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Mechanical microenvironments and protein expression associated with formation of different skeletal tissues during bone healing

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Abstract

Uncovering the mechanisms of the sensitivity of bone healing to mechanical factors is critical for understanding the basic biology and mechanobiology of the skeleton, as well as for enhancing clinical treatment of bone injuries. This study refined an experimental method of measuring the strain microenvironment at the site of a bone injury during bone healing. This method used a rat model in which a well-controlled bending motion was applied to an osteotomy to induce the formation of pseudarthrosis that is composed of a range of skeletal tissues, including woven bone, cartilage, fibrocartilage, fibrous tissue, and clot tissue. The goal of this study was to identify both the features of the strain microenvironment associated with formation of these different tissues and the expression of proteins frequently implicated in sensing and transducing mechanical cues. By pairing the strain measurements with histological analyses that identified the regions in which each tissue type formed, we found that formation of the different tissue types occurs in distinct strain microenvironments and that the type of tissue formed is correlated most strongly to the local magnitudes of extensional and shear strains. Weaker correlations were found for dilatation. Immunohistochemical analyses of focal adhesion kinase and rho family proteins RhoA and CDC42 revealed differences within the cartilaginous tissues in the calluses from the pseudarthrosis model as compared to fracture calluses undergoing normal endochondral bone repair. These findings suggest the involvement of these proteins in the way by which mechanical stimuli modulate the process of cartilage formation during bone healing.

Keywords

Mechanical environment; Tissue differentiation; Pseudarthrosis; FAK; RhoA

1 Introduction

There is now widespread evidence, both clinical and laboratory-based, that bone healing depends on the mechanical conditions at the injury site (Einhorn 1998; Epari et al. 2006;

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Goodship and Kenwright 1985). Elucidating the mechanisms underlying the mechanobiology of bone healing will likely have profound clinical impact. For example, knowledge of an optimal set of mechanical conditions to promote osseous healing would inform design and selection of fracture-fixation systems for treating a given type of injury or deformity, while knowledge of the molecular mechanisms involved in the sensing and transduction of mechanical cues could identify pharmaceutical targets for enhancing bone repair. However, neither the desired mechanical conditions nor the molecular mechanisms have been defined.

Theories on the mechanobiology of bone healing have put forward several features of the mechanical conditions local to the injury site, the mechanical microenvironment, as the stimuli responsible for guiding formation of different skeletal tissues. These features include shear strain and interstitial fluid flow (Lacroix and Prendergast 2002; Prendergast et al. 1997), strain and hydrostatic pressure (Claes et al. 1997; Claes and Heigele 1999), shear strain only (Gomez-Benito et al. 2005), and tensile strain and hydrostatic pressure (Carter et al. 1998). Tests of these theories, largely using numerical simulation to estimate the mechanical microenvironment and then to predict the healing outcome, have produced somewhat contradictory results (Isaksson et al. 2006; Steiner et al. 2013). Experimental evidence obtained by using digital image correlation to measure the strain microenvironment has pointed to the importance of shear strain (as represented by the octahedral shear strain) and, to a lesser extent, tensile strain (as represented by the maximum principal strain) (Morgan et al. 2010). Although this experimental approach measured strains within only a single plane within the specimen, it has an advantage over the numerical simulations in that it does not require assumptions about the material properties of the heterogeneous mixture of tissues at the injury site. However, this prior study considered only a subset of types of skeletal tissues (woven bone, cartilage, fibrocartilage) that can form during bone healing.

In vivo data on the molecular mechanisms of the mechanobiology of bone healing are scarce, yet some insights can be gleaned from the large body of in vitro data on mechanotransduction. Of the numerous potential molecular mediators that have been identified, the aforementioned findings concerning shear strain suggest the role of proteins and molecular complexes that regulate cell shape and physical connections between cells and the extracellular matrix. Focal adhesion proteins and their associated cytoskeletal proteins are primary regulatory molecules in this regard and have all been implicated in the machinery of mechanotransduction (Alenghat and Ingber 2002). Conditional inactivation of focal adhesion kinase (FAK) in cells expressing type I collagen was found to abolish mechanically induced bone formation surrounding a unicortical implant (Leucht et al. 2007). Of note also is the role of the actin cytoskeleton in modulating cell shape and cell differentiation. Cell shape changes during differentiation (McBeath et al. 2004), and forcing certain changes in shape can regulate stem cell fate (Gao et al. 2010; Guilak et al. 2009; McBeath et al. 2004; Sordella et al. 2003). Thus, mechanical stimuli may regulate cell differentiation by inducing changes in cell shape via cytoskeletal reorganization (Cohen and Chen 2008; Ingber 2006). Indeed, shape changes have been linked to lineage commitment of progenitor cells through activity of ras homolog gene family member A (RhoA) and other members of this family of GTP-binding proteins (Arnsdorf et al. 2009; McBeath et al. 2004; Woods et al. 2007), which act as molecular switches linking membrane receptors to focal

adhesion assemblies and the cytoskeleton (Etienne-Manneville and Hall 2002; Kaibuchi et al. 1999). The extent to which these mechanobiologically implicated proteins are involved in bone healing is unknown at present.

The overall goal of this study was to identify both the features of the mechanical microenvironment most strongly associated with tissue formation during bone healing and the expression of proteins such as FAK and RhoA during this mechanobiological process. This study used an *in vivo* model of mechanically induced pseudarthrosis. We used this model because the healing outcome involves formation of a wide range of tissue types (woven bone, cartilage, fibrocartilage, fibrous tissue, clot) and the applied mechanical loading is well controlled. The specific objectives of this study were (1) to identify associations between the strain microenvironment and the formation of different skeletal tissues and (2) to examine protein expression during this process of tissue differentiation.

2 Materials and methods

2.1 Experimental design

All animal care and experimental protocols were followed in accordance with NIH guidelines and were approved by Boston University's Animal Care and Use Committee. Two *in vivo*, surgical models were used: mechanically-generated pseudarthrosis and closed, stabilized fracture. Both models used retired-breeder, male Sprague-Dawley rats (>500 g; age ~ 6 – 7 months) according to the supplier (Charles River Laboratories, Cambridge, MA). For the analyses of the strain microenvironment and its association with tissue formation (as observed by histology), calluses formed from the pseudarthrosis model were harvested at post-operative days (PODs) 7, 14, 21, and 35 ($n=12/\text{POD}$). A subset of these calluses from the pseudarthrosis model ($n=6/\text{POD}$) was used for immunohistochemical analysis of protein expression.

Immunohistochemistry was also performed on tissues from closed, stabilized fractures ($n=6$) harvested on POD 14—the point at which the fracture callus reaches peak volume (Gerstenfeld 2006)—and on the distal epiphyses ($n=6$) of these fractured femora. These data were used for the purpose of comparing the immunohistochemical outcomes in the tissues that form during pseudarthrosis development to those from tissues that form as part of the normal endochondral repair process following fracture and from mature cartilaginous and osseous tissues. In contrast to the pseudarthrosis model, no motion was applied in the closed fracture model except that which occurs during the normal course of healing following intramedullary pin fixation. For these fracture calluses, cartilage formation is restricted to the periosteal callus, and nearly full bony union is achieved within 4 weeks (Einhorn 1998). Comparison of the pseudarthrosis and fracture tissue thus allows examination of changes in protein expression induced by a protocol of mechanical stimulation that produces cartilage and yet creates a marked and persistent deviation from the normal outcome of bone healing. Given that previous work with the pseudarthrosis model found little to no cartilage formation in the absence of the applied bending stimulation (i.e., locking screws in place continuously) and comparatively small amounts of tissue formation overall (Salisbury Palomares et al. 2009), these “continuous fixation”

calluses were deemed poor candidates for examining protein expression during chondrocyte proliferation and differentiation, and they were not included in the study design.

2.2 In vivo models

For the pseudarthrosis model, rats underwent production of a mid-diaphyseal, transverse, ~ 1.5 mm-wide femoral osteotomy, stabilized with a custom-designed external fixator, as described previously (Salisbury Palomares et al. 2009). This fixator contains a central hinge flanked by two locking screws and is attached to the lateral aspect of femur via four bicortical pins such that the hinge is centered at the osteotomy gap. When the locking screws are removed, the fixator allows rotation of the distal half of the femur in the sagittal plane (Supplementary Material Figure S1).

Similar to previously established models (Cullinane 2002; Salisbury Palomares et al. 2009), mechanical stimulation in the form of a cyclic bending motion was applied after a latency period of 7 days. The animals were first anesthetized with isoflurane (4% induction, 2% maintenance), and the external fixator was attached to a servomotor-driven linkage system that effects $\pm 15^\circ$ of angular displacement of the distal half of the femur at a frequency of 1 Hz for 15 min. The proximal half of the femur is held stationary during this time. This stimulation protocol was administered on five consecutive days followed by 2 days of rest each week. After each stimulation period, the locking screws were reinserted. Animals were allowed to ambulate freely in their cages during all other times. Twelve animals were excluded at some point during the in-life portion of the study because of signs of infection, pin displacement, or surgical complications, resulting in group sizes of 12 animals per POD.

For the closed fracture model, fractures were produced in the femora, according to the protocol of Bonnarens and Einhorn (Bonnarens and Einhorn 1984). Based on examination of the radiograph films, one animal was excluded because of signs of pin displacement.

The operated limb of all animals was radiographed once per week under general anesthesia, and again at the time of euthanasia. Animals were euthanized via CO₂ asphyxiation followed by bilateral pneumothorax. All tissues harvested were wrapped in gauze soaked with phosphate-buffered saline (PBS) and were immediately stored in airtight containers at -20°C until further use. Additional details regarding the surgical and mechanical stimulation protocols can be found in Supplementary Material S.1.

2.3 Strain measurement

Displacements and strains induced on the mid-sagittal plane of the calluses from the pseudarthrosis model were quantified using a method similar to that described previously (Morgan et al. 2010). Briefly, this method involved first removing the medial half of the callus to expose the mid-sagittal plane. The external fixator and pins were left in place during harvesting of the callus as well as during removal of the medial half. The newly exposed mid-sagittal plane was speckled with black enamel paint to allow tracking of the movement of the tissue as the bending motion was applied. The motion was applied by mounting the callus in an identical manner to the same linkage system used during the in vivo stimulation. A series of digital images (0.0118 mm/pixel) was taken as one complete bending cycle is applied. The specimens were kept hydrated with $1\times$ PBS during this

process. More details on specimen preparation and image capture are provided in Supplementary Material S.2.

The series of digital images was analyzed in neighboring pairs (e.g., image 1 and image 2, image 2 and image 3, etc.) using a technique of digital image correlation, sequential maximum likelihood estimation (SMLE) (Gokhale et al. 2005; Morgan et al. 2010). In this technique, the first image of each pair is discretized into quadrilateral regions of side length ~ 50 pixels. The in-plane displacement vector $\mathbf{u}(\mathbf{x})$ occurring at each node in this resulting mesh is estimated by minimizing the functional

$$\Pi(\mathbf{u}) = \int_{\Omega} (I_1(\mathbf{x}) - I_2(\mathbf{x} + \mathbf{u}))^2 dx + \int_{\Omega} \omega (\nabla \mathbf{u} \cdot \nabla \mathbf{u})^2 d\mathbf{u} \quad (1)$$

where Ω is the domain of the element, $\mathbf{x}$ is the position, $I_1(\mathbf{x})$ and $I_2(\mathbf{x} + \mathbf{u})$ are the first and second images of the pair, and ω is a regularization parameter to restrict large displacement gradients (Gokhale et al. 2005; Oberai et al. 2003). Improvements in glare reduction during image acquisition, achieved by mounting diffusers to the surrounding light sources, allowed refinement of the spatial resolution of the displacements as compared to previous publication (Morgan et al. 2010). Specifically, the first image of each pair was discretized into quadrilateral regions of side length ~ 20 pixels, then the displacement results for the coarser (50-pixel side length) mesh were interpolated over this finer mesh and used as the initial guess when minimizing Eq. (1) over the entire, finer mesh.

From the resulting displacements, the in-plane Green-Lagrange strain tensor, and subsequently the maximum principal strain, minimum principal strain, shear strain (octahedral), and in-plane dilatation were computed for each quadrilateral. The error in the resulting strain measurements was found to be 0.54% (Supplementary Material S.3). The strain microenvironment for each quadrilateral was defined as the maximum values of maximum principal strain ($E_{\max}$), octahedral shear strain (E_{oct}), and dilatation ($E_{\text{dil,max}}$) over the entire bending cycle, and the minimum values of minimum principal strain ($E_{\min}$) and dilatation ($E_{\text{dil,min}}$) over the entire bending cycle. Tissue tearing was observed in images taken during the bending application; therefore, strains exceeding the range of -30 to 30% were excluded to reduce the effect of tissue tearing on the statistical analyses (Morgan et al. 2010).

3 Histology

Tissues, including the medial halves of the calluses from the pseudarthrosis model and the fracture calluses, inclusive of the distal femoral epiphysis, were fixed (4% paraformaldehyde at 4°C for 7 days), decalcified (14% ethylenediaminetetraacetic acid (EDTA) for 8 weeks), and embedded in paraffin. Care was taken to ensure proper alignment of the specimen during embedding such that the entire mid-sagittal plane could be sectioned intact. Sections were cut at $10\ \mu\text{m}$ thickness and were mounted on glass slides and dried overnight.

Slides were deparaffinized using xylenes and rehydrated with graded ethanol solutions. Slides were then stained with Safranin-O and Fast Green with Hematoxylin counterstain and

imaged under light microscopy. The following tissue types were identified visually based on stain color and morphology: cortical bone, trabecular bone, cartilage, fibrocartilage, fibrous tissue, and clot. A polarized light filter, which highlights the presence of collagen fibrils, was used to aid in distinguishing between cartilage and fibrocartilage. Any tissue not characterized by one of the preceding tissue types was included in an additional category designated “Other Tissue”. For the histological sections of the calluses from the pseudarthrosis model, the regions of each tissue type were assigned a grayscale value (Fig. 1). Void space was also labeled but then excluded from further consideration. An additional specimen from the POD 7, POD 21, and POD 35 groups were excluded because misalignment of the specimen during sectioning resulted in incomplete mid-sagittal sections.

4 Statistical analyses of the strain microenvironment

In preparation for statistical comparisons of the strain microenvironment to the patterns of tissue formation, the strain fields and the labeled, grayscale histology sections were sampled at 4200 grid points throughout the mid-sagittal plane (Morgan et al. 2010) (Fig. 2a). More details on the method of sampling are provided in Supplementary Material S.4. This sampling produced 4200 matching pairs of strain magnitude and tissue type for each type of strain for each callus from the pseudarthrosis model. These pairs allowed comparison of strain fields with histology that coexist at a given POD, i.e., “within-timepoint” comparisons. Recognizing that these comparisons might reflect simply the strains that the differentiated tissues experience, as opposed to the strains that are associated with subsequent tissue differentiation, we also made comparisons across neighboring timepoints (e.g., the strains occurring at POD 7 to the histological distribution of tissues at POD 14). To enable the “across-timepoint” comparisons, the sampled strain fields at a given POD were averaged over all specimens. This produced for each specimen 4200 matching pairs of tissue type and average strain magnitude at that grid point for the given POD, for each strain type (Fig. 2b–f).

Using these matching pairs, a histogram of the distribution of each tissue type with respect to the magnitude of each strain type, in terms of relative frequency, was determined for each specimen for both within- and across-timepoint comparisons. The relative frequency was defined as the number of grid points experiencing a given value of strain and occupied by a tissue type normalized by the total number of points occupied by that tissue type. These histograms were used to identify, for each type of tissue and type of strain, the strain magnitude corresponding to the peak relative frequency (Fig. 2g). Thus, for each specimen, the strain microenvironment for a given type of tissue was defined collectively by these five magnitudes (one per strain type). Given the multivariate nature of this definition of the strain microenvironment, this microenvironment was compared among tissue types via multivariate analysis of variance (MANOVA). MANOVA tests for differences among groups (e.g., tissue types) in the multivariate space defined by the set of original outcome measures (e.g., five types of strain). In this space, each of the k tissue types ($k=5$: woven bone, cartilage, fibrocartilage, fibrous tissue and clot tissue) for each specimen is described in terms of a vector of strain values. Each vector component corresponds to one type of strain, and the magnitude of the component is the strain magnitude corresponding to the peak relative frequency for that tissue type for that specimen. MANOVA defines a set of $k - 1$

“canonical variates” (CVs), or composite variables, that are mutually orthogonal linear combinations of the original outcome measures. These linear combinations are constructed such that the separation among groups is maximized, and in the linear combination for a given CV, the magnitude of the coefficient on a given original outcome measure indicates the relative importance, or weight, of that outcome measure to the CV. The CVs are ordered in terms of the amount of variation among groups that each explains, with the first CV accounting for the greatest variation. A two-factor MANOVA was performed with tissue type and POD, as well as the interaction between tissue type and POD, as the factors (JMP 11, SAS, Cary, NC). When tissue type was found to be significant, Tukey’s or Dunn’s post hoc tests (depending on the distribution of the data) were performed on the canonical variates to determine pairwise differences in strain microenvironment among tissue types.

Logistic regression analysis on the matching pairs, with tissue type as the dependent variable and strain as the independent variable, was used to determine associations between each of the five types of strain and tissue type for each specimen (JMP 11). The strength of the associations was evaluated using the generalized r^2 values and receiver operating characteristic (ROC) curve (Hosmer et al. 2013; Nagelkerke 1991). Although the r^2 value in logistic regression does not assess goodness of fit in a manner analogous to linear regression, and the r^2 values are low compared to typical values seen for linear regression, this parameter was used to compare the logistic regression models among strain types (Hosmer et al. 2013). The outcomes of logistic regression were also used to evaluate the probability of encountering a given tissue type at a given value of each of the five types of strain. For linear logistic regression models, the probability is constrained to be a linear function of strain magnitude. As such, quadratic models were also investigated and were found to be superior to the linear models by all measures of the goodness of fit.

In the MANOVAs and logistic regressions, the matching strain-type/tissue-type pairs corresponding to cortical bone were excluded from the analysis, given that the purpose of the analyses was to identify associations between strains and newly formed tissue types. A significance level of 0.05 was used for all statistical analyses.

4.1 Immunohistochemistry

Serial, mid-sagittal sections (adjacent to those stained for histology) underwent immunohistochemical staining with primary antibodies for Rho-GTPases RhoA (sc-32039, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) and CDC42 (sc-8401, 1:50), the focal adhesion complex-related protein FAK (sc-932, 1:50) and proliferating cell nuclear antigen (PCNA) (sc-56, 1:50). Protein expression was visualized using a DAB Vector Stain Kit (Vector Labs, Burlingame, CA). Antigen retrieval in Rodent Decloaker solution (Biocare, Concord, CA) was performed in a pressure cooker at 95°C for 20 min followed by cooling at 85°C for 10 min. Staining for RhoA, CDC42, and FAK was performed using an Intelli-PATH autostainer (Biocare) at room temperature with a 2-hr incubation in primary antibody, followed by 30-min incubation in an appropriate secondary antibody (Vector Labs, 1:250). PCNA staining was performed manually with antigen retrieval in sodium citrate buffer solution (pH 6.0) in a microwave for 30min at 92°C, with an overnight incubation in primary

antibody at 4°C. Non-immune slides, run without primary antibody, served as a control. Hematoxylin was used as a counterstain for all slides.

5 Results

5.1 Associations between the mechanical microenvironment and tissue formation

Strain magnitudes varied throughout the callus, for each strain type and at all timepoints (Fig. 2), and similar patterns were seen among the specimens at each timepoint. The highest strains were concentrated at the periphery of the osteotomy gap, in the regions aligned with the centerline of the gap. From here, the magnitudes of the strains decreased in the proximal, distal, and medial directions. At the earlier timepoints, the strain magnitudes were much lower in the center vs. periphery of the osteotomy gap, but over time, this difference largely dissipated. Also over time, the width (measured in the proximal-to-distal direction) of the regions of high strain decreased, as bone and cartilaginous tissues formed along the osteotomy fronts.

The different types of skeletal tissues were observed to form in different strain microenvironments. The MANOVAs performed across timepoints indicated that, for all pairs of timepoints investigated, the strain microenvironment in which formation of woven bone occurred differed from that in which formation of the soft tissues occurred ($p < 0.001$, Fig. 3). Distinct strain microenvironments were also observed for fibrocartilage vs. fibrous tissue vs. clot tissue ($p < 0.007$). Examination of the coefficients of the linear combinations that relate the MANOVA canonical variates to the individual strain types (Table 1) as well as the graphical depiction of the MANOVA results (Fig. 3) revealed that no single type of strain or small subset of types of strain was associated with the differences in strain microenvironment among tissue types. Instead, each strain type was implicated: whereas the differences between the microenvironments for woven bone vs. soft tissue were most strongly associated with $E_{\min}$ and $E_{\text{dil},\min}$, each of the strain types contributed at least moderately to the differences in microenvironments among soft tissues. The results of the within-timepoint MANOVAs were similar, except no differences in strain microenvironment were found between fibrocartilage and clot tissue ($p = 1.000$), and $E_{\max}$ was only weakly associated with the differences in strain microenvironment among the other tissue types.

Results of the logistic regressions also revealed associations between formation of the various types of tissues and the strain microenvironment. These associations were strongest for E_{oct} , $E_{\min}$, $E_{\max}$, as indicated by the generalized r^2 values and areas under the ROC curves (AUCs) (Fig. 4). For the across-timepoint analyses using E_{oct} , $E_{\min}$, and $E_{\max}$, the average AUC values for woven bone, cartilage, fibrocartilage, fibrous tissue, and clot tissue were in the range of 0.819–0.822, 0.799–0.810, 0.835–0.845, 0.762–0.765, and 0.751–0.791, respectively. Both r^2 and AUC values were higher for the across- vs. within-timepoint regressions for cartilage and fibrocartilage ($p < 0.001$) and equivalent for woven bone, fibrous tissue, and clot ($p > 0.448$). The average ($\pm$ standard deviation) magnitudes of E_{oct} corresponding to the peak probability of encountering woven bone, cartilage, fibrocartilage, fibrous tissue, and clot tissue were 0.022 (± 0.023), 0.178 (± 0.045), 0.214 (± 0.056), 0.236 (± 0.043), and 0.088 (± 0.063), respectively, for the regressions of POD 14 histology against

POD 7 strains (Figs. 5, 6). A similar ordering was found at the other timepoints and also for $E_{\max}$ and $E_{\min}$ (Fig. 6).

6 Effect of the mechanical microenvironment on protein expression

Prior to the onset of the bending stimulation (POD 7), positive staining for all proteins was observed throughout all newly formed tissues (woven bone, cartilage, clot, and fibrous tissue) in the calluses of the pseudarthrosis model (Fig. 7); however, with the application of the stimulation, spatial variations in protein expression emerged and differed among tissue types. The expression pattern in woven bone was characterized by differences between older and more newly formed tissue and by differences in staining of cells within the trabeculae vs. differences in the marrow spaces (Fig. 8). For example, RhoA expression was limited to the most newly formed trabeculae and to the bone lining cells in the marrow spaces. Expression of FAK and CDC42 was found throughout all of the trabeculae, as well as in the cortical bone, but in the marrow was largely isolated to bone lining cells. The expression pattern in cartilage was characterized primarily by differences in regions with a proliferative (as indicated by PCNA staining) vs. hypertrophic (as indicated by cell morphology) phenotype (Fig. 9). The proliferative chondrocytes were located in a curved band at the convex boundary of the lobe-shaped regions of cartilage, generally aligned tangentially within this band, and stained positive for all investigated proteins. In contrast, the inner, hypertrophic chondrocytes did not stain for FAK or PCNA, staining for CDC42 was less intense, and staining for RhoA in these hypertrophic cells was punctate and appeared to be polarizing to ends of the cells (Fig. 9). Within the fibrocartilage, positive staining was observed for all proteins, with CDC42 and PCNA expression at the later timepoints limited to the cells found along the outer boundary with the fibrous tissue. Within the fibrous tissue, abundant staining of RhoA, FAK, and PCNA and moderate amounts of CDC42 staining were observed in the highly cellular granulation tissue at the early timepoints, and became less frequent over time, with little to no PCNA found at the later timepoints (Fig. 10).

Some differences in the patterns of protein expression were noted in the soft tissues of the calluses from the pseudarthrosis model as compared to those in the fracture calluses. The chondrocyte phenotype in the fracture calluses was predominantly hypertrophic, and intracellular staining for the investigated proteins was similar to, but less prevalent than, that of the hypertrophic chondrocytes in the calluses from the pseudarthrosis model (Fig. 9). In contrast, the proliferative phenotype was scarce. No distinct spatial localization of cells with this phenotype was observed, and these cells did not express RhoA and showed only very weak staining for CDC42 and FAK. Additionally, little to no fibrocartilage formed within the calluses of the fracture specimens. Instead, the formation of new woven bone was commonly observed surrounding the cartilage. The fibrous tissue in the fracture callus resembled mostly to that found in the POD 14 pseudarthrosis model calluses, both in overall histological appearance and protein expression, but with less prevalent staining for RhoA, FAK, and PCNA than was seen in the fibrous tissue of the calluses from the pseudarthrosis model.

Some of the protein expression found within the new tissues formed in the two types of calluses was similar to that found in the native tissues of the distal femoral epiphysis. The

punctate RhoA expression and lack of PCNA expression of the hypertrophic chondrocytes found in both types of calluses closely resembled to that of chondrocytes within the growth plate and the articular cartilage (Fig. 9). However, unlike the hypertrophic chondrocytes of the calluses, chondrocytes throughout all layers of the native articular cartilage did express CDC42, and FAK expression was observed only in chondrocytes adjacent to ligament insertion sites. No difference in any pattern of protein expression was observed between the newly formed woven bone in the calluses as compared to the epiphyseal trabecular bone.

7 Discussion

Defining the mechanisms of the mechano-sensitivity of bone healing is critical for understanding the basic biology and mechanobiology of the skeleton, as well as for enhancing clinical treatment of bone injuries. The goal of this study was to identify both the features of the mechanical microenvironment most strongly associated with tissue formation during bone healing and the corresponding expression of specific proteins that have been implicated in mechanotransduction such as FAK and RhoA. To address this goal, we first measured the strains induced within the calluses of the pseudarthrosis model and carried out complementary histological analyses so as to characterize the strain microenvironment existing prior to and during formation of a given type of skeletal tissue. Multiple types of strain were used to define this microenvironment: maximum and minimum principal strains, octahedral shear strain, and in-plane dilatation. MANOVAs performed on the strain magnitudes corresponding to the peak frequency of each tissue type revealed that the strain microenvironment of skeletal tissue differentiation differs among different tissue types. Interestingly, these differences in strain microenvironment were associated with multiple types of strain. We next considered the entire range of strain magnitudes—rather than only the value corresponding to the peak frequency—and used logistic regression to identify associations between strain type and the formation of each type of tissue. The strongest associations were found between $E_{\max}$, $E_{\min}$, and E_{oct} . For each of these three types of strain, the probability of encountering woven bone was highest in regions of the callus that experienced the smallest strain magnitudes, whereas that of encountering fibrous tissue was highest in regions that experience the highest strain magnitudes. The other tissues were associated with intermediate magnitudes. Finally, we carried out immunohistochemical analyses and found ample expression of RhoA, FAK, and CDC42. Staining was greatest in the newly formed tissues of the calluses from the pseudarthrosis model as compared to both the older tissues and the tissues in the fracture calluses and distal femoral epiphyses. The results of all of these analyses indicate that formation of different types of skeletal tissues in our pseudarthrosis model occurs in distinct mechanical microenvironments, that the type of tissue formed is correlated with the local magnitudes of normal and shear strains, and that these processes involve known molecular mediators of mechanotransduction.

This study has a number of strengths. Five different types of skeletal tissue and five types of strain were investigated so as to provide a wide-ranging investigation of the association between the strain microenvironment and tissue differentiation. The within-timepoint analyses were carried out using strain data, and histological measurements were carried out on the same specimens to avoid the confounding effects of inter-specimen variability. In addition, as compared to an earlier study (Morgan et al. 2010), we used a smaller bending

angle to reduce the incidence of tissue tearing and improved the spatial resolution of our strain measurements by 2.5-fold. The higher resolution affords more accurate measurement in regions of large strain gradients, which is critical considering the close proximity of the different tissues within the callus. For example, our results indicate that formation of bone and fibrous tissue occurs at the lowest and highest magnitudes, respectively, of normal and shear strains. In regions of the callus where there is an interface between bone and fibrous tissue, the strains measured at the coarser resolution would be averages of the large and small values occurring on either side of the interface. The finer resolution reduces this artifact and as a result, allows greater distinction between the strain microenvironments for the different tissues as well as greater accuracy in quantifying these environments. This improvement also affected the distribution of the strain data and is the most probable reason for the need for nonlinear logistic regression models as compared to the linear models used previously (Morgan et al. 2010).

This study also has several weaknesses. First, the comparisons of strain microenvironment with histological patterns of tissue formation only demonstrate correlations, and not cause–effect relationships, between the two. Second, the strength of the correlations may have been affected by several technical limitations, including the examination of only a single plane within the callus, the time lag (7 or 14 days) between the POD used for strain measurement and the POD used for histological analyses in the across-timepoint comparisons, the use of different cohorts of specimens for the strain measurements and histological analyses for the across-timepoint comparisons, and the potential for registration errors between the grid points overlaid on the histological sections and the strain fields due to common histological artifacts such as tissue distortion and damage. To extend the strain measurements throughout the volume of the calluses from the pseudarthrosis model, a non-optical technique is required that has sufficiently fast acquisition to accommodate non-quasi-static loading, tissue viscoelasticity, and visualization of both mineralized and non-mineralized tissues. Such a technique would also need to be feasible in vivo to avoid the use of separate cohorts of specimens in the across-timepoint analyses. Although finite element analysis can estimate the distribution of strains—as well as stresses and interstitial fluid velocities—throughout the volume of callus in a non-destructive manner, these estimates can be very sensitive to assumptions made regarding the material properties of the callus tissues (Isaksson et al. 2009). Third, despite the improvement in the spatial resolution of the strain measurements, the resolution is still finite, and some averaging of strains in regions of high-strain gradients occurs. This is likely the cause of the increase in the probability curves of the woven bone at values of high strain (Fig. 5) as well as the large standard deviation in strain magnitudes corresponding to the peak relative frequency for woven bone (Fig. 6); we did observe that the regions of new woven bone with high strain were located along the peripheral boundary of the osseous tissue, adjacent to that of soft tissue. Fourth, despite the use of separate categories for clot vs. fibrous tissue, the latter category still encompassed a heterogeneous collection of tissues ranging from highly cellular granulation tissue to less cellular tissue with a high density of aligned fibers. However, without a specific stain or other marker for granulation tissue, we concluded that we could not reliably classify the fibrous tissue into subcategories. This heterogeneity in this category may be the cause of the comparatively broad peaks in the probability curves (Fig. 5) and the lower r^2 values at the later timepoints

(Fig. 4) for fibrous tissue. A related point is that the clot tissue was present in only small volumes at the later timepoints (less than 1% of the callus ROI at POD 21 and 35), and as such, sampling of this tissue is more susceptible to errors in image registration. These errors may have contributed to the relatively inconsistent location of the relative frequency peaks for this tissue among the different timepoints.

The statistical comparisons of strain microenvironment and healing outcome provide insight into prior studies in the mechanobiology of bone healing. A prior experimental study that included only E_{oct} , E_{max} , and $E_{dil,max}$ also found that octahedral shear strain and to a lesser extent maximum principal strain were the strain types most consistently associated with the patterns of tissue formation (Morgan et al. 2010). That study used a very similar pseudarthrosis model but with a larger bending angle, which produced a slightly different histological distribution of tissues. Not only were calluses larger with the larger angle, but also the regions of cartilage were more wedge-shaped and did not extend into the intra-cortical gap. Notably, with the smaller bending angle, a high degree of correlation exists among the values E_{oct} , E_{max} , and E_{min} throughout the mid-sagittal plane of the callus (Pearson's correlation coefficients = 0.78–0.89). Thus, investigating additional in vivo models, such as distraction osteogenesis, may allow researchers to probe the relative importance of shear vs. normal strains.

The present results are consistent with a number of prior numerical studies (Gomez-Benito et al. 2005; Hayward and Morgan 2009; Lacroix and Prendergast 2002) and the theory of Prendergast and colleagues (Prendergast et al. 1997), which postulate that shear strain is a dominant strain stimulus for tissue differentiation during skeletal healing and that formation of bone, cartilage, and fibrous tissue is favored at progressively higher values of shear strain. The finding that lower tensile strains are associated with bone formation over formation of any soft tissue is not consistent with the theory of Carter and colleagues (Carter et al. 1998). The present data are also not consistent with studies (Shefelbine et al. 2005; Steiner et al. 2013) that have implemented the theory of Claes and Heigele (Claes et al. 1997; Claes and Heigele 1999) to posit that cartilage formation is favored only in regions of large values of hydrostatic pressure (which may correspond loosely to negative values of dilatation) and is not dependent on the octahedral shear strain. Some of these and other prior studies using numerical simulation of skeletal repair have incorporated physiochemical and/or biological factors such as hypoxia and angiogenesis (Burke et al. 2013; Simon et al. 2011). Our approach in this study considered only mechanical factors; it may be possible in future studies to augment this approach with readouts of local oxygen levels and vessel formation to test these hypotheses. Future tissue-based assessments of the development of vascular tissues and tissue hypoxia relative to determinations of areas of the formation of specific tissue types and the mechanical environments that they are experiencing are certainly feasible using the current experimental approach.

To our knowledge, this study is one of the few to investigate molecular mediators of mechanotransduction during bone healing in vivo. An early study using a rat model of mandibular distraction osteogenesis demonstrated immunolocalization of FAK in the connective tissue within distraction gap, whereas no FAK expression was found in either critical-sized or sub-critical-sized defects to which no tensile displacements were applied

(Tong et al. 2003). Helms and co-workers found that loss of FAK in osteoblasts was shown to impair bone healing, whether in the presence or absence of exogenously applied mechanical loading (Kim et al. 2007; Leucht et al. 2007). In the current study, FAK expression was widespread throughout cells in the fibrous tissue and bone, and correlated with PCNA expression, both in the calluses from the pseudarthrosis model and fracture calluses. That FAK is also observed in the latter type of callus is not entirely surprising since stabilization of the closed fractures with an intramedullary pin does allow deformation of the callus tissues during the animal's daily activities. However, differences between the two types of calluses were noted in the FAK expression within the cartilaginous tissues. In the pseudarthrosis model, the proliferative chondrocytes stained strongly for FAK, whereas very weak staining for FAK was observed in the PCNA-positive chondrocytes in the fracture calluses. Given the difference in histological appearance between the proliferative cartilage in these two types of calluses (Fig. 9), and the evidence that FAK mediates expression of chondrogenic markers in vitro (Kim and Lee 2009; Park et al. 2010; Sanz-Ramos et al. 2012), this contrast in FAK expression suggests the involvement of this kinase in the way in which the controlled, bending stimulation applied to the osteotomy modulates the process of cartilage development during bone healing. The FAK expression observed only in the chondrocytes found adjacent to the ligament insertion sites in the native articular cartilage may also have mechanobiological origins, as forces transmitted through the ligament to the insertion site might create a different strain microenvironment near the insertion site compared to elsewhere in the articular layer.

Differences between the two types of calluses were also seen for RhoA and CDC42 within the cartilage. In general, these two proteins were expressed in proliferative and hypertrophic chondrocytes in both types of calluses, consistent with previous in vitro findings of RhoA and CDC42 expression during all stages of chondrogenesis (Wang and Beier 2005; Wang et al. 2004; Woods and Beier 2006). The change in appearance of RhoA staining from diffuse to globular in the hypertrophic chondrocytes could be related to the positive and negative association of this GTPase with chondrocyte proliferation and hypertrophy, respectively (Wang et al. 2004; Woods and Beier 2006). However, similar to the contrast seen with FAK, strong staining of RhoA and CDC42 was noted in the proliferative chondrocytes of the calluses from the pseudarthrosis model but not in those of the fracture calluses. Although activation of RhoA has been shown to trigger commitment to an osteogenic fate in mesenchymal stem cells in vitro (McBeath et al. 2004), RhoA has also been shown to be critical for loading-induced cell proliferation of periosteal skeletal progenitors in vivo (Sakai 2011). In addition, a study using human chondrosarcoma cells found that the downstream effector of RhoA, Rho kinase (ROCK), directly phosphorylates Sox9 (Haudenschild et al. 2010). CDC42 has been shown to be necessary for terminal differentiation of cartilage and to mediate the upregulation of MMP-9 in articular chondrocytes by fluid-induced shear stress in vitro (Aizawa 2012; Jin et al. 2000; Wang and Beier 2005). Yet, inhibiting each of CDC42 and RhoA inhibits the production of superficial zone protein—a lubricating proteoglycan—by human articular chondrocytes treated with TGF-beta (McNary et al. 2014). Collectively, these data demonstrate that the roles of RhoA and CDC42 differ among cell types and between in vitro and in vivo contexts. Our present data add to the in vivo context for chondrocytes present during bone repair. The mechanical microenvironment

created in the regions of cartilage by the applied bending stimulation may induce expression of RhoA and CDC42 in these cells, which in turn may promote maintenance of their proliferative state.

A sizeable challenge that remains is identifying the mechanisms by which different mechanical microenvironments might regulate formation of different types of skeletal tissues. The finding that the associations between strain type and tissue formation were stronger for the across- vs. within-timepoint comparisons suggests a mechanistic, mechanobiological relationship as opposed to a relationship that merely reflects differences in compliance among the different tissue types. Moreover, the patterns of protein expression that we observed raises the intriguing possibility that FAK, RhoA, and CDC42 signaling may participate in sensing and transducing the mechanical stimuli. RhoA stabilizes the actin cytoskeleton and promotes assembly of focal adhesions, and hence can both maintain and effect changes in cell shape. Activity of RhoA may thus be a reason for the stronger associations of tissue formation with normal and shear strains, which can act to change cell shape, as compared to dilatational strains, which do not. Exploring RhoA and related proteins and downstream effectors in skeletal healing is likely to reveal data on the signaling machinery that perceive and respond to mechanical cues in the microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a The histological progression of tissue formation in the pseudarthrosis model is shown via a representative, mid-sagittal histological section at four post-operative days (PODs). The applied bending stimulation commences on POD 7, with the bending motion applied in the sagittal plane **b** as shown here for the POD 14 histological section, the region(s) of the section corresponding to six different types of tissue (cortical bone, woven bone, cartilage, fibrocartilage, fibrous tissue, and clot tissue) are identified and labeled with a unique grayscale value for ease of subsequent comparison with the strain microenvironment. Other tissues and void space were also considered

Fig. 2.

a The locations of the 4200 grid points used to sample the histology images are shown superimposed on a labeled, grayscale histological section (Fig. 1b). More details on the method of sampling are provided in the Supplementary Material. **(b–f)** average strain field for each of the five types of strain ($E_{\max}$, E_{oct} , $E_{\min}$, $E_{\text{dil,max}}$, and $E_{\text{dil,min}}$) measured at POD 7: The average field was computed by averaging at each grid point the strain values across all specimens ($n=11$). In the plots, the *color* of the square marker at each grid point corresponds to the magnitude of the strain at that grid point averaged across all specimens. Plots of the standard deviation found in the Supplementary Material S.4. **g** histograms of the values of each strain type shown in **(b–f)** observed only for the regions of cartilage (CA) found in the histology section shown in **(a)**. The peak relative frequency is denoted in each graph with an arrow. These five histograms were also constructed for each of the other tissue types, and for every histology section for each across- and within-timepoint comparison (graphs not shown)

Fig. 3.

MANOVA results depicting the first and second canonical variates (CVs) for the across-timepoint comparisons: Each data point represents the strain microenvironment, as defined by the five strain magnitudes (one per strain type) corresponding to the peak relative frequency (Fig. 2g), for one tissue type (*WB* woven bone, *CA* cartilage, *FC* fibrocartilage, *FT* fibrous tissue, *CT* clot tissue) observed in one specimen. For example, the *dark red, filled circles* represent the strain microenvironment observed for cartilage when comparing POD 14 histological sections ($n=12$ specimens) to the POD 7 strains averaged across all POD 7 specimens. The center of the large circle corresponding to a group's data points is positioned at the group mean, the circle diameter represents the 95% confidence interval, and the axis that defines each strain type (e.g., $E_{\max}$) is shown as a ray in the 4D space defined by the four CVs (Table 1). (Note that for ease of presentation in print, this space is projected onto only the 2D space defined by the first two CVs, which together explain more than 87% of the total variation among all groups.) Large distances between groups (e.g., WB at all pairs of timepoints vs. FC for 7→14) indicate very distinct strain microenvironments between the groups, while overlapping groups (e.g., WB at all pairs of timepoints) indicate very similar strain microenvironments. Also, the component (i.e., projection) of a strain type's ray that aligns with the line segment that would connect two group means indicates the extent to which the strain type is associated with differences in strain microenvironment between the groups. For example, the differences in microenvironment between WB and CA were strongly associated with $E_{\min}$ and $E_{\text{dil,min}}$

Fig. 4.

The quality of fit of the logistic regression models is assessed by the (a) generalized r^2 values (Nagelkerke 1991) and b–f the areas under the receiver operating characteristics (ROC) curves (AUCs). In each graph, the generalized r^2 values or AUCs for the across-timepoint comparisons are plotted using the top horizontal axis, and those for the within-timepoint comparisons are plotted using the bottom horizontal axis. Across the top horizontal axis, the numbers before and after the arrow are the PODs at which the strain measurements and histological observations, respectively, were made. Each *symbol* plotted represents the group mean, and *error bars* indicate one SD. The *gray shading* is used to more clearly distinguish between the portions of the graph corresponding to different strain types. These graphs aid in identifying the strength of the association between tissue type and strain type. Lower r^2 values or AUCs indicate weaker associations

Fig. 5.

a, c, e Histograms depicting relative frequency for each tissue type, using the strains measured at POD 7 and the histology data at POD 14. **b, d, f** corresponding nonlinear logistic regression plots indicating the probability of encountering a given type of tissue at a given value of strain. Data are presented as the mean of all specimens and the *error bars*/shaded regions indicate ± 1 SD. The peak relative frequency or probability for each tissue type in each graph is denoted with an *arrow*. The figure shows only the results for the strain types with the strongest correlations to tissue formation ($E_{\max}$, $E_{\min}$, E_{Oct}); the plots for the remaining strain types ($E_{\text{dil,max}}$, $E_{\text{dil,min}}$) are provided in the Supplementary Material S.5

Fig. 6.

For the strain types with the strongest correlations to tissue formation ($E_{\max}$, $E_{\min}$, E_{oct}), the value of strain at the peak probability of occurrence of a given type of tissue, as determined by logistic regression (e.g., Fig. 5b), is shown for each timepoint or pair of timepoints for the within- and across-timepoint comparisons

Fig. 7.

Representative immunohistochemical staining in a section of a POD 7 pseudarthrosis model callus: (*Left*) The location of the region of interest is *boxed* in the *inset* image (Safranin O, Fast Green, and hematoxylin staining), and the region of interest itself is shown enlarged in the remainder of the panel, in order to denote the fibrous tissue (*FT*), cartilage (*CA*), and new woven bone (*WB*) formation within the callus. The *top row* of images of the immunostaining shows the fibrous tissue, the middle row the cartilage, and the bottom row the woven bone. Each column corresponds to a staining for a single protein: RhoA, CDC42, FAK, and PCNA. At the far right are the corresponding non-immune controls

Fig. 8.

Representative immunohistochemical staining in regions of woven bone in a POD 14 callus from the pseudarthrosis model: (*Left*) The location of the region of interest is *boxed* in the *inset* image (Safranin O, Fast Green, and hematoxylin staining), and the region of interest itself is shown enlarged in the remainder of the panel, in order to denote the newer bone formation (*N*) and older bone formation (*O*). The *top row* of images of the immunostaining shows the older bone region and the bottom row shows the newer region of bone formation. Each *column* corresponds to a staining for a single protein: RhoA, CDC42, FAK, and PCNA

Fig. 9. Representative immunohistochemical staining in regions of cartilage in a POD 14 pseudarthrosis model callus, a fracture callus at POD 14, and native articular cartilage of the distal femur. The location of the region of interest for each of the three specimens is *boxed* in the *inset* (Safranin O, Fast Green, and hematoxylin staining), and the region of interest itself is shown enlarged in the remainder of the panel or immediately to the right. In the calluses from the pseudarthrosis model, two chondrocyte phenotypes were observed: proliferative (*P*) and hypertrophic (*H*). The hypertrophic phenotype was widespread throughout the fracture calluses, while the proliferative phenotype was scarce. Images of immunostaining for RhoA, CDC42, FAK, and PCNA are provided for both phenotypes in both calluses. Corresponding images for articular cartilage are shown in the *bottom row*

Fig. 10.

Representative images of immunohistochemical staining in regions of fibrous tissue in the pseudarthrosis model callus at POD 14 (*top row*) and POD 35 (*second row*), and in a fracture callus at POD 14 (*third row*). Each *column* corresponds to a staining for a single protein: RhoA, CDC42, FAK, and PCNA

Table 1

In MANOVA, the coefficients in the linear combinations of strain types that define a canonical variate (CV) indicate the relative importance of each strain type in discriminating between the strain microenvironments associated with formation of the different types of skeletal tissues.

	Whole model			Tissue type			POD			Tissue type × POD		
	CV1	CV2	CV3	CV1	CV2	CV3	CV1	CV2	CV3	CV1	CV2	CV3
<i>Across-timepoint</i>												
$E_{\max}$	0.425	1.896	1.192	0.398	1.655	-0.033	2.191	1.231	1.002	0.579	2.771	
$E_{\min}$	-0.976	-0.501	1.937	-0.984	-0.431	1.620	-0.150	1.958	-1.134	-0.940	2.371	
E_{oct}	0.276	0.350	0.327	0.338	0.757	1.620	0.145	0.055	-0.015	-0.080	0.248	
$E_{\text{dli,max}}$	0.451	-0.929	1.924	0.393	-1.547	1.935	-0.915	1.880	0.618	0.492	-0.516	
$E_{\text{dli,min}}$	-1.613	4.119	-0.026	-1.573	3.891	0.959	4.056	-0.695	-0.454	4.640	0.150	
<i>Within-timepoint</i>												
$E_{\max}$	-0.321	0.036	1.208	0.241	0.140	-0.477	0.770	-1.020	n.s.	n.s.	n.s.	
$E_{\min}$	0.489	-0.890	0.889	-0.576	-0.354	1.088	-0.270	-1.150	n.s.	n.s.	n.s.	
E_{oct}	-0.654	0.039	0.423	0.629	0.625	1.504	0.738	0.130	n.s.	n.s.	n.s.	
$E_{\text{dli,max}}$	0.210	-0.245	-1.370	0.003	-1.285	-0.783	-0.420	1.047	n.s.	n.s.	n.s.	
$E_{\text{dli,min}}$	0.543	1.679	0.171	-0.463	1.390	-0.839	-0.063	0.930	n.s.	n.s.	n.s.	

Graphically speaking, these coefficients for a given strain type and CV are the magnitudes of the projections of the strain type's ray in Fig. 3 onto the axis corresponding to the whole model CVs. The highest coefficients (classified as greater than 50% of the absolute value of the maximum coefficient) are printed in bold face. Only the first three canonical variates (CV1, CV2, and CV3) are shown for the whole model, tissue type and the interaction between tissue type and POD, because these three variates together accounted for more than 95% of the variation among groups. Similarly, only the first two canonical variates are shown for POD. n.s. = non-significant interaction