Maximizing Bone Formation in Posterior Spine Fusion Using rhBMP-2 and Zoledronic Acid in Wild Type and NF1 Deficient Mice

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ABSTRACT: Spinal pseudarthrosis is a well described complication of spine fusion surgery in NF1 patients. Reduced bone formation and excessive resorption have been described in NF1 and anti-resorptive agents may be advantageous in these individuals. In this study, 16 wild type and 16 $Nf1^{+/-}$ mice were subjected to posterolateral fusion using collagen sponges containing 5 μg rhBMP-2 introduced bilaterally. Mice were dosed twice weekly with 0.02 mg/kg zoledronic acid (ZA) or sterile saline. The fusion mass was assessed for bone volume (BV) and bone mineral density (BMD) by microCT. Co-treatment using rhBMP-2 and ZA produced a significant increase ($p < 0.01$) in BV of the fusion mass compared to rhBMP-2 alone in both wild type mice (+229%) and $Nf1^{+/-}$ mice (+174%). Co-treatment also produced a significantly higher total BMD of the fusion mass compared to rhBMP-2 alone in both groups ($p < 0.01$). Despite these gains with anti-resorptive treatment, $Nf1^{+/-}$ deficient mice still generated less bone than wild type controls. TRAP staining on histological sections indicated an increased osteoclast surface/bone surface (Oc.S/BS) in $Nf1^{+/-}$ mice relative to wild type mice, and this was reduced with ZA treatment. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1090–1094, 2014.

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Neurofibromatosis type 1 (NF1) is a genetic disease with an incidence of 1 in 3,000 that results from mutations in the *NF1* gene.[1] *NF1* is expressed in many tissues and its deficiency is associated with a range of characteristic features that present with variable penetrance. Orthopedic manifestations are one of the standard diagnostic criteria for NF1. As many as 25% of individuals will go on to develop some degree of scoliosis.[2,3] Spine fusion surgery to correct the curvature and prevent future deformity is not uncommon, however with NF1 this can have an increased complication rate.[4] Pseudarthrosis at the fusion site may develop in as many as 25% of NF1 cases; in these incidents the adjacent vertebrae are united by fibrous tissue rather than with a solid bone bridge.[5] Solid fusion is seen in as few as 7% of cases.[6]

These poor surgical outcomes are likely due to primary defects in NF1 bone metabolism. Up to 30% of adolescents and adults with NF1 show reduced bone mineral density (BMD),[7,8] likely related to reduced bone formation and increased bone resorption.[9] Moreover, poor bone healing is observed in murine models of NF1-deficient and NF1-null fracture repair, and these feature delayed union and pseudarthrosis, respectively.[10,11] Orthopedic models have likewise shown a bone anabolic deficiency as well as excess resorption from abundant osteoclasts.[9] Consequently, combination therapies using recombinant human bone morphogenetic proteins (rhBMPs) together with bisphosphonates have been proposed for the treatment of tibial pseudarthrosis. This strategy is supported by both genetic mouse models of NF1[12,13] and a published case series of NF1 tibial pseudarthrosis patients.[14]

Currently therapeutic development for NF1 spine fusion is hampered by a lack of an appropriate preclinical model to test interventions. Such a system requires both a genetic model of NF1 deficiency as well as an established protocol for spine fusion surgery. We have previously described a novel protocol for spine fusion in mice which we have shown to be safe, reliable and reproducible.[15] This model utilizes rhBMP-2 delivered by surgically implanted collagen sponges to induce a posterolateral fusion. In this study we describe the application of this surgical model to *Nf1* deficient mice ($Nf1^{+/-}$) with and without systemic bisphosphonate treatment. It was hypothesized that bisphosphonate treatment would maximize the retention of rhBMP-2 induced bone yielding a superior fusion outcome as determined by bone volume (BV) and density.

MATERIALS AND METHODS

Nf1 Gene Knockout Mice

Nf1 knockout mice were a gift from L. Parada (UT Southwestern, Dallas, TX).[16] This mouse line possesses no tissue specificity in its *Nf1* deficiency. The mouse line was maintained on a C57BL/6J background and genotyping was performed using a PCR-based method. $Nf1^{+/-}$ heterozygous knockout mice aged 8–10 weeks were compared with wild-type ($Nf1^{+/+}$) littermate controls. Female mice were used for all experiments. All animal experiments were approved by the local institutional ethics committee.

Study Design

Sixteen wild type mice and 16 $Nf1^{+/-}$ mice were used for the experiment. All mice underwent surgery for posterolateral fusion using bilateral paravertebral acellular collagen sponges (ACS, Medronic Australasia, North Ryde, NSW, Australia), each loaded with 5 μg rhBMP-2. This dose was specifically chosen to represent the lower limit of the dose

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used in pediatric surgery (4.2 mg in a 40 kg individual). For each genotype, $n = 8$ were dosed twice weekly with saline and $n = 8$ with five doses of the bisphosphonate zoledronic acid (ZA, Novartis AG, Basel, Switzerland). ZA was given at 0.02 mg/kg (total dose 0.1 mg/kg) by biweekly subcutaneous injection commencing 3 days postoperatively. Mice were culled at 3 weeks and spines harvested for radiographic and histological analyses.

Surgery

The surgical technique was performed with the intention of fusing the lumbar spine from L4 to L6 using a published technique[15] modified from prior approaches in rodents.[17,18] Surgery was performed on mice aged between 8 and 10 weeks old by two surgeons in a single sitting with the aid of a trained animal technician. Anesthesia, preoperative prep, and postoperative observation were performed by the animal technician. The procedure was performed under an operating microscope at 2.5× magnification. A 15 mm incision was made in the skin along the midline, centered over a line running between the iliac crests. The skin was retracted and held with a self-retaining retractor. The paravertebral muscles overlying the articular processes of L4-L6 were separated from the spinal column by scraping a 10 mm blade down the lateral border of the spinous process and pulling the muscles laterally. A pneumatic 1 mm round tip diamond burr was used to decorticate the visible articular processes until punctate bleeding was observed. Two collagen sponges, impregnated with either saline or a BMP/saline solution were placed bilaterally adjacent to the decorticated bone. The fascia was closed with a single line of continuous sutures using 6.0 Vicryl, and the skin was subsequently closed in the same fashion. Mice were placed on a heat pad following surgery and monitored for recovery. Antibiotics were administered in the drinking water for the duration of the experiment.

Radiography

Bone volume (BV; mm^3) and total bone mineral content (TMD; mg/mm^3) of the fusion mass were measured by microCT using software that encompassed the fusion mass within a uniform region of interest (ROI). MicroCT data was acquired using a SkyScan 1174 compact microCT scanner (SkyScan, Kontich, Belgium). Samples were scanned in 70% ethanol at 21.3 μm magnification, 0.5 mm aluminum filter, 50 kV X-ray tube voltage and 800 μA tube electric current. The images were reconstructed using NRecon, version 1.5.1.5 (SkyScan). The bone of the fusion mass was isolated from native bone by means of manually drawn ROI and thresholded using CTAn software version 1.10.03. A global threshold of $0.3\,g/mm^3$ was set for bone tissue. Representative three-dimensional fractures were reconstructed with sagittal slices using CTVol Realistic Visualization software version 2.1.0.0 (SkyScan).

Histology

Harvested vertebrae and surrounding musculature were fixed in 4% paraformaldehyde for 24 h at 4°C on a shaker and were then stored in 70% ethanol until decalcification. Samples were decalcified in 0.34 M EDTA (pH 8.0) solution at 4°C on a shaker for 30 days with solution changes every 2–3 days. Spines were cut axially and the central 5 mm of the fusion mass were isolated and embedded in paraffin. Axial sections were taken through the center of the fusion mass at a thickness of 5 μm. Mounted sections were stained via Picro Sirius Red/Alcian Blue to differentiate bone and cartilage. Adjacent sections were stained for tartrate-resistant acid phosphatase (TRAP) expression to highlight osteoclasts, and counterstained with light green. Bone volume/trabecular volume (BV/TV) for the heterotopic bone and osteoclast number (OcN) and surface area (OcS) was calculated relative to bone surface (BS) in mm using Bioquant software.

Statistical Analyses

The volume and density of bone present at 3 weeks was compared between $Nf1^{+/-}$ and wild type groups and between groups receiving ZA and saline. Statistical analysis and graphing were performed using Graphpad Prism. The influences of genotype and bisphosphonate treatment were examined by non-parametric Kruskal Wallis/Mann–Whitney *U*-tests between the relevant groups with a significance cut-off of 0.05. Error bars on graphs represent the 95% confidence intervals.

RESULTS

$Nf1^{+/-}$ Mice and Wild Type Mice Develop a Robust Fusion Mass in Response to rhBMP-2

Posterolateral inter-transverse spine fusion surgery was performed on 32 mice and, consistent with prior applications of the model,[15] there were no cases of morbidity or mortality. Two surgeons working in tandem were able to complete an operation in ~20 min. Animals showed no post-surgical complications or adverse reactions to bisphosphonate treatment. On examination at 3 weeks, a large, bony fusion mass was palpable, and occasionally visible overlying the lumbar vertebrae. Mice did not display any discomfort or neurological impairment as a result of the overwhelming bone formation, as can occur in human patients.[19]

MicroCT images of harvested mouse vertebrae obtained at 3 weeks were reconstructed from all spines recovered from both wild type and $Nf1^{+/-}$ mice (Fig. 1). Analysis of the total BV of the fusion masses showed a −11% decrease in the BV and density of fusion masses of $Nf1^{+/-}$ mice relative to wild type controls ($p = 0.08$). While this did not reach significance, post-hoc power analysis indicated $\beta = 0.34$ suggestive of significance with a larger sample size. Notably, the effect size for *Nf1* genotype influencing spine fusion mass was less than that previously reported for rhBMP-2 induced ectopic bone nodules.[13]

Evaluation of the Effects of ZA Co-Administration in Wild Type and $Nf1^{+/-}$ Mice

Co-treatment with rhBMP-2 and systemic ZA was examined in the spines of wild type and $Nf1^{+/-}$ mice. Radiography (XR and microCT) obtained at 3 weeks postoperatively showed that co-treatment with ZA led to a more radio-dense fusion mass. The size of the fusion mass, however, remained unchanged. Both the wild type and $Nf1^{+/-}$ mice showed significant improvements in fusion with ZA treatment.

Quantification of BV by microCT showed a +226% increase (wild type) and +174% increase ($Nf1^{+/-}$) ($p < 0.01$, Fig. 2A). In addition, ZA treatment resulted

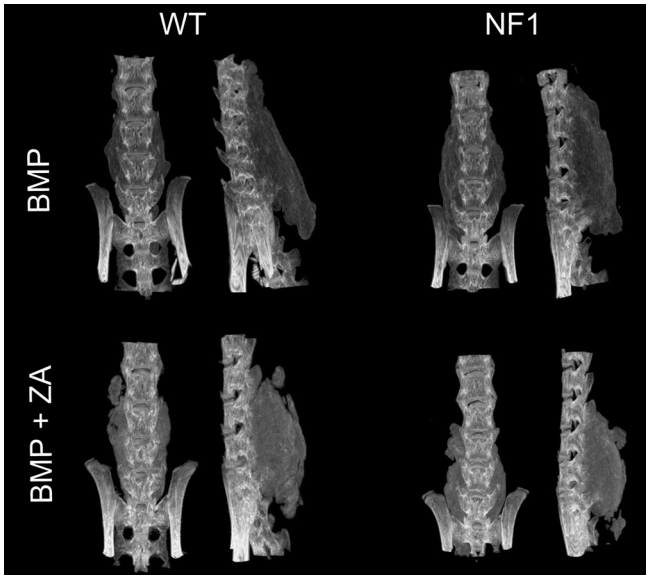

Figure 1. MicroCT reconstructions of harvested spines. ZA treatment led to an increase in the opacity of the rhBMP-2 induced fusion mass relative to controls. Spine fusion was achieved in all samples.

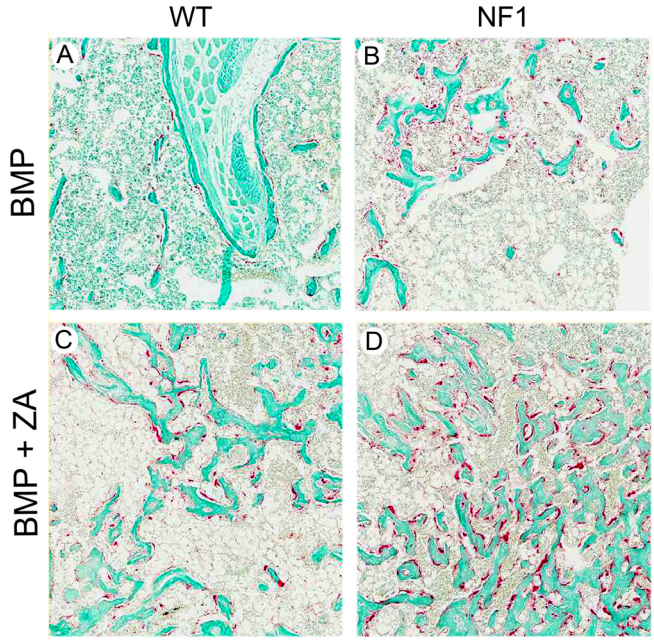

Figure 3. Transverse sections of fusion masses with a TRAP stain, counterstained with fast green.

in a consistent 1% increase in BMD that was statistically significant ($p < 0.01$ for WT and $Nf1^{+/-}$ groups, Fig. 2B). Despite the substantive increases in net bone with ZA treatment, $Nf1^{+/-}$ animals showed a −20% reduction in bone compared to wild type controls ($p = 0.02$).

ZA Treatment Reduces the Resorption of rhBMP-2 Induced Bone in the Fusion Mass of $Nf1^{+/-}$ and Wild Type Mice

Transverse sections were taken from the widest level of the fusion mass and used for osteoclast quantitation (Fig. 3). Osteoclast surface area (OcS) and BS area were calculated via Bioquant software. Analysis revealed an increase in the OcS to BS area ratio (OcS/BS) in $Nf1^{+/-}$ samples relative to wild type controls ($p = 0.05$).

Co-treatment with rhBMP-2 and ZA treatment led to a significant reduction in OcS/BS in both wild type mice ($p < 0.01$) and $Nf1^{+/-}$ mice ($p < 0.01$) with respect to animals treated exclusively with rhBMP-2 (Fig. 4). The decreases in osteoclast surface with ZA treatment were consistent with the increases in net bone identified by radiography. With ZA treatment there was no difference between wild type and $Nf1^{+/-}$ mice with respect to OcS/BS ($p = 0.28$).

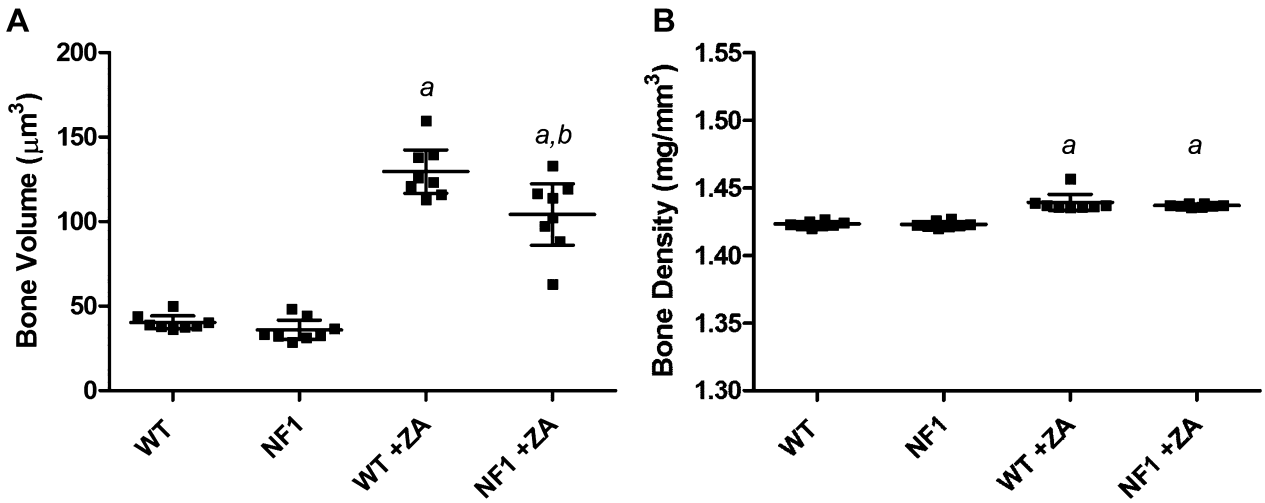

Figure 2. MicroCT assessment of bone volume (A) and bone mineral density (B). BV was significantly increased with ZA treatment (a, $p < 0.01$) and in the ZA treated groups significantly less bone was associated with the $Nf1^{+/-}$ genotype (b, $p = 0.02$). Likewise, BMD was significantly increased with ZA treatment (a, $p < 0.01$) relative to untreated controls of the same genotype.

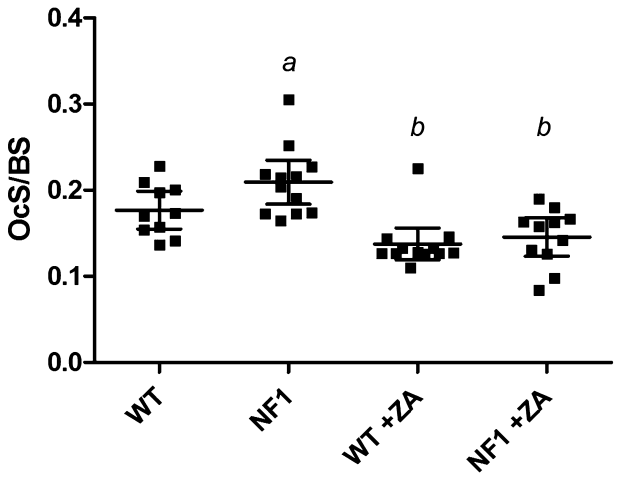

Figure 4. Osteoclast surface relative to bone surface was quantified from tissue sections. Without ZA treatment, $Nf1^{+/-}$ mice showed an increase in OcS/BS relative to wild type controls (a, $p = 0.05$). Treatment with ZA led to significant decreases in OcS/BS for both genotypes (b, $p < 0.01$).

DISCUSSION

The bone phenotype of NF1 is characterized by excessive resorption and decreased osteoblast activity.[9,20,21] This poses a significant challenge in the context of spine fusion surgery, which is commonly used to correct the scoliosis that is associated with the disease. Although an $Nf1^{+/-}$ mouse model has been previously studied in the context of tibial fracture repair,[10] investigation of spine fusion therapies has been limited by the unavailability of a suitable model of posterolateral spine fusion. We have previously described a novel approach for mouse lumbar spine fusion that is safe, efficient, highly reproducible, and analogous to contemporary human surgery.[15] By applying this model to $Nf1^{+/-}$ we have demonstrated that dual treatment with rhBMP-2 and ZA is superior to treatment with rhBMP-2 alone. The combination of rhBMP and bisphosphonate treatment has been previously applied in the context of NF1 pseudarthrosis,[12,14] but this approach has also been shown to be generally effective in other orthopedic models.[22,23]

This study confirms that at a dose of 5 μg bilaterally, rhBMP-2 is able to reliably and reproducibly promote the formation of a large multilevel fusion mass in the lumbar spine in both wild type and $Nf1^{+/-}$ mice. Thus this model does not recapitulate the poor response to rhBMP and complications associated with spine fusion associated with NF1. While the differences in BV between $Nf1^{+/-}$ mice and wild type controls approached significance, the effect size was less than predicted based on prior studies[13] and did not reach significance with the sample size employed.

We propose two possible factors that may have contributed to this lack of difference. First, this could reflect species-dependent effects between humans and mice and may indicate an intrinsic limitation of this model system. Deletion of a single *Nf1* allele has been reported to be less impactful in mice than in individuals with NF1. The $Nf1^{+/-}$ mice do not show reduced bone density or develop spontaneous delayed or non-unions comparable to a clinical NF1 congenital pseudarthrosis.[10,24] We have recently utilized a model of localized double inactivation of *Nf1* in the tibia to model tibial pseudarthrosis[11] and it is possible that such a localized genetic approach could be developed for the spine.

Second, it is possible that employing lower doses of rhBMP-2 could lead to more profound differences in the fusion rate of wild type and $Nf1^{+/-}$ mice. The selected dose of 5 μg per side was comparable to other murine ectopic bone formation studies that model the clinical application of rhBMPs, [25–27] and human patients, particularly those with NF1, typically receive large doses of rhBMPs in order to stimulate a maximal response.[28] Comparable doses were used in ectopic bone studies that showed a difference between wild type and $Nf1^{+/-}$ mice,[13] but use of rhBMPs at a bony location can stimulate a greater response than in an ectopic/intramuscular location.

The addition of the bisphosphonate ZA, a known modulator of bone remodeling, effectively shifted the equilibrium of bone homeostasis in favor of bone formation by inhibiting resorption. The net effect was a measurable and significant increase in fusion mass BV and density when compared to controls receiving rhBMP-2 alone. Notably, a significant difference in fusion mass BV was observed between wild type and $Nf1^{+/-}$ mice treated with ZA. This implies that *Nf1* deficiency is associated with a *bona fide* anabolic deficiency in this mouse model. Nevertheless, inhibition of resorption led to considerable (+174%) increases in bone.

Translation of this concept could yield increased fusion success rates in the treatment of scoliosis in NF1 patients, reducing the incidence of pseudarthrosis which is a source of great morbidity in this population. Additionally, the robust fusion masses seen in wild type mice dosed with ZA suggest that uncomplicated cases of spine fusion may also benefit from bisphosphonate therapy. As an approved anti-resorptive drug, ZA is a candidate for translational application.

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