Published in final edited form as:

J Orthop Res. 2014 November ; 32(11): 1397–1405. doi:10.1002/jor.22677.

Inhibition of beta-catenin signaling by Pb leads to incomplete fracture healing

Eric E Beier^{1,2}, Taylor Buckley¹, Kiminori Yukata¹, Tzong-Jen Sheu¹, Regis O'Keefe¹, Michael J Zuscik¹, and J Edward Puzas^{1,2}

¹Center for Musculoskeletal Research, University of Rochester, School of Medicine and Dentistry, Rochester, New York, 14624, USA

²Department of Environmental Medicine, University of Rochester, School of Medicine and Dentistry, Rochester, New York, 14624, USA

Abstract

There is strong evidence in the clinical literature to suggest that elevated lead (Pb) exposure impairs fracture healing. Since Pb has been demonstrated to inhibit bone formation, and Wnt signaling is an important anabolic pathway in chondrocyte maturation and endochondral ossification, we investigated the impact of Wnt therapy on Pb-exposed mice undergoing bone repair in a mouse tibial fracture model. We established that tibial fracture calluses from Pb-treated mice were smaller and contained less mineralized tissue than vehicle controls. This resulted in the persistence of immature cartilage in the callus and decreased β -catenin levels. Reduction of β -catenin protein was concurrent with systemic elevation of LRP5/6 antagonists DKK1 and sclerostin in Pb-exposed mice throughout fracture healing. β -catenin stimulation by the GSK3 inhibitor BIO reversed these molecular changes and restored the amount of mineralized callus. Overall, Pb is identified as a potent inhibitor of endochondral ossification in vivo with correlated effects on bone healing with noted deficits in β -catenin signaling, suggesting the Wnt/ β -catenin as a pivotal pathway in the influence of Pb on fracture repair.

Introduction

Although more stringent measures have been implemented to curb national incidences of lead (Pb) poisoning, relevant Pb exposures remain a problem for human health. There are approximately half a million children in the United States between the ages of 1 and 5 with elevated blood lead levels ($>5 \mu\text{g/dL}$) according to current Centers for Disease Control and Prevention (CDC) Childhood Lead Program statistics.¹ In addition to children, many adults with occupations involving Pb production and utilization sustain hazardous levels of Pb exposure. This circumstance subsists because Pb has extremely high affinity for hydroxyapatite crystals, and thus becomes incorporated into calcified tissues of the body and persists for many years.² Consequently, the Pb body burden is difficult to remove after exposure.

Corresponding author: J. Edward Puzas, Ph.D., 601 Elmwood Avenue, Rochester, New York 14642, Phone: (585) 275-3664, Fax: (585) 756-4727, Edward_puzas@urmc.rochester.edu.

The authors declare they have no actual or potential competing financial interests.

While environmental toxicants such as heavy metals (Pb, cadmium),³ alcohol,⁴ or cigarette smoke⁵ are associated with negative outcomes of skeletal health, the mechanisms by which they exert their deleterious effects are poorly understood. Reports from the National Health and Nutrition Examination Surveys indicate that elevated Pb may increase the risk of bone fracture.⁶⁻⁸ In a murine closed tibial model of fracture healing, a prominent finding in Pb-intoxicated animals was an increase of immature cartilage that persisted in the callus during bone healing.⁹ One explanation for this is a delayed turnover of the callus by inhibition of endochondral bone formation, which could be achieved by inhibition of mesenchymal stem cell differentiation.¹⁰ In support of this, Pb-treated animals had a significant decrease in osteoprogenitor frequency.⁹

Endochondral ossification is the process where bone formation occurs through a cartilaginous intermediate during longitudinal growth and development and also during fracture healing.^{11; 12} Sequential steps occur through chondrocyte proliferation, extracellular matrix synthesis, chondrocyte hypertrophy, vascular invasion, and osteoblastic bone formation. These cell types are fed by mesenchymal stem cell (MSC) progenitors from the periosteum and bone marrow.¹³ This is controlled by systemic and local factors that modulate expression of stage-specific differentiation markers. The cartilage matrix is comprised of proteins like type II collagen (Col2) and aggrecan, whose infrastructure is orchestrated through collagenases such as MMP13 (collagenase-3).¹⁴ During terminal hypertrophy, chondrocytes express markers including type X collagen (ColX) and alkaline phosphatase.¹⁵ Pb had been implicated in altering both chondrogenic proliferation^{16; 17} and accelerating hypertrophy in committed chondrocytes.¹⁸

Wnt pathways are known to be active and necessary for proper fracture healing, with many signaling constituents upregulated early, from 3 to 5 days post injury,¹⁹ and during late stages of fracture healing,^{20; 21} in both mice and humans.^{22; 23} Wnt signaling molecules are present particularly in the proliferating chondrocytes and osteoblasts during repair.²⁴ Accordingly, increased β -catenin activity and inhibition of GSK-3 β by lithium chloride have been demonstrated to accelerate fracture healing,^{22; 25} whereas conditional β -catenin knockout in chondrocytes²⁶ and osteoblasts²² delay healing. Other GSK-3 β inhibitors such as 6-bromindirubin-3'-oxime (BIO) *in vitro* and *in vivo* has been demonstrated to increase osteoblast differentiation and increase proliferation of human MSC.²⁷ Previously, Pb has been implicated to repress Wnt signaling through elevation of antagonists DKK1 and sclerostin.²⁸ As decreased osteoprogenitors are congruent with some of the major disruptions found in Pb-inhibition of fracture repair, and positive Wnt signaling can promote osteoprogenitor function, we hypothesize that Pb impacts bone repair at least partially via inhibition of Wnt/ β -catenin signaling. In addition, we postulate that BIO-stimulation of β -catenin will attenuate these Pb-induced deficits of fracture repair.

Here, we present the results of a tibial fracture study in which mice were exposed to Pb and administered BIO to examine changes in the callus architecture, including volume of mineralized callus, proportion of cellular tissues, and underlying signaling changes. Furthermore, we employed an *in vivo* model of localized ectopic bone formation to understand the impact of Pb on new endochondral bone growth. Our combined findings indicate that Pb can reduce the amount of mineralized callus during fracture healing and

depress new bone growth, and that activation of β -catenin rescues callus volume. These results implicate the Wnt pathway as a central molecular mechanism of Pb inhibition of skeletal repair.

Materials and Methods

Animal exposures and fracture surgery

All mice used in this study were cared for according to the guidelines provided by the University of Rochester Medical Center Institutional Animal Care and Use Committee. Normal or Pb-treated drinking water was provided *ad lib* beginning at 12 weeks of age. After 6 weeks of Pb exposure we sedated female C57/Bl6 mice, prepared the surgical site with 70% ethanol, and made an incision above the left knee. We inserted a 0.25 mm sterile pin (Fine Science Tools, Foster City, CA) into the tibial marrow space. We temporarily removed the needle, osteotomized the tibia with a scalpel at the mid-shaft, and then re-inserted the pin. We trimmed the pin proximally at the level of the tibial plateau and closed the wound with 3 USP 4–0 nylon sutures.

We confirmed intramedullary fixation and characterized fractures using a Faxitron cabinet X-ray system (Faxitron, Wheeling, IL). We performed radiographs following surgery and repeated the exams at 7-day intervals to follow healing and confirm maintenance of fixation.

Blood Pb determination

Whole blood was collected from mice by subcutaneous bleeding at 0, 7, 14, and 21 days post-fracture. We analyzed blood Pb by anodic stripping voltammetry using the Lead Care II system. Remaining blood was clotted at room temperature for 20 min, centrifuged, and serum stored at -80°C for specific protein determination. The data presented are a longitudinal representation of the final group of mice.

Circulating Pb at the time of surgery in 230 ppm Pb-treated mice was between 15–18 $\mu\text{g/dL}$ (Table 1). At 14 days post-fracture, blood Pb levels had increased by 5 $\mu\text{g/dL}$ and 3 $\mu\text{g/dL}$ in mice also given BIO.

Reagents

BIO (GSK3 inhibitor IX: Calbiochem, MA) was dissolved in DMSO (vehicle 5% v/v) and diluted in corn oil for intraperitoneal (IP) injection into mice undergoing fracture healing (0.5 mg/kg). We did injections every third day starting on day 1 post fracture.

We measured mouse serum for protein levels of DKK1 (R&D Systems, Minneapolis, MN), sclerostin (ALPCO Diagnostics, Salem, NH), and MMP13 (Biorbyt, San Francisco, CA) using standard ELISA methods according to procedures provided from the manufacturer.

MicroCT assessment of fracture callus

Fracture calluses of treated mice were quantified over time by microCT using a Scanco vivaCT40 scanner with a 55kVp source as described previously.⁵ Briefly, we scanned tibiae

at a resolution of 12 μm with a slice increment of 10 μm . All images were reconstructed at the same threshold to give 3-dimensional structural rendering of the mineralizing calluses.

Histology/Histomorphometry/Immunohistochemistry

We harvested fractured tibiae at 7, 10, and 14 days. Subsequently, we fixed the tissues in 10% formalin for 24 hr and decalcified in 10% EDTA at pH 7.2 for 2 weeks. The samples were processed, embedded in paraffin, and cut into 3- μm sagittal sections. Then, we stained three contiguous sections (100 μm apart) from each specimen with Alcian Blue/Hematoxylin/Orange G (ABH) according to standard protocols.

We conducted quantitative analysis on the sagittal ABH/OG-stained sections by histomorphometric analysis using Osteometrics software. Parameters included the areas of woven bone, cartilage, and total fracture callus, and calculated the amount of mesenchymal tissue by subtracting bone and cartilage area from the total callus area. We expressed bone, cartilage, and mesenchymal tissue areas each as a percentage of the total fracture calluses. Sections were stained with a polyclonal anti-mouse antibody specific for β -catenin (1:50, Cell Signaling Technologies, Danvers, MA) or sclerostin (1:100; R&D Systems), or matched isotype control antibody. The primary antibody was visualized with an HRP-conjugated rabbit anti-goat antibody (Jackson ImmunoResearch Laboratories). Sections were counterstained with hematoxylin.

RNA isolation and quantitative PCR

We excised tibial fracture calluses, flushed them of marrow elements, and flash froze them in liquid nitrogen. Calluses were homogenized using the TissueLyser II system (Qiagen, Valencia, CA), and mRNA was isolated using the RNeasy Fibrous Tissue mini kit (Qiagen). mRNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and qPCR was performed using the Rotor Gene Real-Time DNA Amplification System (Corbett Research, Sydney, Australia). We amplified cDNA samples with specific primers (Table 2) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Data were normalized to β -actin expression for each sample.

In vivo calvaria model of local bone formation

We used a localized model of bone formation using parathyroid hormone (PTH) as initially described by Boyce et al.²⁹ 12-week-old female C57BL/6 mice were treated with 0, 55, or 230 ppm Pb through their drinking water for 6 weeks total. After 4 weeks of exposure, mice received injections of 40 $\mu\text{g}/\text{kg}$ human PTH fragment 1-34 (Bachem, Bubendorf, Switzerland) once daily for 3 days into the subcutaneous tissue over the left calvaria. We sacrificed animals after 2 weeks, excised the calvaria, and removed soft tissues. We examined the response on calvarial bone morphology and net bone formation using quantitative histomorphometry. In addition, we performed immunohistochemistry for expression of sclerostin protein.

Statistics

Data are presented as mean $\pm$ SEM. We compared the differences between the groups using unpaired Student's t-test. We compared more than two groups using two-way analysis of

variance between groups (ANOVA). P-values less than 0.05 were considered to be statistically significant.

Results

Pb alters callus architecture in mouse tibial fractures

It is documented that Pb is associated with increased occurrence of bone fractures³⁰ and delays in fracture healing.⁹ To assess whether Wnt signaling is an underlying mechanism for this effect, we stimulated β -catenin signaling in the fracture callus through administration of BIO of Pb-treated mice to test whether this improved the alteration in callus structure. By day 14 post-fracture, all animals showed radiographic evidence of callus formation (Fig. 1A). However, radiographs of Pb-exposed animals demonstrated a marked increase in radiolucency within the fracture callus compared with controls (arrows). At day 21 post-fracture, control and BIO-treated groups showed evidence of remodeling of the fracture callus by disappearance of the fracture line. However, mice exposed to Pb still showed presence of a fracture line at day 21, which was resolved in Pb-treated mice given BIO administration.

To quantify mineralized callus formation we performed microCT imaging from each group at 7, 14, and 21 days post-fracture (Fig. 1B). We found that mineralized bone callus volume was significantly reduced with Pb exposure on day 7 (−18.4%) and day 14 (−37.6%) compared with vehicle controls (Fig. 1C). When Pb-exposed mice were treated with BIO, this deficit in callus volume trended towards control levels on day 7 (ns +1.24-fold) achieving a statistically significant difference by day 14 (+1.87-fold).

Activation of β -catenin signaling improves Pb inhibition of fracture healing

To confirm that Pb alters the tissue composition of fracture callus and that Wnt/ β -catenin signaling is involved, we determined the proportion of bone, cartilage, and mesenchymal tissue on day 10 post-fracture (Fig. 2A). In our vehicle-treated animals we saw the normal pattern of newly formed bone through a cartilage intermediate, as evidenced by the areas of Alcian Blue–stained cartilage throughout the callus. However, in the 230 ppm Pb-treated group, there was an increase in cartilage tissue (+2.07-fold) present in the callus compared to controls (Fig. 2B), which may account for the large radiolucency identified by X-ray. This increase in cartilage was at the expense of bone tissue (−36.3%) in Pb-exposed mice. These results, an increase in unmineralized cartilage and decrease in bone tissue, suggest a significant delay in endochondral ossification with Pb exposure and are in accord with what was previously published.⁹ When β -catenin signaling is increased with BIO administration, we saw a reduction of this cartilage tissue (−37.9%), with a corresponding higher proportion of bone (+1.60-fold) without change in the fibrous tissue area compared to Pb alone. BIO-treated fractures had significantly elevated fibrous tissue (+1.38-fold) compared to controls.

To verify activation of Wnt signaling with our BIO treatments, we performed immunostaining of the β -catenin protein in the medial fracture calluses. Expression of β -catenin was concentrated in the osteoprogenitor cells that line the newly formed callus bone (Fig. 2C). We found that injections of BIO over 10 days increased levels of β -catenin in the

fracture callus. Pb-exposed fractures had lower β -catenin levels than what was found in vehicle control mice. When BIO was given to the Pb-exposed mice, this reverted β -catenin staining toward control levels indicating that our BIO treatment was having the intended effect. In corroboration with the microCT and radiographic results, these results establish that Pb has a direct impact on fracture callus architecture, which is improved with activation of β -catenin signaling, suggesting that the Wnt pathway may be responsible for the altered healing in Pb-exposed mice.

Pb decelerates chondrocyte hypertrophy and callus turnover, which is increased by β -catenin signaling

In order to understand the impact of Pb and BIO on cellular phenotypes within the fracture callus, we performed qPCR for genetic biomarkers for pro-chondrocytic, pro-osteogenic, and pro-mesenchymal cell function. Calluses from Pb-exposed mice had more immature cartilage as demonstrated by increased *col2* expression compared to vehicle controls (Fig. 3A). Activation of β -catenin with BIO resulted in more hypertrophic chondrocytes: reduced *col2* expression in Pb-treated mice, and elevated *colX* expression compared to vehicle controls (Fig. 3B). Osteoblastic *osteocalcin* expression was reduced in Pb-treated mice compared to vehicle controls, and recovered toward control levels with BIO treatment (Fig. 3C).

Stromal cell-derived factor 1 (*sdf-1*) is a chemokine protein essential for chemotaxis recruitment of MSC to the fracture site during bone repair.³¹ BIO treatment led to a distinct +4 fold elevation of *sdf-1* compared to vehicle controls (Fig. 3D). *Sdf-1* levels in fracture calluses from Pb-exposed mice were significantly reduced, and enhanced with addition of BIO treatment.

To determine whether antagonists DKK1 and sclerostin were involved with depressed levels of β -catenin by Pb, we tested serum levels of these proteins throughout bone repair. In vehicle control animals, DKK1 levels dropped early (−61.3%) by day 7 post-fracture, whereas sclerostin levels continued to lower through day 21 (−52.7%) post-fracture (Fig. 4A, B). These trends were absent in Pb-exposed mice, and protein levels were significantly elevated at time of fracture surgery, and remained +2–3 fold elevated throughout healing (Fig. 4A, B). BIO significantly lowered levels of DKK1 at day 7 post-fracture and sclerostin at day 21 post-fracture in Pb-exposed mice, which corresponds with the increase we observed in β -catenin expression in the callus. Levels of MMP13 enzyme peaked at day 14 post-fracture, and BIO treatment significantly elevated MMP13 levels at day 7 and 14 post-fracture compared to vehicle control (Fig. 4C). Together, these phenotypic trends suggest delayed mineralization with Pb intoxication which maybe be associated with the measured increase in Wnt antagonists.

Pb inhibits PTH-induced bone formation and is associated with elevated sclerostin levels

We wanted to investigate whether Pb had a direct effect on ectopic bone formation, and its relationship with sclerostin. Histological images of calvarias are presented in Figure 5A from control, 55 ppm, and 230 ppm Pb-treated mice. Above the green line is the area of newly remodeled bone. After intermittent PTH injections the percentage of new bone on the

periosteal surface was 14.3% in control mice (Fig. 5B). Pb exposure caused a dose-dependent decrease in new bone growth (–31.1% at 55 ppm and –38.6% at 230 ppm) compared to controls. It has been suggested that sclerostin is associated with PTH's induction of bone formation in calvaria.³² In agreement with those findings, sclerostin expression was discovered to be relatively low in control PTH-injected calvaria (Fig. 5C). Interestingly, sclerostin staining was elevated in Pb-exposed mouse calvaria, which correlates with the decrease in bone formation observed in panel B.

Discussion

Despite the significant clinical correlation of elevated Pb with increase risk and delayed healing of bone fractures,^{6, 8} there has yet to be an investigation into the underpinning mechanism of deficient healing in Pb-exposed individuals. As Pb is a risk factor for osteoporosis, and the hallmark symptom of that is fragility fracture, and the industrial world populous has a detectable amount of Pb in their skeleton, it's essential to understand the impact of Pb on bone repair. Because of this, we proposed to further characterize the effect of Pb on callus formation and progression, while evaluating the role β -catenin signaling plays in that process. We used a closed tibial model of healing to illustrate the effect of Pb on cellular and molecular aspects of bone repair. Khalil et al.³⁰ published a study indicating that increased Pb exposure increases fracture risk by decreasing bone strength and decreasing neuromuscular control, i.e. chance of falling. They showed that hipbone density was decreased 7% in the high-level Pb group ($>8 \mu\text{g/dL}$), which correlated with a dose-dependent increase in incidence of fracture over time. Similar behaving metal cadmium at low levels was found to diminish bone density and increased fracture risk in a human population.³³

Our first aim tested whether Pb suppression of Wnt signaling could be an important component in the delay in endochondral ossification and conversion of cartilage tissue to bone. Results from this trial established that Pb effectively reduced the size of the mineralized callus at 7 and 14 days post-fracture. Decreased mineralized callus could result in increased instability and reduce functional strength during a critical phase of healing. In fact, Pb exposure has shown reduced bone mineral properties and bone strength in mice^{34, 35} and rats.²⁸ To further characterize the effect of Pb on endochondral bone repair, we investigated the relative gene expression levels in soft calluses. In accordance with a decreased mineral callus, we observed cartilage that was more immature cartilage (elevated *col2*), depressed osteoblastic activity (decreased osteocalcin), and reduced osteoprogenitor activity (decreased *sdf-1*). This concurs with discoveries that Pb exposure of mice with tibial fractures leads to delayed cartilage mineralization and decreased osteoprogenitors at 14 days.⁹

Induction of β -catenin signaling has previously been shown to improve fracture healing through genetic models or systemic therapy. We demonstrated that systemic injection of BIO successfully elevated β -catenin levels in the fracture callus during healing (Fig. 2C). This BIO administration recovered the decrease in mineralized callus volume in Pb exposed mice. Moreover, activation of β -catenin enhanced mesenchymal cells activity, chondrocyte maturation, and fracture callus turnover. Recent efforts have concentrated around

understanding the importance of Wnt signaling in fracture repair. Inhibitors of LRP5/6 such as DKK1,²⁴ and decoy receptors such as sfrp1, prevent bone repair.³⁶ Moreover, pre-clinical studies using anti-DKK1 monoclonal antibodies (such as BHQ880, which was developed to reduce osteolysis in multiple myeloma³⁷) have been shown to temporally enhance fracture repair when given before day 4 post-fracture.²¹ Anti-sclerostin antibody also improves bone callus and bone strength during healing, but the timing of dose is not pertinent.³⁸ These neutralizing antibodies show promise in advancing orthopedic care in their ability to effectively enhance the fracture-healing response, especially in nonunion or pathogenic conditions.

To select the dose and methodology of Wnt therapy, we conducted a pilot study in TOPgal transgenic mice (data not shown). TOPgal mice have the TCF/LEF responsive promoter directing expression of β -galactosidase incorporated into their genome.³⁹ We administered BIO to male mice via IP injected or oral gavage at a concentration of 0.0, 0.1, 0.5, and 3.0 mg/kg in 5% DMSO (v/v) dissolved in corn oil. We performed x-gal staining in tibial bone histology and concluded that the most effective dose for elevating Wnt signaling based on the quantity of positive staining was the 0.5 mg/kg dose via IP injection every third day.

It is extremely rare for nonunion to occur in a closed, stabilized bone fracture in a mouse no matter what the genetic alteration or pharmaceutical treatment.^{40; 41} This is because of the multiple redundancies built into the fracture healing process. Hence, the observation that the Pb-induced deficit in mineralized callus was recovered by day 21 as we reported previously is somewhat predictable. In addition, one important component of fracture healing not examined in this initial study was the contribution of angiogenesis and vascular infiltration. Pb has been indicated to induce vascular endothelial growth factor via PKC/AP-1 pathway signaling.⁴² Also, Pb induces ROS and activates ERK signaling, which are associated with increased angiogenesis.⁴³ Thus we would hypothesize that there could be an acceleration of vascularization of the callus and not rule out this contributory effect towards the recovery of callus volume in Pb-exposed mice at 21 days. However, the fact that we get a reproducible disruption in callus architecture with Pb exposure, and that stimulated β -catenin signaling improves that is quite profound.

Pb exerts deleterious effects on the skeleton during development,⁷ during postnatal bone accrual,⁴⁴ and in mature animals.^{28; 35} Here, we characterized its effect on bone repair, which is an archetypal clinical outcome of the skeletal pathologies elucidated above. To our knowledge, this is the first mechanistic discovery of how Pb may interact to impair bone healing, and furthermore offers a therapeutic strategy as a means to combat bone-related Pb toxicities. Together with the findings that elevated Pb exposure leads to osteoporotic phenotype and increased fragility fracture risk, we find that Pb-exposed patients are at risk for a delayed in healing. Thorough examination of bone healing in Pb-exposed populations, and elucidating further understanding of molecular mechanisms is necessary.

Acknowledgments

We thank R. Tierney and S. Mack for assistance with histology and M. Thullen for microCT imaging. T Buckley performed ectopic bone formation assay. K Yukata and T Sheu assisted with animal care and fracture surgeries.

This work was supported by Public Health Service grants NIH T32 ES07026 and R01 ES012712, T32 AR053459, P01 ES011854, and P30 ES301247.

References

- Centers for Disease C and Prevention. Blood lead levels in children aged 1–5 years - United States, 1999–2010. MMWR Morbidity and mortality weekly report. 2013; 62:245–248. [PubMed: 23552225]
- Dowd TL, Rosen JF, Mints L, et al. The effect of Pb(2+) on the structure and hydroxyapatite binding properties of osteocalcin. *Biochimica et biophysica acta*. 2001; 1535:153–163. [PubMed: 11342004]
- Abbas S, Khan K, Khan MP, et al. Developmental exposure to As, Cd, and Pb mixture diminishes skeletal growth and causes osteopenia at maturity via osteoblast and chondrocyte malfunctioning in female rats. *Toxicological sciences : an official journal of the Society of Toxicology*. 2013; 134:207–220. [PubMed: 23604592]
- Duckworth AD, Bennet SJ, Aderinto J, et al. Fixation of intracapsular fractures of the femoral neck in young patients: risk factors for failure. *The Journal of bone and joint surgery. British volume*. [PubMed: 21586782]
- Kung MH, Yukata K, O'Keefe RJ, et al. Aryl hydrocarbon receptor-mediated impairment of chondrogenesis and fracture healing by cigarette smoke and benzo(a)pyrene. *Journal of cellular physiology*. 2012; 227:1062–1070. [PubMed: 21567390]
- Campbell JR, Auinger P. The association between blood lead levels and osteoporosis among adults--results from the third national health and nutrition examination survey (NHANES III). *Environmental health perspectives*. 2007; 115:1018–1022. [PubMed: 17637916]
- Ballew C, Khan LK, Kaufmann R, et al. Blood lead concentration and children's anthropometric dimensions in the Third National Health and Nutrition Examination Survey (NHANES III), 1988–1994. *The Journal of pediatrics*. 1999; 134:623–630. [PubMed: 10228299]
- Nash D, Magder LS, Sherwin R, et al. Bone density-related predictors of blood lead level among peri- and postmenopausal women in the United States: The Third National Health and Nutrition Examination Survey, 1988–1994. *American journal of epidemiology*. 2004; 160:901–911. [PubMed: 15496543]
- Carmouche JJ, Puzas JE, Zhang X, et al. Lead exposure inhibits fracture healing and is associated with increased chondrogenesis, delay in cartilage mineralization, and a decrease in osteoprogenitor frequency. *Environmental health perspectives*. 2005; 113:749–755. [PubMed: 15929899]
- Zhang X, Schwarz EM, Young DA, et al. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *The Journal of clinical investigation*. 2002; 109:1405–1415. [PubMed: 12045254]
- Mackie EJ, Ahmed YA, Tatarczuch L, et al. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *The international journal of biochemistry & cell biology*. 2008; 40:46–62. [PubMed: 17659995]
- Einhorn TA. The science of fracture healing. *Journal of orthopaedic trauma*. 2005; 19:S4–6. [PubMed: 16479221]
- Yoo JU, Johnstone B. The role of osteochondral progenitor cells in fracture repair. *Clinical orthopaedics and related research*. 1998:S73–81. [PubMed: 9917628]
- Inada M, Wang Y, Byrne MH, et al. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:17192–17197. [PubMed: 15563592]
- Pelttari K, Steck E, Richter W. The use of mesenchymal stem cells for chondrogenesis. *Injury*. 2008; 39(Suppl 1):S58–65. [PubMed: 18313473]
- Zuscik MJ, Pateder DB, Puzas JE, et al. Lead alters parathyroid hormone-related peptide and transforming growth factor-beta1 effects and AP-1 and NF-kappaB signaling in chondrocytes. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2002; 20:811–818. [PubMed: 12168672]

17. Zuscik MJ, Ma L, Buckley T, et al. Lead induces chondrogenesis and alters transforming growth factor-beta and bone morphogenetic protein signaling in mesenchymal cell populations. *Environmental health perspectives*. 2007; 115:1276–1282. [PubMed: 17805416]
18. Holz JD, Beier E, Sheu TJ, et al. Lead induces an osteoarthritis-like phenotype in articular chondrocytes through disruption of TGF-beta signaling. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2012; 30:1760–1766. [PubMed: 22517267]
19. Hadjiargyrou M, Lombardo F, Zhao S, et al. Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair. *The Journal of biological chemistry*. 2002; 277:30177–30182. [PubMed: 12055193]
20. Chen Y, Alman BA. Wnt pathway, an essential role in bone regeneration. *Journal of cellular biochemistry*. 2009; 106:353–362. [PubMed: 19127541]
21. Komatsu DE, Mary MN, Schroeder RJ, et al. Modulation of Wnt signaling influences fracture repair. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2010; 28:928–936. [PubMed: 20063381]
22. Chen Y, Whetstone HC, Lin AC, et al. Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. *PLoS medicine*. 2007; 4:e249. [PubMed: 17676991]
23. Kakar S, Einhorn TA, Vora S, et al. Enhanced chondrogenesis and Wnt signaling in PTH-treated fractures. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2007; 22:1903–1912.
24. Kim JB, Leucht P, Lam K, et al. Bone regeneration is regulated by wnt signaling. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2007; 22:1913–1923.
25. Clement-Lacroix P, Ai M, Morvan F, et al. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:17406–17411. [PubMed: 16293698]
26. Huang Y, Zhang X, Du K, et al. Inhibition of beta-catenin signaling in chondrocytes induces delayed fracture healing in mice. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2012; 30:304–310. [PubMed: 21818768]
27. Hoffman, MDTM.; Benoit, D. 6-Bromoindirubin-3'-oxime (BIO) induces proliferation of human mesenchymal stem cells (hMSCs) [Abstract]. *Bioengineering Conference*; 2011.
28. Beier EE, Maher JR, Sheu TJ, et al. Heavy metal lead exposure, osteoporotic-like phenotype in an animal model, and depression of Wnt signaling. *Environmental health perspectives*. 2013; 121:97–104. [PubMed: 23086611]
29. Boyce BF, Aufdemorte TB, Garrett IR, et al. Effects of interleukin-1 on bone turnover in normal mice. *Endocrinology*. 1989; 125:1142–1150. [PubMed: 2788075]
30. Khalil N, Cauley JA, Wilson JW, et al. Relationship of blood lead levels to incident nonspine fractures and falls in older women: the study of osteoporotic fractures. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2008; 23:1417–1425.
31. Kitaori T, Ito H, Schwarz EM, et al. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis and rheumatism*. 2009; 60:813–823. [PubMed: 19248097]
32. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone*. 2005; 37:148–158. [PubMed: 15946907]
33. Staessen JA, Roels HA, Emelianov D, et al. Environmental exposure to cadmium, forearm bone density, and risk of fractures: prospective population study. *Public Health and Environmental Exposure to Cadmium (PheeCad) Study Group. Lancet*. 1999; 353:1140–1144. [PubMed: 10209978]
34. Monir AU, Gundberg CM, Yagerman SE, et al. The effect of lead on bone mineral properties from female adult C57/BL6 mice. *Bone*. 2010; 47:888–894. [PubMed: 20643234]

35. Gruber HE, Gonick HC, Khalil-Manesh F, et al. Osteopenia induced by long-term, low- and high-level exposure of the adult rat to lead. *Mineral and electrolyte metabolism*. 1997; 23:65–73. [PubMed: 9252971]
36. Gaur T, Wixted JJ, Hussain S, et al. Secreted frizzled related protein 1 is a target to improve fracture healing. *Journal of cellular physiology*. 2009; 220:174–181. [PubMed: 19301255]
37. Fulciniti M, Tassone P, Hideshima T, et al. Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. *Blood*. 2009; 114:371–379. [PubMed: 19417213]
38. Ominsky MS, Li C, Li X, et al. Inhibition of sclerostin by monoclonal antibody enhances bone healing and improves bone density and strength of nonfractured bones. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2011; 26:1012–1021.
39. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development*. 1999; 126:4557–4568. [PubMed: 10498690]
40. Flick LM, Weaver JM, Ulrich-Vinther M, et al. Effects of receptor activator of NFkappaB (RANK) signaling blockade on fracture healing. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2003; 21:676–684. [PubMed: 12798068]
41. Wang Q, Huang C, Xue M, et al. Expression of endogenous BMP-2 in periosteal progenitor cells is essential for bone healing. *Bone*. 2011; 48:524–532. [PubMed: 21056707]
42. Hossain MA, Bouton CM, Pevsner J, et al. Induction of vascular endothelial growth factor in human astrocytes by lead. Involvement of a protein kinase C/activator protein-1 complex-dependent and hypoxia-inducible factor 1-independent signaling pathway. *The Journal of biological chemistry*. 2000; 275:27874–27882. [PubMed: 10882716]
43. Jing Y, Liu LZ, Jiang Y, et al. Cadmium increases HIF-1 and VEGF expression through ROS, ERK, and AKT signaling pathways and induces malignant transformation of human bronchial epithelial cells. *Toxicological sciences : an official journal of the Society of Toxicology*. 2012; 125:10–19. [PubMed: 21984483]
44. Kafourou A, Touloumi G, Makropoulos V, et al. Effects of lead on the somatic growth of children. *Archives of environmental health*. 1997; 52:377–383. [PubMed: 9546761]

or

Figure 1. Pb reduces mineralized callus formation in mouse tibial fractures

We exposed 12-week old mice to 0 or 230 ppm Pb for 6 weeks, and then performed tibial fracture surgery. Treatment with IP injection of 0.5 μ g/kg BIO or vehicle (5% DMSO) was commenced and continued every third day out to day 21. Images are representative radiographs (**A**) and 3D microCT reconstructions (**B**) from each treatment group, selected based on mean callus BV. Radiolucency in fracture calluses (arrows) on day 14 and day 21 is prominent in Pb-exposed mice and is less in control animals and Pb treatment plus BIO. (**C**) Quantification of mineralized callus volume at 7, 14, and 21 days post-fracture is

presented. Bar = 1 mm. Data represent mean $\pm$ SEM of 3 mice/group. * p < 0.05 vs H₂O, # p < 0.05 vs Pb.

Figure 2. Histologic analysis of the fracture calluses in Pb- and BIO-treated mice

(A) Representative ABH histology sections from day 10 post-fracture. Note the increased alcian blue staining in the fracture callus of Pb-exposed mice. (B) We measured the percent bone tissue, cartilage tissue, and fibrotic tissue and are presented as a ratio to the entire callus. (C) Representative immunohistochemical staining for β -catenin protein is presented from each group. Arrows highlight elevated expression in mesenchymal tissue following BIO treatments. Bar = 50 μ m in (A), 500 μ m in (C). Data represent mean $\pm$ SEM of 3 mice/group, * p < 0.05 vs H₂O, # p < 0.05 vs Pb.

Figure 3. BIO recovers Pb inhibition of cartilage maturation markers during soft callus phase of fracture healing

mRNA was extracted from calluses on 10 days post-fracture, 4 hrs after administration of BIO (or vehicle), and qPCR was performed to assess gene expression. Levels of col2a1 (A), col10 (B), sdf1 (C), and osteocalcin (D) were adjusted to relative β -actin expression and normalized to vehicle control. $n=3$, $*p<0.05$ v H2O, $\#p=0.05$ v Pb.

Figure 4. Pb induces Wnt antagonists throughout fracture healing, which are lowered with BIO administration

Serum was collected from mice 0, 7, 14, and 21 days post-fracture and ELISAs were performed to assess protein expression. Levels of DKK1 (A), sclerostin (B), and MMP13 (C) were measured according to standard protocols and normalized to day 0 vehicle control. n=3, * $p < 0.05$ v H2O, # $p = 0.05$ v Pb.

Figure 5. Pb exposure reduces new bone growth in an *in vivo* model of ectopic bone formation

We exposed 12-week old mice to 0, 55, or 230 ppm Pb for 6 weeks. Then, PTH injections were given for 3 consecutive days, and mouse calvaria were harvested 2 weeks later. (A) Representative ABH histology sections from each group are displayed, and (B) the percentage of new bone formed is represented graphically. (C) We performed immunohistochemistry for sclerostin expression levels on subsequent calvaria slides from each group. Positive osteocytes are red-brown colored. Bar: 100 μ m. Data represent mean $\pm$ SEM for three trials. * $p < 0.05$ vs control.

Table 1

Blood Pb levels in mice at fracture and 2 weeks post-fracture. ($\mu\text{g Pb/dL blood}$).

	Day 0	Day 14 post-fracture
H2O	0.64 ± 0.17	0.45 ± 0.09
BIO	0.92 ± 0.14	0.31 ± 0.12
230 ppm Pb	18.40 ± 1.78	23.75 ± 3.02
230 Pb+BIO	15.82 ± 2.97	18.52 ± 2.76

Pb determination in whole blood of exposed mice (n=4/group). Data are presented mean $\pm$ SEM,

*
 $p < 0.05$.

TABLE 2

Primers sets used for Quantitative PCR

Gene	Forward Primer	Reverse Primer
Actin	TGTTACCAACTGGGACGACA	CTGGGTCATCTTTCCAGGT
Col2a1	ACTGGTAAGTGGGGCAAGAC	CCACACCAAATTCCTGTTCA
ColX	CTTTGTGTGCCTTTCAATCG	GTGAGGTACAGCCTACCAGTTT
OC	AGGGAGGATCAAGTCCCG	GAACAGACTCCGGCGCTA
Sdf1	CGCCAGAGCCAACGTCAAGC	TTCGGGTCAATGCACACTTG