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Constitutive activation of MEK1 in osteoprogenitors increases strength of bone despite impairing mineralization

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Abstract

Recent clinical studies have revealed that a somatic mutation in *MAP2K1*, causing constitutive activation of MEK1 in osteogenic cells, occurs in melorheostotic bone disease in humans. We have generated a mouse model which expresses an activated form of MEK1 (MEK1DD) specifically in osteoprogenitors postnatally. The skeletal phenotype of these mice recapitulates many features of melorheostosis observed in humans, including extra-cortical bone formation, abundant osteoid formation, decreased mineral density, and increased porosity. Paradoxically, in both humans and mice, MEK1 activation in osteoprogenitors results in bone that is not structurally compromised, but is hardened and stronger, which would not be predicted based on tissue and matrix properties. Thus, a specific activating mutation in MEK1, expressed only by osteoprogenitors postnatally, can have a significant impact on bone strength through complex alterations in whole bone geometry, bone micro-structure, and bone matrix.

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CONTRIBUTION OF AUTHORS

J.L.F., R.C.B., P.D.R., and K.M.T. contributed to the design of the experiment. J.L.F., R.C.B., S.U., M.U., J.S.N. and K.M.T. researched data, contributed to discussion, wrote manuscript and reviewed/edited manuscript. E.K. contributed to the discussion and reviewed/edited the manuscript. R.C.B. monitored and maintained compliance of all animal procedure with the UK IACUC. K.M.T., J.S.N., J.L.F., R.C.B. and M.U. provided statistical analyses and/or interpretation of the data. All authors have approved the final version of the submitted manuscript.

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Keywords

Melorheostosis; mitogen-activated protein kinase kinase 1 (MAP2K1); osteoid; MEK1; osteoprogenitor; bone strength; bone structure; Raman spectroscopy

1 INTRODUCTION

An activating mutation in the mitogen-activated protein kinase kinase 1 (*MAP2K1*) gene has recently been found to account for a significant number of cases of melorheostosis, a rare condition of the skeleton in humans (1) which develops after birth, affects ossification centers, and presents radiologically as “dripping candle wax” (2, 3). The identified mutations predicted substitutions in two residues of the protein (MEK1) encoded by *MAP2K1* that map to a negative regulatory element, suggesting constitutive somatic activation of MEK1 in bones affected in melorheostosis. Histological analysis revealed that in melorheostotic bone harboring the MEK1 mutation, there was increased osteoid, more bone cells, and higher amounts of vascular pores compared to unaffected bone (4).

A number of genetically modified mouse models have been developed to study germline mutations in various components of the ERK/MAPK pathway (termed RASopathies) (5). Skeletal abnormalities observed in these mouse models include craniofacial defects, growth retardation and low bone mass (6). Studies have shown that MAPKs promote the bone-forming potential of osteoblasts and regulate bone mass *prenatally* (7–9). Indeed, loss of MEK1/2 specifically in osteoprogenitors prenatally results in severe osteopenia and cleidocranial dysplasia. Postnatal deletion of MEK1/2 causes significantly reduced bone mass (10). Loss of MEK signaling also decreased activation of essential genes in osteoblastogenesis, such as RUNX2, ATF4, and β -catenin (10). In contrast, phosphorylated ERK has also been shown to bind to the promotor of osteocalcin in osteoblasts by its association with RUNX2 to promote osteoblastogenesis (11). To understand the role of activated MEK1 in *postnatal* bone development, as occurs in the human condition of melorheostosis, we developed a mouse model (the MEK1DD-Ob mouse) that expresses a constitutively active *MAP2K1* transgene postnatally specifically in osteoprogenitors. Phenotypically, the MEK1DD-Ob mouse recapitulates many features of the human condition of melorheostosis and sheds light on how MEK1 activation impacts bone remodeling, extra-cortical bone formation, bone mineral density, and the overall structural strength of bone.

2 RESEARCH DESIGN AND METHODS.

2.1 Generation of the MEK1DD-Ob mouse

To generate a mouse model representative of the human condition of melorheostosis, we developed transgenic mice expressing in osteoprogenitor cells a mutant form of rat mitogen-activated protein kinase kinase 1 (*MAP2K1*), wherein serine residues 218 and 222 have been mutated to aspartic acid, rendering the protein product, MEK1, constitutively active. The mutant protein is termed “MEK1DD”. The *Osx-Cre* transgenic mouse (B6.Cg-Tg(Sp7-tTA, tetOEGFP/cre)1Amc/J; Jax labs) was crossed with R26Stop^{fllox}MEK1DD mouse (C57BL/6-

Gt(ROSA)26Sor^{tm8}(Map2k1,EGFP)Rsky/J*; Jax labs) to achieve expression of the mutant transgene in osteoprogenitor cells. Mice expressing the transgene have the genotype: R26Stop^{flox}MEK1DD^{+/-}/*Osx-Cre*^{+/-} (referred to as the MEK1DD-Ob mouse). Control littermates have the genotype R26Stop^{flox}MEK1DD^{-/-}/*Osx-Cre*^{+/-}. The R26Stop^{flox}MEK1DD conditional allele is targeted to the *Gt(ROSA)26Sor* locus and has a *loxP*-flanked Neo-STOP cassette preventing transcription of the downstream MEK1DD and EGFP sequences. Cre-mediated recombination of *loxP* sites, which is repressed by doxycycline in *Osx-Cre* mice, allows transcription of the MEK1DD and EGFP sequences. MEK1DD-Ob mice were obtained at the expected Mendelian frequency when dams consumed chow containing doxycycline (Bio-Serv, S3888) from the time of conception until MEK1DD-Ob and control littermate pups were weaned onto regular chow diet without doxycycline (12–15), resulting in expression of the transgene postnatally. After weaning, mice were maintained on regular chow diet (minus doxycycline) for approximately 18 weeks. Calcein injections were given at 10 and 4 days prior to euthanasia at 22 weeks of age (16). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

2.2 Micro-computed tomography (μCT) analysis

While secured vertically in a holder tube (6mm in diameter × 30mm in length) and immersed in phosphate buffer saline (PBS), the left femurs were scanned using a high resolution μCT scanner (Scanco μCT 50, Scanco Medical AG, Brüttisellen, Switzerland) with the following parameters: peak voltage = 70 kVp, tube current = 0.114 mA, integration time = 300 ms, sampling rate = 1160 samples per 500 projections per 180° rotation of the sample tube, and isotropic voxel size = 6 μm. A thin aluminum filter (0.1 mm) was in the X-ray path, and the manufacturer's recommended beam hardening correction for 1200 mgHA/cm³ was used. The scan regions included the mid-point of the diaphysis (axial length = 1.86 mm) and the distal femur metaphysis (axial length = 3.72 mm). The periosteal and endosteal surfaces were contoured using the Scanco auto-contour, dual-threshold contouring script. Applying a Gaussian noise filter (Sigma = 0.8 and Support = 2), the mid-shaft was segmented from air and marrow using a global threshold of 876.4 mgHA/cm³ to evaluate the structure and tissue mineral density (TMD) of cortical bone (e.g., cortical cross-sectional area, total cross-sectional area, tissue mineral density, moment of inertia) as determined by the standard Scanco midshaft algorithm. The calculation of cortical thickness (Ct.Th) came from the plate model or triangular method, not the distance transformation method (i.e., the largest sphere method). Then, using the periosteal and endosteal contours to define the region of interest, the global threshold was inverted to 900.5 mgHA/cm³ (Sigma = 0.8, Support = 2) to segment pores within the cortex and evaluate intra-cortical porosity using the Scanco trabecular algorithm. The trabecular bone in the metaphysis (contours were ~2 voxels away from the endosteal cortex) was segmented using a global threshold of 429.4 mgHA/cm³ (Sigma = 0.2, Support = 1) to evaluate the micro-architectural properties (e.g., bone volume fraction, trabecular connectivity, thickness, separation) and TMD. Because trabecular bone was observed within the medullary canal, just the endosteal contours defined the region of interest; and then medullary canal (Med) was evaluated using the same thresholds as used for trabecular bone in the distal metaphyseal region.

2.3 Three-point bending test

As previously described (17–20), each hydrated left femur was loaded-to-failure at 3 mm/min in three-point bending (Instron 8800 DynaMight) with a constant span of 8 mm. From the resulting force (measured using a 100 N load cell) vs. displacement (measured using the LVDT) curve, the slope of the elastic linear portion and the peak force endured by the bone were determined to calculate the stiffness and structural-dependent strength, respectively. The yield point in the mechanical test was determined by the 0.2% offset method implemented in a custom MATLAB script, and the associated properties such as yield force and post-yield displacement were calculated. We estimated the material properties, modulus, strength, and toughness, using the structural properties of diaphyseal bone from μ CT (21).

2.4 Raman analysis

Raman spectra were acquired at 10 sites from the anterior surface of each broken femur (Fig. S1 in supplemental materials) using InVia confocal Raman microscopes (Renishaw, Gloucestershire, UK) with a holographic grating (1200 lines/mm) providing $\sim 1 \text{ cm}^{-1}$ spectral resolution and an 830 nm diode laser source. Each spectrum was obtained as the average of 5 consecutive spectra with each collected for 10 seconds using a 20X objective (NA=0.40) to focus the light. Data collection range was between 785 cm^{-1} and 1740 cm^{-1} . Daily silicon and laser power measurements ($\sim 35 \text{ mW}$) before and after data collection ensured wavenumber calibration and light throughput, respectively.

All spectra were processed to determine Raman measurements of bone matrix composition following our published method (22). Briefly, Raman spectra were processed using LabSpec 5 software (Horiba Jobin Yvon, Edison, NJ), and then matrix properties were calculated from each processed spectrum per bone using a custom MATLAB script. First, the algorithm averaged the Raman raw spectra collected at ten sites per bone specimen. Then, by subtracting a 5th-order polynomial function from the base of the raw spectrum, background fluorescence was removed from all averaged spectra. Next, the spectra were further smoothed to minimize noise using a proprietary algorithm provided by the LabSpec 5 software. From the averaged and denoised spectrum per specimen, we calculated the following RS properties from peak intensities: mineral-to-matrix ratio ($\nu_1\text{PO}_4/\text{Amide I}$, $\nu_1\text{PO}_4/\text{Proline}$, $\nu_1\text{PO}_4/\text{Amide III}$, and $\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$), Type-B carbonate substitution ($\text{CO}_3/\nu_1\text{PO}_4$), crystallinity (the inverse of the line-width of the $\nu_1\text{PO}_4$ peak at half the height from baseline or half-maximum; $1/\text{FWHM}$), collagen crosslinks/matrix maturity ratio (calculated as the intensity at $\sim 1670 \text{ cm}^{-1}$ per intensity at $\sim 1690 \text{ cm}^{-1}$ or I_{1670}/I_{1690}), and the newly developed I_{1670}/I_{1610} and I_{1670}/I_{1640} ratio associated with collagen conformational change (23). These sub-peaks of the Amide I were identified using the second-derivative spectrum (i.e., not fixed but rather the locations varied by several wavenumbers among the bones), and the sub-peak ratios were directly calculated without band-fitting (deconvolution is an ill-posed mathematical problem in the case of bone).

2.5 Bone Histomorphometry

Right femurs were harvested, cleaned, fixed in ethanol, dehydrated and embedded in methylmethacrylate. Three micron sections, cut with a microtome, were stained with

Mason-Goldner trichrome stain for measurement of static and dynamic parameters of bone structure, formation and resorption, as described previously (16). Measurements of bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), % osteoid volume (OV/BV), % osteoid surface (OS/BS), osteoid thickness (O.Th), osteoblast number/bone length (N.Ob/B.Pm), osteoblast surface/bone surface (Ob.S/BS), erosion surface/bone surface (ES/BS), erosion depth (E.De), osteoclast number/bone length (N.Oc/B.Pm), osteoclast surface/bone surface (Oc.S/BS), mineral apposition rate (MAR), double labels/bone surface (dLS/BS), single labels/bone surface (sLS/BS), mineralization surface/bone surface (MS/BS), bone formation rate (BFR), mineralization lag time (MLT) and osteoid maturation time (OMT) were obtained using Osteomeasure XP system (OsteoMetrics, Inc., Decatur, GA).

2.6 Biochemical Measurements

Serum obtained from blood collected at sacrifice was used to measure the following: serum procollagen type 1 N-terminal propeptide (PINP) using the Rat/Mouse PINP Enzyme immunoassay (Immunodiagnosics Systems, Inc., Fountain Hills, AZ; #AC-33F1); serum C-terminal telopeptides of type I collagen (RatLAPs) using the RatLAPs ELISA (Immunodiagnosics Systems, Inc., Fountain Hills, AZ; #AC-06F1).

2.7 Statistical Analysis

Pooling females and males, we used unpaired, two-tailed t-tests with Welch's correction to determine whether there was a significant difference in each property between the genotypes (p -value < 0.05). In the event that the sample of a given property did not pass the Shapiro-Wilk normality test for either genotype, the Mann-Whitney test was used instead to determine whether the difference between genotypes was significant (p -value < 0.05). The results are presented as the mean $\pm$ sample standard deviation (SD) unless otherwise noted. Analysis of covariance (ANCOVA) using a general linear model determined whether the relationship (slope and intercept) between peak moment and cross-sectional geometry of the femur mid-shaft (i.e., section modulus) was different between the two genotypes. The statistical analyses were performed using GraphPad Prism (v6, GraphPad Software, Inc., San Diego, CA).

3 RESULTS

3.1 Postnatal expression of an activated MAP2K1 transgene in osteoprogenitors increased the serum marker of bone resorption, but not bone formation, and increased the amount of osteoid in bone

Post-weaning induction of Cre recombinase in osteoprogenitors of MEK1DD-Ob mice resulted in the expression of the *MAP2K1* transgene in bone. Using RNA isolated from tibiae of 22-week old mice, transcript for the mutant transgene was not detected in control littermates, but was detected in MEK1DD-Ob mice (Fig. 1A, arrow). While male and female mice were of different body size (male $>$ female) in each group, as expected, there was no significant difference in size or body mass between MEK1DD-Ob and control mice (Fig. 1B). The bone formation marker, PINP, was assessed in serum, and there were no demonstrable differences in the systemic concentration of this biomarker between

MEK1DD-Ob and control mice (Fig. 1C). However, serum C-terminal telopeptides of type I collagen (i.e., ratLAPS, a systemic marker indicative of bone turnover) was higher in serum from MEK1DD-Ob when compared to control mice (Fig. 1D). These data suggested that expression of an activated *MAP2K1* transgene in osteoprogenitors may lead to a high bone turnover, uncoupled phenotype.

Dynamic and static histomorphometry of the distal femur metaphysis revealed no significant differences in bone formation rate and in the number of osteoblasts and osteoclasts, respectively, between the MEK1DD-Ob mouse and controls, with the exception of single-labeled surface per bone surface (sLS/BS) being less in MEK1DD-Ob mice compared to control (Table S1 in supplemental materials). In contrast, striking differences were noted for MEK1DD-Ob mice related to mineralization parameters (Table 1). MEK1DD-Ob mice exhibited increased osteoid thickness (O.Th) and mineralization lag time (Mlt), suggesting that under-mineralized bone formation was increased in the MEK1DD-Ob bone. The increase in osteoid is visually apparent when control bone (Fig. 1E) is compared to the bone from a MEK1DD-Ob mouse (Fig. 1F).

3.2 The distal femur metaphysis of the MEK1DD-Ob mice had higher trabecular bone volume but lower tissue mineral density compared to the control mice

Microarchitecture assessments by μ CT of the trabecular bone within the distal femur metaphysis (Fig. 2 A–B) revealed a significantly higher bone volume fraction (BV/TV) for the MEK1DD-Ob than for the control mice (Fig. 2C). This was due to the mutation increasing both the number of trabeculae (Fig. 2C) and the thickness of the trabeculae (Table 2). Accompanying these differences in microarchitecture, connectivity density was also higher (Fig. 2C) while trabecular spacing was lower for the mutant mice than for control littermates (Table 2). Moreover, in the distal femur metaphysis of MEK1DD-Ob mice, trabecular structure was more plate-like than rod-like (lower SMI) (Table 2). Matching the osteoid phenotype, the tissue mineral density (Tb.TMD) of trabecular bone was lower in the MEK1DD-Ob distal femurs than control femurs (Table 2).

3.3 In the diaphyseal region, there was higher cortical porosity and higher trabecular bone volume in the medullary canal for the MEK1DD-Ob mice than for the control mice

Two distinct features were apparent when comparing the μ CT images of the femur diaphysis from the MEK1DD-Ob mice to the control mice: cortical porosity (yellow arrows in Fig. 3A) and trabecular-like bone in the medullary canal (red arrows in Fig. 3A). In three-dimensional (3D) renderings of the femur mid-shaft merged with 3D renderings of the pores (Fig. 3B), the difference in cortical porosity (Ct.Po) between the genotypes was even more apparent with some pores exhibiting extensive elongations along the long-bone axis (yellow arrows in Fig. 3B). When quantified, Ct.Po was significantly greater in MEK1DD-Ob mice compared to control mice (Fig. 3C) as indicated by a higher pore number (Po.N) (Table 2). As for the two-dimensional, cross-sectional properties of the femur mid-shaft, the moment of inertia (minimum principal component or I_{min}) was not different between the genotypes (Fig. 3C). As such, there were no differences in the periosteal perimeter (Ps.Pm), total cross-sectional area (Tt.Ar), and anterior-to-posterior width (Table 2). The bone cross-sectional area (Ct.Ar) however was larger for MEK1DD-Ob mice than for the control mice, but there

was not a significant difference in the cortical thickness (Ct.Th), a three-dimensional measurement.

Because μ CT images revealed what appeared to be trabecular-like bone within the medullary canal of the femur diaphysis (see Fig. 3A and Fig. S1 in supplemental materials), we selected this canal as the region of interest to clarify if this additional bone was higher at the mid-point of the diaphysis when the activated *MAP2K1* transgene is expressed in osteoprogenitors. As shown in the 3D renderings of the cortex merged with renderings of medullary canal (Fig. 4A), trabecular bone was consistently present in both female and male MEK1DD-Ob mice, indicative of active bone formation within the medullary canal. Assessments of this bone showed that medullary trabecular bone volume fraction (Med BV/TV) was almost exclusively present within the central mid-shaft of the MEK1DD-Ob mice (Fig. 4B). This trabecular bone may actually extend throughout the entire medullary cavity of the femur (Fig. S1). The medullary bone of the MEK1DD-Ob mouse was well-organized and made up of multiple trabecular-like elements (Fig. 4A). Similar to the distal femur metaphysis, there were more and thicker trabeculae in the canal of the MEK1DD-Ob mice (Fig. 4B). Unlike the distal femur metaphysis, the difference in tissue mineral density of trabecular-like bone within the medullary cavity (Ma.TMD) between the genotypes was not significant (Table 2). Together, these μ CT data show that the constitutive activation of MEK1 in bone cells caused not only trabecular-like bone to form throughout the medullary space of long bones, but also additional pores to form within the cortex.

3.4 With an overly active MEK1 protein being expressed by osteoprogenitors, the mouse femur was stronger in bending

Based on the μ CT assessments showing, in general, decreased cortical thickness, increased cortical porosity and decreased cortical tissue mineral density, the expectation was that the bone strength of the MEK1DD-Ob mouse would be impaired. In contrast to this expectation, the maximal force required to break the femur of MEK1DD-Ob mice in three-point bending was greater than that required to fracture the femur of control mice (Table 3). MEK1DD-Ob mice also demonstrated increased peak stress compared to control (Fig. 5A) such that for a given geometry of the diaphysis (i.e., moment of inertia per distance between centroid and outer bone surface in the anterior-posterior direction or section modulus), the MEK1DD-Ob bone was stronger than the wild-type bone (Fig. 5B). There were no differences in post-yield displacement and toughness between the genotypes (Table 3). Thus, the overall effect of postnatally overexpressing MEK1 in osteoprogenitors was an increase in bending strength of the femur, despite micro-structural and matrix mineralization deficits typically associated with weaker bone.

3.5 The activated MEK1 in the cells of the osteoblast lineage altered the helical structure of type 1 collagen

Because the higher peak stress of MEK1DD-Ob bone was discordant to the lower Ct.TMD, we analyzed the anterior surface of the broken femur mid-shafts using Raman micro-spectroscopy (RS) to identify potential differences in matrix composition and organization between the genotypes (Fig. S2). Two of the 4 mineral-to-matrix ratios by RS (Fig. 6B and Table 2) confirmed the apparent mineralization defect in the MEK1DD-Ob mice (Fig. 6A

and Table S1), but since both the Amide I peak and the Proline peak are sensitive to collagen fibril orientation (24) and to the extent to which collagen I has advanced glycation end-products (22) or is denatured (25), the difference in $\nu_1\text{PO}_4/\text{Amide I}$ and $\nu_1\text{PO}_4/\text{Proline}$ between the genotypes could reflect changes in the organic matrix caused by the activated MEK1. This possibility is supported by the lower Amide I sub-peak ratios (Fig. 6C) and the lower hydroxyproline-toproline ratio (Fig. 6D) for the mutant bone than for the wild-type bone. There were not any significant genotype-related differences in the crystal structure of the mineral phase with respect to crystallinity and type B carbonate substitutions (Table 3). Thus, along with the decrease in cortical TMD (Fig. 6A) and increase in osteoid (Table 1), the induction of an activated MEK1 in osteoprogenitors apparently also affected the helical order or secondary structure of type 1 collagen.

4 DISCUSSION

Melorheostosis develops postnatally in humans and recent evidence shows that many cases of melorheostosis result from somatic mutations in *MAP2K1*, which in turn results in constitutive activation of this oncogene that encodes the protein kinase MEK1 (1). The cell-type harboring the mutation in melorheostosis is assumed to be of osteoblastic origins, and the burden of the disease may be related to the percent of cells expressing the mutant allele and the overall expression level observed in affected bone (1, 26). Recognizing these features of melorheostosis in humans, we developed and characterized a mouse model wherein an activated mutant of MEK1 is expressed in osteoprogenitors postnatally.

The MEK1DD-Ob mouse postnatally expresses a single copy of a mutant *MAP2K1* allele which results in constitutively active MEK1 production in osteoprogenitors. The phenotype of the MEK1DD-Ob mouse mimics several features of the human condition of melorheostosis. For instance, the intra-medullary bone observed in the MEK1DD-Ob mouse is highly reminiscent of the intramedullary hyperostosis observed in melorheostosis (27). The intra-medullary bone of the MEK1DD-Ob mouse demonstrated characteristics of trabecular bone in metaphyseal regions and was highly organized, suggesting it had undergone extensive remodeling, also a hallmark of melorheostotic bone (1). In addition, a distinguishing feature of melorheostosis attributable to *MAP2K1* mutations and MEK1 activation is increased osteoid relative to mineralized bone tissue (3). Similarly, the osteoid surface was also higher in the trabecular compartment of MEK1DD-Ob compared to control mice (Table 1 and Fig. 1F). The mechanisms contributing to the excessive under-mineralized bone (osteoidosis) formed in melorheostosis in humans and in the MEK1DD-Ob mouse are not fully known. It is possible there is an underlying defect in mineralization attributable to the activating mutation. This is supported by the finding that decreased mineralization was observed in affected osteoblasts obtained from individuals with melorheostosis when studied in culture (1).

While the MEK1DD-Ob mouse in many regards mimics the human condition of melorheostosis, melorheostosis occurs due to a somatic mutation involving only a subset of osteoprogenitors expressing the mutant form of MEK1; whereas, all osteoprogenitors in the MEK1DD mouse are capable of expressing the constitutively active form of MEK1. Therefore, the MEK1DD-Ob mouse displays a phenotype that could involve all bones of the

skeleton, which is not the situation in humans, where only segmental portions, principally of long bones, are involved, creating the hallmark radiological finding of “dripping candle-wax” bone. While parameters measured related to the melorheostosis phenotype were highly significantly different between control and MEK1DD-Ob mice, due to the modest number of mice required, subtle sex-related differences in the phenotype may not be detectable. Moreover, changes over time in the phenotype with aging are not known. Measures, such as serum markers of bone turnover at one time point and histomorphometry performed at the distal femur metaphysis, may not be fully sufficient to explain all of the changes in bone metabolism that confer the MEK1DD-Ob phenotype.

Melorheostotic bone has been described as dense, rigid bone that can even dull osteotomes and drill bits when biopsied (1), yet the underlying attributes of affected bone which contribute to these features is not well understood. Indeed, the finding that affected bone is harder to cut than unaffected bone seems counterintuitive because affected bone is characterized by i) osteoidosis, as previously discussed, ii) elevated vascular-lacunar porosity compared to unaffected bone, iii) reduced degree of mineralization, and iv) reduced tissue-level hardness (resistance to the onset of plastic deformation) (4). This same dichotomy was observed in the MEK1DD-Ob mouse whose long bones were more resistant to bending with higher yield stress than “normal” bone (Fig. 5), yet displayed decreased cortical tissue mineral density (Fig. 6A) and increased porosity (Fig. 4), attributes which would typically be associated with a more fracture-prone bone. The reasons behind the enhanced performance of bone in MEK1DD-Ob mice is likely multifactorial. For instance, the additional bone formed within the medullary canal may function to strengthen bone due to a scaffolding effect, though presumably this was factored out in the material property calculations. Also, Raman spectroscopy revealed that the MEK1 activation in osteoprogenitors possibly caused changes in the secondary structure of type 1 collagen (e.g., more helical collagen I relative to non-helical collagen I as reflected by the lower Amide I sub-peak ratios) that might lend more durability and strength to bone.

CONCLUSIONS

These findings provide essential information about how a specific mutation in the MAPK pathway, operative only in the osteoprogenitor lineage, can strengthen bone through changes at the macro- and micro-architectural levels, as well as via alterations to the ultrastructural composition of bone matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Kang H, Jha S, Deng Z, Fratzl-Zelman N, Cabral WA, Iovovic A, et al. Somatic activating mutations in MAP2K1 cause melorheostosis. *Nat Commun.* 2018;9(1):1390. [PubMed: 29643386]
2. Freyschmidt J Melorheostosis: a review of 23 cases. *Eur Radiol.* 2001;11(3):474–9. [PubMed: 11288855]
3. Jha S, Fratzl-Zelman N, Roschger P, Papadakis GZ, Cowen EW, Kang H, et al. Distinct Clinical and Pathological Features of Melorheostosis Associated With Somatic MAP2K1 Mutations. *J Bone Miner Res.* 2018.
4. Jha S, Fratzl-Zelman N, Roschger P, Papadakis GZ, Cowen EW, Kang H, et al. Distinct Clinical and Pathological Features of Melorheostosis Associated With Somatic MAP2K1 Mutations. *J Bone Miner Res.* 2019;34(1):145–56. [PubMed: 30138550]
5. Hernandez-Porras I, Guerra C. Modeling RASopathies with Genetically Modified Mouse Models. *Methods Mol Biol.* 2017;1487:379–408. [PubMed: 27924582]
6. Tajan M, Paccoud R, Branka S, Edouard T, Yart A. The RASopathy Family: Consequences of Germline Activation of the RAS/MAPK Pathway. *Endocr Rev.* 2018;39(5):676–700. [PubMed: 29924299]
7. Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *The Journal of cell biology.* 2007;176(5):709–18. [PubMed: 17325210]
8. Matsushita T, Chan Y, Kawanani A, Balmes G, Landreth G, Murakami S. Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. *Molecular Cellular Biology.* 2009;29(21):5843–57. [PubMed: 19737917]
9. Greenblatt MB, Shim JH, Glimcher LH. Mitogen-activated protein kinase pathways in osteoblasts. *Annu Rev Cell Dev Biol.* 2013;29:63–79. [PubMed: 23725048]
10. Kim JM, Yang YS, Park KH, Oh H, Greenblatt MB, Shim JH. The ERK MAPK Pathway Is Essential for Skeletal Development and Homeostasis. *Int J Mol Sci.* 2019;20(8).
11. Li Y, Ge C, Franceschi RT. Differentiation-dependent association of phosphorylated extracellular signal-regulated kinase with the chromatin of osteoblast-related genes. *J Bone Miner Res.* 2010;25(1):154–63. [PubMed: 19580458]
12. Eleftheriou F, Yang X. Genetic mouse models for bone studies--strengths and limitations. *Bone.* 2011;49(6):1242–54. [PubMed: 21907838]
13. Zanolini S, Canalis E. Notch1 and Notch2 expression in osteoblast precursors regulates femoral microarchitecture. *Bone.* 2014;62:22–8. [PubMed: 24508387]
14. Rodriguez-Carballo E, Gamez B, Sedo-Cabezon L, Sanchez-Feutrie M, Zorzano A, Manzanares-Céspedes C, et al. The p38alpha MAPK function in osteoprecursors is required for bone formation and bone homeostasis in adult mice. *PLoS One.* 2014;9(7):e102032. [PubMed: 25007355]
15. Yu C, Xuan M, Zhang M, Yao Q, Zhang K, Zhang X, et al. Postnatal deletion of beta-catenin in osterix-expressing cells is necessary for bone growth and intermittent PTH-induced bone gain. *J Bone Miner Metab.* 2017.
16. Thrailkill KM, Nyman JS, Bunn RC, Uppuganti S, Thompson KL, Lumpkin CK Jr., et al. The impact of SGLT2 inhibitors, compared with insulin, on diabetic bone disease in a mouse model of type 1 diabetes. *Bone.* 2016.
17. Nyman JS, Even JL, Jo CH, Herbert EG, Murry MR, Cockrell GE, et al. Increasing duration of type 1 diabetes perturbs the strength-structure relationship and increases brittleness of bone. *Bone.* 2011;48(4):733–40. [PubMed: 21185416]
18. Thrailkill K, Bunn RC, Lumpkin C Jr., Wahl E, Cockrell G, Morris L, et al. Loss of insulin receptor in osteoprogenitor cells impairs structural strength of bone. *J Diabetes Res.* 2014;2014:703589. [PubMed: 24963495]

19. Nyman JS, Kalaitzoglou E, Clay Bunn R, Uppuganti S, Thrailkill KM, Fowlkes JL. Preserving and restoring bone with continuous insulin infusion therapy in a mouse model of type 1 diabetes. *Bone reports*. 2017;7:1–8. [PubMed: 28736738]
20. Thrailkill KM, Clay Bunn R, Nyman JS, Rettiganti MR, Cockrell GE, Wahl EC, et al. SGLT2 inhibitor therapy improves blood glucose but does not prevent diabetic bone disease in diabetic DBA/2J male mice. *Bone*. 2016;82:101–7. [PubMed: 26211996]
21. Uppuganti S, Granke M, Makowski AJ, Does MD, Nyman JS. Age-related changes in the fracture resistance of male Fischer F344 rat bone. *Bone*. 2016;83:220–32. [PubMed: 26610688]
22. Unal M, Uppuganti S, Leverant CJ, Creecy A, Granke M, Voziyani P, et al. Assessing glycation-mediated changes in human cortical bone with Raman spectroscopy. *J Biophotonics*. 2018;11(8):e201700352. [PubMed: 29575566]
23. Unal M, Jung H, Akkus O. Novel Raman Spectroscopic Biomarkers Indicate That Postyield Damage Denatures Bone's Collagen. *Journal of Bone and Mineral Research*. 2016;31(5):1015–25. [PubMed: 26678707]
24. Makowski AJ, Pence IJ, Uppuganti S, Zein-Sabatto A, Huszagh MC, Mahadevan-Jansen A, et al. Polarization in Raman spectroscopy helps explain bone brittleness in genetic mouse models. *J Biomed Opt*. 2014;19(11):117008. [PubMed: 25402627]
25. Nyman JS, Uppuganti S, Unal M, Leverant CJ, Adabala S, Granke M, et al. Manipulating the Amount and Structure of the Organic Matrix Affects the Water Compartments of Human Cortical Bone. *JBMR PLUS*. 2019.
26. Jha S, Laucis N, Kim L, Malayeri A, Dasgupta A, Papadakis GZ, et al. CT analysis of anatomical distribution of melorheostosis challenges the sclerotome hypothesis. *Bone*. 2018;117:31–6. [PubMed: 30218789]
27. Ihde LL, Forrester DM, Gottsegen CJ, Masih S, Patel DB, Vachon LA, et al. Sclerosing bone dysplasias: review and differentiation from other causes of osteosclerosis. *Radiographics*. 2011;31(7):1865–82. [PubMed: 22084176]

HIGHLIGHTS

- MEK1 activation in osteoprogenitors in mice resembles melorheostosis in humans.
- Osteoidosis is a prominent feature of MEK1 activation in osteoprogenitors.
- MEK1DD-Ob mice demonstrate increased bone strength, but impaired mineralization.

Figure 1.

Genotype and phenotype of control and MEK1DD-Ob mice. Upon extracting RNA from the tibia, the mutant transgene was only detected in MEK1DD^{+/-}/Osx-Cre^{+/-} (MEK1DD-Ob) mice (Aa) and not in MEK1DD^{-/-}/Osx-Cre^{+/-} control littermates (Ab). The body weight prior to euthanasia was not different between the genotypes (B). While the serum marker of bone formation (P1NP) was not different (C), the serum marker of bone resorption was higher for the MEK1DD-Ob than for the Control mice (D). Mason-Goldner trichrome stain of sections of the distal femur metaphysis indicated that the bone tissue of the Control mice had some osteoid (E), whereas there was pronounced osteoid accumulation in the trabeculae of MEK1DD-Ob mice (F). Red-brown staining represents osteoid. Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Figure 2.

Micro-computed tomography evaluations of distal femur. More trabecular bone was apparent in the cross-sections of the metaphysis (A) and 3D renderings of the compartment (B) for MEK1DD-Ob mice than for the control littermates, regardless of sex. As such, bone volume fraction, trabecular number, and connectivity density was higher when the activated MEK1 protein was expressed (C). Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Figure 3.

Micro-computed tomography evaluations of the femur mid-shaft. Porosity in the cortex (yellow arrows) and bone in the medullary canal (red arrows) were apparent in the cross-sections of the diaphysis (A) and 3D renderings of the mid-shaft (B) when comparing MEK1DD-Ob mice to the control littermates, regardless of sex. While the moment of inertia was not different, the MEK1DD-OB mice had femurs with thinner, more porous cortices than did the control mice (C). Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Figure 4.

Micro-computed tomography evaluations of the medullary canal of the femur. Trabecular-like bone in the medullary canal (green coloring) was apparent in 3D renderings of the mid-shaft (A) when comparing MEK1DD-Ob mice to the control littermates, regardless of sex. When quantified, there was higher bone volume fraction, higher trabecular number, and higher trabecular thickness (left to right) for MEK1DD-Ob mice than for the control mice (B). Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Figure 5.

Material property and mechanical behavior of the femur in bending. When activated MEK1 was postnatally expressed in osteoprogenitors, the cortical bone tissue was stronger (A) such that the femur mid-shaft experienced a higher peak moment for a given cross-sectional geometry or section modulus (B). That is, the intercept was higher for the MEK1DD-Ob mice than control mice, while the slope of the linear regression was not different between the genotypes. Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Figure 6.

Matrix properties of the femur mid-shaft. As assessed by μCT , cortical tissue mineral density was lower for the MEK1DD-Ob cortices than for control cortices (A). As assessed by Raman spectroscopy, the mineral-to-matrix ratio (B), the Amide I sub-peak ratio (C), and hydroxyproline-to-proline ratio (D) were lower for the cortical bone from the MEK1DD-Ob mice than from control mice. Black circle = control female; blue circle = control male; black triangle = MEK1DD-Ob female; blue triangle = MEK1DD-Ob male.

Table 1.

Differences in mineralization parameters of the mouse femur between the two genotypes as assessed by histomorphometry.

Mineralization* Parameters	Groups	Mean $\pm$ SD	p-value
OS/BS (%)	Control (4F/4M)	0.58 $\pm$ 0.50	0.0002^a
	MEK1DD-Ob (6F/4M)	5.70 $\pm$ 5.52	
OV/BV (%)	Control	0.09 $\pm$ 0.10	< 0.0001^a
	MEK1DD-Ob	2.08 $\pm$ 2.46	
O.Th (μ m)	Control	2.21 $\pm$ 0.68	< 0.0001
	MEK1DD-Ob	6.32 $\pm$ 1.74	
MS/BS (%)	Control	8.97 $\pm$ 1.49	0.1332
	MEK1DD-Ob	6.92 $\pm$ 3.68	
Mlt (Days)	Control	0.27 $\pm$ 0.20	< 0.0001^a
	MEK1DD-Ob	8.26 $\pm$ 6.44	
Omt (Days)	Control	4.43 $\pm$ 1.64	0.0003
	MEK1DD-Ob	11.25 $\pm$ 3.98	

^aP-value from a Mann-Whitney test, otherwise from a two-sided t-test with Welch's correction.

Abbreviations: % osteoid surface (OS/BS%), % osteoid volume (OV/BV%), osteoid thickness (O.Th), mineralization surface/bone surface (MS/BS), mineralization lag time (Mlt) and osteoid maturation time (Omt).

Table 2.

Differences in selected properties of the mouse femur (mean $\pm$ SD) between the two genotypes as assessed by μ CT and calipers.

Property	Units	Control n = 4F / 4M	MEK1DD-Ob n = 8 F / 7M	p-value
<i>Metaphysis of distal femur</i>				
Tb.Th	mm	0.0451 $\pm$ 0.004	0.0494 $\pm$ 0.005	0.0240
Tb.Sp	mm	0.23 $\pm$ 0.06	0.17 $\pm$ 0.03	0.0034^a
SMI	0:plates 3:rods	1.51 $\pm$ 0.72	-0.04 $\pm$ 0.80	<0.0001^a
Tb.TMD	mg HA/cm ³	949 $\pm$ 18	907 $\pm$ 17	<0.0001
<i>Cortex of femur diaphysis</i>				
Ct.Ar ^b	mm ²	0.886 $\pm$ 0.107	1.039 $\pm$ 0.156	0.0123
Tt.Ar	mm ²	2.064 $\pm$ 0.202	2.112 $\pm$ 0.260	0.6300
Ct.Ar/Tt.Ar	mm ²	0.429 $\pm$ 0.026	0.495 $\pm$ 0.076	0.0069
J	mm ⁴	0.483 $\pm$ 0.100	0.541 $\pm$ 0.123	0.1901 ^a
I _{min} /c _{min}	mm ³	0.236 $\pm$ 0.035	0.256 $\pm$ 0.044	0.2480
Ps.Pm	mm	5.57 $\pm$ 0.31	5.62 $\pm$ 0.38	0.7249
Ec.Pm	mm	4.32 $\pm$ 0.26	4.25 $\pm$ 0.40	0.6176
Po.N ^c	1/mm	3.17 $\pm$ 0.42	4.35 $\pm$ 0.62	<0.0001
Po.Th	mm	0.03 $\pm$ 0.02	0.04 $\pm$ 0.02	0.0901 ^a
<i>Intact femur (caliper measurements)</i>				
Axial length	mm	15.21 $\pm$ 0.28	15.10 $\pm$ 0.26	0.4238
Ant.-post. Width	mm	1.31 $\pm$ 0.06	1.31 $\pm$ 0.07	0.4943
<i>Medullary canal of femur diaphysis^d</i>				
Ma.Conn.D	1/mm ³	8.1 $\pm$ 8.6	138.9 $\pm$ 102.9	<0.0001^a
Ma.SMI	0:plates 3:rods	3.17 $\pm$ 0.31	1.27 $\pm$ 1.50	<0.0001^a
Ma.Tb.Sp	mm	0.701 $\pm$ 0.162	0.298 $\pm$ 0.072	0.0001
Ma.avBMD	mg HA/cm ³	25 $\pm$ 18	171 $\pm$ 136	<0.0001^a
Ma.TMD	mg HA/cm ³	1004 $\pm$ 52	984 $\pm$ 24	0.3330

^aP-value from a Mann-Whitney test, otherwise from a two-sided t-test with Welch's correction

^bRegion of interest defined by periosteal contour (outer) to determine cross-sectional properties related to structure

^cRegion of interest defined by outer contour and endosteal contour (inner) to determine pore number and thickness using inverse threshold.

^dRegion of interest defined by inner contour to isolate the medullary canal (Ma).

Abbreviations: trabecular thickness (Tb.Th), trabecular number (Tb.N), structural model index (SMI), tissue mineral density (TMD) of trabecular bone (Tb.TMD), cortical bone area (Ct.Ar), total area of diaphysis including medullary canal (Tt.Ar), bone area fraction (Ct.Ar/Tt.Ar), polar moment of inertia (J), section modulus (I_{min}/c_{min}), periosteal perimeter (Ps.Pm), endosteal perimeter (Ec.PM), pore number (Po.N), pore thickness (Po.Th), anterior (Ant.) and posterior (post.), connectivity density (Conn.D), trabecular spacing (Tb.Sp), and apparent volumetric bone mineral density (avBMD).

Table 3.

Differences in selected properties of the mouse femur (mean $\pm$ SD) between the two genotypes as assessed by mechanical testing and Raman spectroscopy.

Property	Units	Control n = 4F / 4M	MEK1DD-Ob n = 8F / 7M	p-value
<i>Three-point bending of femur diaphysis</i>				
Stiffness	N/mm	107.0 $\pm$ 24.0	127.5 $\pm$ 27.9	0.0461
Yield force	N	16.8 $\pm$ 3.0	20.7 $\pm$ 5.2	0.0195
Peak force	N	19.2 $\pm$ 3.8	25.3 $\pm$ 5.1	0.0024
Post-yield displacement	mm	0.54 $\pm$ 0.12	0.47 $\pm$ 0.30	0.1901 ^a
Work-to-fracture	N-mm	10.32 $\pm$ 1.32	11.87 $\pm$ 3.25	0.0714
Yield displacement	mm	0.19 $\pm$ 0.06	0.18 $\pm$ 0.03	0.4374 ^a
Modulus	GPa	7.2 $\pm$ 2.0	8.0 $\pm$ 2.0	0.2899
Yield stress	MPa	138.2 $\pm$ 11.1	163.5 $\pm$ 37.2	0.0162
Toughness	MJ/m ³	4.4 $\pm$ 0.4	4.9 $\pm$ 1.4	0.5579 ^a
Post-yield toughness	MJ/m ³	3.8 $\pm$ 0.4	4.2 $\pm$ 1.5	0.9748 ^a
<i>Anterior surface of femur diaphysis</i>				
$\nu_1\text{PO}_4/\text{Amide I}$	–	29.9 $\pm$ 3.7	26.8 $\pm$ 2.4	0.0238
$\nu_1\text{PO}_4/\text{Amide III}$	–	16.32 $\pm$ 3.22	15.46 $\pm$ 3.63	0.5668
$\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$	–	14.1 $\pm$ 2.0	14.7 $\pm$ 1.8	0.5096
$\text{CO}_3/\nu_1\text{PO}_4$	–	0.16 $\pm$ 0.01	0.16 $\pm$ 0.00	0.0946
Crystallinity	cm	0.0542 $\pm$ 0.0008	0.0541 $\pm$ 0.0015	0.4758 ^a
I_{1670}/I_{1610}	–	4.00 $\pm$ 1.63	3.09 $\pm$ 0.49	0.1624
I_{1670}/I_{1690}	–	1.92 $\pm$ 0.13	1.81 $\pm$ 0.08	0.0305

^aP-value from Mann-Whitney test, otherwise from t-test with Welch's correction

Abbreviations: phosphate (PO₄), carbonate (CO₃), and oxygen-methylene wagging vibration (CH₂-wag), symmetric stretch vibration (ν_1).