Knee-Loading Modality Drives Molecular Transport in Mouse Femur

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Abstract—Mechanical loading is well known to stimulate bone remodeling. Load-driven interstitial fluid flow and molecular transport have been postulated to play a role in the enhancement of bone formation. In order to evaluate load-driven molecular transport in a lacunocanalicular network, we conducted fluorescence recovery after photobleaching (FRAP) experiments using lacunae stained with uranine (376 Da). Loads were applied to a mouse femur *ex vivo* with a novel knee-loading modality, where the distal epiphysis was loaded with a sinusoidal force at 2 Hz. The lacunae in the diaphysis located 25% (∼4 mm) proximal to the loading site were photobleached and sequentially imaged, and a time constant for fluorescence recovery was determined both with and without knee loading. The time constant was estimated as the period to recover 63% of fluorescent intensity using a best-fit exponential curve. The results reveal that the applied loads shortened the time constant from 33 ± 9 s with non-loading control to 25 ± 11 s with knee loading ($p = 0.0014$). The strain in the measurement site was < 100 μstain along the femoral midshaft, which was an order of magnitude smaller than the minimum effective strain threshold for bone remodeling. Taken together, the current study supports the notion that molecular transport in cortical bone is enhanced by the loads applied to the epiphysis without inducing significant *in situ* strain in the diaphysis.

Keywords—Mechanical loading, Lacuna, Fluorescent recovery after photobleaching, Interstitial fluid flow.

INTRODUCTION

Studies on bone remodeling during the past 100 years have revealed a determining role for dynamic mechanical loads.[11,18,26] Mechanical loads strengthen bone, whereas long-term bed rest, cast immobilization and microgravity of space flight induce bone loss and mineral changes.[1,6,7] Dynamic deformation of porous bone matrix is considered to stimulate interstitial fluid flow and molecular transport.[15,31] Elucidation of the details of the effects of dynamic deformation on flow and transport should contribute to our understanding of bone remodeling and the development of a safe, efficient loading modality.[24] We have, therefore, conducted a real-time, *in situ* transport analysis in response to dynamic deformation.

Transport of varying molecules such as ions, nutrients, and waste materials within lacunae is mainly governed by a process of diffusion and convection. Mechanical loads are considered to facilitate their transport through a network of canaliculi as well as Haversian and Volkmann's canals.[5,8] A typical lacuna is ∼20 μm in length and ∼5 μm in thickness, while the diameter of individual canaliculi ranges from 50 to 100 nm.[2,16,30] Because of the complex hierarchical architecture of the lacunocanalicular network, experimental evaluation is mandatory to validate transport models.[17,22] Histological inspections of bone cross-sections support the idea that mechanical loads stimulate transport of dye molecules in the lacunocanalicular network.[13,21] Such approaches are, however, not suited to monitor real-time *in situ* responses.

In the present study, we employed fluorescence recovery after photobleaching (FRAP) to evaluate the apparent transport of molecular markers with and without dynamic loading. FRAP is a fluorescence-based technique for estimating a site-specific diffusion profile *in situ*.[14,19] After fluorescently labeled molecules are allowed to permeate a bone sample, an intense laser is focused on a lacuna to cause irreversible photobleaching of fluorescent molecules. This creates a dark spot in the photobleached lacuna, which gradu-

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ally recovers fluorescence due to the transport of neighboring fluorescent molecules. The rate of fluorescence recovery is dependent on diffusional mixing and convection around the lacuna as well as local and global bone architectures.[28] In this study we used a time constant of fluorescence recovery as an indicator of load-driven alterations in molecular transport.

A unique loading method, a so-called "knee-loading modality," was employed, in which mechanical loads were given to the distal epiphysis of a mouse femur using a custom-made piezoelectric loader. The knee-loading modality is one of the joint-loading modalities. In our previous mouse studies, elbow loading enhanced bone formation in the ulna and knee loading strengthened the tibia and the femur.[25,29] These loading modalities appear to stimulate osteogenesis in the diaphysis without inducing obvious *in situ* strain at the site of bone formation.[23] In this study, we examined whether knee loading would facilitate molecular transport in the femoral diaphysis without significantly deforming its cortical bone. The results supported the view that molecular transport in the lacunae can be enhanced by a remotely applied loads with this novel knee-loading modality.

MATERIALS AND METHODS

Bone Sample

Femurs of C57BL/6 mice (female, ∼14 weeks old) with a body weight of ∼20 g were used. The procedures for the use of mice were approved by IACUC. A 0.2 ml saline solution consisting of 10 mg/ml uranine (fluorescein sodium salt, $C_{20}H_{10}O_5Na_2$, 376.27 Da) was injected into the tail vein. Thirty minutes after injection the mice were anesthetized with 2% isoflurane

and sacrificed using cervical dislocation. The femurs were surgically harvested and maintained in the αMEM culture media supplemented with 10% fetal calf serum and a mixture of 1 U/ml penicillin and 1 μg/ml streptomycin.

Knee Loading

A custom-made piezoelectric mechanical loader was used to apply lateral loads to the distal epiphysis of the femur *ex vivo*. The loader was mounted on a Zeiss LSM 510 multi-photon fluorescence microscope system (Fig. 1). The harvested bone was immobilized in the sample holder using super glue (Henkel Loctite Co.), and the loading rod was placed at the distal epiphysis. The rod was slid by the piezoelectric actuator (LPD12060X, Megacera Inc.), which was regulated with a voltage amplifier (PZD700 M/S, TREK) through a BNC-2110 interface (National Instruments). A strain gauge (EA-06-015DJ-120/LE, Measurements Group Inc.) glued on the top surface of the loading rod was used as a force sensor. In the FRAP experiments, a sinusoidal voltage of 40 V (peak-to-peak) was applied to the piezoelectric actuator. Based on force calibration with the strain gauge this force was estimated as 1.7 N.

Strain Measurements

The strain, which was induced at 25% (∼4 mm along the length of the femur) proximal to the loading site with the applied force, was measured using four mice with a strain gauge (Model EA-06-015DJ-120, Measurements Group Inc., NC). Prior to strain measurements, the periosteal bone surface was cleaned with a cotton swab and degreased with degreasing solvent (M-Line CSM-1, Measurements Group Inc.).
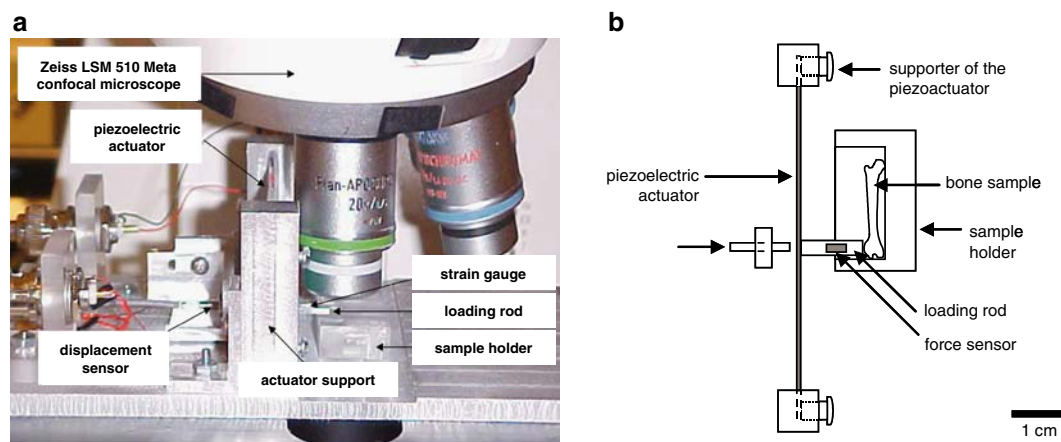

FIGURE 1. System setup for fluorescence recovery experiments. (a) The piezoelectric mechanical loader mounted on a Zeiss LSM 510 multi-photon fluorescence microscope system. (b) Schematic diagram illustrating application of mechanical loads on the distal epiphysis of a femur *ex vivo*.

A strain gauge was cut into a size of 0.7 mm in width and 2.8 mm in length to fit for the mouse femur *ex vivo*. It was glued to the anterior surface along the length of the femur using cyanoacrylate glue. The knee was loaded with 1, 2 and 4 N forces (peak-to-peak) at 2 Hz in the lateral–medial direction. Voltage signals from the strain gauge were sent to the computer via a signal-conditioning amplifier (2210, Measurement Group Inc.), and Fourier transform was applied to remove the background noise from the signal.

Imaging and Photobleaching

The lacunae on mouse femurs *ex vivo* were imaged using a Zeiss LSM 510 multi-photon microscope system. All measurements were conducted within 1 h after harvest. The imaging system was equipped with a femto-second pulse laser (Spectraphysics), and the images were collected with an excitation wavelength of 800 nm and a band pass emission filter for 505–550 nm. We used a $20\times$ air plan-apochromat objective lens (NA, 0.75). In the FRAP experiments, 12 lacunae in total were photobleached and each lacuna was used for both the static (non-loading control) and the loaded (knee loading) configurations. In order to avoid a potential damage of bone tissues by over photobleaching, we first confined a photobleaching area to a single lacuna using a size-adjustable elliptical marker and then bleached the marked area to achieve $\sim75\%$ reduction of fluorescent intensities. Photobleaching was conducted for 5 s with a high laser power, where an acousto-optic tunable filter was set to 80%. A series of time-lapse images were taken at 5-s intervals after photobleaching.

Determination of Apparent Diffusion Coefficients and Time Constants

An apparent diffusion coefficient, λ, was determined:

$$\lambda = \frac{1}{t}\ln\frac{c_0 - c(t)}{c_0 - c_b} \tag{1}$$

where $c(t)$ = fluorescence intensity at time t, c_0 = intensity prior to photobleaching, and c_b = intensity right after photobleaching. Recovery of fluorescence intensity was also modeled using an exponential function in a form of $\{1 - \exp(-t/\tau)\}$, where τ was defined as a time constant for fluorescence recovery. In this study, we used τ as a measure of load-driven alteration in molecular transport and conducted a paired *t*-test for statistical significance.

RESULTS

Loading Force to the Femoral Epiphysis

The force applied to the distal epiphysis of the femur was estimated using the strain gauge attached to the loading rod (Fig. 1). The relationship between the input voltage to the piezoelectric loader and the predicted force with the strain gauge attached on the loading rod was modeled by a best-fit linear regression line ($r^2 = 0.98$). Based on this calibration, the loading force was estimated as 0.042 N per voltage to the loader. In this study sinusoidal loads at the loading frequency of 2 Hz with 1–4 N (peak-to-peak) force were employed.

Strain Measurements

In response to the loads at 2 Hz with the knee-loading modality, the strain in the femur 25% (~4 mm) proximal to the distal end of the femur was measured as 26.8 ± 7.8, 43 ± 7.7 and 93.8 ± 8.2 μstrain with 1, 2 and 4 N force, respectively (Fig. 2). The best-fit regression line for the force–strain relationship was $y = 22.7x + 1.5$ with $r^2 = 0.99$. Note that the background strain level without any loads was ~10 μstrain.
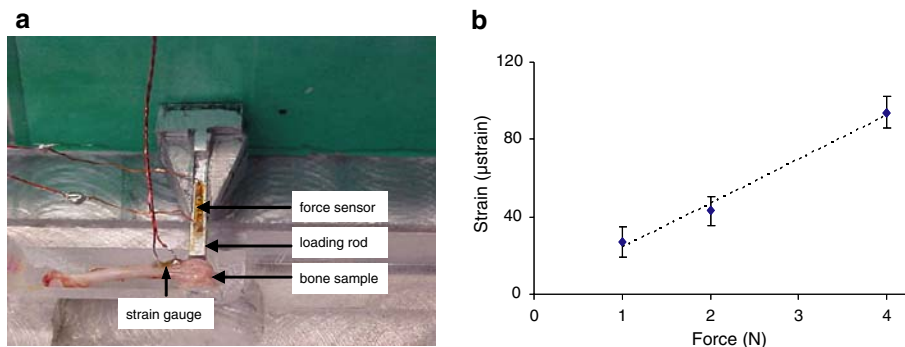

FIGURE 2. Strain measurements. (a) Strain gauge attached to the femur ~4 mm proximal to the loading site. (b) Measured strain (26.8 ± 7.8, 43 ± 7.7, 93.8 ± 8.2 μstrain) in response to 1, 2 and 4 N forces.
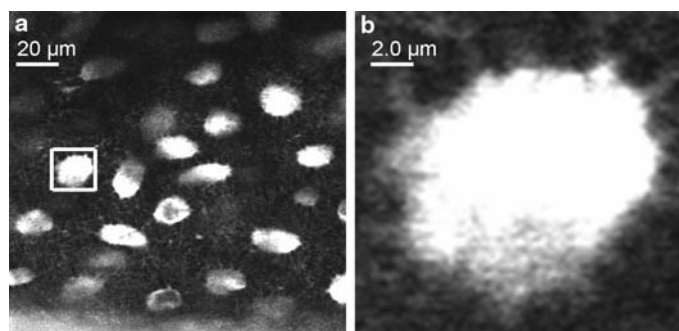
FIGURE 3. Fluorescent images of lacunae. (a) Cluster of lacunae in the midshaft of the mouse femur. The dimension was 16.6 ± 3.5 μm (mean ± SEM; major axis) and 7.6 ± 1.6 μm (minor axis). (b) Enlarged lacuna boxed in the image A.

Imaging of Lacunae

A cluster of lacunae stained with uranine were captured in cortical bone ∼4 mm proximal to the loading site and ∼50 μm deep from the outer surface (Fig. 3). Their dimension was 16.6 ± 3.5 μm (major axis; $n = 14$) and 7.6 ± 1.6 μm (minor axis; $n = 14$). The zoomed image showed faint staining representing a lacunocanalicular network.

Photobleaching and Apparent Diffusion Coefficients

The lacuna images were shown before and after photobleaching to demonstrate a recovery of fluorescent intensity (Fig. 4). In this example, fluorescence recovery was examined first by applying the loads with the knee-loading modality and the fluorescence recovery was monitored. The same lacuna was then used without any loads (non-loading control). The recovery of fluorescence intensity was plotted as a function of time with and without the loading, and the apparent diffusion coefficient was determined. The time constant for fluorescence recovery was determined as 43.1 s (non-loading control) and 37.0 s (knee loading). The logarithmic ratio defined in Eq. (1) showed an increase in the apparent diffusion coefficient with the knee-loading modality.

Time Constant of Fluorescent Recovery with and without Knee Loading

The observed time constants of fluorescence recovery clearly showed enhanced molecular transport with knee loading (Fig. 5). We conducted a series of recovery measurements using 12 independent lacunae in the femoral diaphysis with and without the loading. For one half of the lacunae, the time constants were determined in the non-loading control first and then in the loading. For the other half of the lacunae, the experimental order was reversed to calibrate any effect of irreversible photobleaching processes. In both cases, the time constant for fluorescent recovery was significantly shortened with loading. The mean and the standard deviation of time constants were 33 ± 9 s (non-loading control) and 25 ± 11 s (knee loading) for 12 pairs of lacunae, and the p-value in a paired t-test was 0.0014 ($n = 12$).

DISCUSSION

This study demonstrates for the first time the load-driven enhancement of molecular transport in the mouse femur *ex vivo* in response to knee loading. The loads were applied to the distal epiphysis of the femur, and the measured stain revealed that the site of the photobleached lacunae in the diaphysis was virtually with no strain. In response to knee loading, the time constant for fluorescence recovery was shortened by approximately one fourth of the non-loading control. Although there was a significant variation in the time constants among the unloaded lacunae ranging from 15 to 43 s, the dynamic loading reproducibly reduced the time constant and thereby enhanced the apparent molecular transport. The results clearly reveal that molecular transport is facilitated by external loads without inducing substantial *in situ* bone strain.

Enhancement of molecular transport, shown in the current study, is consistent with recent findings of the osteogenic potentials with the joint-loading modalities.[25,29] It has been reported that elbow loading stimulated bone formation in the mouse ulna, and knee loading was effective in elevating bone formation in the mouse tibia and the femur. The local strain in cortical bone, in which bone formation was enhanced in the ulna or the tibia, was measured at <100 μstrain, suggesting that *in situ* strain above the minimum effective threshold at ∼1000 μstrain is not necessary with these joint-loading modalities. The current fluorescence recovery data were obtained in the femur
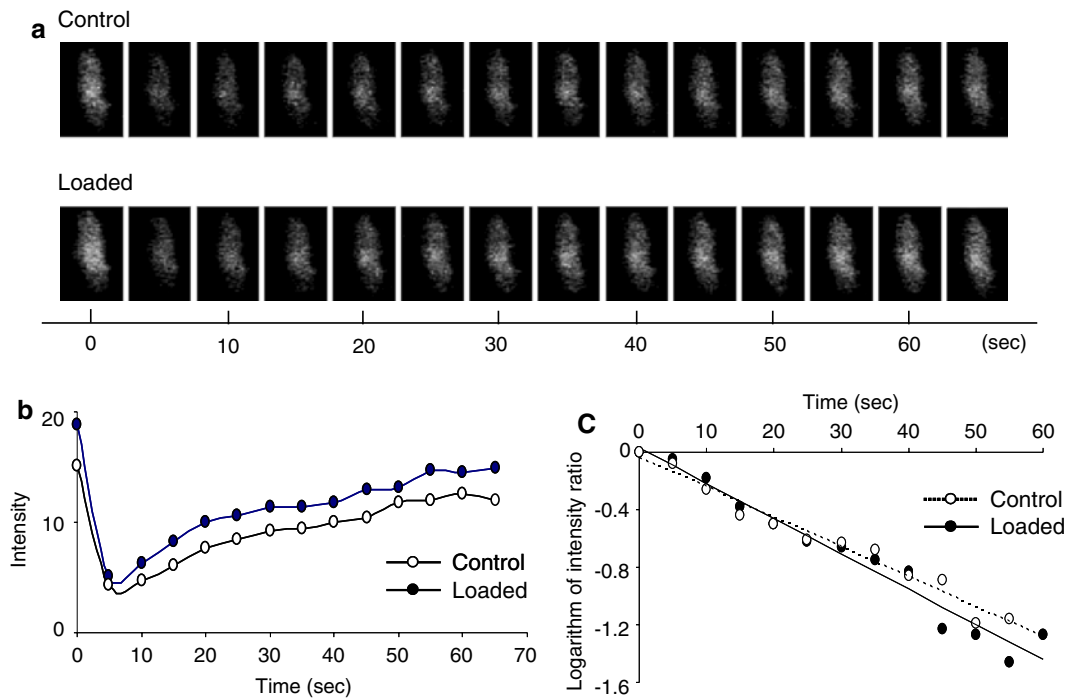
FIGURE 4. Fluorescent recovery with and without knee loading. (a) Time-serial images of the lacuna before and after photo-bleaching. The top and the bottom series correspond to non-loading control and knee loading, respectively. (b) Representative fluorescent recoveries. The white and the black circles represent the data corresponding to control (non-loading) and knee loading, respectively. According to a best-fit curve analysis, the correlation coefficients (r^2) were 0.98 (knee loading) and 0.97 (control). (c) Apparent diffusion coefficients. The negative slopes indicate the apparent diffusion coefficient of 0.021 (1/s) (non-loading control, $r^2 = 0.97$) and 0.025 (1/s) (knee loading, $r^2 = 0.96$).

ex vivo, and the results support the notion that load-driven molecular transport does not require *in situ* strain above 100 μstrain.

The observed enhancement of molecular transport in response to the applied loads indicates periodic load-driven fluid mixing through the canalicular network. Among analytical and computational models, some studies emphasize the effects of pressure gradients and convective flows and others stress the role of

instantaneous mixing of solutes in the lacuna.[12,22,27] A pioneering work on load-induced fluid flow was conducted using a rat tibia with the four-point bending, and it demonstrated a clear correlation between *in situ* strain and load-driven molecular transport.[13] Unlike the previous report with the strained rat tibia, the mouse femur in the current study was *ex vivo* and the site of FRAP measurements was under virtually no strain. Therefore, the results herein indicate that knee
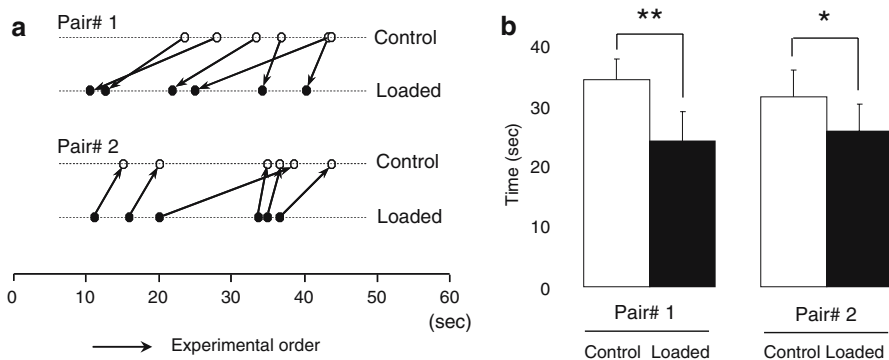

FIGURE 5. Comparison of the time constants for fluorescence recovery with and without knee loading. (a) Time constants for 12 pairs of lacunae. The arrow indicates the order of FRAP measurements. (b) Mean and SEM of time constants: 33 ± 2.7 s (non-loading control; $n = 12$) and 25 ± 3.2 s (knee loading; $n = 12$). The single and the double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively.

loading can enhance molecular transport with a mechanism different from four-point bending.[25,29] The described knee-loading modality may remotely displace fluid from the epiphysis towards the metaphysis and the diaphysis and stimulate molecular transport in cortical bone. A frequency response of the time constant may contribute to elucidation of the mechanism underlying the observed molecular transport.

Molecular transport should be affected by many factors such as a molecular weight, charge of fluorescent dyes, location and dimension of lacunae, connectivity of canaliculi, and loading conditions.[22] We employed a loading frequency of 2 Hz, since this frequency was shown to enhance bone formation with the elbow-loading modality.[25] Other loading frequencies may exhibit differential sensitivity in molecular transport. It is possible that the variations observed in the time constant may result from structural heterogeneity among lacunae. In order to determine the unequivocal effects of mechanical loads, we photobleached and imaged the same lacuna for the measurements with and without knee loading. The reproducible shortening of recovery time confirmed load-driven enhancement of molecular transport. The fluorescence recovery experiments in this study were performed using the femur *ex vivo*, and the femur *in vivo* may present altered sensitivity because of the effects of removed connective tissues and blood pressure.

The current FRAP study focused on the fluorescent measurement on the xy-plane, and the depth of photobleaching along the z-axis was estimated as ~ 4.5 μm from the following relationship:

$$\Delta Z = 1.77\lambda \Big/ \left[4\sin^2\frac{\alpha}{2}\left(1 - \frac{1}{3}\tan^4\frac{\alpha}{2}\right) \right] \qquad (2)$$

where λ = wavelength (800 nm), $\alpha = \sin^{-1}(NA/n)$, NA = numerical aperture (0.75), and n = refractive index of the medium (1.4). Note that this depth defines a width at a half-maximum of the point-spread function and therefore the region wider than this depth was photobleached in a lower intensity. Since a typical thickness of lacunae was ~ 5 μm, it appeared that the lacuna was almost entirely photobleached along the z-axis. On the xy-plane, the photobleached region was confined within the lacuna by controlling the laser scanning area. The mechanical drift of the focal plane due to the dynamic loading was estimated < 0.1 μm, which was $\sim 2\%$ of the photobleaching depth.

Bone is a mechanosensory system, and the effect of dynamic loading is a complex interplay among viscoelastic musculoskeletal tissues, lacunocanalicular networks, and non-Newtonian interstitial fluid. Molecular transport is considered to play a role in bone adaptation and load-driven bone formation. Potential mediators include alteration in molecular transport, intramedullary pressure,[9] blood perfusion,[20] electric stimulation[3,4] and streaming potentials.[10] With our unique knee loading approach, the current fluorescence recovery study provides new evidence for evaluating the mechanism of load-driven molecular transport and bone formation.

REFERENCES

[1]Adams, G. R., V. J. Caiozzo and K. M. Baldwin. Skeletal muscle unweighting: Spaceflight and ground-based models. *J. Appl. Physiol.* 95:2185–2201, 2003.

[2]Anderson, E. J., S. Kaliyamoorthy, J. Iwan, D. Alexander and M. L. Knothe Tate. Nano-microscale models of periosteocytic flow show differences in stresses imparted to cell body and processes. *Ann. Biomed. Eng.* 33(1):52–62, 2005.

[3]Bassett, C. A. L. and R. O. Becker. Generation of electric potentials in bone in response to mechanical stress. *Science* 137:1063–1064, 1962.

[4]Becker, R. O.. Stimulation of partial limb regeneration in rats. *Nature* 235:109–111, 1972.

[5]Burger, E. H. and J. Klein-Nulend. Mechanotransduction in bone – role of the lacuno-canalicular network. *J. FASEB* 13:S101–S112, 1999.

[6]Burr, D. B., A. G. Robling and C. H. Turner. Effects of biomechanical stress on bones in animals. *Bone* 30:781–786, 2003.

[7]Carmeliet, G., L. Vico and R. Bouillon. Space flight: A challenge for normal bone homeostasis. *Crit. Rev. Eukaryot. Gene. Expr.* 11:131–144, 2001.

[8]Gururaja, S., H. J. Kim, C. C. Swan, R. A. Brand and R. S. Lakes. Modeling deformation-induced fluid flow in cortical bone's canalicular–lacunar system. *Ann. Biomed. Eng.* 33:7–25, 2005.

[9]Jendrucko, R. J., W. A. Hyman, P. H. Newell and B. K. Chakrabarty. Theoretical evidence for the generation of high pressure in bone cells. *J. Biomech.* 9:87–91, 1976.

[10]Johnson, M. W., D. A. Chakkalakal, R. A. Harper, J. L. Katz and S. W. Rouhana. Fluid flow in bone in vitro. *J. Biomech.* 15:881–885, 1982.

[11]Kaspar, D., W. Seidl, C. Neidlinger-Wike and L. Claes. In vitro effects of dynamic strain on the proliferative and metabolic activity of human osteoblasts. *J. Musculoskelet. Neuronal. Interact.* 1:161–164, 2000.

[12]Knothe Tate, M. L.. Mixing mechanisms and net solute transport in bone. *Ann. Biomed. Eng.* 29:810–811, 2001.

[13]Knothe Tate, M. L., R. Steck, M. R. Forwood and P. Niederer. In vivo demonstration of load-induced fluid flow in the rat tibia and its potential implications for processes associated with functional adaptation. *J. Exp. Biol.* 203:2737–2745, 2000.

[14]Leddy, H. A. and F. Guilak. Site-specific molecular diffusion in articular cartilage measured using fluorescence recovery after photobleaching. *Ann. Biomed. Eng*. 31:753–760, 2003.

[15]Mak, A. F. and J. D. Zhang. Numerical simulation of streaming potentials due to deformation-induced hierarchical flows in cortical bone. *J. Biomech. Eng*. 123:66–70, 2001.

[16]McCreadie, B. R., S. J. Hollister, M. B. Schaffler and S. A. Goldstein. Osteocyte lacuna size and shape in women with and without osteoporotic fracture. *J. Biomech*. 37:563–572, 2004.

[17]Mishra, S. and M. L. Knothe Tate. Effect of lacunocanalicular architecture on hydraulic conductance in bone tissue: Implications for bone health and evolution. *Anat. Rec. A. Discov. Mol. Cell. Evol. Biol*. 273:752–762, 2003.

[18]Murphy, N. M. and P. Carroll. The effect of physical activity and its interaction with nutrition on bone health. *Proc. Nutr. Soc*. 62:829–838, 2003.

[19]Patel, R. B., J. M. O'Leary, S. J. Bhatt, A. Vasnja, and M. L. Knothe Tate. Determing the permeability of cortical bone at multiple length scales using fluorescence recovery after photobleaching techniques. *51st Ann. Meeting. ORS* 141, 2004.

[20]Piekarski, K. and M. Munro. Transport mechanism operating between blood supply and steocytes in long bones. *Nature* 269:80–82, 1977.

[21]Robling, A. G. and C. H. Turner. Mechanotransduction in bone: Genetic effects on mechanosensitivity in mice. *Bone* 31:562–569, 2002.

[22]Steck, R., P. Niederer and M. L. Knothe Tate. A finite element analysis for the prediction of load-induced fluid flow and mechanochemical transduction in bone. *J. Theor. Biol*. 220:249–259, 2003.

[23]Su, M., L. Yang, R. S. Praveen, H. H. Jiang and H. Yokota. Measurement of bone strain using electronic speckle pattern interferometry. *J. Holograph. Speckle* 2:1–6, 2005.

[24]Tami, A. E., P. Nasser, O. Verborgt, M. B. Schaffler and M. L. Knothe Tate. The role of interstitial fluid flow in the remodeling response to fatigue loading. *J. Bone. Miner. Res*. 17:2030–2037, 2002.

[25]Tanaka, S. M., H. B. Sun and H. Yokota. Bone formation induced by a novel form of mechanical loading on joint tissue. *Biol. Sci. Space* 18:41–44, 2004.

[26]Turner, C. H.. Three rules for bone adaptation to mechanical stimuli. *Bone* 23:399–407, 1998.

[27]Wang, L., S. C. Cowin, S. Weinbaum and S. P. Fritton. Modeling tracer transport in an osteon under cyclic loading. *Ann. Biomed. Eng*. 28:1200–1209, 2000.

[28]Wang, L., Y. Wang, Y. Han, S. C. Henderson, R. J. Majeska, S. Weinbaum and M. B. Schaffler. In situ measurement of solute transport in the bone lacunar–canalicular system. *Proc. Natl. Acad. Sci. USA* 102(33):11911–11916, 2005.

[29]Yokota, H. and S. M. Tanaka. Osteogenic potentials with joint loading modality. *J. Bone. Miner. Metab*. 23:302–308, 2005.

[30]You, L., S. C. Cowin, M. B. Schaffler and S. Weinbaum. A model for strain amplification in the actin cytoskeleton of osteocytes due to fluid drag on pericellular matrix. *J. Biomech*. 34:1375–1386, 2001.

[31]You, J., C. E. Yellowley, H. J. Donahue, Y. Zhang, Q. Chen and C. R. Jacobs. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. *J. Biomech. Eng*. 122:387–393, 2000.