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Phenotypic integration of skeletal traits during growth buffers genetic variants affecting the slenderness of femora in inbred mouse strains

Karl J. Jepsen,

Leni and Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, Box 1188, One Gustave L. Levy Place, New York, NY 10029, USA

Bin Hu,

Leni and Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, Box 1188, One Gustave L. Levy Place, New York, NY 10029, USA, e-mail: b.hu@mssm.edu

Steven M. Tommasini,

New York Center for Biomedical Engineering, CUNY Graduate School, Department of Biomedical Engineering, City College of New York, New York, NY 10031, USA e-mail: steven.tommasini@stonybrook.edu

Hayden-William Courtland,

Leni and Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, Box 1188, One Gustave L. Levy Place, New York, NY 10029, USA, e-mail: hayden-william.courtland@mssm.edu

Christopher Price,

Leni and Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, Box 1188, One Gustave L. Levy Place, New York, NY 10029, USA, e-mail: cprice@udel.edu

Matthew Cordova, and

Leni and Peter W. May Department of Orthopaedics, Mount Sinai School of Medicine, Box 1188, One Gustave L. Levy Place, New York, NY 10029, USA, e-mail: aiymfromaz@gmail.com

Joseph H. Nadeau

Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA e-mail: jhn4@case.edu

Abstract

Compensatory interactions among adult skeletal traits are critical for establishing strength but complicate the search for fracture susceptibility genes by allowing many genetic variants to exist in a population without loss of function. A better understanding of how these interactions arise during growth will provide new insight into genotype-phenotype relationships and the biological controls that establish skeletal strength. We tested the hypothesis that genetic variants affecting growth in width relative to growth in length (slenderness) are coordinated with movement of the inner bone surface and matrix mineralization to match stiffness with weight-bearing loads during postnatal growth. Midshaft femoral morphology and tissue-mineral density were quantified at ages of 1 day and at 4, 8, and 16 weeks for a panel of 20 female AXB/BXA recombinant inbred mouse strains. Path Analyses revealed significant compensatory interactions among outer-surface expansion rate, inner-surface expansion rate, and tissue-mineral density during postnatal growth, indicating that

genetic variants affecting bone slenderness were buffered mechanically by the precise regulation of bone surface movements and matrix mineralization. Importantly, the covariation between morphology and mineralization resulted from a heritable constraint limiting the amount of tissue that could be used to construct a functional femur. The functional interactions during growth explained 56-99% of the variability in adult traits and mechanical properties. These functional interactions provide quantitative expectations of how genetic or environmental variants affecting one trait should be compensated by changes in other traits. Variants that impair this process or that cannot be fully compensated are expected to alter skeletal growth leading to underdesigned (weak) or overdesigned (bulky) structures.

Introduction

There is tremendous interest in identifying disease susceptibility genes because genetic information holds great promise for identifying at-risk individuals early in life and for discovering novel biological pathways that can be used for personalized treatment. Many complex systems have developed robust, regulatory mechanisms to buffer themselves against the deleterious effects of genetic variants (Rutherford 2000). Function is maintained in these systems because genetic variants affecting one trait are compensated by the covariation of other traits. These buffering mechanisms, which are often not incorporated into genetic analyses, complicate the search for disease susceptibility genes because they allow many genetic variants to exist in a population without loss of function (Csete and Doyle 2002). Genetic or environmental variants that alter buffering mechanisms can compromise system function (Nadeau and Topol 2006) and expose cryptic genetic variants (Rutherford and Lindquist 1998). One approach to studying these buffering mechanisms is to examine how healthy systems establish function when faced with variants that could lead to dysfunction.

The skeleton is an example of a complex system that relies on buffering mechanisms to establish and maintain mechanical function. Bone must be sufficiently stiff and strong to support the forces engendered during daily activities, otherwise the structure will be weak and susceptible to fracture. The compensatory relationship among traits leading to functionality is called phenotypic integration (Olson and Miller 1958; Pigliucci and Preston 2004). Phenotypic integration, which has been studied primarily in the context of evolutionary changes in bone size and shape (Cheverud 1982, 1996; Klingenberg et al. 2003, 2004; Olson and Miller 1958), has recently been described in a context that is relevant to skeletal fragility (Jepsen et al. 2007; Tommasini et al. 2008).

Genetic variation in bone width provides a model to study how the skeletal system utilizes buffering mechanisms to establish and maintain mechanical function. Bone slenderness, a measure of cross-sectional size (e.g., width, total area) relative to length (Fig. 1a), is a heritable trait that results from variation in the biological processes regulating the relationship between growth in width (subperiosteal expansion) and growth in length. Genetic variants that impair growth in width leading to a slender bone (narrow relative to length) threaten mechanical function because small reductions in width could lead to disproportionately large reductions in fracture resistance (strength) (van der Meulen et al. 2001). However, compensatory changes in morphology and mineralization strengthened slender long bones under daily load activities (Jepsen et al. 2007). Importantly, this buffering occurred at the expense of increased brittleness under extreme load conditions, indicating that fracture susceptibility may arise within the normal range of functionally adapted structures. Finding that compensatory interactions among traits may be a source of fracture susceptibility is a departure from conventional reductionist approaches which assume that individuals within a population are at risk of fracturing for the same structural and thus biological reasons. However, an emergent property of compensatory trait interactions is that individuals acquire a specific set of adult traits, a concept recently

shown in the human femoral neck (Zebaze et al. 2007). Consequently, fracture susceptibility may depend on acquiring an “at-risk set of traits” or a set of traits that deviates from an ideal, perfectly adapted trait set. Thus, the genetic basis of fragility may be better examined in the context of sets of traits and the relationship among traits rather than individual traits.

Because prior work examined the adult skeleton (Jepsen et al. 2007; Tommasini et al. 2008), it was unclear how and when these functional interactions arose during growth (Cheverud 1996). Studying phenotypic integration during growth will provide new insight into the biological mechanisms that directly regulate the establishment of bone strength. Bone structure is adapted to forces during growth by the coordinated activities of bone-forming (osteoblasts) and bone-resorbing (osteoclasts) cells, suggesting that mechanical forces mediate the compensatory relationship among traits (Forwood et al. 2004; Frost 1987; Moro et al. 1996; Ruff et al. 2006; Sumner and Andriacchi 1996; van der Meulen et al. 1996). This process of functional adaptation is commonly referred to as Wolff’s Law (Ruff et al. 2006). Prior work examining adult mouse strains showed a strong correlation between body weight and femoral stiffness (Jepsen et al. 2007). Because stiffness is inversely related to the amount of strain (deformation) a structure undergoes while loaded, the data suggested that femora were functionally adapted so peak tissue-level strains were similar across the panel, consistent with the theory of elastic similarity (McMahon 1973). This theory provides an end point for the system (i.e., similar peak tissue-level strains) as well as a framework for hypothesizing a biomechanical mechanism explaining the genetically varying growth patterns (Fig. 1b). Under physiologic bending and torsional loads, bone diameter defines the amount of strain that bone cells perceive and the contribution of each increment of bone apposition to overall stiffness during growth. Each increment of tissue deposited on the outer surface of a small-diameter bone will have a dramatically lower mechanical benefit to overall stiffness compared to the same amount of tissue deposited on a large-diameter bone. To maintain elastic similarity, genetic variants that impair the expansion rate of the outer surface during growth and that lead to small-diameter femora are hypothesized to require (1) compensatory reductions in marrow (endosteal) expansion to increase bone mass and (2) compensatory increases in matrix mineralization to increase tissue stiffness. We tested this hypothesis by determining whether matrix mineralization and the relative expansion of the outer and inner surfaces are functionally coordinated during growth. Functional interactions among traits were examined using a panel of AXB/BXA recombinant inbred (RI) mouse strains, as described previously (Nadeau et al. 2003). Genetic recombination and segregation during meiosis randomizes allelic segregation, thereby creating new and usually nonpathologic combinations of bone traits (Bailey 1986; Jepsen et al. 2007). The pattern of trait interactions during growth can be rigorously tested by examining how traits covary or correlate across the RI panel.

Methods

Inbred mouse strains

Female A/J, C57BL/6 J (B6), and 20 AXB/BXA RI mouse strains ($n = 8-17/\text{age}/\text{strain}$) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and examined at 1 day and 4, 8, and 16 weeks of age. These ages were chosen based on prior work showing differences in the rates of bone surface movements during early (birth to 4 weeks) and later (4 to 16 weeks) phases of growth (Price et al. 2005). The handling and treatment of mice was approved by the Institutional Animal Care and Use Committee.

Bone morphology

Femoral length (L_e) was measured from the proximal femoral head to the distal condyles using digital calipers (0.01-mm resolution). Midshaft (diaphyseal) cross-sectional morphology (Fig. 1) for the 1-day, 4-week, and 8-week time points was determined from nondecalcified plastic

embedded sections, as described previously (Jepsen et al. 2003). For the 16-week time point, midshaft cross-sectional morphology was measured at high resolution (8.7- μ m voxel size) using an eXplore Locus SP Pre-Clinical Specimen Micro-computed Tomography system (GE Healthcare, London, Ontario, Canada). Data for 16-week-old mice were reported previously (Jepsen et al. 2007) and are included in the current study to relate skeletal growth patterns to adult traits and mechanical properties. For the histologic and tomographic images, the morphologic traits that were quantified included the amount of tissue (cortical area, Ct.Ar; marrow area, Ma.Ar; total area, Tt.Ar; cortical thickness, Ct.Th) and the spatial distribution of tissue (polar moment of inertia, Jo). Moment of inertia is a measure of the proximity of the tissue to the geometric centroid of the cross-section. Total area, which reflects the biological activity on the outer surface, was defined as the sum of the cortical and marrow areas. Marrow area was included as a measure of the biological activity on the inner surface. Traits were quantified for each cross-section and the values were averaged.

Measures of the age changes in bone size and shape were also calculated. For each RI strain, the rate of growth in width ($dTt.Ar/dt_{0-4}$) was calculated as total area at 4 weeks ($Tt.Ar_4$) minus the average Tt.Ar at 1 day ($Tt.Ar_0$) and normalized for the age interval (dt_{0-4}). Marrow expansion rate ($dMa.Ar/dt_{0-4}$) used a similar formula but substituted Ma.Ar for Tt.Ar. Measures representing the relationship between traits included bone slenderness, the cortical area-body weight relationship, and the relative cortical area. Slenderness was calculated as total area/length ($Tt.Ar/Le$), where slender refers to bones with low values of $Tt.Ar/Le$ and robust refers to bones with large values of $Tt.Ar/Le$. The relationship between cortical area (Ct.Ar) and body weight (BW) was determined from a linear regression of data from the 4-, 8-, and 16-week time points. The relative cortical area ($Ct.Ar/Tt.Ar$) was calculated as the ratio of cortical area to total area, and is a measure of the relative expansion of the outer and inner surfaces.

Tissue-mineral density

Micro-computed tomography (microCT) was also used to quantify tissue-mineral density (TMDn) for femora at 4 and 16 weeks of age, as described previously (Jepsen et al. 2007). TMDn (mg/cc) was determined by converting gray-scale values to mineral density values using a density calibration factor, and then averaging mineral content values over all thresholded “bone” voxels. The density calibration factor was determined for each scan using a phantom containing air, water, and a hydroxyapatite standard (SB3; Gammex RMI, Middleton, WI, USA). TMDn is a measure reflecting the amount of mineral incorporated within the bone matrix and should not be confused with bone-mineral density (BMD) which averages gray-scale values over total volume or projected bone areas.

Whole-bone mechanical properties

Mechanical properties of 16-week-old mice were reported previously (Jepsen et al. 2007) and are included here to show how variable growth patterns affect mechanical function (stiffness) of adult femora. Following microCT analysis, femora from 16-week-old mice were loaded to failure in 4-point bending, as described previously (Jepsen et al. 2001). Stiffness was calculated from a linear regression of the initial portion of the load deflection curve. Femora were tested at room temperature and kept moist with phosphate-buffered saline during all tests.

Narrow sense heritability

The narrow sense heritability, h^2 , which estimates the proportion of phenotypic variation attributable to additive genetic effects, was determined for each trait using the method described by Belknap (1998). The value of h^2 was estimated for the RI panel using the R^2 value from a one-way analysis of variance (ANOVA). For the relationship between cortical area and body weight, a Ct.Ar/BW ratio was calculated for each sample in the 4-, 8-, and 16-week time groups using a linear regression-based method. Cortical area for each sample was calculated as

$$\text{Ct.Ar}_i = x_j \text{BW}_i + y_j \quad (1)$$

where i is the sample number and j refers to the RI strain. x_j and y_j are the slope and intercept, respectively, calculated for each RI strain by regressing Ct.Ar and BW measured at 4, 8, and 16 weeks. Normalizing for body weight gives

$$\text{Ct.Ar}_i / \text{BW}_i = x_j + y_j / \text{BW}_i \quad (2)$$

A Ct.Ar/BW ratio was calculated for each sample and h^2 was determined for each time point.

Correlation analysis

Pearson correlation coefficients were calculated for all trait-trait comparisons to identify the bone traits that cosegregate after randomization of A/J and B6 genomes among the RI strains. A correlation matrix among traits was constructed at each age. Statistically significant correlations were identified by establishing a threshold correlation magnitude, which was determined using permutation tests (Churchill and Doerge 1994).

Path analysis

Because complex systems comprise multiple traits, many of which interact, the overall organization of these interactions in the context of function can be understood as a network. Networks of functional relationships can be examined using Path Analysis (Jepsen et al. 2007; Sharkey and Lang 2007; Wright 1921), which is a well-established and widely used multivariate analysis technique (Grace 2006) that uses conditional covariances to test for causal relationships among traits (Wright 1921). Because the relationship between two traits will change in sign and in significance when placed in the context of function and when the effects of confounding variables are taken into consideration (Jepsen et al. 2007), Path Analysis provides a rigorous approach to understand how variability in one trait is compensated by directed changes in other traits. Functional interactions are described in a statistical sense based on the pattern of statistical dependency among the traits. Independent (x) and dependent (y) traits are selected based on a *priori* knowledge or hypotheses of how a system functions. The hypothesized direction of the relationship specified between traits (i.e., a change in x causes a change in y) is critical as this dictates the computation of the covariance matrix and determines the overall goodness of fit for the model. The directionality between two traits within the network provides the basis for implying functional relationships (Sieberts and Schadt 2007) and can be specified based on the postulated nature of causality. If causality is not known, directions can be specified based on the temporal sequence of events which would establish causality. The causal nature of trait associations can be confirmed by conducting Path Analyses with the directions reversed.

The data examined using Path Analysis were the mean values of each RI strain. All data were Z-transformed relative to the overall mean and standard deviation of the RI panel, as described previously (Jepsen et al. 2007). This was done for each age group separately. Z transformation standardizes the variables so each trait at each age shows a mean of zero and a standard deviation of one. The Z-transformed data make it easier to interpret the path coefficients derived in the Path Analysis, as the coefficients represent how two traits relate in terms of the number of standard deviation units. Three Path Analyses were conducted to test whether compensatory interactions among traits established during growth can be explained based on the hypothesized biomechanical mechanism (Fig. 1b).

Path Model A—The first Path Model tested the hypothesis that genetic variants affecting the outer-surface expansion rate during growth are compensated by changes in the rate of marrow expansion and the amount of mineral incorporated into the matrix. The independent variables included the expansion rate of the outer surface during postnatal growth ($dTt.Ar/dt_{0-4}$), body weight (BW_4), and femoral length (Le_4) at 4 weeks. Bone length was included to statistically control for variation in growth in width relative to growth in length (i.e., slenderness). Dependent variables included marrow expansion rate ($dMa.Ar/dt_{0-4}$) and tissue-mineral density ($TMDn_4$), a measure of matrix mineralization that correlates with tissue stiffness. The subscripts indicate the age of the mouse, where 0 refers to birth (1 day), 4 refers to 4 weeks, and 16 refers to 16 weeks. Directed paths from $dTt.Ar/dt_{0-4}$ to $dMa.Ar/dt_{0-4}$ and to $TMDn_4$ tested whether marrow expansion rate and matrix mineralization are functionally related to outer-surface expansion rate. A directed path from $dMa.Ar/dt_{0-4}$ to $TMDn_4$ tested if changes in morphology were further compensated by changes in mineralization. Nonsignificant paths were removed to maximize the degrees of freedom.

Path Model B—The second Path Model tested whether genetic variants affecting bone growth patterns are deterministic of the set of acquired adult traits that are needed to establish function. We tested whether variation in slenderness relative to body weight during postnatal growth (4 weeks of age) resulted in compensatory changes in adult cortical thickness, $Ct.Th_{16}$, and matrix mineralization, $TMDn_{16}$. $Ct.Th_{16}$ was incorporated as a measure of the relative expansions of the outer and inner surfaces. The number of traits used in the Path Model was minimized by merging bone length and outer-surface expansion rate into a single slenderness variable, $Tt.Ar/Le_4$. Paths between $Tt.Ar/Le_4$ and $TMDn_{16}$ and $Ct.Th_{16}$ were directed in accordance with those described in Path Model A. Adult cortical area ($Ct.Ar_{16}$), moment of inertia (Jo_{16}), and stiffness ($Stiff_{16}$) were incorporated into the model to provide a biomechanical basis for how slender femora were strengthened by compensatory changes in morphology and tissue quality. Directed paths from $Tt.Ar/Le_4$ to Jo_{16} and to $Ct.Ar_{16}$ tested whether variation in outer-surface expansion during postnatal growth defined the set of adult traits contributing to mechanical function (stiffness).

Path Model C—A third Path Model tested whether the functional interactions observed for the AXB/BXA RI panel predict the set of traits for adult female A/J and B6 mice as well as six other inbred strains (C3H/HeJ, AKR/J, 129s1/SvImJ, DBA/2 J, BALB/cByJ, SM/J) that were reported previously (Jepsen et al. 2003). $Ct.Th$ and $TMDn$ were examined because these traits were coadapted to compensate for variability in bone slenderness. The Path Model was modified to allow for variable $Ct.Ar$ - BW relationships by treating $Ct.Ar$ as an independent variable. This analysis used data for adult mice only. The direction of paths was based on those described in Path Model A.

All Path Models were compared to the measured data using maximum-likelihood estimation and overall fit was determined by a χ^2 test (LISREL v. 8.8; Scientific Software International, Lincoln Park, IL, USA). Because Path Analysis favors the *a priori*, theory-based model, models are rejected if the observed data and the expectations derived from the model do not match (i.e., if $p < 0.05$). Thus, χ^2 values with an associated p value greater than 0.05 means that the model adequately fits the data. The root-mean-square error of approximation (RMSEA), which takes the number of degrees of freedom of the model into consideration, was included as an additional fit index, with $p < 0.05$ indicating a close fit.

Engineering model of skeletal growth

Beam theory was used to evaluate how compensatory changes in morphology and mineralization during growth contributed to mechanical function. For cylindrical structures of similar length loaded in bending, whole-bone stiffness is proportional to the product of tissue

stiffness (E) and the rectangular moment of inertia (I) (Sumner and Andriacchi 1996). Tissue stiffness was assumed to increase during growth proportional to the degree of mineralization (Price et al. 2005). Tissue-stiffness values were based on the tensile modulus measured for an independent cohort of adult A/J and B6 mice (Courtland et al. 2008). Expansion of the outer and inner surfaces was modeled to fit the growth patterns leading to slender (A/J-like) and robust (B6-like) femora. The four models examined included a B6-like growth pattern coupled with reduced mineralization, an A/J-like growth pattern coupled with increased mineralization (i.e., fully coadapted), an A/J-like growth pattern without the compensatory increase in mineralization, and an A/J-like growth pattern without compensatory changes in morphology and mineralization (i.e., no coadaptation).

Results

Slenderness is established during postnatal growth

Randomizing A/J and B6 genomes resulted in wide variation in diaphyseal size among the RI panel throughout growth. RI femora ranged from robust (B6-like) to slender (A/J-like) (Fig. 2a). Femoral slenderness (Tt.Ar/Le) showed a normal distribution for each age group, with an overall mean value that was intermediate between A/J and B6. This variation was expected for a complex trait regulated by multiple genes. Slenderness measured at 4 weeks correlated positively with slenderness measured at 8 ($R^2 = 0.78$, $p < 0.001$) and at 16 ($R^2 = 0.79$, $p < 0.001$) weeks of age (Fig. 2b), indicating that genetic variation in slenderness was established early in life. Although RI mice that tended to be bigger (i.e., heavier) also tended to have bigger bones (i.e., larger Tt.Ar), Tt.Ar/Le correlated weakly with body weight at each age (Fig. 2c).

Functional interactions among traits are present during postnatal growth

Traits that cosegregated after randomization were identified by correlation analysis (Table 1). A permutation test indicated that correlation coefficients greater than 0.66 were significant at the $p < 0.05$ level. More than 81% of the significant correlations observed at 16 weeks of age were also observed at 1 day, 4 weeks, and 8 weeks of age, indicating that the functional relationships observed among adult traits were present during postnatal growth. The narrow sense heritability (h^2) increased with age to values greater than 0.72 for body weight and for all bone traits examined (diagonals in Table 1). The narrow sense heritability for slenderness, which increased from 0.78 at 4 weeks to 0.94 at 16 weeks of age, was one of the highest heritabilities for the traits examined in this study.

The amount of tissue used to establish functionality is genetically regulated

Cortical area (Ct.Ar) was regressed against body weight (BW) for all age groups to determine whether the RI strains used similar amounts of tissue to build mechanically functional femora. Mean Ct.Ar values for the RI panel increased significantly with BW across the 4-, 8-, and 16-week age groups ($R^2 = 0.81$, $p < 0.0001$) (Fig. 3). The slope of each age group was not significantly different from the slope determined across all age groups ($p > 0.3$, ANCOVA). The narrow sense heritability for the Ct.Ar-BW relationship was 0.67 at 4 weeks, 0.68 at 8 weeks, and 0.69 at 16 weeks of age, indicating that additive genetic effects explained approximately two-thirds of the variation in this relationship. Using data at 4, 8, and 16 weeks, B6 femora showed a significantly greater Ct.Ar-BW slope compared to A/J femora, which had a slope and y intercept similar to the mean values of the RI panel (Fig. 4a, b). Since body weight was not measured at 1 day of age, the intercept was calculated at the average BW value for the 4-week age group. The Ct.Ar-BW relationship varied among the RI strains such that nine RI strains showed Ct.Ar-BW slopes and intercepts that were not different from the average curve (i.e., A/J-like) (Fig. 4c); four RI strains showed similar intercepts and either larger ($n = 3$) (B6-like) or smaller ($n = 1$) slopes (Fig. 4d); six RI strains showed slopes that were not different

from the average curve but had larger ($n = 2$) or smaller ($n = 4$) intercepts (Fig. 4e); and one RI strain showed a larger intercept and a reduced slope (Fig. 4f).

The outer-surface expansion rate relative to body size is functionally related to marrow expansion rate and the degree of matrix mineralization

The near-perfect goodness of fit for the first Path Model and the large R^2 values for the structural equations (Fig. 5a) indicated that the functional relationships among the traits were consistent with the hypothesized biomechanical mechanism (Fig. 1b). The strong, positive path coefficient (1.04) between $dTt.Ar/dt_{0-4}$ and $dMa.Ar/dt_{0-4}$ and the strong, negative path coefficient $[-0.68 = -0.86 \text{ (direct)} + 1.04 \times 0.17 \text{ (indirect)}]$ between $dTt.Ar/dt_{0-4}$ and $TMDn_4$ indicated that when body weight and bone length were held fixed, a 1-SD (standard deviation) decrease in outer-surface expansion rate was associated with a 1.04-SD decrease in marrow expansion rate and a 0.68-SD increase in mineralization. Thus, RI femora showing reduced outer-surface expansion rate relative to body weight during postnatal growth also showed reduced marrow expansion rate but increased matrix mineralization. At the other extreme, RI femora showing greater outer-surface expansion rate relative to body weight during postnatal growth showed increased marrow expansion rate but reduced matrix mineralization. The goodness of fit was not affected when $dTt.Ar/dt$ and $dMa.Ar/dt$ were switched, indicating that no clear causal association could be determined between the outer-surface and marrow expansion rates.

Covariation during postnatal growth defines adult trait sets and mechanical function

The second Path Model tested how precise control of postnatal skeletal growth patterns facilitated the development of a femoral structure that was sufficiently stiff to support weight-bearing loads (Fig. 5b). Substituting $Tt.Ar/Le_4$ for bone length and outer-surface expansion rate had no effect on the goodness of fit, confirming that $Tt.Ar/Le_4$ was a valid trait substitution to evaluate postnatal growth patterns using Path Analysis. The reduced form of the structural equations showed that $TMDn_{16}$ and $Ct.Th_{16}$ were negatively related to $Tt.Ar/Le_4$, consistent with the relationships defined in the first Path Model. The structural equations showed that variation in slenderness relative to body weight during postnatal growth accounted for 56-63% of the variation in adult mineralization and cortical thickness. The use of $Ct.Th_{16}$ as a measure of the relative expansion of the outer and inner surfaces was supported by observing significant positive correlations between $Ct.Th_{16}$ and the relative expansion of these two surfaces ($dCt.Ar/dt$) measured from 4 to 8 ($R = 0.61, p < 0.005$) and from 8 to 16 ($R = 0.64, p < 0.002$) weeks of age.

The strong, positive path coefficient between $Tt.Ar/Le_4$ and Jo_{16} was expected because Jo and $Tt.Ar$ both correlate with bone diameter. Substituting $Tt.Ar_{16}$ or the average outer bone diameter for Jo_{16} had no effect on the goodness of fit or the R^2 values for the structural equations, as expected (data not shown). The weak path coefficient between BW_4 and $Ct.Ar_{16}$ was surprising given that the bivariate correlation analysis showed a strong linear relationship between these two traits across growth (Fig. 3). The correlation between BW_4 and $Ct.Ar_{16}$ showed a similarly strong bivariate regression ($R^2 = 0.70, p < 0.0001$; data not shown). This analysis indicated that the relationship between $Ct.Ar$ and BW was mediated through a reciprocal relationship between tissue distribution (Jo) and cortical thickness. An advantage gained by using Path Analysis is that these complex relationships are generally hidden in bivariate regressions. Thus, Jo covaried with $Ct.Th$ in a way that maintained a similar $Ct.Ar$ - BW relationship across the RI panel, regardless of whether the genetic background resulted in robust (large Jo , small $Ct.Th$) or slender (small Jo , large $Ct.Th$) femora. Finally, the variation in adult stiffness was nearly fully explained ($R^2 = 0.84$) by $Ct.Ar_{16}$ and $TMDn_{16}$. Adding connections from BW_4 , $Ct.Th_{16}$, or Jo_{16} to $Stiff_{16}$ did not change the goodness of fit or the R^2 value of the structural equation. This analysis showed that genetic variation in bone

slenderness during postnatal growth was associated with highly coordinated changes in morphology and mineralization that resulted in the acquisition of sets of adult bone traits that matched whole-bone stiffness with body weight.

Functional interactions generalize to other inbred mouse strains

The third Path Model tested whether the functional interactions observed for the AXB/BXA RI panel predicted the set of traits for other inbred mouse strains. The modified Path Model (Fig. 6a) fit the data extremely well and the structural equations for Ct.Th and TMDn showed R^2 values of 0.96 and 0.70, respectively, indicating that treating Ct.Ar as an independent variable to allow for variable Ct.Ar-BW relationships had no effect on the model fit nor the functional interactions observed in Path Models A and B. The structural equations were rederived with nontransformed data using multivariate regression analysis because Z transformation is specific to the AXB/BXA RI panel and the constant in the structural equations had to be nonzero to extend the regressions to other strains. The structural equations predicted Ct.Th (Fig. 6b) and TMDn (Fig. 6c) extremely well for the inbred mouse panel. No differences in the slopes or intercepts ($p > 0.2$, ANCOVA) were observed for either Ct.Th or TMDn when comparing the linear regressions for the RI panel with the inbred mouse panel, suggesting that similar functional interactions exist across inbred mouse strains exhibiting a wide range in bone sizes and shapes.

Engineering model of skeletal growth

The engineering beam model revealed that without compensatory changes in cortical thickness and matrix mineralization, the slender (A/J-like) femora showed 55% lower stiffness (EI) by 112 days of age compared to the robust (B6-like) femora (Fig. 7). Compensatory increases in cortical thickness and mineralization dramatically improved the stiffness of the slender femora to within 14% of the robust femora, which is well within the error associated with estimating femoral stiffness assuming a circular cross-section. Compensatory increases in cortical thickness alone, however, had only a small benefit to global stiffness, indicating that the compensatory increase in mineralization was a critical factor contributing to the functionality of a slender structure.

Discussion

Origins of functionality during growth

A panel of AXB/BXA RI strains was examined to study how traits covary within the skeletal system during growth to buffer genetic variants affecting bone slenderness. The continuous variation in trait values across the RI panel confirmed that genetic randomization perturbed bone in a natural, nonpathologic manner (Nadeau et al. 2003). The interstrain variation provided a statistical advantage over single-gene and single environmental perturbation experiments by allowing us to use regression analysis and multivariate models to rigorously study how multiple traits vary simultaneously (i.e., covary) during growth after confounding variables like body size were taken into consideration. Our data showed that the compensatory relationships observed among adult bone traits (Jepsen et al. 2007) were established early during postnatal growth. Precisely regulating skeletal growth patterns may be a critical function of a complex system that builds mechanically functional structures using limited resources and to ensure that peak tissue-level strains fall within a narrow range (i.e., elastic similarity) (McMahon 1973). This level of biological organization has the benefit of allowing function to be achieved by different sets of traits (Marder and Goillard 2006; Nadeau et al. 2003). Observing a narrow range of trait sets across the RI panel is entirely consistent with the idea that buffering defines the range of phenotypic variation expressed in a population (Rutherford 2000). Prior theoretical studies attempted to find a set of genes leading to an “optimal” bone structure, but found instead that multiple outcomes were supported by different gene sets

(Nowlan and Prendergast 2005). These results are entirely consistent with our data, which showed that phenotypic integration during growth allowed for multiple functional outcomes and thus for many genetic variants to exist in a population without deleterious mechanical effects.

Functional adaptation

The results provided new insight into functional adaptation, which is a critical developmental process that ensures a bone is sufficiently stiff and strong to support applied loads. Given that whole-bone stiffness correlated significantly with body weight across the RI panel (Jepsen et al. 2007), the results indicated that precise coordination of outer-surface expansion rate, marrow expansion rate, and matrix mineralization during postnatal growth was required to successfully adapt sets of traits to match weight-bearing loads. Similar associations between bone size and the amount of cortical bone have been observed recently for the human femoral neck (Zebaze et al. 2007). The engineering model showed that regulating mineral content during postnatal growth was critical to fully compensate femora that were slender relative to body weight (Fig. 7). Prior work rarely incorporated the contribution of tissue quality (mineralization) to functional adaptation (Brear et al. 1990; Carrier and Leon 1990; Heinrich 1999; Sumner and Andriacchi 1996). Although bone morphology is the primary determinant of the development of bone stiffness and strength during growth for an individual (Sumner and Andriacchi 1996), our work suggested that regulation of matrix mineralization (and by correlation tissue-level stiffness) was the primary determinant explaining differences in functional adaptation among individuals with variable slenderness. The relatively low R^2 value (40%) for TMDn at 4 weeks of age (Fig. 5a) may result from partial volume effects associated with noninvasive imaging or it may reflect increased variation in the degree of mineralization among mice during a period of rapid growth.

Biological constraints defining phenotypic integration

Complex systems have to work within biological constraints. For skeletal structures there are limits to the magnitude and speed in which bone can be formed, resorbed, and mineralized. Examination of data across growth provided new insight into an important biological constraint contributing to phenotypic integration. The second Path Analysis indicated that the RI strains inherited a constraint limiting the amount of tissue (i.e., Ct.Ar) that could be used to build a functional structure during growth. This constraint has also been observed for human bone (Zebaze et al. 2007). Moment of inertia (a measure of the spatial distribution of tissue) covaried with cortical thickness in such a way that all RI femora had the same Ct.Ar relative to body weight, regardless of slenderness. The Ct.Ar-BW relationship showed a large narrow sense heritability, indicating that this relationship was genetically regulated, consistent with prior work (Klein et al. 2002). Heritability of the Ct.Ar-BW relationship explained why RI strains with large-diameter femora tended to have thin cortices, thus avoiding excessive bulk. Furthermore, heritability of this relationship limited the amount of morphologic compensation for RI strains with small-diameter femora, and thus may explain why femora that were slender relative to body weight also showed compensatory increases in matrix mineralization. Finding that the amount of tissue used to establish mechanical functionality was highly regulated or constrained (Frost 1987) is consistent with the theory that bone maximizes stiffness using minimum mass (Currey and Alexander 1985).

Variation in the slopes and intercepts of the Ct.Ar-BW relationship among the RI strains indicated that this relationship was established and sustained during growth in different ways. The biological factors regulating the Ct.Ar-BW relationship are not fully understood, but mutant mouse models suggested that this relationship is modified by many factors, such as serum IGF-1 levels (Rosen et al. 1997, 2004; Yakar et al. 2002) and mutations in the gene coding for LRP5 (Akhter et al. 2004). Genetic factors that regulate the Ct.Ar-BW relationship

may be important for studies of skeletal fragility because this relationship may represent a set-point for the system (Frost 1987). Different inbred mouse strains show variable Ct.Ar-BW relationships (Jepsen et al. 2003; Turner et al. 2000; Wergedal et al. 2005). When the Ct.Ar-BW relationship was treated as an independent variable in the Path Analysis, the structural equations accurately predicted cortical thickness and TMDn for inbred mouse strains showing wide variability in bone size and shape. The data indicated that inheritance of a particular Ct.Ar-BW relationship was central to phenotypic integration and therefore to the set of traits acquired as an adult. Although only one skeletal site was examined in this study, we expect that other sites, including corticocancellous structures, also rely on phenotypic integration to establish function. However, the nature of the relationships and the biological constraints will vary depending on the applied loads, the size and shape of the skeletal structure, and the temporal sequence of biological (modeling/remodeling) events occurring during growth (Tanck et al. 2006).

Biological regulators of trait covariation

The Path Analyses provided important insight into the biological factors that are involved in regulating skeletal growth patterns to establish mechanical function. Growth in length, which is an endochondral process, is regulated by mechanical and endocrine factors that overlap with growth in width (Rauch 2005). However, the factors that coordinate growth in width *relative* to growth in length (i.e., slenderness) are not fully understood. Slenderness was established in RI femora as early as 4 weeks of age, indicating that genetic factors established the relationship between growth in width and length early in life and maintained this relationship throughout growth, despite dramatic temporal changes in hormone levels and mechanical forces during this postnatal time frame (Seeman 2004). The large narrow sense heritability (Table 1) combined with the weak correlation between slenderness and body weight (Fig. 2c) indicated that the relationship between transverse bone expansion and longitudinal growth was defined primarily by genetic factors rather than mechanical factors.

The large path coefficient between the outer- and inner-surface expansion rates (Fig. 5a) suggested that a strong biological signal is conveyed across the cortex to precisely coordinate the relative movement of these two surfaces during growth. The mid-diaphysis of the femur grows by expanding and drifting in space (Enlow 1963), and consequently, movement of the outer and inner surfaces involves the coordination of the relative activities of osteoblastic bone formation and osteoclastic resorption. Although osteoblasts and osteoclasts communicate via endocrine and cytokine factors, the processes that regulate this communication to precisely expand or contract these surfaces relative to weight-bearing loads are not understood fully. Our data showed that mineralization increases during growth, consistent with prior studies (Carrier and Leon 1990; Currey and Butler 1975; Heinrich 1999; Papadimitriou et al. 1996; Price et al. 2005), and also showed that the degree of mineralization varied with the rate of outer-surface expansion relative to body size. This suggested that osteoblasts (or osteocytes) precisely modulate the degree of matrix mineralization during postnatal growth in concert with mechanical loading demands. The biological factors that are responsible for the overall degree of coordination are expected to involve both global (e.g., hormones, mechanical forces) and local signals (e.g., tissue strain).

Hierarchically structured genetic buffering mechanisms

Phenotypic integration may have evolved to buffer genetic or environmental variants that would otherwise threaten system function (Marder and Goillard 2006) or to allow for plasticity in organ- or organismal-level design so fitness matches environmental demands (Pigliucci 2001). The ability of a complex system to buffer itself against gene variants that threaten system function has been studied primarily in the context of compensatory genetic factors such as modifier genes (Nadeau and Topol 2006), epistasis (Cheverud et al. 2004), and modularity

(Klingenberg et al. 2004). The mechanism responsible for integrating bone traits to establish mechanical function, however, may not be fully explained by compensation at the gene level. Genetic randomization reduced the likelihood that each RI strain inherited a common set of genes that coordinated skeletal traits in a consistent manner across the panel (Nadeau et al. 2003). Thus, random sets of genes gave rise to nonrandom sets of traits. Furthermore, the coordination of bone surface expansion and matrix mineralization required to establish function involves the precise regulation of the activity of multiple cell populations working on different bone surfaces. This level of coordination likely involves a feedback control mechanism (Csete and Doyle 2002; Marder and Goillard 2006), suggesting that organ-level buffering mechanisms in bone may involve biological controls at multiple levels of structural hierarchy. Thus, the set of traits acquired by each RI mouse strain appears to result from an interplay between functional adaptation (Wolff's Law) and genetic variants affecting growth in width relative to growth in length. We assumed that variation in growth in width relative to length (slenderness) was the primary variant driving the compensatory changes in other traits. This was assumed based on the temporal sequence of surface movements and matrix deposition (i.e., Tt.Ar is established before Ma.Ar and TMDn) (Price et al. 2005). However, reversing the direction of arrows in the Path Models had no effect on the goodness of fit, indicating that our data could not establish the causal nature of these interactions. This remains a limitation of this study and indicates that the relative contributions of genetic and environmental factors to skeletal function are tightly coupled.

Conclusions

We examined how the skeletal system compensates for genetic variants affecting bone slenderness as an experimental model to identify traits that covary during growth to establish function. Path Analyses showed that establishing mechanical function in a complex system subjected to genetic variations requires a tremendous amount of order in the way bone is constructed during growth. Although variation in traits among inbred mouse strains has been attributed largely to genetic factors, our data showed that trait values are also determined in large part by strong adaptive processes operating during growth. Thus, the relationship between genotype and phenotype is complicated by hierarchically structured buffering mechanisms. We showed that variation in bone size and shape arises during postnatal growth from genetic variants affecting body weight, genetic variants affecting slenderness, constraints imposed on the amount of tissue that can be used to construct a functional structure (i.e., the Ct.Ar-BW relationship), and biological processes that covary morphologic and compositional traits. Genetic variants altering the biological processes that covary traits would be expected to compromise bone stiffness and strength. Genetic variants that alter covariation and improve stiffness and strength, however, would be expected to have tremendous therapeutic value. Thus, a novel approach to identifying gene variants that impair or promote bone strength is to focus on the processes that regulate the relationship between traits, because it is the relationship between traits that determines the set of traits which defines function. Understanding the biological processes that coordinate these traits is critical for determining how genetic background contributes to fracture susceptibility.

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Fig. 1.

a The partially rendered three-dimensional micro-computed tomography image illustrates the region of analysis, and the midfemoral cross-section illustrates the morphologic traits that were quantified. **b** Schematic of the hypothesis illustrates how variation in outer surface (subperiosteal) expansion rate is functionally related to marrow expansion rate and the degree of matrix mineralization

Fig. 2.

a Midfemoral cross-sections for representative members of the AXB/BXA RI panel are shown at four ages during growth. A/J and B6 are shown for comparison. **b** Slenderness measured at 4 weeks of age correlated significantly with slenderness measured at 8 and 16 weeks of age. **c** Slenderness correlated weakly with body weight at all ages (16 weeks shown below)

Fig. 3.
Cortical bone area (Ct.Ar) increased linearly with body weight at 4, 8, and 16 weeks of age.
Each data point represents the mean value for each RI strain

Fig. 4. Variation in the relationship between cortical area and body weight (Ct.Ar-BW) for **a** A/J, **b** B6, and **c-f** representative RI strains. Individual strains are compared to the average curve derived using mean values for all RI strains. All curves include data at 4, 8, and 16 weeks of age

Fig. 5.

a Path Model A tested for functional relationships between outer-surface expansion rate, marrow expansion rate, and mineralization from 0 (day 1) to 4 weeks of age. **b** Path Model B tested for functional relationships between variation in slenderness relative to body size at 4 weeks of age and the set of adult traits (16 weeks) that contribute to whole-bone stiffness (Stiff)

Fig. 6.

a Path Model C generalized the relationships observed in Path Models A and B by treating cortical area as an independent variable. No change in the overall fit of the model was observed. The structural equations derived from the generalized Path Model accurately predicted **b** cortical thickness and **c** tissue-mineral density for an independent panel of inbred mouse strains

Fig. 7. The effects of compensatory changes in morphology and mineralization on the development of bone stiffness (EI) were simulated for idealized femora with cylindrical cross-sections. The stiffness for slender femora with no covariation and only morphologic covariation was dramatically reduced compared to the stiffness achieved by slender and robust femora that were fully coadapted

Table 1
Correlation coefficients among traits at 1 day and 4, 8, and 16 weeks of age

	BW	Le	Tt.Ar	Ma.Ar	Ct.Ar	Jo	Ct.Th	Tt.Ar/Le
<i>1 day</i>								
BW	ND	ND	ND	ND	ND	ND	ND	ND
Le		ND	ND	ND	ND	ND	ND	ND
Tt.Ar			0.55	0.89	0.79	0.96	0.37	ND
Ma.Ar				0.46	0.42	0.75	-0.09	ND
Ct.Ar					0.41	0.90	0.85	ND
Jo						0.57	0.55	ND
Ct.Th							0.30	ND
Tt.Ar/Le								ND
<i>4 weeks</i>								
BW	0.60	0.87	0.61	0.57	0.60	0.59	0.39	0.40
Le		0.63	0.61	0.51	0.71	0.64	0.63	0.36
Tt.Ar			0.76	0.97	0.91	0.98	0.57	0.96
Ma.Ar				0.75	0.79	0.92	0.37	0.96
Ct.Ar					0.66	0.96	0.84	0.81
Jo						0.72	0.68	0.92
Ct.Th							0.39	0.44
Tt.Ar/Le								0.78
<i>8 weeks</i>								
BW	0.70	0.79	0.61	0.47	0.77	0.65	0.54	0.48
Le		0.69	0.59	0.46	0.75	0.64	0.42	0.41
Tt.Ar			0.84	0.97	0.92	0.99	0.46	0.98
Ma.Ar				0.80	0.80	0.94	0.29	0.98
Ct.Ar					0.77	0.95	0.71	0.84
Jo						0.81	0.55	0.95
Ct.Th							0.47	0.41
Tt.Ar/Le								0.86
<i>16 weeks</i>								
BW	0.73	0.81	0.56	0.33	0.80	0.61	0.75	0.40
Le		0.73	0.52	0.31	0.75	0.59	0.72	0.31

	BW	Le	Tt.Ar	Ma.Ar	Ct.Ar	Jo	Ct.Th	Tt.Ar/Le
Tt.Ar								
Ma.Ar			0.92	0.95	0.87	0.99	0.21	0.97
Ct.Ar				0.94	0.67	0.90	-0.10	0.97
Jo					0.83	0.92	0.68	0.76
Ct.Th						0.89	0.31	0.94
Tt.Ar/Le							0.77	0.06
								0.94

Narrow sense heritability (h^2) shown on the diagonal (all h^2 values are significant, $p < 0.0001$). Bold correlations ($R > 0.66$) indicate significance at $p < 0.05$. ND not determined