METHODS ARTICLE

Guidelines for Dual Energy X-Ray Absorptiometry Analysis of Trabecular Bone-Rich Regions in Mice: Improved Precision, Accuracy, and Sensitivity for Assessing Longitudinal Bone Changes

Jiayu Shi, DDS,^{1,*} Soonchul Lee, MD, PhD,^{2,3,*} Michael Uyeda, MD,^{3,4} Justine Tanjaya, DDS,¹ Jong Kil Kim, BS,¹ Hsin Chuan Pan, DDS,¹ Patricia Reese, DDS,¹ Louis Stodieck, PhD,⁵ Andy Lin, PhD,⁶ Kang Ting, DMD, DMedSci,^{1,3} Jin Hee Kwak, DDS, MS,^{1,†} and Chia Soo, MD, FACS^{3,4,†}

Trabecular bone is frequently studied in osteoporosis research because changes in trabecular bone are the most common cause of osteoporotic fractures. Dual energy X-ray absorptiometry (DXA) analysis specific to trabecular bone-rich regions is crucial to longitudinal osteoporosis research. The purpose of this study is to define a novel method for accurately analyzing trabecular bone-rich regions in mice via DXA. This method will be utilized to analyze scans obtained from the International Space Station in an upcoming study of microgravity-induced bone loss. Thirty 12-week-old BALB/c mice were studied. The novel method was developed by preanalyzing trabecular bone-rich sites in the distal femur, proximal tibia, and lumbar vertebrae via high-resolution X-ray imaging followed by DXA and micro-computed tomography (micro-CT) analyses. The key DXA steps described by the novel method were (1) proper mouse positioning, (2) region of interest (ROI) sizing, and (3) ROI positioning. The precision of the new method was assessed by reliability tests and a 14-week longitudinal study. The bone mineral content (BMC) data from DXA was then compared to the BMC data from micro-CT to assess accuracy. Bone mineral density (BMD) intra-class correlation coefficients of the new method ranging from 0.743 to 0.945 and Levene's test showing that there was significantly lower variances of data generated by new method both verified its consistency. By new method, a Bland–Altman plot displayed good agreement between DXA BMC and micro-CT BMC for all sites and they were strongly correlated at the distal femur and proximal tibia ($r=0.846, p<0.01$; $r=0.879, p<0.01$, respectively). The results suggest that the novel method for site-specific analysis of trabecular bone-rich regions in mice via DXA yields more precise, accurate, and repeatable BMD measurements than the conventional method.

Introduction

DUAL ENERGY X-RAY absorptiometry (DXA) is used clinically for the diagnosis and monitoring of osteoporosis in patients. In the realm of research, DXA has become the most commonly used method for measuring the bone mineral density (BMD) of small animals used in

the study of a wide spectrum of metabolic bone disease studies.^{1–5} It is a simple, fast, low-radiation, and cost-effective alternative to micro-computed tomography (micro-CT) for quantitative analysis of changes in trabecular bone in living subjects; which enables researchers to obtain BMD values at more time points in longitudinal studies than micro-CT.^{6–8}

¹Division of Growth and Development and Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California.

²Department of Orthopedic Surgery, CHA Bundang Medical Center, CHA University, School of Medicine, Gyeonggi-do, Republic of Korea.

³Department of Orthopedic Surgery and the Orthopedic Hospital Research Center, University of California, Los Angeles, Los Angeles, California.

⁴Division of Plastic and Reconstructive Surgery, Department of Surgery, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California.

⁵Aerospace Engineering Sciences, University of Colorado, Boulder, Colorado.

⁶Institute for Digital Research and Education Statistical Consulting Group, University of California, Los Angeles, Los Angeles, California.

*These authors contributed to the work equally and should be regarded as co-first authors.

†Co-senior authors.

Furthermore, due to the relative compact size and light weight of small animal DXA densitometers, DXA is the ideal method to monitor serial changes in BMD in mice housed on board the International Space Station (ISS). The novel method described in this study was designed to be implemented in our ongoing project "Systemic Therapy of NELL-1 for Spaceflight-induced Osteoporosis," collaborating with the Center for the Advancement of Science In Space (CASIS) and the National Aeronautics and Space Administration (NASA) to evaluate a candidate systemic therapy to prevent or treat microgravity-induced bone loss.

Although there are benefits to using DXA versus micro-CT for measuring BMD, many publications using various animal models, including mouse, have described elements that hinder the precision and accuracy of measurements made via DXA.^{9,10} Also, highly variable and fluctuating longitudinal results have been observed in our previous studies, which used large nonspecific regions of interest (ROIs), including the entire bone (e.g., entire femur or lumbar vertebrae) (data not shown). These observations call into question the accuracy and precision of what our literature review found to be the most widely used method of DXA analysis (i.e., conventional method). Possible sources of error include the following: the relatively small body dimensions of mice, difficulty in positioning mice to match the same position they had in previous DXA scans of a longitudinal study, and uncertainty in determining bone edges due to the relatively low resolution of DXA images.¹¹

Trabecular bone-rich regions, such as vertebrae and the metaphysis of long bones, are more important in osteoporosis research in contrast to cortical bone-rich regions because decreased trabecular BMD is the major cause of osteoporotic fractures.¹² Moreover, trabecular bone has a greater response to ovariectomy than cortical bone due to its high turnover rate. The induced osteoporotic state observed in trabecular bone better allows researchers to monitor the effects of medical therapies by measuring the changes in trabecular bone.^{13,14}

Clinically, proper acquisition of DXA BMD measurements requires great attention to subject positioning and image analysis. Poorly performed DXA analysis can lead to errors in diagnosis and therapy.¹⁵ Like DXA studies in humans, DXA studies of small animals can be greatly affected by improper positioning. It is especially important to obtain high quality images of consistently well-positioned subjects in longitudinal studies.¹⁶

Presently, no standardized published protocol exists for precise and accurate placement of ROIs over trabecular bone-rich regions in DXA images used in the assessment of mouse BMD. Nor is there a standardized protocol for proper mouse positioning during DXA scans. The purpose of this study was to develop a standardized method for performing precise and accurate site-specific DXA analyses of trabecular bone-rich regions, in mice. This will enhance the utility of DXA in longitudinal studies of bone loss. To our knowledge, this has not yet been published.

Materials and Methods

Animals and experimental design

Thirty female 12-week-old BALB/c mice were purchased from Charles River Laboratories and handled according to the guidelines of the Chancellor's Animal Research Committee

at the University of California, Los Angeles. The mice were housed in pathogen-free ventilated cages in a light and temperature-controlled environment, and fed *ad libitum*.

To develop the new method of DXA analysis specific to trabecular bone-rich regions, the anatomy of trabecular bone-rich sites in the distal femur, proximal tibia, and lumbar vertebrae (L5, L6) were preanalyzed via high-resolution cabinet X-ray imaging (Faxitron LX-60; Cross Technologies Plc; Fig. 1A). X-ray images of 30 mice were used to ascertain the best mouse position and to customize ROI for DXA analysis of the aforementioned sites. Based on our literature review of the methods used to measure changes in BMD via DXA, the most widely used method of analysis (i.e., conventional method) utilizes large nonspecific ROIs, containing the entire bone (Fig. 2A Improper position), rather than small ROIs specific to trabecular bone-rich sites (Fig. 2B).^{17–20} The conventional method did not contain specific animal positioning instructions for DXA, with one exception: the PIXImus 2 densitometer user manual stated that mice were to be laid prone with heads placed in the tray headrest.

Next, to test the benefits of the proposed method, precision and accuracy were evaluated. First, we scanned cylindrical phantoms of known mineral densities using DXA and micro-CT and compared the results to determine that there was a one-to-one correlation between DXA and micro-CT bone mineral content (BMC) measurements. Because DXA and micro-CT images are 2D and 3D, respectively, the units of DXA BMD and micro-CT BMD are different (DXA BMD: g/cm², micro-CT BMD: g/cm³). To analyze a one-to-one correlation between DXA and micro-CT, it was necessary that the compared parameters were the same. Therefore, we focused on BMC as a more appropriate parameter to evaluate the comparison between DXA and micro-CT. BMC (g) was calculated by multiplying the BMD by ROI in DXA or volume of interest (VOI) in micro-CT. The result showed that both DXA and micro-CT produced the same BMC results, as verified by statistics. Hence, we concluded that they were comparable to each other (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec). Second, three individuals (a medical doctor and two graduate students) analyzed DXA images from 30 mice to assess inter-observer reliability and all DXA measurements were replicated by one of the observers for intra-observer reliability evaluation. Third, DXA was performed once every 2 weeks throughout a 14-week-long longitudinal study. Then, each DXA image was analyzed using both the conventional method and the novel method, and the variance of the two sets of data was compared. Next, accuracy was assessed by comparing both the DXA BMC (g) and BMD (g/cm²) of the conventional method and new method to corresponding *ex vivo* micro-CT BMC (g) and BMD (g/cm³).

Additionally to test the significance of animal position, the following were analyzed: (1) The hip joint angle, knee joint angle, and spine curvature in both the standardized position and the conventional position (which is a nonstandardized random placing of the mouse without the overlapping of bones) were compared to determine whether there was significant angle difference between two positioning methods; (2) The BMDs of trabecular bone-rich sites measured under the standardized position or the conventional position using

FIG. 1. Key anatomic landmarks and proper DXA ROI placement steps. Anatomic analysis via high-resolution X-ray imaging. (A). For the distal femur and proximal tibia the EPD and EPA were defined as the distance between “Line 2” and “Line 3” and the angle between “Line 3” and “Line 4” respectively. To ascertain the patella’s location the knee joint was set to a 90° angle and the PD measured from the patella proximal tip to “Line 3.” To predict the location of lumbar vertebrae L5 and L6, the distance from the IC to the intervertebral space of L5/L6 was measured (ID). To fit the ROI in the vertebral body we measured the height (H) and width (W) of L5 and L6. The height was measured between the end plates and the width was measured at the cranial margin of each vertebral body. Following the anatomic analysis, ROIs were placed on each trabecular bone-rich region (B). For the distal femur and proximal tibia each ROI was placed at the articular edge of the bone and aligned perpendicular to the longitudinal axis of the bone (Step 1). Then, the ROI was moved proximally (femur) or distally (tibia) as depicted in Step 2. The final step involved rotating the ROI (Step 3) based on the angles measured during the anatomic analysis. Proper lumbar ROI positioning required initial ROI placement parallel to IC at the middle of the lumbar vertebrae (Step 1). The ROI was then moved cranially to meet the cranial boarder of L6 (Step 2). Arrows in B show the direction of movement or rotation. Line 1: longitudinal axis of femur or tibia, Line 2: perpendicular line to “Line 1” that starts from the end of each long bone, Line 3: parallel line to “Line 2” that starts from the most proximal point of epiphyseal plate in femur and the most distal point of epiphyseal plate in tibia, Line 4: the line that connects the anterior and posterior margin of epiphyseal plate. DXA, dual energy X-ray absorptiometry; EPA, epiphyseal plate angle; EPD, epiphyseal plate distance; IC, intercrystal line; ID, intercrystal distance; PD, patella distance; ROI, region of interest. Scale bar = 2 mm. Color images available online at www.liebertpub.com/tec

the same customized ROI were compared to determine whether there was any significant effect on BMD by the animal position.

High-resolution X-ray imaging

High-resolution Faxitron X-ray images of the knee joint and lumbar vertebrae were obtained using a setting of 47 kV. The sedated mice were positioned in the Faxitron just as they were during DXA scans, which was the standard position described in the new method. The location of key anatomic landmarks (e.g., epiphyseal plate) in the distal femur, proximal tibia, and lumbar vertebrae (L5, L6) were defined using the Faxitron images and applied to the DXA analysis.

Distal femur and proximal tibia. Four lines were defined to describe the anatomy of epiphyseal plate of the distal femur and proximal tibia: (1) Line 1, the longitudinal axis of femur or

tibia; (2) Line 2, a line perpendicular to “Line 1” starting from the end of femur or tibia; (3) Line 3, a line parallel to “Line 2” starting from the most proximal point of the epiphyseal plate in femur and the most distal point of epiphyseal plate in tibia; and (4) Line 4, the line connecting the anterior and posterior margin of the epiphyseal plate (Fig. 1A).

Epiphyseal plate distance (EPD in Fig. 1A) and epiphyseal plate angle (EPA in Fig. 1A) were analyzed by measuring the distance between “Line 2” and “Line 3” and the angle between “Line 3” and “Line 4.” Patella distance (PD in Fig. 1A) was defined as the distance from the most proximal end of patella to “Line 3,” and was measured after the knees were flexed to 90°. This was done to identify the location of patella during DXA analysis of distal femur (Fig. 1A).

Lumbar vertebrae. The height and width of L5 and L6 were measured separately. Lumbar body height was defined

FIG. 2. Mouse positioning and ROI locations in DXA and micro-CT. Proper mouse position is necessary for optimal DXA images (A). The knee joint should be set to 90° to pull down the patella and make the knee joint line obvious. The spine should be as straight as possible. Improper mouse position makes ROI placement difficult. The conventional method ROI (A: improper position) consisted of the whole bone. Knowing the location of the trabecular bone-rich regions to analyze and the location of the structures to exclude from analysis (e.g., epiphyseal plate and patella) allowed small ROIs specific to trabecular bone-rich regions to be properly positioned consistently. The ideal ROI location for distal femur, proximal tibia, and lumbar vertebrae L6 are shown (B). For the practical anatomical landmark, IC can be defined on DXA image. Also, the indentation of the bone line (arrow) can be used to determine the end of the proximal tibia. To make a proper comparison between DXA and micro-CT, the same ROI was chosen in micro-CT as DXA for the distal femur, proximal tibia, and lumbar vertebrae (C). micro-CT, micro-computed tomography. Scale bar in A=1 cm; B=2 mm; C=1 mm. Color images available online at www.liebertpub.com/tec

and measured as the distance between proximal and distal epiphyseal plate of each vertebra. The width was measured as the length along the upper margin of the vertebral body. Since the intercrystal line (IC in Fig. 1A) is the clearest landmark on DXA images, it was used to provide a rough estimate for intervertebral space L5/6.²¹ Intercrystal distance (Fig. 1A), which extends from the IC to the top of the intervertebral space L5/6, was measured to determine the exact location of the ROI for L5 and L6. The IC was defined by the line joining the superior aspect of the iliac crests (Fig. 1A).

Dual energy X-ray absorptiometry

DXA image acquisition using the new method. The DXA images (Fig. 2A) were acquired using a PIXImus 2 mouse densitometer (GE Lunar PIXImus) after sedation. The highest quality images were obtained by strictly following the novel method. The steps to improved DXA image acquisition are as follows: (1) Fast mice overnight to prevent

calcified food pockets from appearing in the image. (2) Clean the whole imaging area to avoid any noise caused by animal feces, fur, and so on. (3) Lay the mouse prone with the head in the circular groove of the specimen tray. (4) Ensure that the tail of the mouse does not cross any of the long bones. (5) Move the legs of the mouse away from its body. (6) Apply gentle traction to the tail and back to straighten the spine and ensure that the skull is parallel to the sagittal plane. (7) Set the front legs at a 45° angle to the spine to prevent overlap in image. (8) Abduct the femurs so that when viewing a prone mouse from above the femurs appear at a 90° angle to the spine. (9) Set knees to an angle of 90°. (10) Check every image immediately after each DXA scan, and rescan poor quality images (Fig. 2A).

Analysis of DXA images using the new method. To assess the reliability of the anatomic guidelines, the instructive mean distances and angles acquired from the anatomic evaluation were applied during DXA image analysis to put

the ROI on the trabecular bone-rich regions (Fig. 2B). The absolute BMD and BMC values were obtained in g/cm^2 and g , respectively.

For the distal femur and proximal tibia, the length of the ROIs was 1.8 mm (the smallest length allowed by the software), and the width of the ROIs was set just long enough to cover metaphyseal trabecular bone width (Fig. 2B). First, the edge of the ROI was aligned with the articular edge of the distal femur or proximal tibia; then it was shifted and rotated to avoid the epiphyseal plate (Fig. 1B). The articular edge of the distal femur was recognized directly by the yellow bone-edge line, which was automatically drawn in the DXA image. The articular edge of proximal tibia could be identified by the indentation of bone edge line, as noted by red arrow and line in Figure 2B.

For lumbar vertebrae, the length of the ROI on the lumbar vertebrae was 1.8 mm and the width of the ROI was set based on the anatomical analysis of vertebral body. Consequently, the rectangular DXA ROIs were placed inside of L5 and L6 as much as possible. The ROIs were initially placed parallel to the IC at the center of lumbar vertebrae, then they were moved cranially (Fig. 1B). The final ROI position at each skeletal site is shown in Figure 2B. The new DXA method tutorial video is available with the supplementary data (Supplementary Video S1).

Postmortem micro-CT evaluation

To verify the accuracy of DXA analysis, we directly compared the BMC obtained from DXA with BMC obtained from micro-CT. In addition, DXA and micro-CT BMD measurements were correlated as an indicator of relative accuracy of the proposed method versus conventional method. Mice were sacrificed after DXA and the skeletal components of interest were harvested. Samples were then placed in 4% paraformaldehyde for 48 h and transferred to 70% ethanol. The femurs, tibiae, and lumbar vertebrae were scanned using micro-CT (SkyScan 1172; Bruker MicroCT N.V.) at an image resolution of $10\ \mu\text{m}$ (55 kV and 181 mA radiation source, 0.5 mm aluminum filter). After 3D image reconstruction, 3D morphometric analyses were performed using CTAn software provided by manufacturer. The same ROIs were chosen for DXA and micro-CT to ensure that the BMC measurements were of comparable volume. The ROI in the distal femur (Fig. 2C) was composed of 180 transverse slices taken from the distal end of the epiphyseal plate extending 1.8 mm proximally because we used the 1.8 mm length ROI in DXA. The proximal tibia ROI (Fig. 2C) comprised 180 slices beginning just distal to the epiphyseal plate proceeding 1.8 mm distally. This ROI included both the tibia and fibula, following that of DXA, which does not discriminate the tibia and fibula on DXA images. For every slice, the micro-CT ROI covered both the trabecular bone and cortical bone to include the same VOI as the DXA ROI. Lumbar vertebrae ROI (Fig. 2C) width was 1.8 mm and the length of the ROI covered the total vertebral body without including the epiphyseal plate. The thickness of the lumbar ROIs included the posterior column of each vertebral body.

Statistics

The precision of DXA data generated by the new method was assessed by intra-class correlation (ICCs) coefficients.

The ICCs for one-way random effect models were used to assess intra-observer reliability, and ICCs for two-way random effect models were used to assess inter-observer reliability. To model the trajectory of DXA BMD over time, a linear mixed model was used, with a random intercept term to account for repeated measures within each mouse. The distal femur, proximal tibia, and lumbar vertebrae BMD trajectories of the new method and conventional method were modeled separately. Levene's equal variance test was performed to determine whether there was a difference between the variances of the two data sets.

The accuracy of the DXA BMD and BMC data was assessed by Pearson's correlation coefficient (r) and the standard error of estimate (SEE) of a linear regression. Accuracy was also assessed using Bland–Altman plots comparing DXA BMC and micro-CT BMC measurements for each site. Paired sample t -test was used to compare the two data sets measured in the same animal. All statistical analyses were carried out using Stata 14 (StataCorp). Only p -values less than 0.05 were considered statistically significant.

Results

Anatomic analysis

Anatomic analysis data were acquired from high-resolution X-ray images of 60 femurs (30 left, 30 right), 60 tibiae (30 left, 30 right), and 60 lumbar vertebrae (30 L5, 30 L6). The X-ray images revealed that the proximal end of the patella was located 0.47 mm (standard deviation [SD] ± 0.09) inferior to the epiphyseal plate of the distal femur when the knee was flexed 90° (Fig. 1A). Thus, placing the DXA ROI distal to the femur growth plate excluded the patella from the analysis. The mean distances and angles measured for the distal femur, proximal tibia, and lumbar vertebrae are shown in Table 1.

Precision of the new method

Reliability test. The inter-observer reliability tests showed substantial agreement across sites. The distal femur had the highest inter-observer reliability with an ICC of 0.945 followed by proximal tibia (ICC=0.932) and L6 (ICC=0.801). L5 had the lowest inter-observer reliability with an ICC of 0.743. Intra-observer reliability showed similar results to inter-observer reliability across skeletal sites (Table 2).

Longitudinal analysis of BMD for 14 weeks. After obtaining the longitudinal DXA data from the new method (i.e., localized ROI) and the conventional method (i.e., broad ROI), linear mixed models were used to reflect the growth trajectory of DXA BMD at each location. Regardless of the method, models that included the quadratic effects of time yielded the best model fit for distal femur and linear function for proximal tibia and lumbar vertebrae ($p < 0.01$ at all sites). All three sites using both methods showed significant changes in DXA BMD over time compared to the baseline (Supplementary Fig. S2 and Supplementary Table S1).

Although both methods had similar linear mixed models, when the new method was applied to the longitudinal DXA study the BMD gradually increased over time. In contrast, the conventional method had a higher variance in percent-change/2 weeks than the new method. The range of

TABLE 1. RESULTS OF ANATOMIC ANALYSIS

Sites	Measurements	Mean ($\pm$ SD)	Maximum	Minimum
Distal femur	EPD (mm)	3.81 ($\pm$ 0.15)	4	3.58
	EPA ($^{\circ}$)	14.62 ($\pm$ 1.8)	17	11
Proximal tibia	EPD (mm)	2.36 ($\pm$ 0.08)	2.5	2.25
	EPA ($^{\circ}$)	7.54 ($\pm$ 1.44)	10	5
Lumbar vertebrae				
L5	Height (mm)	3.02 ($\pm$ 0.09)	3.14	2.85
	Width (mm)	1.64 ($\pm$ 0.10)	1.76	1.47
L6	Height (mm)	3.04 ($\pm$ 0.09)	3.21	2.92
	Width (mm)	1.51 ($\pm$ 0.05)	1.59	1.42
	ID (mm)	1.13 ($\pm$ 0.79)	2.07	-0.81

EPA, epiphyseal plate angle; EPD, epiphyseal plate distance; ID, distance between the intervertebral space of L5/6 and intercrystal line; SD, standard deviation.

nonoutliers (i.e., all data between the T-bars above and below the box plot in Fig. 3B) was similar between the new and conventional method, but the conventional method had more outliers for the distal femur and proximal tibia. Lumbar vertebrae had a much larger range of nonoutliers for the conventional method than the new method (Fig. 3B). Levene's test of equal variances was performed to determine whether there was a significant difference between the variances from each method; it showed that the data obtained by the conventional method had significantly higher variances at all sites ($p < 0.05$) (Table 3).

Accuracy of the new method

Correlation coefficients between DXA BMC and micro-CT BMC were 0.846 for the distal femur, 0.879 for the proximal tibia, and 0.678 for lumbar vertebrae respectively and significant with $p < 0.01$ for all sites (Fig. 4A). Because DXA BMD and micro-CT BMD are in different units (g/cm^2 and g/cm^3 , respectively) the results showed a similar trend, but a lesser correlation than that of DXA versus micro-CT BMC ($r = 0.752$ for distal femur, $r = 0.764$ for proximal tibia, and $r = 0.465$ for lumbar vertebrae; $p < 0.01$ at all sites) (Supplementary Fig. S3).

The linear regression of new method DXA BMC against micro-CT BMC for the distal femur, proximal tibia, and lumbar vertebrae resulted in SEEs of 0.00026, 0.00024, and 0.0006 g, respectively. The SEEs of all three sites were smaller than the SD of micro-CT BMC measurements (SD: 0.00047 g for distal femur, 0.00049 g for proximal tibia, and 0.00079 g for lumbar vertebrae).

A Bland-Altman plot was generated to determine whether difference in the BMCs obtained by DXA and micro-CT were dependent on the mean BMC values. The mean difference between DXA BMC and micro-CT BMC was -0.000161 g for the distal femur, -0.000043 g for the proximal tibia, and 0.00013 g for the lumbar vertebrae. There was good agreement at all three sites. All data differences between new method DXA BMC and micro-CT BMC were within the 95% confidence interval, except one measurement from the proximal tibia. The data were scattered with no obvious patterns in the Bland-Altman plot of the distal femur or proximal tibia. The difference between DXA and micro-CT BMC was not significantly correlated to mean BMC ($p > 0.05$). The plot of the lumbar vertebrae showed that micro-CT BMC tended to become significantly higher than DXA BMC ($p < 0.05$) when mean BMC was high (Fig. 5).

The significance of animal positioning on BMD measurement

The angles measured under standardized position or conventional position were significantly different for hip joint, knee joint, and lumbar curvature (paired sample t -test, $p < 0.05$, respectively; Supplementary Table S2). The BMD between the standardized position and the conventional position were significantly different for the distal femur and the proximal tibia ($p = 0.013$, $p < 0.001$, respectively). For the lumbar vertebrae, the BMD between the standardized position and the conventional position were different, but not statistically significant (Supplementary Table S3).

TABLE 2. INTER- AND INTRA-OBSERVER RELIABILITY OF DXA BMD MEASUREMENT AT VARIOUS TRABECULAR BONE-RICH SITES

Sites	Inter-observer reliability		Intra-observer reliability	
	ICC	95% CI	ICC	95% CI
Distal femur	0.945	0.923, 0.972	0.964	0.939, 0.995
Proximal tibia	0.932	0.887, 0.973	0.952	0.924, 0.988
L6	0.801	0.764, 0.843	0.835	0.810, 0.858
L5	0.743	0.647, 0.831	0.783	0.683, 0.879

BMD, bone mineral density; CI, confidence interval; DXA, dual energy X-ray absorptiometry; ICC, intra-class correlation.

FIG. 3. Longitudinal change of DXA BMD for 14 weeks at distal femur, proximal tibia, and lumbar vertebrae. To evaluate the precision of the new method, DXA BMD was analyzed longitudinally for 14 weeks. The longitudinal analysis of DXA BMD changes via the conventional method had higher variance compared to the new method for all sites (i.e., distal femur, proximal tibia, and lumbar vertebrae), as shown in (A). The *solid line* fluctuates significantly more than the *dashed line*, which reflects the greater BMD percent-change/2 weeks acquired by conventional method versus the new method. The box plots of percent-change/2 weeks showed a significant variance difference at all sites (B). For distal femur and proximal tibia, the range of nonoutliers (distances between T-bars above and below the box plot) for BMD percent change per 2-week interval was similar between the conventional and new method, but there are more outliers in conventional method. There were no femur BMD measurement outliers for measurements obtained via the new method, but for the conventional method, 3.97% of the measurements were outliers. Tibial BMD measurement outlier percent was 0.74% by the new method, and 4.29% by the conventional method. Lumbar vertebral BMD measurement outlier percent was 2.21% for the new method and 0.71% for the conventional method, but the conventional method had a much wider range of nonoutlier measurements compared to the new method. Levene's test of equal variances was performed to compare the variance from each method ($p < 0.05$ significance). BMD, bone mineral density.

TABLE 3. RESULTS OF BMD PERCENTAGE CHANGES OF EVERY 2 WEEKS IN DXA BY NEW AND CONVENTIONAL METHODS

Sites	Terms	New method	Conventional method
Distal Femur	Mean (%)	2.77	2.64
	SD	0.076 ^a	0.108
	CV	2.726	4.089
Proximal Tibia	Mean	2.89	2.97
	SD	0.0727 ^b	0.089
	CV	2.516	2.994
Lumbar Vertebrae	Mean	3.96	1.94
	SD	0.099 ^a	0.147
	CV	2.507	7.576

^aSignificant at $p < 0.01$ compared to SD of conventional method.

^bSignificant at $p < 0.05$ compared to SD of conventional method. CV, coefficient of variation.

In addition, the difference of SD was assessed using Levene's test to assess the equality of variances. The results showed that there were no significant differences in variances between the BMD data measured in the standardized and the conventional positions.

Discussion

The dynamic measurement of BMD in live subjects is an important step in musculoskeletal tissue engineering research. Several techniques are available for analyzing BMD and BMC *in vivo*. These measurements provide the essential information for identifying the efficacy of candidate for the new drug. DXA is currently the most widely used noninvasive technique to assess BMD and BMC in animal research due to its simple, quick, low-radiation, and cost-effective features. In this study, a new method for precise and accurate DXA analysis of specific trabecular bone-rich regions was derived based on a detailed analysis of the anatomy of the distal femur, proximal tibia, and lumbar vertebrae (L5, L6) (Fig. 6). By following the new method or using it as a guide to customize one's own ROI, the ROI can be made and positioned accurately and precisely over the same trabecular bone-rich regions over time to obtain consistent and reproducible DXA results.

DXA is incapable of analysis specific to trabecular bone because a DXA scan yields a 2D projection image that represents the full thickness of the mouse, similar to an X-ray image. Thus, any DXA ROI containing trabecular bone will also contain the tissues that are above and below the trabecular bone within the ROI. This includes cortical bone since trabecular bone is always contained within cortical bone. The conventional way to assess changes in BMD via DXA has been to measure the BMD of the entire bone for which changes in trabecular bone are being assessed (e.g., the entire femur). However, data generated this way have been correlated with micro-CT BMD, which we found to have a low correlation (Supplementary Fig. S3B).

Most studies concerning the precision and accuracy of BMD measurements made via DXA on rodents were based on rats.^{22,23} Moreover, the studies that reported the precision and accuracy of DXA in mice were with regard to total body BMD and total body fat percent. Such measurements were

obtained by positioning the entire mouse within a single large ROI, except for the head.^{9,24} Based on our review of current literature, the studies that used broad ROIs to measure BMD via DXA in mice specified ROIs that contained whole bones (e.g., whole femur).^{17–20} These ROIs were not specific to trabecular bone-rich regions (Fig. 2A). Similar studies have been performed on isolated mouse limbs *ex vivo*, yet these studies also used ROIs not specific to trabecular bone-rich regions of bone.²⁵

Our study analyzed specific trabecular bone-rich regions via DXA using the conventional method and obtained BMC values that did not correlate well with the same values obtained via micro-CT (Fig. 4B). Factors such as lack of consistent mouse positioning, casual DXA ROI placement, and unmatched DXA ROIs versus micro-CT ROIs likely contributed to the low correlation coefficients. Therefore, the conventional DXA BMD acquisition method could not serve as a reliable way to track mice BMD changes in specific regions of bone. This clearly demonstrated the need for a method to improve upon the precision and accuracy of DXA BMD measurements. To our knowledge, this is the first study designed to develop a standard protocol to obtain precise and accurate BMD measurements specific to trabecular bone-rich regions, via DXA; which is of great concern to osteoporosis investigators.

The trabecular bone-rich regions such as metaphyseal part of long bones (forelimb and hind limb bones) and vertebral body are the most appropriate for bone metabolism research because it has higher bone turnover rate. In this study, we selected the distal femur, proximal tibia, and lumbar vertebrae for the representative areas because most of the articles analyzed these areas for their micro-CT analysis among the various trabecular bone-rich regions.^{19,26–32} Moreover, anatomically these areas are relatively larger in marrow cavity and hence have a greater quantity of trabecular bone; this makes quantification easier and more reproducible. For example, in the femur and tibia, distal femur is bigger than the proximal femur and proximal tibia is bigger than the distal tibia. Femurs and tibiae also constitute the appendicular bones prone to osteoporotic fractures. The lumbar vertebral body is an important area for bone research because it represents the axial bone most prone to osteoporotic compression fractures and the vertebral bodies also become increasingly larger from cranial to caudal until the end of the lumbar vertebrae, thereby making L5 and L6 the largest lumbar vertebral bodies. Especially in small mammals such as the mouse, this larger bone size significantly aids in increasing the accuracy of research involving loss or gain of the trabecular bone.

We observed that mice laying prone on a flat surface do not have femurs and tibiae parallel to the frontal plane. This yields a view of the femur and tibia that is not the true lateral view of the femur or tibia. The EPD and EPA of the femur and tibia were measured via X-ray imaging using the same view as the DXA scan, not the true lateral view (Fig. 1A). The same views were used for both DXA and X-ray imaging to apply the measurements obtained via X-ray to DXA images. Examination of the X-ray images revealed that the patella was positioned inferior to the epiphyseal plate of the femur when the knee was flexed to an angle of 90°; this happens via patella tracking, which helps prevent it from being included in distal femur DXA ROI (Fig. 1A).

FIG. 4. Correlation between DXA BMC and micro-CT BMC. Correlation analysis of the new method showed that the BMC of DXA was significantly correlated with the BMC of micro-CT at all sites, distal femur, proximal tibia, and lumbar vertebrae (A) ($p < 0.01$). The correlation coefficient of the new method was greatest for the proximal tibia (0.879), followed by distal femur (0.846), and lumbar vertebrae (L5, L6) with 0.678. However, conventional method DXA BMC was not significantly correlated with micro-CT BMC ($p > 0.05$ in all sites) (B). Pearson's correlation coefficients were used ($p < 0.05$ significance; r = correlation coefficient). BMC, bone mineral content.

However, flexing the knee past 90° made the joint line unclear, and made the proximal tibia and distal femur indistinguishable. Proper positioning of the spine was also important. Analysts could position lumbar ROIs more reliably when the spine was straight (i.e., not bent laterally).

Analysts should carefully avoid the epiphyseal plate when defining trabecular bone-rich ROIs because the epiphyseal plate is adjacent to the metaphysis, and it is well known that rodent epiphyseal growth plates never close.^{33,34} Unlike trabecular bone, which is spongy, the epiphyseal plate has a provisional zone of calcification. Therefore, BMD measurements will be influenced if this region is included in analyses. Because of the close proximity of the epiphyseal plate and patella to trabecular bone-rich regions, minor changes in the position of site-specific DXA ROIs can cause

significant changes in BMD measurements. Positioning of site-specific ROIs following strict animal positioning guidelines is critical to gathering precise analytical results. For example, a BMD increase of 15.67% was observed when the ROI was rotated slightly to include the epiphyseal plate and patella (Supplementary Fig. S4). This suggested an analytical error because the expected change in mouse BMD of 4–6 weeks post-ovariectomy was only 10–15%.^{35–39} An error increase in BMD of over 10% could impact the SD greatly.

In the analysis of the significance of animal positioning on BMD measurement, we found that there were significant differences in the distal femur and proximal tibia by the animal positioning, but not in the lumbar vertebrae. This is likely because the difference in lumbar curvature was not as significant as in distal femur or proximal tibia between the

FIG. 5. Results of Bland–Altman plot for accuracy test. The Bland–Altman plot displayed individual dots scattered around the bias without obvious patterns, which means the differences were not significantly related to the difference between DXA and micro-CT results for distal femur (A) and proximal tibia (B) ($p > 0.05$). The plot of the lumbar vertebrae (C) showed that the BMC of micro-CT tended to become higher than the BMC of DXA when the BMC became higher, and the related difference between two measurements gets larger ($p < 0.05$). However, there was good agreement between the DXA and micro-CT measurements for all sites. All the differences between DXA BMC and micro-CT BMC were within the 95% confidence interval, with the exception of one sample from the proximal tibia. Linear regression test was used ($p < 0.05$ significance).

two animal positioning methods. Therefore, we can conclude that selection of proper ROI size and that the location of ROI is more important for the lumbar vertebrae analysis than the animal position as long as the spine is not curved too much. Moreover, the results show that there were no

significant differences in variances between BMD data measured in the standardized and the conventional animal positions. This suggests that positioning does not determine precision, but rather that a small ROI is important for precision. Also, we can conclude that the standardized animal position is important for accuracy because the BMD means between the standardized position and the conventional position were significantly different. In spite of the abundance of BMD studies in humans, we could not find comprehensive research addressing changes in BMD throughout longitudinal studies of mice. Several studies reported gradual increases in BMD after the mice had fully matured.^{38,40–42} Likewise, when strictly following the new method, our longitudinal study showed a gradual BMD increase of 2–4% every 2 weeks according to both linear and quadratic models.

Advances in micro-CT technology have made it the gold standard of bone densitometry.⁴³ Several studies have used *in vivo* micro-CT for noninvasive evaluation of trabecular bone,^{44–46} proving it to be a precise and accurate tool for the monitoring of changes in bone stereology, bone volume, and microarchitecture.^{47–50} However, frequent *in vivo* micro-CT scans are limited due to the high radiation doses, and *ex vivo* micro-CT can only be used to measure changes in BMD at the various time points of a longitudinal study by sacrificing animals at each time point. The results of this study show that strict adherence to the new method can improve the precision and accuracy of DXA BMD measurements; which will reduce the need for micro-CT and the number of animals used in longitudinal research.

To make a proper comparison between DXA and micro-CT the same ROI was chosen using both apparatuses. The DXA BMC and micro-CT BMC measurements from the femur and tibia were strongly correlated; whereas lumbar vertebrae were moderately correlated (Fig. 4A). The DXA BMD new method was compared to micro-CT BMD obtained using a polymorphic volume limited to the bone marrow space (1.8 mm from the epiphyseal plate) and the same strong correlation between DXA BMD and micro-CT BMD was found for femur and tibia, but less correlation for lumbar vertebrae (data not shown). This is consistent with previous findings; as stated by Bolotin, the lumbar vertebrae is known to have most variable DXA BMD values because it can be affected more by extra-osseous abdominal fat than the knee joint.⁵¹

This study was funded by, and collaborated with CASIS. The method developed by this study will be implemented in an ongoing research study of a novel medical therapy for microgravity-induced osteoporosis. Although advancements in space science have made prolonged space flights possible, microgravity-induced bone loss has placed limits on extended space flights.^{52–54} Presently, there is no approved drug to prevent or treat microgravity-induced bone loss. NASA ranked microgravity-induced osteoporosis and its complications as the foremost disease risk among astronauts.⁵⁵ DXA was chosen to quantify the changes in BMD throughout the longitudinal medical therapy study on the ISS because it is fast, easy to perform, economical, and the apparatus is space efficient. The method developed by this study will be used for the BMD measurement in the space. Forty mice will be sent to the ISS for 9 weeks, and BMD will be dynamically tracked by DXA every other week by

FIG. 6. Flowchart of new DXA method. The flow chart shows the steps of new DXA method of scanning procedures and analysis procedures as a brief guideline.

astronauts onboard the ISS. For the spaceflight study, DXA is not only the sole method of BMD analysis that can be carried out onboard the ISS, but also the only radiographic equipment available in the same model in all locations involved in the research project (the animal vendor, the ISS, the Kennedy Space Center in Florida, and the university). To enhance the accuracy and reproducibility of the DXA measurement in our upcoming high-priority project, we needed to conduct this study to standardize the scan and analysis method.

The limitations of this study are addressed as follows. First, this study did not account for bone growth during the study period, but the effect of growth were assumed to be insignificant because we just studied the epiphysis length of the femur and tibia for measuring the accurate anatomic location to position the ROI. As such, it was important to determine whether the length of the epiphysis of the bones would change with growth instead of the whole bone size. After puberty when the bone growth is almost complete, the length of the epiphysis will not change much because the cartilage portion in the epiphysis specifically has already completely become bone and the direction of bone growth is toward the diaphysis. Also through literature reviews, we found that after 12 weeks of age, the bone size increase in female mice is small. Despite strain and sex, the femoral length of mice only increased $\sim 0.5\text{--}0.7 \text{ mm}$ from 3 to 6 months of age,^{41,56,57} while the whole femur length is about 15.5–16 mm during this period, thus the increasing length is just around 3.1–4.5% of the total femur size. Therefore, during the 3-month period the bone growth in mice long bones is not discernable on DXA scans and therefore would be unlikely to introduce variances in the final outcome. For

the lumbar vertebrae, the size change of L5 and L6 of BALB/c mice from 12 to 24 weeks is only around 0.20 mm (data not shown), and similar result is validated by Buie *et al.* for L3 height.⁵⁸ Therefore, the whole size change across 3-month period just equals to one smallest measurement unit (0.18 mm) of the ROI movement on the DXA software interface, which is a 1-click difference in the analysis procedure; the resultant error is significantly less than the conventional method where the error is much greater than “just a click away.”

Second, even when utilizing the new method, the epiphyseal plate and/or the surrounding bony structures including the proximal fibula and fabella could not be completely excluded. Nevertheless, their effects on BMD were found insignificant because the proportion of these structures was much smaller compared to that of distal femur or proximal tibia. Based on our calculation using micro-CT, the BMD value at the distal femur was just 1.14% different with or without the surround bony structure (i.e., fabella). For tibia, the BMD value was just 2.74% different with or without the inclusion of fibula. Nevertheless, the added BMD by surrounding bony structures in the selected ROI is an inevitable innate limitation of the DXA densitometry that exists regardless of the analysis method. The new standardization of method proposed in this study was found to improve the precision, accuracy, and sensitivity compared with the conventional method.

In conclusion, the new method developed by this study verifiably leads to more precise and accurate BMD measurements obtained from DXA analysis of ROIs specific to trabecular bone-rich regions in mice. It was designed for longitudinal studies of the trabecular bone-rich regions of

the distal femur, proximal tibia, and lumbar vertebrae. Analysts should find the new method easy to learn and quick to perform.

Acknowledgments

This work was supported by the Center for the Advancement of Science in Space (CASIS) GA-2014-154, NIH/NIAMS R01 AR061399-01A1, and NIH/NIAMS R01 AR066782-01 and AAOF OFDFA award for Dr. Jin Hee Kwak. The authors would like to thank Dr. Philip Ender and Dr. Joni Ricks at the UCLA Institute for Digital Research and Education Statistical Consulting Group for their constructive advice on biostatistics. The authors would also like to thank Dr. Aldons Lusis at the UCLA Gonda Neuroscience and Genetics Research Center, and Pia Ang, Dr. Mehdi Cheheltenan, and Rachel Lim for their technical support.

Disclosure Statement

No competing financial interests exist.

References

- Gargiulo, S., Gramanzini, M., Megna, R., *et al.* Evaluation of growth patterns and body composition in C57Bl/6J mice using dual energy X-ray absorptiometry. *BioMed Res Int* 253067, 2014.
- Grier, S.J., Turner, A.S., and Alvis, M.R. The use of dual-energy x-ray absorptiometry in animals. *Invest Radiol* 31, 50, 1996.
- Ammann, P., Rizzoli, R., Slosman, D., and Bonjour, J.P. Sequential and precise in vivo measurement of bone mineral density in rats using dual-energy X-ray absorptiometry. *J Bone Miner Res* 7, 311, 1992.
- Griffin, M.G., Kimble, R., Hopfer, W., and Pacifici, R. Dual-energy X-ray absorptiometry of the rat: accuracy, precision, and measurement of bone loss. *J Bone Miner Res* 8, 795, 1993.
- Sievanen, H., Kannus, P., and Jarvinen, M. Precision of measurement by dual-energy X-ray absorptiometry of bone mineral density and content in rat hindlimb in vitro. *J Bone Miner Res* 9, 473, 1994.
- Leitner, M.M., Tami, A.E., Montavon, P.M., and Ito, K. Longitudinal as well as age-matched assessments of bone changes in the mature ovariectomized rat model. *Lab Anim* 43, 266, 2009.
- Kallai, I., Mizrahi, O., Tawackoli, W., Gazit, Z., Pelled, G., and Gazit, D. Microcomputed tomography-based structural analysis of various bone tissue regeneration models. *Nat Protoc* 6, 105, 2011.
- Larkin, A., Sheahan, N., O'Connor, U., *et al.* QA/acceptance testing of DEXA X-ray systems used in bone mineral densitometry. *Radiat Prot Dosimetry* 129, 279, 2008.
- Nagy, T.R., and Clair, A.L. Precision and accuracy of dual-energy X-ray absorptiometry for determining in vivo body composition of mice. *Obes Res* 8, 392, 2000.
- Brommage, R. Validation and calibration of DEXA body composition in mice. *Am J Physiol Endocrinol Metab* 285, E454, 2003.
- Lochmuller, E.M., Jung, V., Weusten, A., Wehr, U., Wolf, E., and Eckstein, F. Precision of high-resolution dual energy X-ray absorptiometry of bone mineral status and body composition in small animal models. *Eur Cell Mater* 1, 43, 2001.
- Simkin, A., Ayalon, J., and Leichter, I. Increased trabecular bone density due to bone-loading exercises in postmenopausal osteoporotic women. *Calcif Tissue Int* 40, 59, 1987.
- Thompson, D.D., Simmons, H.A., Pirie, C.M., and Ke, H.Z. FDA guidelines and animal models for osteoporosis. *Bone* 17, 125S, 1995.
- Verhulp, E., van Rietbergen, B., and Huiskes, R. Load distribution in the healthy and osteoporotic human proximal femur during a fall to the side. *Bone* 42, 30, 2008.
- Watts, N.B. Fundamentals and pitfalls of bone densitometry using dual-energy X-ray absorptiometry (DXA). *Osteoporos Int* 15, 847, 2004.
- Katikaneni, R., Ponnappakkam, A., Miller, E., Ponnappakkam, T., and Gensure, R.C. A new technique for precisely and accurately measuring lumbar spine bone mineral density in mice using clinical dual energy X-ray absorptiometry (DXA). *Toxicol Mech Methods* 19, 225, 2009.
- Tsujio, M., Mizorogi, T., Kitamura, I., *et al.* Bone mineral analysis through dual energy X-ray absorptiometry in laboratory animals. *J Vet Med Sci* 71, 1493, 2009.
- Fujioka, M., Uehara, M., Wu, J., *et al.* Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. *J Nutr* 134, 2623, 2004.
- Bartell, S.M., Kim, H.N., Ambrogini, E., *et al.* FoxO proteins restrain osteoclastogenesis and bone resorption by attenuating H₂O₂ accumulation. *Nat Commun* 5, 3773, 2014.
- Cawley, N.X., Yanik, T., Woronowicz, A., Chang, W., Marini, J.C., and Loh, Y.P. Obese carboxypeptidase E knockout mice exhibit multiple defects in peptide hormone processing contributing to low bone mineral density. *Am J Physiol Endocrinol Metab* 299, E189, 2010.
- Rao, R.D., Bagaria, V.B., and Cooley, B.C. Posterolateral intertransverse lumbar fusion in a mouse model: surgical anatomy and operative technique. *Spine J* 7, 61, 2007.
- Rozenberg, S., Vandromme, J., Neve, J., *et al.* Precision and accuracy of in vivo bone mineral measurement in rats using dual-energy X-ray absorptiometry. *Osteoporos Int* 5, 47, 1995.
- Kastl, S., Sommer, T., Klein, P., Hohenberger, W., and Engelke, K. Accuracy and precision of bone mineral density and bone mineral content in excised rat humeri using fan beam dual-energy X-ray absorptiometry. *Bone* 30, 243, 2002.
- Iida-Klein, A., Lu, S.S., Yokoyama, K., Dempster, D.W., Nieves, J.W., and Lindsay, R. Precision, accuracy, and reproducibility of dual X-ray absorptiometry measurements in mice in vivo. *J Clin Densitom* 6, 25, 2003.
- Franco, G.E., Litscher, S.J., O'Neil, T.K., Piette, M., Demant, P., and Blank, R.D. Dual energy X ray absorptiometry of ex vivo HcB/Dem mouse long bones: left are denser than right. *Calcif Tissue Int* 76, 26, 2005.
- Bouxsein, M.L., Myers, K.S., Shultz, K.L., Donahue, L.R., Rosen, C.J., and Beamer, W.G. Ovariectomy-induced bone loss varies among inbred strains of mice. *J Bone Miner Res* 20, 1085, 2005.
- Bucay, N., Sarosi, I., Dunstan, C.R., *et al.* osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 12, 1260, 1998.
- He, Y.X., Zhang, G., Pan, X.H., *et al.* Impaired bone healing pattern in mice with ovariectomy-induced osteoporosis: a drill-hole defect model. *Bone* 48, 1388, 2011.
- Hohman, E.E., and Weaver, C.M. A grape-enriched diet increases bone calcium retention and cortical bone properties in ovariectomized rats. *J Nutr* 145, 253, 2015.

30. Iwaniec, U.T., Yuan, D., Power, R.A., and Wronski, T.J. Strain-dependent variations in the response of cancellous bone to ovariectomy in mice. *J Bone Miner Res* **21**, 1068, 2006.
31. Nam, S.H., Jeong, J.H., Che, X., *et al.* Topically administered Risedronate shows powerful anti-osteoporosis effect in ovariectomized mouse model. *Bone* **50**, 149, 2012.
32. Willey, J.S., Livingston, E.W., Robbins, M.E., *et al.* Risedronate prevents early radiation-induced osteoporosis in mice at multiple skeletal locations. *Bone* **46**, 101, 2010.
33. Mehta, G., Roach, H.I., Langley-Evans, S., *et al.* Intrauterine exposure to a maternal low protein diet reduces adult bone mass and alters growth plate morphology in rats. *Calcif Tissue Int* **71**, 493, 2002.
34. Roach, H.I., Mehta, G., Oreffo, R.O., Clarke, N.M., and Cooper, C. Temporal analysis of rat growth plates: cessation of growth with age despite presence of a physis. *J Histochem Cytochem* **51**, 373, 2003.
35. Bonnet, N., Laroche, N., Beaupied, H., *et al.* Doping dose of salbutamol and exercise training: impact on the skeleton of ovariectomized rats. *J Appl Physiol* (1985) **103**, 524, 2007.
36. Park, E., Jin, H.S., Cho, D.Y., *et al.* The effect of Lycii Radicis Cortex extract on bone formation in vitro and in vivo. *Molecules* **19**, 19594, 2014.
37. Cui, Y., Niziolek, P.J., MacDonald, B.T., *et al.* Lrp5 functions in bone to regulate bone mass. *Nat Med* **17**, 684, 2011.
38. Blais, A., Malet, A., Mikogami, T., Martin-Rouas, C., and Tome, D. Oral bovine lactoferrin improves bone status of ovariectomized mice. *Am J Physiol Endocrinol Metab* **296**, E1281, 2009.
39. Stunes, A.K., Westbroek, I., Gustafsson, B.I., *et al.* The peroxisome proliferator-activated receptor (PPAR) alpha agonist fenofibrate maintains bone mass, while the PPAR gamma agonist pioglitazone exaggerates bone loss, in ovariectomized rats. *BMC Endocr Disord* **11**, 11, 2011.
40. Datta, N.S., Samra, T.A., and Abou-Samra, A.B. Parathyroid hormone induces bone formation in phosphorylation-deficient PTHR1 knockin mice. *Am J Physiol Endocrinol Metab* **302**, E1183, 2012.
41. Glatt, V., Canalis, E., Stadmeier, L., and Bouxsein, M.L. Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. *J Bone Miner Res* **22**, 1197, 2007.
42. Philip, B.K., Childress, P.J., Robling, A.G., *et al.* RAGE supports parathyroid hormone-induced gains in femoral trabecular bone. *Am J Physiol Endocrinol Metab* **298**, E714, 2010.
43. Genant, H.K., Gordon, C., Jiang, Y., Lang, T.F., Link, T.M., and Majumdar, S. Advanced imaging of bone macro and micro structure. *Bone* **25**, 149, 1999.
44. Kapadia, R.D., Stroup, G.B., Badger, A.M., *et al.* Applications of micro-CT and MR microscopy to study pre-clinical models of osteoporosis and osteoarthritis. *Technol Health Care* **6**, 361, 1998.
45. Gross, G.J., Dufresne, T.E., Smith, T., *et al.* Bone architecture and image synthesis. *Morphologie* **83**, 21, 1999.
46. Borah, B., Gross, G.J., Dufresne, T.E., *et al.* Three-dimensional microimaging (MRmicroI and microCT), finite element modeling, and rapid prototyping provide unique insights into bone architecture in osteoporosis. *Anat Rec* **265**, 101, 2001.
47. Feldkamp, L.A., Goldstein, S.A., Parfitt, A.M., Jesion, G., and Kleerekoper, M. The direct examination of three-dimensional bone architecture in vitro by computed tomography. *J Bone Miner Res* **4**, 3, 1989.
48. Kuhn, J.L., Goldstein, S.A., Feldkamp, L.A., Goulet, R.W., and Jesion, G. Evaluation of a microcomputed tomography system to study trabecular bone structure. *J Orthop Res* **8**, 833, 1990.
49. Odgaard, A., and Gundersen, H.J. Quantification of connectivity in cancellous bone, with special emphasis on 3-D reconstructions. *Bone* **14**, 173, 1993.
50. Goulet, R.W., Goldstein, S.A., Ciarelli, M.J., Kuhn, J.L., Brown, M.B., and Feldkamp, L.A. The relationship between the structural and orthogonal compressive properties of trabecular bone. *J Biomech* **27**, 375, 1994.
51. Bolotin, H.H. DXA in vivo BMD methodology: an erroneous and misleading research and clinical gauge of bone mineral status, bone fragility, and bone remodelling. *Bone* **41**, 138, 2007.
52. Lang, T., LeBlanc, A., Evans, H., Lu, Y., Genant, H., and Yu, A. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. *J Bone Miner Res* **19**, 1006, 2004.
53. LeBlanc, A., Schneider, V., Shackelford, L., *et al.* Bone mineral and lean tissue loss after long duration space flight. *J Musculoskelet Neuronal Interact* **1**, 157, 2000.
54. McCarthy, I., Goodship, A., Herzog, R., Oganov, V., Stussi, E., and Vahlensieck, M. Investigation of bone changes in microgravity during long and short duration space flight: comparison of techniques. *Eur J Clin Invest* **30**, 1044, 2000.
55. Vico, L., Collet, P., Guignandon, A., *et al.* Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. *Lancet* **355**, 1607, 2000.
56. Beamer, W.G., Donahue, L.R., Rosen, C.J., and Baylink, D.J. Genetic variability in adult bone density among inbred strains of mice. *Bone* **18**, 397, 1996.
57. Willingham, M.D., Brodt, M.D., Lee, K.L., Stephens, A.L., Ye, J., and Silva, M.J. Age-related changes in bone structure and strength in female and male BALB/c mice. *Calcif Tissue Int* **86**, 470, 2010.
58. Buie, H.R., Moore, C.P., and Boyd, S.K. Postpubertal architectural developmental patterns differ between the L3 vertebra and proximal tibia in three inbred strains of mice. *J Bone Miner Res* **23**, 2048, 2008.

Address correspondence to:

Jiayu Shi, DDS

Division of Growth and Development
and Section of Orthodontics

School of Dentistry

University of California, Los Angeles
Los Angeles, CA 90095

E-mail: jiayushi@ucla.edu

Received: August 19, 2015

Accepted: March 2, 2016

Online Publication Date: April 11, 2016