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Multiple Increased Osteoclast Functions in Individuals with Neurofibromatosis Type 1

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Abstract

Skeletal abnormalities including scoliosis, tibial dysplasia, sphenoid wing dysplasia, and decreased bone mineral density (BMD) are associated with neurofibromatosis type 1 (NF1). We report the cellular phenotype of NF1 human-derived osteoclasts and compare the *in vitro* findings with the clinical phenotype.

Functional characteristics (e.g. osteoclast formation, migration, adhesion, resorptive capacity) and cellular mechanistic alterations (e.g. F-actin polymerization, MAPK phosphorylation, RhoGTPase activity) from osteoclasts cultured from peripheral blood of individuals with NF1 (N=75) were assessed. Osteoclast formation was compared to phenotypic, radiologic, and biochemical data.

NF1 osteoprogenitor cells demonstrated increased osteoclast forming capacity. Human NF1-derived osteoclasts demonstrated increased migration, adhesion, and *in vitro* bone resorption. These activities coincided with increased actin belt formation and hyperactivity in MAPK and RhoGTPase pathways. Although osteoclast formation was increased, no direct correlation of osteoclast formation with BMD, markers of bone resorption, or the clinical skeletal phenotype was observed suggesting that osteoclast formation *in vitro* cannot directly predict NF1 skeletal phenotypes.

While *NF1* haploinsufficiency produces a generalized osteoclast gain-in-function and may contribute to increased bone resorption, reduced BMD, and focal skeletal defects associated with

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NF1, additional and perhaps local modifiers are likely required for the development of skeletal abnormalities in NF1.

Keywords

bone resorption; osteoporosis; Ras; bone mineral density; neurofibromatosis type 1

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder presenting in the pediatric period with a range of both malignant and nonmalignant manifestations. NF1 results from mutations in *NF1*, a tumor suppressor gene. Somatic mutations in the normal *NF1* allele and loss of heterozygosity (LOH) in specific cell types induce many features of NF1 including neoplasia [Wallace et al., 1990; Atit et al., 1999; Atit et al., 2000; Ingram et al., 2000; Rutkowski et al., 2000; Ajuebor et al., 2001; Cichowski and Jacks, 2001; Zhu et al., 2002; Bajenaru et al., 2003; Yang et al., 2003; Johannessen et al., 2005].

However, data from particular cell lineages [Atit et al., 2000; Ingram et al., 2000; Bajenaru et al., 2003; Johannessen et al., 2005; Wu et al., 2006] indicate that *NF1* gene dosage (i.e. heterozygosity versus nullizygosity) can alter cellular fate and function. Recent studies have explored the critical contributions of *Nf1* haploinsufficient cells to the microenvironment of nullizygous Schwann cells in plexiform neurofibromas and nullizygous glial cells in optic gliomas [Atit et al., 1999; Atit et al., 2000; Ingram et al., 2000; Rutkowski et al., 2000; Zhu et al., 2002; Bajenaru et al., 2003; Yang et al., 2003; Johannessen et al., 2005]. Now, clinical data from NF1 patients and experimental studies in *Nf1*^{+/-} mice indicate that *NF1* (or *Nf1*) haploinsufficiency itself results in a range of non-malignant phenotypes including learning and spatial-visual deficits, renal vascular hypertension, cerebrovascular disease and osteopenia [Kurien et al., 1997; Cormier et al., 1999; Fossali et al., 2000]. Collectively, these data argue that *NF1* gene dosage critically modulates multiple and heterogeneous disease phenotypes. Thus, delineation of the unique cellular and biochemical underpinnings of *NF1* pathologies are crucial to the development of targeted and efficacious molecular therapies.

Individuals with NF1 are at risk for both generalized and focal skeletal abnormalities. The generalized skeletal abnormalities include decreased bone mineral density (BMD) [Illes et al., 2001; Kuorilehto et al., 2005; Lammert et al., 2005; Dulai et al., 2007; Stevenson et al., 2007; Yilmaz et al., 2007], increased fracture rates in adults [Tucker et al., 2009], and short stature [Clementi et al. 1999]. One study showed that all postmenopausal women with NF1 experienced either osteoporosis or osteopenia [Kuorilehto et al., 2005], putting them at risk for fractures [Roberts et al., 2010].

The focal skeletal abnormalities occur at a lower rate (e.g. sphenoid wing dysplasia, focal short angle scoliosis, tibial dysplasia) suggesting that modifying physiological processes – such as *NF1* loss of heterozygosity – may induce localized pathology. For example, NF1 patients with anterolateral bowing of the long bones have increased incidence of pseudarthrosis, and *NF1* LOH has been detected in pseudarthrosis tissue at these fracture sites. Potentially, then, the localized bone defects result from genetic second hits [Stevenson et al., 2006] and regional cellular variations.

The clinical observations of multiple localized defects, LOH in pseudarthrotic tissue, and generalized skeletal dysplasia suggest that individuals with NF1 have both local and general

disregulation in the mechanisms of bone resorption, remodeling, and repair. Bone resorption, remodeling, and repair depend on the coordinated function of osteoclasts, multinucleated marrow-derived cells which demonstrate hyperactivity in skeletal pathologies such as osteoporosis [Lane 2006]. Importantly, established data in mice demonstrate that *Nf1* mutations create cytokine hypersensitivity in multiple myeloid lineages, including marrow-derived myeloid progenitor cells, mast cells, and osteoclasts [Zhang et al., 1998; Ingram et al., 2000; Yang et al., 2006]. Osteoclasts and osteoclast progenitors derived from both human NF1 patients and from *Nf1* haploinsufficient (*Nf1*^{+/-}) mice have enhanced resorption capacities and aberrant morphology *in vitro* [Yang et al., 2006; Heervä et al., 2010]. However, it is unclear whether bone-related clinical manifestations in NF1 patients correlate with these cellular alterations. In the present study, we analyze multiple functions of osteoclasts generated from the peripheral blood of a large cohort of individuals with NF1, and we evaluate potential relationships between their clinical and biochemical data and *in vitro* osteoclast phenotypes.

MATERIALS AND METHODS

Recruitment of NF1 individuals

Seventy five individuals with NF1, ages 1 to 25 years, were enrolled at an NF1 clinic at the University of Utah. All individuals fulfilled NF1 clinical diagnostic criteria [Stumpf et al., 1988; Gutmann et al., 1997]. Details of skeletal abnormalities were recorded, and any reports of radiographs of the spine, limbs and sphenoid wing were reviewed. Individuals with spinal curvature were categorized into 3 groups: individuals with curves between 9-20 and >20 degrees without surgical intervention, and individuals with scoliosis requiring surgical intervention. Peripheral blood was obtained to generate NF1 patient-derived osteoclasts, and a cohort of 39 individuals (age range from 2 to 48 years) without NF1 donated peripheral blood to generate control osteoclasts. This study was approved by the Institutional Review Board at the University of Utah and Indiana University. Informed consent was obtained from all participants.

Dual energy X-ray absorptiometry (DXA)

Images on a subset of NF1 individuals were obtained using a DXA instrument (Hologic QDR-4500A, Waltham, MA) under well-established standard protocols as previously described [Stevenson et al., 2007]. Whole body subtotal measurements were obtained by subtracting the head region from the whole body. BMD z-scores were generated on NF1 individuals by utilizing age- and gender-matched healthy controls selected from DXA reference data on 293 individuals living in the same geographic region.

Generation of osteoclasts from human NF1 individuals

Ten mL of heparinized peripheral blood was obtained. Peripheral blood mononuclear cells (MNCs) were separated by Ficoll-Hypaque (Sigma, St Louis, MO) and differentiated into osteoclasts [Fujikawa et al., 2001]. MNCs (5×10^4 /well in a 96 well plate) were cultured in α -MEM supplemented with 10% fetal bovine serum (FBS, Biomed, Foster City, CA) in the presence of 50 ng/mL human recombinant Receptor Activator for Nuclear Factor κ B Ligand (RANKL; Peprotech, Rocky Hill, NC) and 30 ng/mL recombinant Macrophage Colony Stimulating Factor (M-CSF, Peprotech) for 7 days with media replacement on days 4. The cells were plated on plastic dishes and washed with α -MEM. Adherent cells were fixed in 10% formaldehyde in phosphate-buffered saline (PBS), treated with ethanol-acetone (50:50), and stained for tartrate resistant acid phosphatase (TRACP), as described previously [Yang et al., 2006]. Osteoclast number and morphology were evaluated with a Nikon TE2000-S microscope (Nikon Inc.). Images were taken by a QImaging camera and QCapture-Pro software (Fryer Company Inc., Cincinnati, OH). Multinucleated, TRACP⁺

cells containing more than three nuclei were scored as mature osteoclasts. The area of multinucleated TRACP⁺ osteoclasts (% of field) was calculated with Metamorph offline software. In order to avoid potential variation in culture condition, osteoclast differentiation was qualitatively evaluated by calculating the fold-change in osteoclast number for each NF1 sample compared to control samples obtained on the same day. One to three investigators independently scored samples in a blinded fashion.

Thymidine incorporation assay

Proliferation was assessed by [³H]thymidine incorporation of osteoclasts and osteoclast precursors. See figure legend for Figure 1C for details.

Osteoclast migration assay

Cells cultured in the presence of M-CSF and RANKL for four day were used for migration assays as described previously [Yang et al., 2006]. See figure legend for Figure 3B for details.

Osteoclast adhesion assay

Four day M-CSF and RANKL cultured cells (1×10^5 cells/ml) were placed into 24-well plates coated with 20 μ g/ml α v β 3 (Takara Inc. Shiga, Japan) and supplemented with 30 ng/ml M-CSF, as described previously [Yang et al., 2006]. Thirty minutes later, non-adherent cells were lifted gently with pipetting and rinsed with PBS. The adherent cells were fixed with 1% paraformaldehyde and counted under microscopy [Yang et al., 2006].

Immunofluorescence microscopy

To evaluate cellular mechanisms underlying osteoclast adhesion and degradation activity, immunofluorescence microscopy was used as previously described [Sun et al., 2005; Yang et al., 2006; Yan et al., 2008]. After 4 days of culture in the presence of M-CSF and RANKL as described above, the cells were lifted from the culture dish and then replated on coverslips and grown on the coverslips for an additional 2 days in the presence of M-CSF and RANKL. Immunofluorescence images were acquired by phase contrast microscopy using a Nikon TE2000-S microscope (Nikon Inc.), QImaging camera, and QCapture-Pro software (Fryer Company Inc., Cincinnati, OH). Actin cytoskeletal organization in podosomes, was enumerated as progressively larger patterns of clusters, rings, and ultimately belts [Yan et al., 2008]. Percent area of belt-forming cells per field was calculated. See figure legend for Figure 3D,E for additional details.

Bone resorption assay

Bone resorption was examined on cultured cells taken after 4 days culture in the presence of M-CSF and RANKL placed on dentine slices as described previously [Yang et al., 2006; Takeshita et al., 2002]. The number of resorptive areas or “pits” per low power field was counted using reflective light microscopy. The area (mm^2) of each pit was measured (width $\times$ length) using QCapture Pro (Version 5.1), and the area of pit as a percentage of each field was evaluated with Metamorph software. See figure legend for Figure 2 for additional details.

Immunoblotting and kinase activity

Cultured cells taken after 4 days culture in the presence of M-CSF and RANKL were washed with PBS and deprived of serum and growth factors for 4 hours and then stimulated with 30ng/mL M-CSF for indicated time points (0, 1, and 5 minutes) and utilized to assess mitogen-activated protein kinase (MAPK) and RhoGTPase activation as previously

described [Yang et al., 2000; Yang et al., 2003; Ming et al., 2007]. See figure legend for Figure 5 for additional details.

Pyridinium crosslink analysis (bone resorption markers)

Urine from two first morning voids was obtained from a subset of individuals with NF1 for extraction and analysis of pyridinium crosslinks, pyridinoline (Pyd) and deoxy-pyridinoline (Dpd), by high performance liquid chromatography (HPLC) as previously described [Stevenson et al., 2008]. Control data for comparison came from a cohort of healthy individuals from the same geographic region. Two first-morning urine samples were obtained and the average of the two samples was used in the analysis.

Statistical analysis

Two-tailed unpaired Student's t-tests were used to evaluate statistical differences. P values less than 0.05 were considered statistically significant. Data for Pyd, Dpd, and Dpd/Pyd ratios, and age from the NF1 individuals with and without “hyper osteoclast formation” values, as defined as a > 2 fold increase in osteoclast formation (higher osteoclast formation) and <1.5 fold increase in osteoclast formation (“normal” osteoclast formation), were compared with each other and with the pyridinium crosslink control group using linear regression models. P-values are generated from t-tests for regression model coefficients.

RESULTS

Osteoclast formation is enhanced in NF1 peripheral blood cultures

The peripheral blood of 70 out of 75 NF1 individuals demonstrated nearly twice or more of the control area of multinucleated osteoclasts (Fig 1A, and Fig 1B, mean fold increase 2.07 ± 0.1 SEM, $p < 0.0001$). We suggest that the increased number and size of multinucleated osteoclasts in NF1 samples represent enhanced osteoclast formation, and herein the area of multinucleated osteoclasts will be termed “osteoclast formation”. This result coincides with previous data acquired from the study of *Nf1* +/- mice [Yang et al., 2006], wherein peripheral blood- and marrow-derived osteoclasts demonstrate a hyperproliferative capacity in the presence of M-CSF and RANKL, and similar to results in a cohort of 17 individuals with NF1 [Heervä et al., 2010]. The fold-change increase in osteoclast formation also coincided between groups of NF1 individuals with known presence or absence of specific skeletal phenotypes (Table I).

NF1 osteoclasts and osteoclast precursors demonstrate altered proliferation

Human NF1 derived macrophages demonstrated increased [^3H]thymidine incorporation in the culture containing M-CSF (30ng/ml) on day 5 (mean CPM versus control mean CPM; SEM; $p = 0.01$, $n = 4$ each group, Figure 1C). However, in a longer culture period (day seven), a reduced [^3H]thymidine incorporation was observed in NF1 culture as compared with the control cultures (data not shown). The reduced proliferation on day seven cultures in NF1 individuals suggests an enhanced maturation and terminal differentiation of the dysregulated NF1 osteoclasts.

NF1 osteoclasts hyperactively resorb bone

NF1 osteoclasts created larger dentine pit areas than control osteoclasts (Fig 2A), as seen in representative photomicrographs (Fig 2B). This data suggested that osteoclasts generated from NF1 patients have increased bone resorptive activity *in vitro*.

NF1 pre-osteoclasts hyperactively migrate and adhere

A marked increase in the number of migrated pre-osteoclasts was observed in NF1 compared to control cells with an approximate 5-fold increase over controls (* $p < 0.01$) (Fig 3A-B). It has been reported that osteoclasts adhere to bone surface via integrin $\alpha v \beta 3$ binding that critically influences bone resorptive activity [Aubin 1992; McHugh et al., 2000; Sanjay et al., 2001]. To mimic this physiological process *in vitro*, macrophage adhesion assays were conducted and showed an increase in adhesion of NF1-derived osteoclast precursors (Fig 3C).

NF1 osteoclasts form altered cytoskeletal structures

While comparable numbers of clusters and actin rings were observed in control and NF1 osteoclasts (data not shown), NF1 osteoclasts demonstrated increased belt formation (Figure 3D-E, * $p < 0.05$).

No direct relationship detected between increased NF1 osteoclast formation and reduced whole body subtotal bone mineral density (BMD) or increased urinary pyridinium crosslink concentrations

To evaluate whether the increased osteoclast formation correlates with the observed reduced whole body subtotal BMD, the fold change of human NF1 derived osteoclast formation over control osteoclast formation was plotted against the whole body subtotal BMD data (z-score) of sixty NF1 individuals (Fig 4A). Although in general, NF1 individuals have increased osteoclast formation and decreased whole body subtotal BMD, no statistically significant direct correlation existed between the degree of categorically determined osteoclast formation and numerically determined whole body subtotal BMD z-score.

To evaluate bone resorption *in vivo*, urinary pyridinium crosslink concentrations were measured. As previously documented in Stevenson et al. [2008], in which there is an overlap of NF1 participants in this report, there were statistically significant increases in Dpd ($p = 0.015$) and the Dpd/Pyd ratio ($p < 0.0001$) between NF1 individuals and healthy controls. No significant difference in Pyd was observed between the NF1 and healthy controls. To evaluate the correlation of osteoclast formation with urinary pyridinium crosslink concentrations, individuals with NF1 were divided into two groups: NF1 patients with ≥ 2 fold increase in osteoclast formation (higher osteoclast formation) and < 2 fold increase in osteoclast formation (normal osteoclast formation). No statistical differences were found in the overall Pyd concentration among the three groups ($p = 0.271$ for NF1-High osteoclasts vs. Control, $p = 0.603$ for NF1-Normal osteoclast formation group vs. Control, and $p = 0.207$ for NF1-High osteoclast formation group vs. NF1-Normal osteoclast formation group) (Fig 4B). There was an increase in Dpd between the NF1-Normal osteoclast formation group and the control group ($p = 0.034$), but not between the NF1-Normal osteoclast formation group and the NF1-High osteoclast formation group ($p = 0.528$). The increase of the Dpd concentration between the NF1-High formation group and control did not reach statistical significance ($p = 0.132$) (Figure 4C). Individuals with NF1 had an increased ratio of Dpd/Pyd compared to controls ($p < 0.0001$ and $p = 0.018$ for the high and normal osteoclast groups respectively). There were no statistical differences in the Dpd/Pyd ratio between the two osteoclast formation groups among NF1 individuals ($p = 0.38$) (Fig 4D).

NF1 osteoclast precursors show heightened mitogen-activated protein kinase (MAPK) and RhoGTPase activation

Compared to controls, M-CSF-stimulated NF1 osteoclast precursors produced heightened extracellular signal-regulated kinases (ERK) phosphorylation at two minutes and five minutes (representative data from four independent assays, Fig 5A). Consistent with the data

demonstrating increased migration and actin belt formation, NF1 osteoclast precursors demonstrated higher Rac1 phosphorylative capacity (Figure 5B), which is similar to that in murine *Nf1*^{+/-} osteoclasts [Yan et al., 2008].

DISCUSSION

Skeletal abnormalities including reduced BMD are seen in children with NF1 [Illes et al., 2001; Kuorilehto et al., 2005; Lammert et al., 2005; Dulai et al., 2007; Stevenson et al., 2007; Yilmaz et al., 2007]. Osteoclasts derived from *Nf1*^{+/-} mice demonstrate hyperactive physiology *in vitro* and *in vivo* [Yang et al., 2006; Schindeler et al., 2008], and similar to the murine-derived data and data from a cohort of NF1 individuals reported by Heervä, et al. [2010], we show that human NF1 derived osteoclasts demonstrate multiple osteoclast gain-in-functions and biochemical hyperactivities. On a functional level, NF1 osteoclast proliferation, migration, adhesion, and bone resorptive capacity, processes which are critical to bone homeostasis, are increased. In addition, we examined mechanistic alterations on the cellular level, including F-actin polymerization, MAPK phosphorylation, and RhoGTPase activity. These data suggest a potential cellular mechanism contributing to decreased BMD and the focal skeletal dysplasias of NF1.

Blood from individuals with NF1 produces an increase in the number of osteoclasts *in vitro*. These data, while not definitive as *in vivo* evidence, are indicative of an enhanced pool of circulating precursor cells that may proliferate and differentiate to osteoclasts upon cytokine stimulation. Moreover, human NF1 derived osteoclast precursors demonstrate increased incorporation of thymidine in response to M-CSF *in vitro*, a direct measure of cellular proliferative capacity. Interestingly, NF1 osteoclasts examined later on culture day five demonstrate a decreased incorporation of thymidine, indicating rapid progression of NF1 osteoclasts toward terminal differentiation.

Likewise, human NF1 derived osteoclasts demonstrate increased migratory capacity *in vitro*, which may induce increased biological activity at sites of fracture. While M-CSF stimulation of NF1 osteoclasts shows heightened migration in tissue culture, NF1 osteoclasts also show increased adherence to the integrin $\alpha v \beta 3$. Hypothetically, dysregulated migration and increased adhesion may synergistically alter osteoclast-osteoblast interactions at fracture sites, tipping the balance in favor of pathological osteoclastic resorption as evidenced by the increased pit formation *in vitro* in NF1 cultures. The increased bone resorptive activity may contribute to the predisposition to localized pseudarthrosis in some individuals with NF1.

Extending this inquiry, we have mechanistically examined NF1 osteoclast migration and adhesion. Bone resorption by osteoclasts is linked to the migration and adhesion of these cells to a local bone surface. The initiation of bone resorption by osteoclasts is dependent on their ability to bind to the integrin $\alpha v \beta 3$ on the bone surface. Following adhesion, osteoclasts form a specialized cell-extracellular matrix to initiate degradation of bone matrix [Boyle et al., 2003]. The coalescence of multiple small actin-based adhesion structures in osteoclasts creates the podosome, which organizes into progressively larger patterns identified as clusters, rings, and, ultimately, belts. These belts form a sealing zone mediating interactions with the extracellular matrix [Boyle et al., 2003]. NF1 osteoclasts demonstrated increased podosomal belt formation compared to control osteoclasts, suggesting a cellular mechanism through which osteoclast adhesive and degradation activity is enhanced. Importantly, these data coincide with hyperactivity found in NF1 osteoclast MAPK and RhoGTPase signaling, two cross-talking signal transduction pathways critical to cellular proliferation and actin polymerization.

While NF1 individuals have increased osteoclast formation from peripheral blood MNCs, we did not uncover a direct relationship between this increase and whole body subtotal BMD or urinary pyridinium crosslink excretion. These data suggest that this increased osteoclast formation *in vitro*, as measured in this study, cannot directly predict generalized NF1 skeletal phenotypes. Likewise, no direct relationship emerged between patients grouped into specific localized skeletal dysplasia subsets and osteoclast formation. There appears to be a trend towards an increase in osteoclast formation based on severity of scoliosis, however, the number of individuals in each group is small and not significantly higher than individuals without a focal skeletal manifestation. These results suggest that while *NF1* haploinsufficiency produces a generalized osteoclast gain-in-function, specific pathogenesises may require additional and perhaps local modifiers, such as a genetic second-hit. This conjecture is supported by data showing *NF1* loss of heterozygosity in pseudarthrosis tissue [Stevenson et al., 2006]. Additionally, we were able to only compare “osteoclast formation” with the NF1 clinical skeletal phenotype, and it is possible that the other functional assays may directly correlate with the clinical phenotype which will require further investigation. Finally, we note that the majority of the aforementioned assays occurred *in vitro* from cells derived from a precursor pool rather than mature osteoclasts, and that the microenvironment *in vivo* may critically alter osteoclast fate and function. Further studies to elucidate microenvironment interactions critical to local skeletal pathology are needed.

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REFERENCES

- Ajuebor MN, Hogaboam CM, Kunkel SL, Proudfoot AE, Wallace JL. The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat. *J Immunol.* 2001; 166:552–558. [PubMed: 11123336]
- Atit RP, Crowe MJ, Greenhalgh DG, Wenstrup RJ, Ratner N. The *Nf1* tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. *J Invest Dermatol.* 1999; 112:835–842. [PubMed: 10383727]
- Atit RP, Mitchell K, Nguyen L, Warshawsky D, Ratner N. The neurofibromatosis type 1 (*Nf1*) tumor suppressor is a modifier of carcinogen-induced pigmentation and papilloma formation in C57BL/6 mice. *J Invest Dermatol.* 2000; 114:1093–1100. [PubMed: 10844550]
- Aubin JE. Osteoclast adhesion and resorption: the role of podosomes. *J Bone Miner Res.* 1992; 7:365–368. [PubMed: 1609624]
- Bajenaru ML, Hernandez MR, Perry A, Zhu Y, Parada LF, Garbow JR, Gutmann DH. Optic nerve glioma in mice requires astrocyte *Nf1* gene inactivation and *Nf1* brain heterozygosity. *Cancer Res.* 2003; 63:8573–8577. [PubMed: 14695164]
- Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature.* 2003; 423:337–342. [PubMed: 12748652]

- Cichowski K, Jacks T. NF1 tumor suppressor gene function: narrowing the GAP. *Cell*. 2001; 104:593–604. [PubMed: 11239415]
- Clementi M, Milani S, Mammi I, Boni S, Monciotti C, Tenconi R. Neurofibromatosis type 1 growth charts. *Am J Med Genet*. 1999; 87:317–323. [PubMed: 10588837]
- Cormier JM, Cormier F, Mayade F, Fichelle JM. Arterial complications of neurofibromatosis. *J Mal Vasc*. 1999; 24:281–286. [PubMed: 10582177]
- Dulai S, Briody J, Schindeler A, North KN, Cowell CT, Little DG. Decreased bone mineral density in neurofibromatosis type 1: results from a pediatric cohort. *J Pediatr Orthop*. 2007; 27:472–475. [PubMed: 17513973]
- Fossali E, Signorini E, Intermite RC, Casalini E, Lovaria A, Maninetti MM, Rossi LN. Renovascular disease and hypertension in children with neurofibromatosis. *Pediatr Nephrol*. 2000; 14:806–810. [PubMed: 10955932]
- Fujikawa Y, Sabokbar A, Neale SD, Itonaga I, Torisu T, Athanasou NA. The effect of macrophage-colony stimulating factor and other humoral factors (interleukin-1, -3, -6, and -11, tumor necrosis factor-alpha, and granulocyte macrophage-colony stimulating factor) on human osteoclast formation from circulating cells. *Bone*. 2001; 28:261–267. [PubMed: 11248655]
- Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, Rubenstein A, Viskochil D. The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. *JAMA*. 1997; 278:51–57. [PubMed: 9207339]
- Heervä E, Alanne MH, Peltonen S, Kuorilehto T, Hentunen T, Väänänen K, Peltonen J. Osteoclasts in neurofibromatosis type 1 display enhanced resorption capacity, aberrant morphology, and resistance to serum deprivation. *Bone*. 2010; 47:583–590. [PubMed: 20541045]
- Illes T, Halmi V, de Jonge T, Dubousset J. Decreased bone mineral density in neurofibromatosis-1 patients with spinal deformities. *Osteoporos Int*. 2001; 12:823–827. [PubMed: 11716184]
- Ingram DA, Yang FC, Travers JB, Wenning MJ, Hiatt K, New S, Hood A, Shannon K, Williams DA, Clapp DW. Genetic and biochemical evidence that haploinsufficiency of the NF1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J Exp Med*. 2000; 191:181–188. [PubMed: 10620616]
- Johannessen CM, Reczek EE, James MF, Brems H, Legius E, Cichowski K. The NF1 tumor suppressor critically regulates TSC2 and mTOR. *Proc Natl Acad Sci U S A*. 2005; 102:8573–8578. [PubMed: 15937108]
- Kuorilehto T, Poyhonen M, Bloigu R, Heikkinen J, Vaananen K, Peltonen J. Decreased bone mineral density and content in neurofibromatosis type 1: lowest local values are located in the load-carrying parts of the body. *Osteoporos Int*. 2005; 16:928–936. [PubMed: 15551055]
- Kurien A, John PR, Milford DV. Hypertension secondary to progressive vascular neurofibromatosis. *Arch Dis Child*. 1997; 76:454–455. [PubMed: 9196366]
- Lammert M, Kappler M, Mautner VF, Lammert K, Storkel S, Friedman JM, Atkins D. Decreased bone mineral density in patients with neurofibromatosis 1. *Osteoporos Int*. 2005; 16:1161–1166. [PubMed: 15988556]
- Lane NE. Epidemiology, etiology, and diagnosis of osteoporosis. *Am J Obstet Gynecol*. 2006; 194(2 Suppl):S3–11. [PubMed: 16448873]
- McHugh KP, Hodivala-Dilke K, Zheng MH, Namba N, Lam J, Novack D, Feng X, Ross FP, Hynes RO, Teitelbaum SL. Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J Clin Invest*. 2000; 105:433–440. [PubMed: 10683372]
- Ming W, Li S, Billadeau DD, Quilliam LA, Dinauer MC. The Rac effector p67phox regulates phagocyte NADPH oxidase by stimulating Vav1 guanine nucleotide exchange activity. *Mol Cell Biol*. 2007; 27:312–323. [PubMed: 17060455]
- Roberts BJ, Thrall E, Muller JA, Boussein ML. Comparison of hip fracture risk prediction by femoral BMD and by the factor-of-risk for hip fracture derived from direct measurements of femoral strength. *Bone*. 2010; 46:742–746. [PubMed: 19854307]
- Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E. Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. *Hum Mol Genet*. 2009; 18:1059–1066.
- Sanjay A, Houghton A, Neff L, DiDomenico E, Bardelay C, Antoine E, Levy J, Gailit J, Bowtell D, Horne WC, Baron R. Cbl associates with Pyk2 and Src to regulate Src kinase activity,

- alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. *J Cell Biol.* 2001; 152:181–195. [PubMed: 11149930]
- Schindeler A, Ramachandran M, Godfrey C, Morse A, McDonald M, Mikulec K, Little DG. Modeling bone morphogenetic protein and bisphosphonate combination therapy in wild-type and Nf1 haploinsufficient mice. *J Orthop Res.* 2008; 26:65–74. [PubMed: 17787010]
- Stevenson DA, Moyer-Mileur LJ, Murray M, Slater H, Sheng X, Carey JC, Dube B, Viskochil DH. Bone mineral density in children and adolescents with neurofibromatosis type 1. *J Pediatr.* 2007; 150:83–88. [PubMed: 17188620]
- Stevenson DA, Schwarz EL, Viskochil DH, Moyer-Mileur LJ, Murray M, Firth SD, D'Astous JL, Carey JC, Pasquali M. Evidence of increased bone resorption in neurofibromatosis type 1 using urinary pyridinium crosslink analysis. *Pediatr Res.* 2008; 2008; 63:697–701. [PubMed: 18317233]
- Stevenson DA, Zhou H, Ashrafi S, Messiaen LM, Carey JC, D'Astous JL, Santora SD, Viskochil DH. Double inactivation of NF1 in tibial pseudarthrosis. *Am J Hum Genet.* 2006; 79:143–148. [PubMed: 16773574]
- Stumpf DA, Alksne JF, Annegers JF, Brown SS, Conneally PM, Housman D. Neurofibromatosis. Conference statement. National Institutes of Health Consensus Development Conference. *Arch Neurol.* 1988; 45:575–578. [PubMed: 3128965]
- Sun Y, Buki KG, Ettala O, Vaaraniemi JP, Vaananen HK. Possible role of direct Rac1-Rab7 interaction in ruffled border formation of osteoclasts. *J Biol Chem.* 2005; 280:32356–32361. [PubMed: 16040606]
- Takeshita S, Namba N, Zhao JJ, Jiang Y, Genant HK, Silva MJ, Brodt MD, Helgason CD, Kalesnikoff J, Rauh MJ, Humphries RK, Krystal G, Teitelbaum SL, Ross FP. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. *Nat Med.* 2002; 8:943–949. [PubMed: 12161749]
- Tucker T, Schnabel C, Hartmann M, Friedrich RE, Frieling I, Kruse HP, Mautner VF, Friedman JM. Bone health and fracture rate in individuals with neurofibromatosis 1 (NF1). *J Med Genet.* 2009; 46:259–265. [PubMed: 19066167]
- Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain J, Brereton A, Nicholson J, Mitchell A, Brownstein B, Collins F. Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science.* 1990; 249(4965):181–186. [PubMed: 2134734]
- Wu X, Estwick SA, Chen S, Yu M, Ming W, Nebesio TD, Li Y, Yuan J, Kapur R, Ingram D, Yoder MC, Yang FC. Neurofibromin plays a critical role in modulating osteoblast differentiation of mesenchymal stem/progenitor cells. *Hum Mol Genet.* 2006; 15:2837–2845. [PubMed: 16893911]
- Yan J, Chen S, Zhang Y, Li X, Li Y, Wu X, Yuan J, Robling AG, Kapur R, Chan RJ, Yang FC. Rac1 mediates the osteoclast gains-in-function induced by haploinsufficiency of Nf1. *Hum Mol Genet.* 2008; 17:936–948. [PubMed: 18089636]
- Yang FC, Chen S, Robling AG, Yu X, Nebesio TD, Yan J, Morgan T, Li X, Yuan J, Hock J, Ingram DA, Clapp DW. Hyperactivation of p21 and PI3K cooperate to alter murine and human neurofibromatosis type 1-haploinsufficient osteoclast functions. *J Clin Invest.* 2006; 116:2880–2891. [PubMed: 17053831]
- Yang FC, Ingram DA, Chen S, Hingtgen CM, Ratner N, Monk KR, Clegg T, White H, Mead L, Wenning MJ, Williams DA, Kapur R, Atkinson SJ, Clapp DW. Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for Nf1+/- mast cells. *J Clin Invest.* 2003; 112:1851–1861. [PubMed: 14679180]
- Yang FC, Kapur R, King AJ, Tao W, Kim C, Borneo J, Breese R, Marshall M, Dinuer MC, Williams DA. Rac2 stimulates Akt activation affecting BAD/Bcl-XL expression while mediating survival and actin function in primary mast cells. *Immunity.* 2000; 12:557–68. [PubMed: 10843388]
- Yilmaz K, Ozmen M, Bora Goksan S, Eskiuyurt N. Bone mineral density in children with neurofibromatosis 1. *Acta Paediatr.* 2007; 96:1220–1222. [PubMed: 17608828]
- Zhang YY, Vik TA, Ryder JW, Srour EF, Jacks T, Shannon K, Clapp DW. Nf1 regulates hematopoietic progenitor cell growth and ras signaling in response to multiple cytokines. *J Exp Med.* 1998; 187:1893–1902. [PubMed: 9607929]

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. *Science*. 2002; 296:920–922. [PubMed: 11988578]

Figure 1. Increased osteoclast differentiation and increased macrophage proliferation of human neurofibromatosis type 1 (NF1) mononuclear cells (MNCs) in response to M-CSF
(A) Representative photomicrographs (magnification, 20) from each group are shown, indicating an increase number and larger area of multinuclear osteoclasts. **(B)** Fold increase of area of multinuclear osteoclasts from culture of 5×10^4 MNCs following M-CSF and RANKL stimulation for 7 days. Data represent the mean $\pm$ SEM for 75 individuals with NF1 compared to 39 unaffected controls. $P < 0.0001$, osteoclasts from NF1 individuals versus unaffected controls. **(C)** After previous culture in the presence of M-CSF for 3 days, MNCs (5×10^4 cells) were starved in 0.2% BSA without M-CSF for six hours and then plated in a 96-well plate in 200 μ l α -MEM containing 10% FBS with or without 30ng/mL M-CSF for 48 hours. They were subsequently pulsed with 1.0 μ Ci of [3 H] thymidine for 6 hours. Cells were then harvested using an automated 96-well cell harvester (Brandel, Gaithersburg, MD) and [3 H] thymidine incorporation was enumerated as counts/minute (CPM). Data represent the mean $\pm$ SEM for 4 individuals with NF1 and 4 unaffected controls. * $P < 0.01$, osteoclasts from NF1 patients versus unaffected controls in the presence of M-CSF.

Figure 2. Osteoclasts from individuals with neurofibromatosis type 1 (NF1) have increased bone resorption

(A) Quantitative evaluation of bone resorption. MNCs were incubated in the presence of M-CSF and RANKL for 4 days and then cells placed on dentine slices and cultured for an additional 3 days and then stained with toluidine blue at the end of culture. The area of pit (% of field) on dentine slides was measured. Results represent the mean area $\pm$ SEM of 5 independent experiments. * $P < 0.01$ for bone resorption of controls versus NF1 individuals.

(B) Representative photomicrographs (magnification, $\times 10$) of each group are shown. The resorbed bone area appears dark purple (stained with 1% toluidine blue).

Figure 3. Increased migration, adhesion, and actin polymerization in neurofibromatosis type 1 (NF1) osteoclast precursors

(A) Pre-osteoclast migration quantitative analysis of NF1 individuals and controls comparing mean number of pre-osteoclasts ($\pm$ SEM) per high power field (HPF). Six fields were scored for each patient. Experiment conducted on eight independent occasions. $*P < 0.05$ for macrophage migration of controls versus NF1 individuals. (B) Representative photomicrograph of the migrating osteoclast progenitors through an 8 μ m polycarbonate filter coated with vitronectin in response to α -MEM, 0.1% bovine serum albumin (BSA), and M-CSF (30ng/ml). (C) Quantitative evaluation (mean $\pm$ SEM) of osteoclast adhesion 30 minutes following incubation with M-CSF in NF1 (n=5) vs. control (n=5) individuals. Triplicates were conducted for each condition. $*P < 0.05$, NF1 versus control macrophages by Student's *t* test. (D) Percent area of belt forming cells per field from 5 fields of each group was quantitated morphologically. Experiment conducted on ten independent occasions. $*P < 0.01$ for controls vs. NF1. (E) Representative photomicrograph of osteoclasts following staining with FITC-conjugated phalloidin at 10X and 40X magnification, respectively. Yellow arrows indicate the belt forming cells.

Figure 4. No direct correlation between osteoclast formation and bone mineral density (BMD) or pyridinium crosslink concentration in individuals with neurofibromatosis type 1 (NF1)
(A) Fold changes of osteoclast formation of sixty NF1 patients over controls are shown on the left side of the Y-axis, whereas, the right side of the Y axis shows the whole body subtotal BMD (Z-score). Each line connects the fold changes of osteoclast formation on the left side with the whole body subtotal BMD on the right side. Comparison of urinary pyridinoline (Pyd) (B), deoxypyridinoline (Dpd) (C), and the Dpd/Pyd ratio (D) between individuals with NF1 with or without hyper osteoclast formation. Black dots indicate healthy controls, red triangles indicate the NF1 patients with hyper osteoclast formation (≥ 2 fold increase than controls), and blue squares indicate the NF1 patients with less than 2 fold increase of osteoclast formation.

Figure 5. MAPK/Rac1 GTPase is hyperactivated in pre-osteoclasts from individuals with neurofibromatosis type 1

(A) Erk phosphorylation was measured at the indicated levels following stimulation with M-CSF (10ng/ml). A representative Western blot from four independent occasions is shown.

(B) Rac1 total protein and Rac1 GTPase activation was examined at baseline and 1 minute following M-CSF (10ng/ml) stimulation in osteoclast progenitors. Data represent three independent occasions.

Fold change of osteoclast formation in individuals with NF1 based on skeletal phenotype over controls

Table 1

Disease Type	Long Bone Dysplasia	Sphenoid Wing Dysplasia	Scoliosis		Non-skeletal Manifestation
			I	II	III
Mean±SE	1.9±0.2	1.8±0.6	1.6±0.3	2.1±0.4	2.3±0.2
n	12	3	10	3	6
					46

Data show osteoclast formation in individuals with NF1 with different types of skeletal manifestations over control groups. P<0.01 when comparing controls with all NF1 groups using ANOVA. N represents case numbers in each skeletal manifestation group (some individuals had more than one manifestation). Individuals were not included if the presence of skeletal manifestations was not known. NF1 individuals with scoliosis were divided into three groups according to the degree of scoliosis [Group I: degree curve ≥9 but ≤20 degrees; Group II: degree curve >20 degrees; Group III: severe scoliosis requiring surgery].