL/6J (B6), and C3H/HeJ (C3H) inbred mouse strains from 1 to 105 days of age ($n = 6–10$ mice/age/strain).

Results—Inter-strain differences in external bone size were observed as early as 1 day of age. Reciprocal and rapid changes in the trabecular bone volume fraction and alignment in the direction of axial compression were observed by 7 days of age. Importantly, the inter-strain difference in adult trabecular bone volume fraction was established by 7 days of age. Early variation in external bone size and trabecular architecture was followed by progressive increases in cortical area

K. J. Jepsen, kjepsen@med.umich.edu.

Compliance with ethical standards

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between 28 and 105 days of age, with the greatest increases in cortical area seen in the mouse strain with the lowest trabecular mass.

Conclusion—Establishing the temporal changes in bone morphology for three inbred mouse strains revealed that genetic variation in adult trabecular traits were established early in postnatal development. Early variation in trabecular architecture preceded strain-specific increases in cortical area and changes in cortical thickness. This study established the sequence of how cortical and trabecular traits co-develop during growth, which is important for identifying critical early ages to further focus on intervention studies that optimize adult bone strength.

Keywords

Bone histomorphometry; Cortical; Genetic variation; Growth; Trabecular

Introduction

Vertebral fractures are the most common age-related osteoporotic fractures and often the first site of fragility fracture [1]. Fracture susceptibility later in life has been attributed to low bone mineral density (BMD) and age changes in bone architecture and material properties leading to reduced bone strength [1, 2]. Reduced BMD in the elderly has also been attributed in part to sub-optimal growth of the cortical and trabecular tissues that comprise fracture-prone structures like the vertebral body [3–6]. Since whole-bone strength depends on the relative proportion of cortical and trabecular tissues [7–9], understanding how both bone types develop during growth will be important for identifying factors that contribute to variation in adult bone mass and strength.

The mouse has proven to be a remarkable model to identify genetic and environmental factors contributing to variation in clinically important traits such as BMD and bone strength [10–13]. We and others have shown that cortical and trabecular bone traits are highly correlated in the lumbar vertebral body of different adult inbred mouse strains [12, 14], such that narrow bones tend to have a proportionally thicker cortex and a higher trabecular bone volume fraction compared to wide bones. Similar associations have been observed for human proximal femurs [15–17], suggesting that both the mouse and human skeletal systems coordinate similar sets of traits to establish whole-bone mechanical function. Although much is known about the development of individual cortical and trabecular traits [18, 19], only a few studies have investigated how these traits develop relative to each other (i.e., co-develop) during growth. Tanck et al. showed that a decline in trabecular bone volume fraction was accompanied by an increase in trabecular anisotropy in a growing pig model [20], which suggested that there is coordination between the amount and alignment of trabecular bone relative to the predominant loading directions. How these changes in trabecular bone traits coincided with the development of the cortical shell was not addressed. Roschger et al. showed that within the first years of life, cortical and trabecular regions of the human lumbar vertebral body underwent rapid changes in mineral particle thickness and orientation, which coincided with changes in trabecular orientation from a radial to a vertical/horizontal microarchitecture. Further, the adult mineral concentration and trabecular alignment were established by approximately 5 years of age [21], suggesting that this morphological/material coordination occurred early during growth. These studies convey the

precision and coordination that occur during growth to establish mechanical function in the skeletal system. However, a basic understanding of how cortical and trabecular morphologies develop during early postnatal growth has not been established. Examining the temporal changes in cortical and trabecular traits during growth is expected to provide new fundamental information about how different inbred mouse strains establish adult mechanical function using different sets of traits. Further, determining the temporal changes in bone traits is expected to reveal rapid growth phases when the system may be more susceptible to genetic or environmental perturbations and may help identify the biomechanical mechanisms early in life that affect whole-bone mechanical function [22].

The goal of this study was to establish a timeline of changes in cortical and trabecular morphological traits during growth of the vertebral bodies for A/J, C57BL/6J (B6), and C3H/HeJ (C3H) inbred mouse strains. These strains show significant differences in adult bone mineral content, BMD, and the relative proportion of adult cortical and trabecular traits [13, 14, 23]. Previous work from our lab showed rapid changes in cortical expansion of the femoral diaphysis between 2 and 4 weeks of age among these inbred mouse strains [24]. This early rapid cortical expansion coincided with the beginning of behavioral changes such as ambulation of the mouse reported by others [25]. These analyses in mouse long bone motivated the current hypothesis that rapid changes in cortical and trabecular traits in the vertebral body would also occur early during postnatal development. Prior work examining mouse lumbar vertebral bodies reported age-related changes in bone traits as young as 4–6 weeks of age [18, 19], which we postulate is after the age in which these interactions are established. Identifying the window of rapid morphological change is expected to benefit future studies focused on the material property changes that coincide with the morphological changes.

Materials and methods

Animals

Female ($n = 6/\text{strain}$) and male ($n = 3/\text{strain}$) A/J, C57BL/6J (B6), and C3H/HeJ (C3H) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at 6–8 weeks of age and used to establish breeding colonies under standard environmental conditions. These three inbred mouse strains represent a range of naturally occurring variation in bone traits relative to bone size [13, 14, 23, 24]. In the adult vertebral body, A/J mice have a narrow external size with low cortical area and high trabecular volume. B6 mice have a wide external size with greater trabecular volume compared to A/J. C3H mice have a wide external size but a thicker cortex and low trabecular volume compared to A/J and B6 [14, 26]. The Institutional Animal Care and Use Committee approved the handling and treatment of mice. All applicable institutional and/or national guidelines for the care and use of animals were followed. Mice were fed a standard rodent chow (Purina Rodent Chow 5001) and water ad libitum, subjected to a 12-h light/dark cycle, and housed at a maximum of five mice per cage in the same room. L3 to L4 vertebrae were harvested from female mice at the following seven ages: 1, 4, 7, 10, and 14 days of age ($n = 10/\text{age}/\text{strain}$) as well as 28 and 105 days ($n = 6/\text{age}/\text{strain}$). Female mice were analyzed to be consistent with previous work that characterized adult mouse lumbar vertebral body traits and mechanical properties [14]. The

ages chosen were similar to previous work examining variation in the development of female femoral morphology during growth [24]. The number of mice used per age group was calculated using a power analysis [27] to detect at least a 20 % difference between strains at a power of 0.85, given a significance level of 0.05 and a standard deviation that was 10 % of the mean.

Histomorphometry

All samples were prepared for plastic resin embedding to allow for consistency in assessing morphological traits across all age points. Vertebrae were fixed with 10 % neutralbuffered formalin for 72 h at room temperature, washed with deionized water, then dehydrated with ethyl glycol monomethyl ether for 48 h and acetonitrile for 24 h. Samples were cleared with methyl salicylate for 24 h and infiltrated with a series of solutions that consisted of methyl methacrylate, dibutyl phthalate, and progressively increasing concentrations of dry benzoyl peroxide. After the methyl methacrylate polymerized at 37 °C for 48 h, a low-speed diamond-coated wafering saw (Buehler, Lake Bluff, IL, USA) was used to cut the L3 vertebrae along the mid-transverse plane and L4 vertebrae along the mid-coronal plane. Three-hundred-micron-thick sections from either orthogonal plane were affixed to glass slides and polished to a reflective surface finish. The auto-fluorescence of bone and surrounding tissue was viewed at $\times 100$ magnification using a 365/397-nm-long pass filter in a fluorescence microscope (Zeiss Axioplan, Zeiss, Thornwood, NY, USA) configured with Apitome image enhancement to generate high-resolution images. Images were captured with a CCD camera and AxioVision Imaging Software (version 4.8) that provided an image resolution of 0.65 $\mu\text{m}/\text{pixel}$.

Bone morphological and microarchitectural measurements

ImageJ software (version 1.49i) was used to analyze morphological traits in the transverse and coronal planes. In the L3 vertebral body mid-transverse sections, total cross-sectional area (Tt.Ar) and cortical area (Ct.Ar = Tt.Ar – Med.Ar (medullary area = marrow area + trabecular area)) were measured in the region between the vertebral pedicles (left and right sides) and the depth of the vertebral body (dorsal-ventral direction) as seen in Suppl. Figure 1A. L4 mid-coronal sections were used to quantify the trabecular architecture to capture the wide variation in trabecular architecture along the cranial-caudal direction in A/J, B6, and C3H inbred mouse strains [14]. For this analysis, cortical and trabecular tissues were manually segmented. The auto-fluorescence of marrow tissue in the mid-coronal images was blackened using Adobe Photoshop Elements 4.0 software, and then, images were smoothed with a median filter algorithm, thresholded, and binarized to quantify the fraction of bone tissue from surrounding surfaces (ImageJ software). The percentage of trabecular bone area (%Tb.Ar) was measured as the trabecular bone area within the secondary spongiosa of the centrum (Suppl. Figure 1B). The %Tb.Ar measured in 2D sections was representative of the trabecular bone to total bone volume fraction (BV/TV) derived from three-dimensional (3D) volumes [28]. Trabecular thickness (Tb.Th) was measured for 20 random trabeculae within the secondary spongiosa (Suppl. Figure 1B) using BoneJ software [29]. This method calculates the thickness of individual trabeculae by fitting a sphere within the structure and recording the value of the largest diameter. The average value across all trabeculae in the region of interest was calculated and recorded for each section.

The trabecular degree of anisotropy (DA) was also measured in the cranial-caudal (Cr-Ca) direction within the secondary spongiosa. Trabecular anisotropy was estimated with the mean intercept length (MIL) method [30, 31] using BoneJ software. The value of DA ranged from 0 (isotropic) to 1 (anisotropic). The region of interest to measure anisotropy and other trabecular traits in the mid-coronal plane was consistent for the three mouse strains at each age. Cortical and trabecular traits were measured for three sections per bone and then averaged for each sample. Means and standard deviations were calculated for each strain at each age.

In vivo bone labeling

Additional female A/J, B6, and C3H mice ($n = 5/\text{strain}$) were purchased from Jackson Laboratory at 3–3.5 weeks of age. At this age, mice continue to grow in body size and external bone size; therefore, we quantified whether there are differences in the cortical bone growth of the vertebral body among the three strains. Dynamic parameters were not quantified for the trabecular bone since three-dimensional information could not be generated in this study that would capture the complexity by which the system coordinates the amount (BV/TV) and alignment (anisotropy) of the trabecular bone (see “Results” section and Fig. 4). After a 3-day acclimation period, mice received a series of intraperitoneal injections of the following three different fluorescent bone labels (Sigma-Aldrich, St. Louis, MO, USA) over the course of 1 week: day 1 90 mg/kg Xylenol Orange, day 3 10 mg/kg Calcein Green, and day 5 20 mg/kg Alizarin Red. Mice were sacrificed on day 7 at 4.5 weeks of age. L3 and L4 vertebrae were prepared for plastic embedding as described earlier. Two-hundred-micron-thick sections were cut along the mid-transverse plane and at the distal end approximately 20 % above the caudal growth plate, where vertebral processes were not present. Sections were affixed to glass slides and polished to a reflective surface. Bone labels were viewed using a fluorescence microscope configured with Aipitome image enhancement (Zeiss Axioplan, Zeiss, Thornwood, NY, USA) and filters with an excitation/emission of 485/540 and 558/631 nm. An auto-fluorescent image overlay of the sample using a 365/397-nm-long pass filter identified the proximity of bone labels relative to the size and shape of the cortical or trabecular bone.

Dynamic measures of cortical bone formation of the lumbar vertebral body were determined from bone labels along the dorsal, side, and ventral (near the spinal canal) regions of caudal end sections using ImageJ software. This section represented a complete cross section of the total area of the vertebral body without pedicles. We determined the average osteoblastic activity through mineral apposition rate (MAR; $\mu\text{m}/\text{day}$), which was calculated as the distance between the calcein and alizarin bone labels divided by 2 days. Orange bone labels from a week prior to sacrifice were difficult to detect consistently around the cortex and therefore were not included in MAR measures. It was assumed that the orange label was resorbed as the cortical shell expanded rapidly between 3 and 4 weeks of age. Labeling with no distance between the lines was classified as a point of mineralization with no apposition. Other standard measures included the mineralized surface relative to bone surface (MS/BS; in percent) where mineralizing surface (MS) = $dLS + sLS/2$; dLS is the doublelabeling surface perimeter, sLS is the single-labeling perimeter, and BS is the total bone surface. The amount of bone surface that was actively mineralized or bone formation rate (BFR/BS) was

calculated as $MAR \times (MS/BS)$ ($\mu\text{m}/\text{day}$) [32, 33]. Cortical bone formation measures were compared among strains at similar regions of interest within caudal sections.

Statistical analysis

All results were expressed as mean $\pm$ standard deviation. Differences in morphological traits and mineral apposition rate values among the three mouse strains at each age were determined using one-way ANOVA with Tukey's post hoc tests. In this cross-sectional study, different cohorts of mice were collected at each age point for each inbred strain. Therefore, differences in trait values between two ages were determined using Student's *t* test. Differences were considered statistically significant at $p < 0.05$ (GraphPad software 5.0, San Diego, CA).

Results

Qualitative morphological changes of the lumbar vertebral body during growth

Overall morphological development of the vertebral body during growth was qualitatively similar for A/J (Fig. 1a), B6 (Fig. 1b), and C3H (Fig. 1c) mouse strains. At 1 day of age, the primary ossification center exhibited a cuboidal appearance and lacked a distinct cortex. However, a mesh-like trabecular architecture was clearly visible at this early age. Between 4 and 7 days of age, there appeared to be less trabecular bone and a thinner region of calcified cartilage (as a result of endochondral ossification). At 7 days of age, the trabecular region appeared to be organized along the cranial-caudal direction. By 14 days of age, a well-defined cortical shell was evident, and the trabecular region showed an increase in trabecular alignment along the direction of axial compression (cranial-caudal). In particular, cranial and caudal growth plates continued to expand the bone structure in length while the sides of the vertebral body were joined to the transverse processes. Regions of endochondral ossification as observed by hypertrophic chondrocytes were apparent on the left and right sides of the vertebral body prior to 14 days of age but were no longer apparent afterward. This observation suggested that early variation in cross-sectional bone size was attributed to expansion in width through an endochondral ossification process. By 28 days of age, the vertebral body had acquired general structural features that were largely consistent with that of the adult structure.

Inter-strain variation in bone formation of the lumbar vertebral body

Fluorochrome labeling in both mid-transverse (Fig. 2a) and caudal end (Fig. 2b) sections of lumbar vertebral body samples revealed that bone tissue was deposited in a lamellar fashion on all surfaces between 3.5 and 4.5 weeks of age. Further, the pattern of fluorescent labels indicated that growth of the vertebral body and the spinal canal involved cortical expansion toward the ventral surface and sides of the vertebral body. Clear distinctions of the cortical and trabecular bone were observed including regions where trabeculae merged with the cortical shell (Fig. 2a (a), b (a)).

Within caudal end sections, the MAR along the ventral surface of A/J mouse samples was 56 % greater than the side regions ($p = 0.03$) but not different from the dorsal surface of the vertebral body, which is adjacent to the spinal canal (Table 1). MAR and BFR indices for B6

mice were 142 and 134 % greater along the ventral ($p = 0.02$) and side ($p = 0.002$) surfaces, respectively, compared to the dorsal surface. Bone formation indices were not different within C3H mice vertebral body samples. MAR along the ventral surface at this age was not different among the three mouse strains ($p > 0.39$). Along the dorsal surface, MAR and BFR indices were on average 172 % greater in C3H mice compared to B6 mice ($p = 0.001$); however, C3H showed no difference in MAR along this surface compared to A/J mice. Bone formation indices for expansion on the sides of the vertebral body were 30 % greater for C3H mice compared to A/J mice ($p = 0.01$) but not different from B6 mice ($p = 0.11$).

Postnatal development of the cortical shell

The three strains showed similar increases in body weight at each age point (Suppl. Table 1). Since no differences in body weight were found among the inbred strains, traits were not adjusted for body weight; adjustment of select traits for body weight did not affect the inter-strain differences (data not shown). Significant differences in Tt.Ar were observed among the inbred mouse strains as early as 1 day of age when values were 15.3, 15.4, and 18.4 % of the adult for A/J, B6, and C3H mice, respectively (Fig. 3a and Suppl. Table 1). Each strain showed progressive increases in the vertebral body traits. Tt.Ar increased rapidly between 1 and 10 days of age and was 62.8, 87.8, and 65 % of the adult value by 28 days of age for A/J, B6, and C3H mice, respectively. Tt.Ar was significantly smaller for A/J mice compared to both B6 and C3H mice throughout growth. B6 had a larger Tt.Ar than C3H from 10 to 28 days of age but then was smaller than C3H by 105 days of age.

All strains showed a steady increase in cortical area during growth (Fig. 3b and Suppl. Table 1). After 7 days of age, A/J vertebral bodies had a consistently smaller Ct.Ar compared to B6 and C3H. Ct.Ar was not different between B6 and C3H mice until 28 days of age, at which point the Ct.Ar of B6 was 45 and 31% larger than A/J and C3H, respectively. By 28 days of age, Ct.Ar was 54.7, 75.2, and 48.9 % of the adult values for A/J, B6, and C3H mouse strains, respectively. By 105 days of age, C3H mice had the largest cortical area among the three strains at a value of $0.385 \pm 0.037 \text{ mm}^2$ as a result of a 105 % increase between 28 and 105 days of age.

Postnatal development of trabecular traits

Inter-strain variation in trabecular thickness was apparent from 1 day of age and remained consistent from 1 to 7 days of age (Suppl. Table 1). For each strain, a gradual increase in the trabecular bone area (%Tb.Ar) was observed between 1 and 4 days of age, which was followed by a rapid decline in %Tb.Ar between 4 and 7 days of age ($p < 0.0001$ within each strain; Fig. 4a). After 7 days of age, all three strains showed a progressive increase in %Tb.Ar and achieved over 85 % of the adult %Tb.Ar by 28 days of age. The variation in %Tb.Ar among the three strains seen at 105 days of age was retained at all postnatal ages beginning at 7 days of age ($p < 0.05$ across all strains).

Between 4 and 7 days of age, all three strains showed an increase in anisotropy which coincided with the large decrease observed for the %Tb.Ar during this time frame. This age interval appeared to be critical in the development of phenotypic variation in trabecular traits among the three strains. The DA was 87, 91, and 90 % of adult values by 7 days of age for

A/J, B6, and C3H, respectively (Fig. 4b). A/J mice showed a peak trabecular alignment in the lumbar vertebral body at 14 days of age, which was not significantly different from the adult value ($p = 0.12$; Student's t test). From 28 days of age into adulthood, C3H had a significantly greater trabecular alignment compared to A/J ($p = 0.0003$) and B6 mice ($p = 0.002$). A/J and B6 mice did not show a difference in anisotropy ($p = 0.31$) at these later ages.

Rate of change in trait development during growth

The rates of changes in cortical and trabecular traits of the lumbar vertebral body for A/J, B6, and C3H mice were calculated by dividing the difference in trait values between successive ages by the age interval and plotting the average rate at the mid-span of each age interval (Fig. 5). Peak increases in Tt.Ar (Fig. 5a) and Ct.Ar (Fig. 5b) were observed between 7 and 14 days of age for each strain. Large changes in %Tb.Ar (Fig. 5c) and DA (Fig. 5d) occurred within the first week of life. A/J mice showed a large positive change in %Tb.Ar as early as 1 to 4 days of age compared to B6 and C3H mice. In addition, the lumbar vertebral body of the three mouse strains continued to show positive changes in total area and cortical area from 14 days of age into adulthood, while trabecular traits showed negligible changes after 21 days of age.

Discussion

Analyzing the temporal changes in bone traits during growth of the lumbar vertebral body in three mouse strains provided important new insight into the coordination that occurs between and within cortical and trabecular tissues that give rise to mechanically functional adult structures. There were clear inter-strain differences in the development of cortical and trabecular bone traits during growth to achieve an adult phenotype in A/J, B6, and C3H mice. First, the inter-strain differences in adult external bone size were present early during growth. Second, the organization of trabecular architecture was established largely by 7 days of age, well before mice are known to begin to ambulate [25]. Each mouse strain showed rapid changes in the %Tb.Ar and DA within a narrow window from 1 to 7 days of age during postnatal growth, which was immediately followed by establishing inter-strain variation in %Tb.Ar. Inter-strain variation in Tt.Ar occurred as early as 1 day of age. Finally, inter-strain variation in Ct.Ar was not observed until after 4 weeks of age. These temporal changes suggested that early variation in external bone size and trabecular architecture preceded the maturation of the cortical shell area. Our empirical data supported previous computational simulations of the coordination of cortical area and trabecular architecture within the lumbar vertebral body [9]. As hypothesized, the age at which individual traits in the mouse lumbar vertebral body rapidly changed occurred very early during growth, similar to the mouse femoral diaphysis [24]. The first 2 weeks of postnatal life appeared to be a critical window for interpreting the genetic basis of phenotypic differences that arise among inbred mouse strains.

Bone traits examined as early as 1 day of age in inbred mouse strains advanced our understanding of developmental patterns that are important for achieving adult mechanical function. Between 4 and 7 days of age, a decrease in %Tb.Ar in the lumbar vertebral body

coincided with an increase in cortical area. This pattern among traits was similar to previous studies that examined bone trait maturation in mouse vertebral bodies from 1 to 20 months of age [19] or 6 to 48 weeks of age [18]. In addition, the decrease in %Tb.Ar prior to the rise of inter-strain variation in %Tb.Ar at 7 days of age coincided with an increase in trabecular anisotropy. This pattern was similar to a previous study that examined the development of trabecular microarchitecture in 6–230-week-old porcine lumbar vertebral bodies [20]. We also observed that the variation in Tb.Th was apparent at 1 day of age and remained consistent until 7 days of age. The timing of this coordination between %Tb.Ar and trabecular DA suggested that osteoblast and osteoclast activities are highly coordinated to remove trabecular tissue that leads to a trabecular architecture that is aligned relative to the primary loading direction. It is unclear whether cellular activity was a result of selective resorption of existing trabeculae which contributes to a decrease in %Tb.Ar or whether there is a highly coordinated remodeling in the secondary spongiosa to provide an early internal structural integrity. Investigating the dynamics of architectural development would require high-resolution in vivo computed tomography (CT) imaging and histological staining on plastic sections to retain bone morphology and visualize bone formation and resorption cell activity at multiple ages particularly between 7 and 14 days of age. Gerstenfeld et al. showed that there are spatial and genetic differences in osteoclast activity among A/J, B6, and C3H mouse strains in long bones at 2 weeks of age [34]. Our data identified the postnatal time frame for further investigation of the cellular dynamics including along the growth plate and hypertrophic chondrocyte activity that contributes to the development of variations in adult trabecular bone mass and architecture [35, 36].

Sequential bone labeling advanced our understanding of how bone is formed in cortical and trabecular regions of the lumbar vertebra. Cortical MAR and BFR were measured during growth between 3.5 and 4.5 weeks of age, given that this age was associated with the continued increase in external size, which is a critical determinant of bone strength. This age also marked the beginning of a rapid increase in Ct.Ar, at least for the C3H mice; therefore, we focused on cortical dynamic growth measures for these samples. Differences in how external bone size was achieved during growth among the three mouse strains were interpreted from the inter-strain variation in the in vivo labeling indices in the caudal end of the vertebral body. The differences in the set of index values within a strain suggested genetic differences in osteoblast and osteoclast activities. In C3H mice, high MAR and BFR values along the different regions of the vertebral body section suggested increased cortical thickening throughout the cortex. The osteoblast activity at this age interval is consistent with the thick cortex seen in adult C3H mice. In B6 mice, MAR and BFR values indicated greater periosteal expansion along the ventral and side regions of the caudal end of the vertebral body compared to the dorsal region during this age interval, which suggests an outward expansion in bone size or widening of the total cross section as seen in adulthood. In A/J mice, the BFR values tended to be low along the ventral and side regions of the vertebral body section compared to the other mouse strains suggesting a slower periosteal expansion. However, there was not a difference in values across the section. Adult A/J mice are known to have a narrow bone size compared to the other mouse strains. Here, the index values suggest that there is growth in bone size, but the shape of the bone may have been

established at an early age compared to B6 or C3H mice, and expanding at a rate so that the narrow phenotype is maintained.

Previous studies that analyzed developmental stages to determine the origin of cortical and trabecular bone traits primarily focused on the metaphyseal region of long bones along the periphery of the growth plate [37–39]. Metaphyseal trabecular bone adaptation to mechanical load was shown to undergo “corticalization” or become cortical bone, which was important for endosteal expansion and cortical elongation of long bone in this specific region in adult rabbits [38] and during growth in pigs [39]. The vertebral body structure appeared to develop differently compared to the long-bone metaphysis. The bone labeling patterns showed that the direction of bone growth of the vertebral body was toward the ventral surface, which may be a result of expansion of the spinal canal combined with cortical thickening. Additionally, by 4.5 weeks of age, the labels suggested that growth of the cortical shell occurred by lamellar bone deposition. Prior to this age, it is unclear how the periphery of the primary ossification center consolidated to form a cortical shell. Given that the vertebral body continuously expands outward during early growth, whereas the metaphyseal region of long bones undergoes cortical wasting [37, 38], our data suggest that bone structures in the skeletal system may develop in different ways depending on site-specific differences in whole-bone microarchitectural organization as well as mechanical function. Investigating early trait developmental patterns of different bone structures would provide additional insight into this hypothesis.

Although inter-strain differences in movement and ambulation were not quantified in this study, we compared the morphological changes reported in this study to the sequence of behavioral changes during growth reported by others [25]. As early as 2 days of age, mice have increased strength for mobility through limb reflexes to orient the body and seek nourishment from the mother [25]. Because strength for mobility increases with age, we speculate that this early loading activity along with genetic factors contributed to the change in %Tb.Ar and DA seen between 1 and 4 days of age for each mouse strain. At 7 days of age, when mice tend to show the ability to right themselves to normal stance position on all four paws and crawl in a straight line, we observed rapid changes in trabecular area and anisotropy as well as a rise in inter-strain variation in %Tb.Ar. Between 9 and 15 days of age, when mice have locomotor movements similar to an adult [25], rapid increases in total area and cortical area occurred as well as the rise of interstrain variation in cortical traits continued to develop. This suggested that these early stages of behavioral development including an increase in body size and weight with age provide necessary loading forces on the skeletal system that coincide with establishing phenotypic variation in both trabecular traits and cortical traits. This also advocates for the importance of studying both genetic and environmental factors to understand the development of adult mechanical function [12, 40, 41].

Importantly, temporal changes in traits during growth observed or quantified in this study could not establish a causal nature of how cortical and trabecular traits interact. However, the distinct timing of change in both cortical and trabecular bone traits suggested a highly coordinated development between the different bone types during growth. Both cortical and trabecular traits are genetically regulated, and sets of traits are developmentally and

functionally interdependent to establish a particular adult phenotype [41]. Mapping the temporal sequence in which cortical and trabecular traits co-develop during postnatal growth simply established that early variation in external bone size and trabecular architecture may be a principal event contributing to the characteristic adult phenotypes of these three inbred mouse strains. Identifying that trabecular bone volume fraction and anisotropy were highly coordinated further emphasized the importance of multiple trait interactions that are essential to achieving function within a bone type and should be included in biomechanical and genetic analyses. Our findings did not exclude whether trabecular traits continue to adapt (or co-adapt) with subsequent changes in the cortical shell. Therefore, examining both tissue types is necessary to understand variation in adult bone mechanical function.

There were several limitations to this study. Trait values were not adjusted for body weight since values were not different among strains at different age points. Dynamic bone indices were not quantified for the trabecular bone since 3D information could not be generated in this study. In addition, 2D histological preparations were used for all samples to maintain consistency in the analysis of changes in traits during growth. This preparation was particularly important for bone samples younger than 4 weeks of age with low mineral content that could not be reliably quantified using our desktop micro-CT system. However, this posed a limitation in understanding whether there were differences in trait development and interactions that would occur in a 3D space. Advanced X-ray technology in a high-resolution imaging system may allow quantification of architectural and compositional bone traits in 3D volumes, including changes in tissue mineral density in both the cortical and trabecular regions over time [42]. The 3D histomorphometric methods such as serial milling-imaging operations [43] may be beneficial to quantify the dynamics of the trabecular bone and provide a better understanding of the complex 3D drift patterns of trabecular bone in conjunction with trabecular alignment over time. Examining tissue-level properties during development including mineralization [21] will be important to test whether tissue-level mechanical properties are also coordinately regulated relative to variation in external bone size. The focus of this study was on morphological development; therefore, mechanical properties were not measured during growth. For future work, finite element analysis may be used to estimate how rapid changes in trait development affect the mechanical properties and load sharing between cortical and trabecular regions. This approach may help explain why structures like C3H achieve function with a high cortical area but low trabecular bone fraction.

To determine whether patterns of bone trait development seen among the three inbred mouse strains examined here can be generalized to other inbred strains, it is essential to examine traits across more genetically diverse mouse populations. Examining trait development in multiple inbred mouse strains, recombinant inbred (RI) mouse strains, or outbred strains would provide further understanding of how cortical and trabecular traits functionally interact, given a wide range of natural genetic variation. In addition, investigating temporal changes in bone traits in male specimens would provide insight into whether there is a sex-specific difference in the timing in which traits develop during growth [44].

In conclusion, this was the first study to report the development of cortical and trabecular morphologies throughout postnatal growth for the lumbar vertebral body. We identified

patterns in the development of cortical and trabecular morphological traits from 1 day of age to adulthood that explained when and how differences in adult bone traits arise for three inbred mouse strains. We identified a critical window between 7 and 14 days of age when rapid morphological changes occurred for each inbred mouse strain. Further, it was at this age interval that inter-strain variation observed in adult trabecular traits was established. Reporting of the temporal sequence of normal trait development will be beneficial for efforts aimed at interpreting how bone adapts to targeted (transgenic) or naturally occurring genetic or environment perturbations and how the traits together affect whole-bone mechanical function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Auto-fluorescent images of the lumbar vertebral body in female **a** A/J, **b** B6, and **c** C3H mouse strains. Transverse and coronal mid-sections from each strain at 1, 4, 7, 14, 28, and 105 days during postnatal development. Embedded abbreviations indicating direction: *D* dorsal, *V* ventral, *Cr* cranial, *Ca* caudal. *Scale bar* 1 mm

Fig. 2.

Fluorescent microscopy images of in vivo bone labels within the L4 vertebral body in a 4.5-week-old A/J mouse. Sections were taken at the **a** mid-transverse plane and **b** a transverse slice approximately 20 % above the caudal end growth plate. Intraperitoneal injections were used to administer Xylenol Orange at 7 days pre-sacrifice, followed by Calcein Green at 5 days, and finally Alizarin Red at 3 days pre-sacrifice. Bone labeling patterns seen in these images are representative for all three strains. **a** Cross section of the lumbar vertebrae with *white arrows* indicating cortical expansion was observed toward the ventral surface and along the sides of the vertebral body. **a** Periosteal expansion occurred along the ventral

surface of the vertebral body (*arrows*) (window *a*). A cortical shift was observed along opposite sides of the spinal canal toward the ventral side of the bone (*open arrows*) (window *b* and window *c*). Trabeculae also showed lamellar mineral deposition and in a similar direction as seen in the cortex. **b** In the caudal end slice, we observed periosteal expansion along the sides of the vertebral body (window *a*) and bone deposition toward the endocortical surface along the dorsal region of the vertebral body (window *b*). *Scale bar* 1 mm

Fig. 3.
a Cross-sectional total area and **b** cortical area values from the mid-transverse section of the mouse lumbar vertebral body in three inbred strains—A/J (*white triangle*), B6 (*black circle*), and C3H (*white square*) during growth from 1 to 105 days of age. Data are represented as mean $\pm$ SD. Overall significant differences among strains at a given age as determined by an ANOVA are indicated by asterisk ($p < 0.05$). The *pound sign* represents the specific comparisons where significant differences were found among all three mouse strains at a given age point. Differences among individual strains analyzed from Tukey's post hoc tests are shown in Suppl. Table 1

Fig. 4.

a Percentage trabecular bone area (%Tb.Ar) and **b** trabecular anisotropy values in the direction of axial compression (cranial-caudal) for A/J (*white triangle*), B6 (*black circle*), and C3H (*white square*) mice during growth from 1 to 105 days of age. Data are represented as mean $\pm$ SD. Overall significant differences among strains at a given age as determined by an ANOVA are indicated by asterisk ($p < 0.05$). The *pound sign* represents the specific comparisons where significant differences were found among all three mouse strains at a given age. Differences among individual strains analyzed from Tukey's post hoc tests are shown in Suppl. Table 1

Fig. 5. Rate of change in **a** total area, **b** cortical area, **c** percent of trabecular bone area, and **d** trabecular anisotropy during growth for A/J, C57BL/6J (B6), and C3H/HeJ (C3H) mouse strains. The rate of change at each age was calculated as the average trait value between successive age intervals taken from 1 to 105 days of age

Table 1

Indices of cortical bone formation along different regions of the caudal end section of the lumbar vertebral body for A/J, B6, and C3H mouse strains at 4.5 weeks of age

Index	A/J (n = 5)	B6 (n = 5)	C3H (n = 5)
Ventral			
MAR (µm/day)	3.29 ± 1.56	2.53 ± 0.96 ^M	2.88 ± 0.37
MS/BS (%)	92.22 ± 29.68	83.82 ± 8.61 ^{c,S,s}	95.49 ± 4.08 ^{b,S}
BFR/BS (µm/day)	2.19 ± 0.49 ^c	2.29 ± 1.01 ^R	2.95 ± 0.38 ^a
Sides (left and right)			
MAR (µm/day)	2.11 ± 0.17 ^c	2.38 ± 0.68 ^M	2.78 ± 0.55 ^a
MS/BS (%)	99.40 ± 6.16	98.97 ± 2.74 ^S	98.49 ± 2.35 ^S
BFR/BS (µm/day)	2.13 ± 0.21 ^c	2.27 ± 0.52 ^R	2.67 ± 0.58 ^a
Dorsal			
MAR (µm/day)	1.95 ± 1.10	1.04 ± 0.54 ^c	2.91 ± 0.51 ^b
MS/BS (%)	82.17 ± 9.84	74.37 ± 6.67	80.34 ± 10.23
BFR/BS (µm/day)	1.97 ± 0.83 ^b	0.97 ± 0.35 ^{a,c}	2.58 ± 0.33 ^b

Data are represented as average value per region ± SD. Comparing dynamic histomorphometric measures among mouse strains, using one-way ANOVA and Tukey's post hoc tests. Significant differences indicated at $p < 0.05$

(across rows)

^aSignificantly different from A/J,

^bsignificantly different from B6, and

^csignificantly different from C3H

(within columns) Comparing similar measures at different regions within a mouse strain:

^Msignificant difference from dorsal region MAR value,

^Ssignificant difference from dorsal region MS/BS values,

^ssignificant difference from side region MS/BS values, and

^Rsignificant difference from dorsal region BFR values