Asfotase-α improves bone growth, mineralization and strength in mouse models of neurofibromatosis type-1

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Individuals with neurofibromatosis type-1 (NF1) can manifest focal skeletal dysplasias that remain extremely difficult to treat. NF1 is caused by mutations in the NF1 gene, which encodes the Ras GTPase-activating protein neurofibromin. We report here that ablating NF1 in bone-forming cells leads to supraphysiologic accumulation of pyrophosphate (PPi), a strong inhibitor of hydroxyapatite formation, and that a chronic extracellular signal–regulated kinase (ERK)-dependent increase in expression of genes promoting PPi synthesis and extracellular transport, namely Enpp1 and Ank, causes this phenotype. NF1 ablation also prevents bone morphogenetic protein–2–induced osteoprogenitor differentiation and, consequently, expression of alkaline phosphatase and PPi, breakdown, further contributing to PPi accumulation. The short stature and impaired bone mineralization and strength in mice lacking NF1 in osteochondroprogenitors or osteoblasts can be corrected by asfotase-α enzyme therapy aimed at reducing PPi concentration. These results establish neurofibromin as an essential regulator of bone mineralization. They also suggest that altered PPi homeostasis contributes to the skeletal dysplasias associated with NF1 and that some of the NF1 skeletal conditions could be prevented pharmacologically.

Mutations in the NF1 gene cause NF1, a genetic disorder with an incidence of 1/3,500 live births worldwide. This condition is characterized by malignant and nonmalignant pathologies, including skeletal manifestations1–4. Dystrophic scoliosis, tibia bowing, bone fragility, fracture and pseudarthrosis (nonunion following fracture) are skeletal conditions associated with high morbidity in this population5–10. Despite recent progress toward understanding the role of NF1 in skeletal tissues, it is still unclear why and how these bone pathologies arise, raising uncertainty regarding optimal treatment2–3.

Although individuals with NF1 are typically born with heterozygous mutations in NF1, loss of heterozygosity has been detected in pseudarthrosis biopsies11, suggesting that local somatic NF1 loss of function contributes to NF1 skeletal dysplasia. This point is further supported by the relative commonality of defects observed between pseudarthrosis lesions from individuals with NF1 and the skeleton of mice characterized by conditional loss of NF1 in osteoprogenitors. These mice indeed tend to recapitulate, in their entire skeleton, the genetic and cellular consequences of NF1 loss of function that occurs locally in human NF1 pseudarthroses. NF1 inactivation in osteochondroprogenitors in Nf1floX/floX; Prx-cre or Nf1floX/floX; Col2a1-cre mice (herein called Prx-Nf1 KO or Col2-Nf1 KO mice, respectively) led to reduced stature, low bone mass, tibia bowing, diaphyseal ectopic blood vessel formation and hypomineralization associated with weakened bone mechanical properties. Bone cellular parameters also indicated that neurofibromin is required for normal osteoblast differentiation and expression of Tnfsf11, the gene encoding receptor activator of nuclear factor κB ligand, and hence for osteoclastogenesis12–18. The existence of Nf1-deficient osteoblasts in an Nf1 heterozygous bone microenvironment has also been shown to cause bone loss and delayed bone healing in Nf1floX/floX; Col1a1-cre (Col1-Nf1 KO) mice via activation of transforming growth factor-β (TGF-β) signaling19. Notably, each of these NF1 models, as well as bone biopsies from individuals with NF1 pseudarthrosis20, are characterized by excessive deposition of unmineralized bone matrix (osteoid) despite normal serum phosphate and calcium concentrations.

Bone matrix mineralization is a tightly regulated process that requires collagen, calcium and phosphate to form ordered crystals of hydroxyapatite, as well as tissue-nonspecific alkaline phosphatase.

Received 12 December 2013; accepted 1 May 2014; published online 6 July 2014; corrected after print 13 March 2015; doi:10.1038/nm.3583

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(ALP) activity to hydrolyze PPi (a potent inhibitor of mineralization) and generate inorganic phosphate. Extracellular concentrations of PPi are determined by (i) its degradation via ALP, (ii) its synthesis catalyzed by the nucleoside triphosphate pyrophosphohydrolase ENPP1/PC-1 (called ENPP1 herein) and (iii) its transport into the extracellular milieu through the PPi channel ANK. Mineralization is also controlled by Phosphol, a phosphatase that provides intracellular inorganic phosphate to generate PPi, and by glycoproteins such as osteopontin, which inhibits crystal nucleation on collagen fibers in mineralizing vesicles. Multiple growth factors such as TGF-β, activin A, bone morphogenetic protein-2 (BMP2), insulin-like growth factor-1, fibroblast growth factor-2 and fibroblast growth factor-23 are involved in bone and/or cartilage mineralization. A common signaling pathway engaged by these factors is the RAS-ERK pathway, which is constitutively activated in cells lacking neurofibromin, the RAS GTPase-activating protein (RAS-GAP) encoded by NF1 (ref. 35). We thus hypothesized that neurofibromin, via its inhibitory action on RAS-ERK signaling in bone-forming cells, could be an important regulator of bone matrix mineralization and bone mechanical properties. We show here that neurofibromin inhibits the expression of genes increasing PPi, extracellular PP concentration, and that hydrolysis of excess PPi is associated with a higher expression of genes regulating bone matrix mineralization and bone mechanical properties. We thus hypothesized that neurofibromin, via its inhibitory action on RAS-ERK signaling in bone-forming cells, could be an important regulator of bone matrix mineralization and bone mechanical properties.

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**RESULTS**

**Uncontrolled PPi production in NF1-deficient bone cells**

To address whether and how NF1 regulates bone mineralization, we first asked whether NF1 ablation in bone marrow stromal cells (BMSCs) affects extracellular PPi concentrations. BMSCs from Col2-Nf1 KO mice, lacking NF1 in osteochondroprogenitor cells, showed a 60–70% lower NF1 expression compared to those from wild-type (WT) mice (Fig. 1a), consistent with the heterogeneous nature of the cell populations that comprise BMSC cultures. This lower NF1 expression level was accompanied by a significantly higher (70%) extracellular PPi concentration in the conditioned medium of undifferentiated BMSC cultures compared to that of WT controls (Fig. 1b). Addition of a recombinant form of ALP, in the form of sALP-FcD10 (also known as asfotase-α, 0.5 μg ml−1) to induce PPi hydrolysis significantly reduced the amount of PPi detected in both genotypes, confirming the validity of the PPi measurements.

High extracellular PPi concentration can be generated by increased production of PPi, by the ectonucleophosphatase ENPP1 and by increased cellular export through the transporter ANK. Both Ank and Enpp1 mRNA (Fig. 1c) and protein (Supplementary Fig. 1a) levels were higher in Nf1−/− BMSCs compared to WT BMSCs. Expression of the gene encoding osteopontin (Spp1) was also higher in Nf1−/− BMSCs (Fig. 1e), consistent with the reported stimulatory effect of PPi on Spp1 expression. We obtained similar results when comparing Nf1−/− osteoprogenitor cells generated from Nf1lox/lox BMSC cultures infected with a Cre-expressing adenovirus to control Nf1lox/lox BMSC cultures infected with a GFP-expressing adenovirus (Supplementary Fig. 1b), which confirmed that the changes in gene expression measured in BMSCs from Col2-Nf1 KO mice were not caused by fewer osteoprogenitors initially plated. Ank, Enpp1 and Spp1 expression was also significantly higher in long bones, cartilages and epiphyses (cartilage) from 3-week-old Col2-Nf1 KO versus WT mice (Fig. 1d), whereas expression of Runx2 and Alpl, two osteoblast differentiation marker genes, was lower (Supplementary Fig. 1c).

Lastly, MEK inhibition by U0126 (1 μM, 24 h) blunted the increase in Ank, Enpp1 and Spp1 expression observed in Nf1−/− BMSCs, indicating that neurofibrin controls the expression of these genes in a RAS/ERK-dependent fashion (Fig. 1c and Supplementary Fig. 1b).

To assess whether these molecular findings in mice could be replicated in humans, we obtained RNA from adherent human bone marrow cells prepared from bone biopsies from six healthy control subjects without NF1 and nine individuals with NF1 tibial pseudoarthrosis, and measured ENPP1 and ANKH transcript levels by quantitative PCR. Consistent with the mouse data, ENPP1 expression was significantly higher in cultured cells from NF1 pseudoarthrosis tissues (Fig. 1e), despite the small number of available samples and consistent with the cell heterogeneity of these cultures. ANKH expression, however,

**Figure 1** Uncontrolled Ank, Enpp1 and Spp1 expression and increased PPi production in NF1-deficient osteoblasts. (a) NF1 mRNA expression in mouse BMSCs differentiated for 7, 14 and 21 d (n = 3). Vehicle, Na(PO₄)₂ . 0.15 M, pH 7.4. (c) Ank, Enpp1 and Spp1 mRNA expression in BMSCs treated with vehicle (DMSO) or U0126 for 24 h (n = 3). (d) Ank, Enpp1 and Spp1 mRNA expression in long bones, calvarias and epiphyses of 3-week-old WT (blue) and Col2-Nf1 KO (gray) mice (n = 6 per group). (e) ENPP1 and ANKH mRNA expression in human adherent bone marrow cells from control (n = 6 per group) and NF1-related pseudoarthrosis (NF1 PA, n = 9 per group) biopsies. * P < 0.05, determined by one-way analysis of variance (ANOVA) and Student’s t-test. NS, nonsignificant. Data are expressed as mean ± s.d.
was variable between samples and not significantly different between cultures from normal and NF1 pseudarthrosis biopsies (Fig. 1f).

Mice lacking Nf1 in mature osteoblasts (Col1-Nf1 KO mice) have a uniform distribution of nonmineralized matrix throughout trabecular bone compartments\(^{18}\), whereas mice lacking Nf1 in osteochondroprogenitors and chondrocytes (Col2-Nf1 KO mice) are characterized by an osteoid preferentially distributed in the primary spongiosa, where osteoblasts and chondrocytes mineralize their matrix (Fig. 2a). On the basis of these observations and because neurofibromin is expressed in hypertrophic chondrocytes\(^{37,38}\), we hypothesized that this RAS-GAP could also contribute to cartilage mineralization, which is a process important for bone growth and ossification during development and bone healing in adults. In support of this hypothesis, Col2-Nf1 KO chondrocyte high-density micromass cultures generated a typical Alcian blue-positive matrix but did not show signs of mineralization, in contrast to WT chondrocyte cultures (Fig. 2b). In addition, Ank, Enpp1 and Spp1 expression was significantly higher in Nf1-deficient micromass chondrocyte cultures versus WT cultures (Fig. 2c), in agreement with the data obtained from cartilaginous epiphyses, which contain a high proportion of chondrocytes (Fig. 1d). Accordingly, extracellular PP, concentration (Fig. 2d) and Enpp1 enzymatic activity (Fig. 2e) were significantly higher, whereas ALP activity was lower (Fig. 2f) in Nf1-deficient versus WT chondrocytes.

**Lack of Nf1 in BMSCs impairs BMP2 osteogenic action**

BMSCs isolated from Col2-Nf1 KO mice displayed, compared to BMSCs isolated from WT mice, a significantly lower differentiation potential, as determined by lower osteoblast colony-forming unit (CFU-Ob) number, lower tissue-nonspecific ALP activity (Fig. 3a) and lower expression of osteoblast differentiation markers including Runx2, Alpl and Bglap, the gene encoding osteocalcin (Fig. 3b). We obtained similar results using Nf1\(^{-}\)BMSCs infected with a Cre-expressing adenovirus (Supplementary Fig. 1d,e). However, in contrast to what we observed in the case of Ank and Enpp1 expression, MEK inhibition by U0126 (1 µM), tremetinib or PD198306 (0.1 µM and 200 nM, respectively, data not shown) for 24 h did not correct the expression level of Runx2 or Alpl in Nf1-deficient BMSCs (Fig. 3c), indicating that the expression of these two genes is not directly controlled by neurofibromin. Extracellular PP concentration, as well as Ank, Enpp1 and Spp1 expression, remained above or equal to that of WT controls throughout the differentiation period (Fig. 3d,e).

BMFs are known for their ability to promote osteoprogenitor differentiation\(^{39}\) but have had limited effects on the differentiation of Nf1-heterozygous osteoprogenitors and on bone union in Nf1-heterozygous mice\(^{40,41}\). Recombinant human BMP2 (100 ng ml\(^{-}\)) did not stimulate ALP activity or CFU-Ob formation in BMSC cultures from Col2-Nf1 KO mice, although it did, as expected, promote

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**Figure 2**. Altered PP, homeostasis in Nf1-deficient chondrocytes. (a) Hyperostosisis (pink, white arrow) in the primary spongiosa from Col2-N1 KO mice (undecalcified sections stained by von Kossa and van Gieson; n = 6 per group; scale bars, 150 µm). (b) High-density chondrocyte pellets prepared from WT and Col2-N1 KO pups. Proteoglycan production (top, Alcian blue staining) and matrix mineralization (bottom, von Kossa staining) (n = 3; scale bars, 100 µm). (c) Ank, Enpp1 and Spp1 mRNA expression in high-density chondrocyte pellets (n = 3). (d-f) Relative extracellular PP, concentration (d), Enpp1 activity (e) and ALP activity (f) in WT and Col2-N1 KO high-density chondrocyte pellets (n = 3). *P < 0.05, determined by Student’s t-test. Data are expressed as mean ± s.d.

**Figure 3**. Blunted BMP2 response and osteoblast differentiation potential in Nf1-deficient osteoprogenitors. (a) BMSC differentiation analyzed by Alizarin red S staining (differentiation and mineralization, CFU-Ob), crystal violet staining of fibroblast CFUs (cell number, CFU-F, left), soluble Alizarin red S/crystal violet optical density ratio (middle) and ALP activity/crystal violet ratio (right) (n = 3). Blue, WT mice; gray, Col2-N1 KO mice. (b) Runx2, Alpl and Bglap mRNA expression in BMSCs differentiated for 7, 14 and 21 d (n = 3). (c) Runx2 and Alpl mRNA expression in serum-starved BMSCs treated with vehicle (DMSO) or U0126 for 24 h (n = 3). (d) Extracellular PP, concentration/protein concentration in BMSCs differentiated for 7, 14 and 21 d (n = 3). (e) Normalized Ank, Enpp1 and Spp1 mRNA expression in BMSCs differentiated for 7, 14 and 21 d (n = 3). *P < 0.05, determined by one-way ANOVA and Student’s t-test. Data are expressed as mean ± s.d.
CFU-Ob formation and ALP activity in WT BMSC cultures following 2 weeks of treatment (Fig. 4a). Smad1 and Smad5 phosphorylation in response to BMP2 treatment (100 ng ml\(^{-1}\), 1 h) was not affected by Nf1 deficiency (Fig. 4b), indicating that the lack of stimulatory effect of BMP2 on Nf1-deficient BMSC differentiation is not caused by repression of BMP2 receptor expression or by the production of factors inhibiting canonical Smad signaling. Treatment with BMP2 for 2 weeks also failed to increase the expression of Alpl, Runx2 and Col1a1 in BMSC cultures from Col2-Nf1 KO mice (Fig. 4c). However, it significantly increased the expression of Ank and Enpp1 (but not Spp1) (Fig. 4d) and PP, extracellular concentration (Fig. 4e) in both WT and Nf1-deficient BMSCs. CFU-Ob formation, ALP activity (Fig. 4f,g) and the expression of Alpl and Col1a1 (Supplementary Fig. 2a,b) in Nf1-deficient BMSC cultures were higher following a 2-week-long combined treatment with the MEK inhibitor U0126 (1 μM) and BMP2 (100 ng ml\(^{-1}\)), but not with either of these treatments alone. This combination treatment also partially reduced the increased Ank and Enpp1 expression and PP, extracellular concentration detected in vehicle-treated Nf1-deficient BMSC cultures, possibly owing to the antagonistic effect of these two drugs on Ank and Enpp1 expression (Supplementary Fig. 2c,d).

sALP-FcD10 improves bone growth and mineral density in Col2-Nf1 KO mice

If excessive extracellular PP\(_i\) levels cause the mineralization deficit observed in Col2-Nf1 KO mice, then reducing PP\(_i\) concentration should have beneficial effects on matrix mineralization. This is experimentally possible by inhibiting PP\(_i\) generation or increasing its catabolism. We chose the latter approach because PP\(_i\) is a substrate for ALP, and a recombinant form of human ALP is clinically available to treat ALPL-deficient subjects with hypophosphatasia\(^{42,43}\). We thus treated WT and Nf1-deficient BMSCs with vehicle or sALP-FcD10 (0.5 mg ml\(^{-1}\)) in osteogenic conditions for 14 d and assessed matrix mineralization. As predicted, sALP-FcD10 increased matrix mineralization in both genotypes, although the relative increase was more pronounced in cultures from Col2-Nf1 KO than in those from WT mice (Fig. 5a). This pronounced increase occurred despite the persistent differentiation deficit of Nf1-deficient BMSCs in the presence of sALP-FcD10 (Supplementary Fig. 3a). Treatment with sALP-FcD10 reduced Spp1 expression in Nf1-deficient BMSCs (Supplementary Fig. 3a), in agreement with the known stimulatory effect of PP\(_i\) on Spp1 expression\(^{25}\).

On the basis of these encouraging results, we treated Col2-Nf1 KO newborn mice daily with subcutaneous injections of sALP-FcD10 (8.2 mg per kg body weight per d) for 18 d\(^{44,45}\). Col2-Nf1 KO mice have short stature, low bone mass, decreased bone mineralization, cortical thickness and mineral density, and high cortical porosity\(^{37}\). Following this short treatment (dictated by the relatively high death rate of these mice at weaning), we observed a significant 73% increase in the size of mutant mice (Fig. 5b) and a clear increase in vertebral and tibial bone mineral density on radiographs (Fig. 5c,d). Treatment with sALP-FcD10 also significantly increased mid-diaphyseal cortical bone thickness, as measured by three-dimensional microcomputed tomography (μCT) (Fig. 5e), partially rescued the formation of secondary ossification centers, expanded tibia metaphyseal envelopes and increased the amount of calcified matrix in the growth plate hypertrophic zone of Col2-Nf1 KO mice (Fig. 5f). Despite the seemingly pronounced effects of sALP-FcD10 observed by radiography and μCT, tibia cortical tissue mineral density and mineral-to-collagen ratio (Supplementary Fig. 3b,c) were not increased following treatment.

sALP-FcD10 increases bone mineralization in Osx-Nf1 KO mice

Because Col2-Nf1 KO mice manifest severe developmental phenotypes that limit their survival and thus the duration of treatments,
we generated mice in which Nf1 be ablared postnatally in osteoporogenitors expressing Sp7 (also known as Osx) by crossing the inducible Tet-Off–based Osx-cre transgenic mice66 with Nf1<sup>flkx/flkx</sup> mice67. This new mouse model makes it possible to dissect the mechanisms by which postnatal Nf1 ablartion impairs bone homeostasis, without complications arising from developmental phenotypes. Osx-cre; Nf1<sup>flkx/flkx</sup> mice (herein called Osx-Nf1 KO mice) were undistinguishable in size from WT littermates upon doxycycline administration (i.e., Cre recombinase repression) from conception to day 14 (Fig. 6a) and had normal phosphate, calcium and 25-hydroxycholecalciferol (vitamin D) serum concentrations (Supplementary Table 1). Osx-cre–mediated Nf1 ablartion in osteoporogenitors at postnatal day 14 following doxycycline withdrawal, as seen in Osx-Nf1 KO mice, caused hyperosteoidosis (Fig. 6b), lower bone mass (Fig. 6c), higher femoral diaphyseal cortical porosity (Fig. 6d) and lower cortical thickness, midshaft moment of inertia and cortical tissue mineral density compared to WT mice (Fig. 6e–g). Cortical mineral-to-collagen ratio measured by Raman spectroscopy (Fig. 6h).
was also lower in Osx-Nf1 KO mice, and femurs from Osx-Nf1 KO mice were mechanically weaker than those from WT controls, as measured by three-point bending tests (Supplementary Table 2).

To assess the effect of sALP-FcD10 on the skeleton of this mouse model, we administered sALP-FcD10 daily from 2 weeks of age (at the time of Nf1 ablation) for 6 weeks. In Osx-Nf1 KO mice, treatment with sALP-FcD10 significantly increased trabecular bone volume/tissue volume ratio and moment of inertia, as assessed by μCT (Fig. 6c,f), as well as femoral stiffness, modulus and peak force, as measured by three-point bending (Supplementary Table 2), and led to a non-significant trend for increased cortical femoral thickness (Fig. 6e).

Treatment with sALP-FcD10 also improved bone mineralization in Osx-Nf1 KO mice, as measured by a drastic 73% reduction in osteoid volume per bone volume, a 65% reduction in osteoid surface per bone surface, a 53% decrease in osteoid thickness (Fig. 6b) and a 20% increase in mineral-to-collagen ratio (Fig. 6h).

**DISCUSSION**

We show here that the RAS-GAP activity of neurofibromin in the bone mesenchymal lineage restrains the expression of Enpp1 and Ank, two main genes controlling PP, homeostasis, and that increasing PP, catabolism through enzyme therapy considerably improves bone mineralization and bone mechanical properties in mouse models of NF1 skeletal dysplasia. These results, along with suggestive evidence of conservation of function between mice and humans, support the causal role of increased PP, levels in the etiology of NF1-related hyperosteooidosis and position neurofibromin as a critical and obligatory regulator of cartilage and bone mineralization. They also provide preclinical evidence that some of the most clinically challenging NF1-related skeletal maladies might be preventable.

Hyperactive TGF-β signaling has been proposed to cause bone loss and to delay bone healing in mice deficient for Nf1 in mature osteoblasts and heterozygous for Nf1 (ref. 19). TGF-β is also known to stimulate ERK activity and Ank and Enpp1 expression, and to increase PP, concentration in WT chondrocytes. Therefore, Nf1-deficient BMSCs may contribute cell autonomously and/or in a hyperactive TGF-β para-crone fashion to the extrophysiological skeletal accumulation of PP, and to the impaired osteoblast differentiation and matrix mineralization observed in the setting of NF1. The beneficial effect of sALP-FcD10 on bone growth, mineralization and strength observed in this study suggests that PP, accumulation and abnormal mineralization are important components of NF1-related bone dysplasia. However, further studies will be necessary to determine the evolution and contribution of all the cellular defects typical of Nf1-deficient bone cells on bone mass and strength over extended periods of treatment with sALP-FcD10, as this drug does not correct the differentiation phenotype of Nf1-deficient osteoblasts. Although TGF-β blockade might theoretically be used to promote bone union in children with NF1 pseudarthrosis, the cancer-prone status of this pediatric population and the known tumor-suppressor activity of TGF-β signaling limit this therapeutic approach.

Our results, on the other hand, suggest that stimulation of PP, catabolism through enzyme therapy could be applied on a more chronic basis before fracture to strengthen the NF1-related dysplastic bones and prevent their mechanical failure.

The mineralization deficit of Nf1-deficient BMSCs could be detected in immature BMSCs before their differentiation into osteoblasts. Therefore, this phenotype cannot be attributed to the reduced differentiation potential of Nf1-deficient BMSCs, although the latter certainly contributes to the low bone mass phenotype observed in the two NF1 mouse models used in this study. It is also worth noting that BMP2 treatment, without the need for ERK blockade, stimulated the expression of Ank and Enpp1 and increased extracellular PP, concentration in Nf1-deficient BMSC cultures, as shown previously in WT cells. This observation could explain why recombinant human BMP2 alone did not improve bone healing in NF1 mouse models and bone union in individuals with NF1-related pseudarthroses.

Our results indicate that Nf1-deficient BMSCs are not responsive to BMP2 with regard to their differentiation potential and suggest that this defect may in part underlie their inability to differentiate. In addition, the response of Nf1-deficient BMSCs to BMP2 with regard to Ank and Enpp1 expression suggests that neurofibromin is not the sole negative regulator of the RAS-ERK signaling pathway upstream of these two genes. These results also indicate that the stimulatory effect of BMP2 on osteoprogenitor differentiation requires controlled ERK signaling by neurofibromin.

It is unknown to what extent poor matrix mineralization contributes to the low bone mineral density, tibia bowing, poor mechanical properties and possibly pseudarthrosis observed in children with NF1. Although local PP, concentration could not be quantified, the observed increase in the expression of Enpp1 in BMSCs extracted from biopsies of pseudarthroses from patients with NF1, as well as the presence of thick osteoid seams on histological sections supports conservation of function between mice and humans.

Pseudarthrosis and dystrophic scoliosis can currently be treated only by invasive, and often repetitive, surgical orthopedic interventions. Most approaches to date are corrective in nature, and only bracing techniques are available to reduce the incidence and severity of these complications. Of major interest is the possibility that sALP-FcD10, if applied preventatively, might improve mineralization, growth, architectural and mechanical properties of dysplastic bones affected by NF1 and, thus, limit their likelihood of deformation and fracture. This latter point is particularly noteworthy, as the current standard for treatment is limited to avoidance of prophylactic surgery and early long-term bracing to prevent fracture until skeletal maturity is reached. It is worth emphasizing that sALP-FcD10 targets bone and is already successfully used in the clinic to treat children with hypophosphatasia. Therefore, its potential use in the context of NF1-related skeletal dysplasia has an advantage over the development of other experimental drugs that target this and other aspects of the NF1 skeletal pathologies.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank A. Bianchi and F. Cailotto for their help in establishing the PP, measurement protocol and K.S. Campbell for editorial assistance. This work was supported by a Young Investigator Award (2012–01–028) from the Children’s Tumor Foundation (J.r.d.C.N.), the US National Institute of Arthritis and Musculoskeletal and Skin Diseases and National Center for Research Resources, part of the US National Institutes of Health, under award numbers AR055966 (P.E.) and S10 RR027631 (D.S.P.), the National Center for Advancing Translational Sciences of the National Institutes of Health under award number UL1TR001105 (J.J.R.), the Pediatric Orthopaedic Society of North America and Texas Scottish Rite Hospital for Children (J.J.R.), a Career Development Award (no. 1IK2BX001634) from the US Department of Veterans Affairs, Biomedical Laboratory Research and Development Program (D.S.P.), and the US Army Medical Research Acquisition Activity under award W81XWH–11–1–0250 (D.A.S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the US National Institutes of Health or US government.
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BMSCs were isolated from new bones or mice and seeded at a density of 1 × 10^4 cells/well in 12-well plates (or bone tissue were maintained by complete culture medium). At 40% confluence, cells were incubated in complete culture medium (α-MEM, 10% FBS and 100 U/ml penicillin) containing either Ad5-CMV-GFP or Ad5-CMV-Cre (Vector development lab, Baylor College of Medicine). Teeth were dissected and soft tissues removed, then digested by digestion of bone mass (GFP or Ad5-CMV-Cre (Vector development lab, Baylor College of Medicine) or bone tissue were maintained by complete culture medium) for the periods of time indicated in the text. sALP-FcD10 was assayed by an ELISA Assay kit (Eagle Biosciences, cat# VID31-K01), a Phosphate Assay kit (BioVision, cat #K410-500) and a Calcium Assay kit (BioVision, cat #K380-250), respectively, according to the manufacturer's instructions.

**Cell culture.** Mouse BMSCs were extracted from long bones by spinning down diaphyses at 1,500 rpm for 3 min. Cells were then counted, plated at a density of 1 × 10^4 cells/well (12-well plates) or 2 × 10^4 cells/well (6-well plates) and grown for 7 days in DMEM supplemented with 10% FBS, 100 U ml^-1 penicillin, 100 µg/ml streptomycin (Cellgro, Manassas, VA, USA). Day 7, differentiation and mineralization was induced by the addition of 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate, and the medium was refreshed every 2–3 days. BMSC differentiation and mineralization were assessed by ALP activity and Alizarin red S staining, respectively, using standard protocols.

Primary chondrocytes were extracted from 4-day-old pup rib bones. The cartilaginous part of the rib was dissected and soft tissues removed, then digested by collagenase D (3 mg/ml, Roche, USA) and 0.25% trypsin/EDTA (EDTA) (Gibco, USA) in DMEM for 3 h. At confluence, 5 × 10 µl drops of concentrated cells (2 × 10^6 cells/ml) were plated in 6 wells. After 2 h of incubation, 2 ml of complete cell culture medium was delicately added. Cells were differentiated in DMEM supplemented with 10% FBS, 100 IU ml^-1 penicillin, 100 µg ml^-1 streptomycin, 50 µg ml^-1 ascorbic acid and 10 mM β-glycerophosphate.

Human cells extracted from bone marrow or tissue were maintained in alpha MEM supplemented with 10% FBS, 100 U ml^-1 penicillin, 0.1 mg ml^-1 streptomycin, 50 µg ml^-1 ascorbic acid and 10 mM β-glycerophosphate.

**Human subjects.** The study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center, of the Rizzoli Orthopaedic Institute (Bologna, Italy) and of Vanderbilt University. The parents of the subjects provided informed consent. Bone tissues were obtained from 9 patients with NF1 and tibial pseudarthrosis (aged between 7 months and 18 years), and control samples were obtained from 6 children without NF1 who underwent surgery for congenital dysplasia of the hip without any other coexisting pathology (n = 3) or scoliosis (n = 3) (aged between 3.3 and 17 years). Diagnosis of pseudarthrosis was based on radiographic and clinical findings. Diagnosis of NF1 was performed according to the criteria presented at the National Institutes of Health Consensus Development Conference on Neurofibromatosis (http://consensus.nih.gov/1987/1987Neurofibromatosis06html.htm).

**RT-qPCR and genomic PCR.** Total RNA was extracted using TRIozol (Invitrogen, Grand Island, NY, USA), and cDNAs were synthesized from 1 µg of RNA following DNase treatment using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, USA). Quantitative PCR (qPCR) was performed using the TaqMan or SYBR green gene expression assays. The probe and primer sets for mouse Runx2 (Mm00501578_m1), Alpl (Mm00475384_m1), Ank (Mm00455047_m1), Enpp1 (Mm00510097_m1), Sprr1 (Mm00436767_m1), Igf1 (Mm01218180_m1), human ANKH (Hs00219798_m1) and human ENPP1 (Hs01054046_m1) and the normalizers Hprt1 (Mm00446968_m1), human GAPDH (Hs99999905_m1) were obtained from Applied Biosystems (Foster City, CA, USA). The SYBR green primers were Sprr1 (forward: CTCCTGGCCACAGCAAGT, reverse: TGGCAGACAGGAGTGA), Nf1 (forward: GTATTGAATTGAAGCACC TTGTTTTGG, reverse: CTGCCAACAGGTTCCCTCCAG), Bglap (forward: ACCCTGGTCTGCCCCTGCTCT, reverse: GTATGGTTTTGAGGGGTTTCCTCA) and Col1a1 (forward: GATATTGAATTGAAGCACC TTGTTTTGG, reverse: CTGCCAACAGGTTCCCTCCAG). Specificity of amplification was verified by the presence of a single peak on the dissociation curve. Amplification conditions are available upon request. Measurements were performed in triplicate and from at least 3 independent experiments.

For genotyping, genomic DNA was isolated from tail tips by sodium hydroxide digestion, and PCR was performed using primers P1, P2 and P4, as described by Zhu et al. The Col2a1-cre transgene was detected using the fwd: GAGTT GATAACGTGCTGGTGGCCAGATG and rev: TCTCCTGCTGCTAGG CCTCTTCGTCG primers. Whole cell lysates were separated by SDS-PAGE electrophoresis according to standard protocols. Nitrocellulose membranes were probed with the indicated antibodies using standard protocols (monoclonal anti β-actin antibody (Sigma cat# AC-74, dilution 1:5,000), anti-pSmad1/5/6 antibody (Cell Signaling cat#9516S, dilution 1:1,000), anti-Smad1/5/6 antibody (Abcam cat# ab75273, dilution 1:1,000), anti ENPP1/PC-1 (Aviva Systems Biology, cat# 46540, dilution 1:1,000).
Histology. Static histomorphometry measurements were performed as previously described in accordance with standard nomenclature,\textsuperscript{29} using the Bioquant Analysis System (Nashville, TN, USA) on 5 μm undecalciﬁed methylnacrylate sections. Calciﬁed cartilage BV/TV was measured in the growth plate hypertrophic region following von Kossa and van Gieson staining.

X-rays and μCT analyses. Radiographs were obtained using a digital cabinet X-ray system (LX-60, Faxitron X-Ray, USA). μCT analyses were performed using a Scanco μCT 40 system (Scanco Medical, Bassersdorf, Switzerland). Tomographic images were acquired at 55 kVp and 145 mA with an isotropic voxel size of 12 μm and at an integration time of 250 ms with 500 projections collected per 180° rotation.

Raman spectroscopy. Sensitive to the vibrational modes of chemical bonds, Raman spectroscopy (RS) characterizes the biochemical properties of bone tissue, namely mineral-to-collagen ratio (MCR) and crystal structure. Using midshaft vessel perforations as landmarks, spectra were obtained from cortical bone of the femur with 5 accumulations of 20 s exposures to a 20-mW, near-infrared laser (785 nm) at a spot size of ~1.5 μm in diameter. Spectra were processed via least-squares modiﬁed polynomial ﬁt to suppress background ﬂuorescence\textsuperscript{60} and smoothed for noise using a second-order Savitsky-Golay ﬁlter\textsuperscript{41}. Raman shift calibration was accomplished using a neon lamp and a silicon standard. Silicon standard measurements before and after data acquisition ensured wave number consistency across bones. Spectral intensities for known Raman peaks and peak ratios were extracted using custom Matlab software (Mathworks, Natick, MA) to measure mineralization as v1 phosphate (symmetrical stretching at ~960 cm\textsuperscript{-1}) per proline (ring at ~854 cm\textsuperscript{-1}) and crystallinity (crystal grain size and perfection) as the inverse full width at half maximum intensity of the v1 phosphate peak.

Biomechanical testing. Hydrated samples were tested in three-point bending with a span of 8 mm at a rate of 3 mm min\textsuperscript{-1} (ref. 62). Force and displacement were measured from a 100 N load cell and from the linear variable displacement transformer of the material testing system (Dynamight 8841, Instron, Canton, OH). Structural properties were extracted from force-displacement curves by custom Matlab algorithms (Mathworks, Natick, MA). Material properties were calculated by accounting for structure by using cross-sectional area and moment of inertia as measured by μCT.

Statistical analyses. Depending on whether data per group passed the Shapiro-Wilk normality test or whether standard deviations were not different among the groups (Bartlett’s test), one-way analysis of variance (ANOVA) or the Kruskal-Wallis Test (nonparametric) was used to determine whether differences existed in μCT-, Raman- and biomechanically-derived properties among the experimental groups. When differences existed at P < 0.05, post hoc pair-wise comparisons were tested for signiﬁcance in which the P value was adjusted (FDR < 0.05) by Holm-Sidak’s method or Dunn’s method (nonparametric). Statistical analysis was performed using GraphPad PRISM (v6.0a, La Jolla, CA). Data are provided as mean ± s.d. No statistical method was used to predetermine sample size. The investigators were blinded to allocation during experiments and outcome assessment. The experiments were not randomized.

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Corrigendum: Asfotase-α improves bone growth, mineralization and strength in mouse models of neurofibromatosis type-1

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Nat. Med. 20, 904–910 (2014); doi:10.1038/nm.3583; published online 6 July 2014; corrected after print 13 March 2015

In the version of this article initially published, the acknowledgment that Daniel S. Perrien was supported by a Career Development Award from the US Department of Veterans Affairs was omitted. The error has been corrected in the HTML and PDF versions of the article.