Bone 49 (2011) 1311–1316

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

JNK inhibitors increase osteogenesis in Nf1-deficient cells

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ARTICLE INFO

Article history:
Received 18 July 2011
Revised 13 September 2011
Accepted 14 September 2011
Available online 22 September 2011

Edited by T. Jack Martin

Keywords:
Neurofibromatosis type 1
NF1
Bone morphogenetic protein
Mitogen-activated protein kinase
c-Jun-N-terminal kinase inhibitor

ABSTRACT

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder that is associated with a variety of manifestations, including orthopedic complications such as scoliosis and tibial pseudarthrosis. Orthopedic management of these skeletal complications is rendered more challenging due to a lack of standardized adjunctive pharmacotherapies. NF1 leads to disruption of the canonical Ras/Raf-1/MEK/ERK axis, and this has been associated with defects in bone anabolism. The roles of other non-canonical Ras effector pathways, such as the c-Jun N-terminal Kinase (JNK) pathway, are less well understood. In this study we examine the effects of an anthrapyrazolone inhibitor of JNK (SP600125) on inducible osteoprogenitors as well as *Nf1*-deficient and *Nf1*-null primary osteoblasts.

C2C12 cells, which are highly responsive to rhBMP-2, were examined with exogenous rhBMP-2 and a range of SP600125 doses. Based on the expression of early and late bone markers and matrix mineralization, 10 μM SP600125 was found to be pro-osteogenic whether delivered concurrent with or following 2 days of rhBMP-2 treatment. Aberrant JNK activity was identified in *Nf1*-deficient osteoprogenitors (increased rhBMP-2 induced phospho-c-Jun) and in *Nf1*-null mature osteoblasts (increased total c-Jun). Next, SP600125 was used to treat these cells and was found to facilitate osteogenesis in *Nf1*-deficient osteoprogenitors, and in *Nf1*-null osteoblasts when given in conjunction with rhBMP-2. Outcome measures included alkaline phosphatase activity, matrix mineralization, and osteogenic gene expression. In summary, JNK inhibitors represent a class of potentially useful adjunctive agents for orthopedic medicine, particularly in the context of NF1.

Introduction

Neurofibromatosis Type 1 (NF1) is a genetic disorder that affects approximately 1:3000 individuals. The NF1 disease state is associated with a range of clinical manifestations but orthopedic complications such as congenital tibial dysplasia and progressive scoliosis can be among the challenging to manage and can significantly impair the patients' quality of life. Even with bracing and conservative management, fracture is usually inevitable and the resulting deficient bone repair typically results in a pseudarthrosis. Pre-clinical studies have suggested that deficiencies in bone anabolism [1] and excessive bone catabolism [2] may be the underlying cause of the pathology.

Historically, orthopedic treatment of a pseudarthrosis focused primarily on achieving stable fixation, removing any aberrant fibrotic tissue, and providing an anabolic stimulus. One generalized approach to improving repair involves conjunctive treatment with an anti-resorptive drug, such as a bisphosphonate. In a preclinical mouse model of NF1 that shows defective bone repair [1], combination therapy with rhBMP-2 and bisphosphonate led to improved healing [3]. However, this represents a broad approach that does not target the specific signaling deficiencies associated with the NF1 state.

The *NF1* gene encodes the protein neurofibromin, a Ras GTPase [4] that is expressed in a variety of cell types including osteoblasts and osteoclasts [5]. NF1 deficiency is associated impaired osteogenesis as well as increased osteoclast differentiation and function [2,6]. While disruption to the canonical Ras-Raf-1/MEK/ERK axis is the normal mechanism attributed to decreased anabolism [7], phosphatidylinositol 3 phosphate kinase (PI3-K) signaling has been linked to the oversensitivity of the osteoclasts [2]. The role of other non-canonical Ras effecter pathways in mediating the poor bone healing seen in NF1 is unclear.

A recent study identified increased JNK activity in murine $Nf1^{+/-}$ microglia [8]. Treatment with the JNK inhibitor SP600125 ameliorated the phenotype in $Nf1^{+/-}$ microglial cells and in an $Nf1^{+/-}$ mouse model of optic glioma. The relative activity of the JNK pathway and its role in regulating the NF1 bone phenotype has not yet been investigated. However, should the JNK pathway modulate the bone effects, this could represent a new and targeted pathway for therapeutic intervention.

To investigate the importance of JNK signaling in bone, we examined cells isolated from wild type ($Nf1^{+/+}$) mice, *Nf1*-deficient mice ($Nf1^{+/-}$), and *Nf1* null ($Nf1^{null}$) cells generated by treating primary cells from $Nf1^{flox/flox}$ mice with a Cre-expressing adenovirus. JNK

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doi:10.1016/j.bone.2011.09.043

activity based on c-Jun phosphorylation and MAPK activity was assayed by western blot following growth factor stimulation with rhBMP-2. The effects of the JNK inhibitor SP600125 on immortalized inducible osteoprogenitors, as well as primary *Nf1*$^{+/+}$, *Nf1*$^{+/-}$, and *Nf1*^{null} osteoblasts from mice were examined in culture, with and without exogenous rhBMP-2 treatment. Functional outcome measurements included enzymatic assays, matrix mineralization and genetic bone markers. We hypothesized that JNK activity would be overactive in NF1-deficient cells, and that functional osteoblast deficiencies in these cells could be overcome with a specific JNK inhibitor.

Materials and methods

Animal models

Heterozygous Nf1-knockout mice (*Nf1*$^{+/-}$) ubiquitously deficient for one allele were sourced from L. Parada (UT Southwestern, Dallas, TX) and were maintained by backcrossing to wild type C57BL/6J mice [9]. The conditional *Nf1* line (*Nf1*$^{flox/flox}$) generated by Prof Luis Parada was obtained from the National Cancer Institute (NCI) mouse repository (Bethesda, MD, United States) [10]. All animal experiments were approved by the Southwest Area Health Service (SWAHS) Animal Ethics Committee. Genotyping was performed using a PCR-based method to differentiate between heterozygous and wild-type animals.

Cell culture

C2C12 myoblasts are an immortalized myoblastic cell line that is highly sensitive to bone morphogenetic protein stimulation and subsequent osteogenic stimulation [11]. C2C12s were cultured in Dulbecco's modified eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) (Invitrogen). For experimental work, C2C12 cells were seeded on collagen coated plates at a density of 2×10^4 cells/well in 48-well plates and 1.5×10^5 in 6-well plates. BMSCs were seeded at a density of 1×10^5 cells/well in 48-well plates and 2.5×10^5 cells/well in 6-well plates without collagen-coating. Osteogenic differentiation was induced by culturing cells in osteogenic media containing α-MEM, 10% FBS and 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate.

Primary bone marrow stromal cells (BMSCs) were selected as an inducible osteoprogenitor population. Primary BMSCs were obtained from 8 to 12 week old *Nf1*$^{+/-}$ mice and *Nf1*$^{+/+}$ (wild type) littermates. Cells were isolated from the femoral medullary canal by flushing with DMEM using a 21-gauge needle. Cells adherent after 5 days of culture in DMEM with 10% FBS were passaged once for subsequent experiments [12]. Drug interventions were performed in multi-well plates after cells had reached at least 80% confluence in the osteogenic media previously described.

Primary neonatal calvarial cells were examined as a committed osteoprogenitor population. Cells were isolated from *Nf1*$^{flox/flox}$ neonates within 1 week of birth using a modified version of our previously published methodology [13]. In brief, mice were decapitated and the calvaria stripped of all fibrous tissue and washed with PBS. Bones were digested three times for 15 min in Collagenase D (Roche) at 1 mg/ml in α-minimum essential medium (α-MEM) (Invitrogen) at 37 °C. Isolated cells were grown in 10% FBS in α-MEM for 3 to 5 days. All cells were cultured with antibiotics consisting of 100 unit/ml penicillin and 0.1 mg/ml streptomycin and grown in a humidified incubator at 37 °C with 5% CO_2. To generate *Nf1*^{null} cells *in vitro*, *Nf1*$^{flox/flox}$ calvarial cells were treated with Adeno-Cre virus. Cells were seeded at 1×10^5 cells/well of a 6-well plate and transduced with Adeno-Cre virus and Adeno-GFP control viruses (1×10^7 infectious units/ml) (provided by Dr. Gajewski, University of Chicago, USA and Dr. Alexander, Children's Medical Research Institute, Sydney, Australia, respectively) using 30 μl GeneJammer (Stratagene) in α-MEM with 5% FBS. Genejammer is a transfection reagent that has been previously reported to improve adenoviral transduction efficiency [14]. Cells were serum starved for 4 h prior to the 18 hour transduction phase. The previously described osteogenic media was used during drug treatments.

Recombinant proteins and drugs

Cells were cultured with 0, 50, or 200 ng/ml rhBMP-2 (Medtronic), with the media changed every third day. SP600125 inhibitor (Sigma-Aldrich) was trialed at a concentration range of 0–50 μM. DMSO vehicle concentrations were less than 0.1% (v/v) media and consistent in all wells to avoid potentially confounding pro-osteogenic effects of DMSO [15].

Cell viability and alkaline phosphatase (AP) activity assays

Alkaline phosphatase (AP) activity was quantified using *p*-nitrophenylphosphate (*p*NPP) (Sigma-Aldrich) enzymatic assays and normalized to viable cell number measured using the CellTitre 96Aqueous One Solution Cell Proliferation Assay kit (Promega) [13]. Briefly, live cell number was assayed using the Promega reagent as per the manufacturer's instructions. Next, cells were fixed with 4% paraformaldehyde (PFA) for 15 min and washed with an AP wash buffer (0.1 M $NaCO_3$ pH 10, 1 mM $MgCl_2$) and then incubated with 10 mM *p*NPP for 10 min (C2C12s) or 20 min (BMSCs) at room temperature. AP activity was based on A_{405} readings from triplicate assays normalized to cell number.

Matrix mineralization staining

Mineralized calcium deposits were directly assessed by Alizarin Red S staining. Monolayers of differentiated osteoblasts at day 10 (rhBMP2-induced C1C12s and calvarial cells) and day 14 (BMSCs) were fixed with 4% PFA for 15 min at room temperature and then stained with Alizarin Red S for 10 min (40 mM, pH 4.2) and then washed five times with distilled water to remove non-specific staining. Assays were performed in triplicate.

RNA extraction, cDNA preparation, and real-time quantitative PCR (qPCR)

Total cellular RNA was extracted from cells grown in 6-well plates using TRIZOL reagent (Invitrogen) as per the manufacturer's instructions. Superscript III Reverse Transcriptase (Invitrogen) was then used with equivalent amounts of RNA to obtain cDNA. The *Gapdh* (5′-GCCACCCAGAAGACTGTGGATGGC-3′; 5′-GTGGTGGACCTCATGGCCTACATG-3′), *alkaline phosphatase* (*Alp*) (5′-GGGACTGGTACTCGGATAACGA-3′; 5′-TGCAAGGAC ATCGCATATCAG-3′), *osteocalcin* (*Ocn*) (5′-CGGCCCTGAGTCTGACAAA-3′; 5′AAGGTAGTGAACAGACTCCGGC-3′), and *osterix* (*Osx*) (5′-CGTCCTCTCTGCTTGAGGAA-3′; 5′-AAAGGTCAGCGTATGGCTTC-3′) sequences were amplified using SYBR Green I PCR reagent kit (Integrated Sciences) according to the manufacturer's protocol. PCR was performed on the Rotor-Gene 3000 (QIAGEN). Samples were denatured at 94 °C for 2 min followed by 40 amplification cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 60 s for *Alp*, *Ocn* and *Osx* and 35 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s for *Gapdh*. These thermal cycling programs were followed by a final extension at 72 °C for 60 s. PCR reactions were performed in triplicate and normalized to the housekeeping gene *Gapdh*, to control for cDNA loading. Data are presented as mean fold induction (±standard error).

Ras activity pull-downs and western blotting

Activated Ras was detected using Ras activity pull-down assays (New East Bioscience) according to the manufacturer's protocol. Briefly, cells were lysed in RIPA buffer with protease inhibitors. Samples were then incubated at 4 °C for 1 h with the active-Ras antibody and A/G Agarose Beads. The beads were pelleted, washed and resuspended in 30 μl 1× SDS-PAGE loading buffer. The samples were then

heated to 95 °C for 5 min and loaded onto 4–20% gradient Tris-glycine polyacrylamide gels (Biorad).

Western blots were performed as described previously [3]. Primary antibodies were diluted at 1:1000 for anti-Ras (New East Biosciences), anti-phospho-c-Jun (Ser63), anti-c-Jun, anti-p42/44 and anti-phospho-p42/44 (Thr202/Tyr204) antibodies (Cell Signaling Technology). The anti-Gapdh antibody (Millipore) was diluted at 1:5000. Horseradish peroxidase-tagged secondary antibodies (GE Healthcare) were used for detection by chemiluminescence with exposure to Biomax film (Amersham Biosciences). Each western blot was performed once on single freeze thawed samples to prevent protein degradation. The blots were probed first for an individual signaling molecule and reprobed for Gapdh.

Analysis of the blots was performed by band densitometry using ImageJ software, with bands measured in duplicate. Values from cell signaling antibodies were normalized to Gapdh.

Statistical analysis

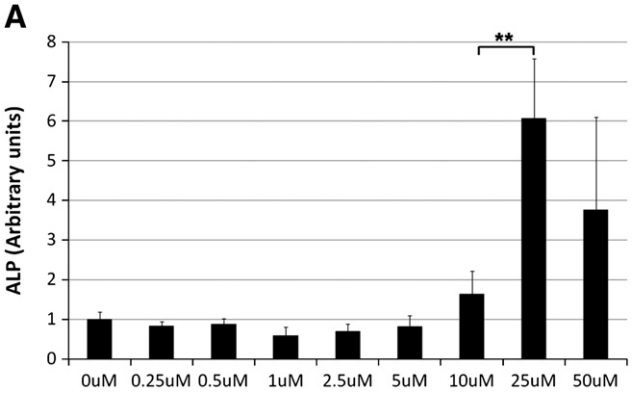
Values are presented as mean ± standard error (SE), and statistical comparisons were made using a two-tailed student's t-test. Values of $p<0.05$ were considered statistically significant.

Results

JNK inhibitors increased osteogenic markers in C2C12 myoblasts

The effects of JNK inhibition on BMP-induced osteogenesis were examined in culture. C2C12s were treated with a low dose of rhBMP-2 (50 ng/ml) with and without JNK inhibitors for 10 days. Consistent with previous reports, this low rhBMP-2 dose poorly stimulated bone markers, [11], however doses of SP600125 greater than 10 μM were able to increase alkaline phosphatase activity by up to 6-fold (Fig. 1A). SP600125 decreased cell viability at concentrations above 10 μM. Alizarin red staining showed matrix mineralization with high concentrations of the SP600125 compound at all time points in the presence of rhBMP-2 (Fig. 1B), suggesting that JNK inhibition did not negatively affect terminal differentiation and matrix production.

Matsuguchi et al. suggested that JNK inhibition could affect the function and/or terminal differentiation of osteoblasts [16]. The effects of SP600125 on early *versus* late osteogenic differentiation were examined by comparing SP600125 dosing on untreated C2C12 progenitors with C2C12s that had been pre-differentiated using rhBMP-2. Contrary to previous findings, both early and late rhBMP-2 treated cells showed increased ALP activity with SP600125 (Fig. 2), suggesting that JNK inhibition promotes and does not prevent osteogenesis in culture.



SP600125	0 μM	0.25 μM	0.5 μM	1 μM	2.5 μM	5 μM	10 μM	25 μM	50 μM
Day 9	-	-	-	-	-	-	+++	-	-
Day 10	-	-	-	-	-	-	+++	+++	-
Day 11	-	-	-	-	-	-	+++	+++	-
Day 12	-	-	-	-	-	-	+++	+	-

Fig. 1. JNK inhibitors in combination with BMP2 (50 ng/ml) increase osteogenic early and late markers in a myoblast cell line. (A) ALP activity at day 3 post-treatment in C2C12s (**$p<0.01$) and (B) mineralization at day 10 post-treatment. — no mineralized wells, + one mineralized well, ++ two mineralized wells, +++ three mineralized wells.

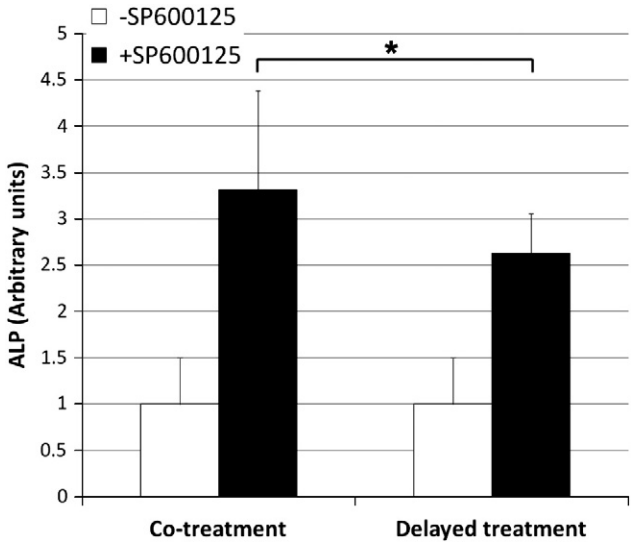


Fig. 2. Co-treatment *versus* delayed inhibition of JNK signaling affects osteogenic differentiation of C2C12 cells. BMP2 and SP600125 were added to C2C12 cells conjunctively in co-treated samples or JNK inhibitors were applied 3 days post-BMP2 treatment in delayed treatment samples. ALP was measured after 7 days of BMP2 treatment. *$p<0.05$.

Increased rhBMP-2 induced JNK activity in Nf1 $^{+/-}$ BMSCs can be rescued with SP600125

To test the hypothesis of increased JNK signaling with NF1-deficiency, growth factor induced signaling was stimulated by 30 min of treatment with 200 ng/ml rhBMP-2. This was shown to activate Ras as measured by pull down assay in wild type BMSCs, but Ras activity was found to already be maximally stimulated and not further increased by rhBMP-2 in *Nf1* $^{+/-}$ BMSCs (Fig. 3).

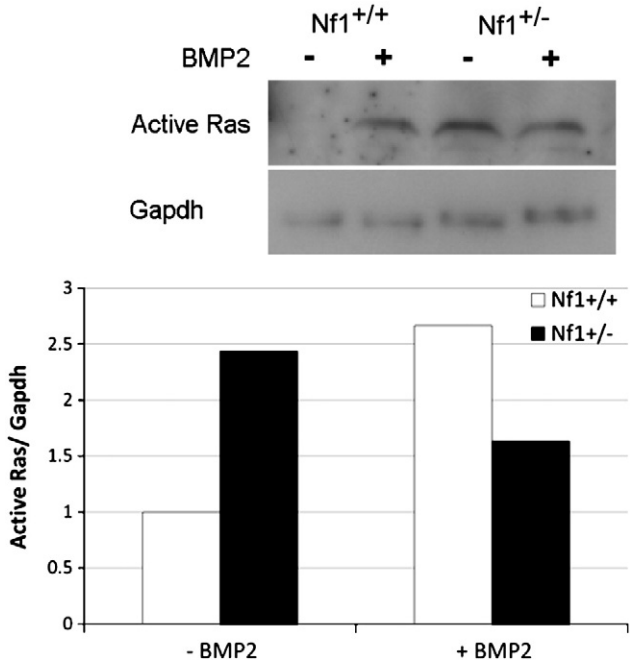


Fig. 3. Ras activation in Wild-type (*Nf1* $^{+/+}$) and *Nf1* $^{+/-}$ BMSCs treated with BMP2 (200 ng/ml).

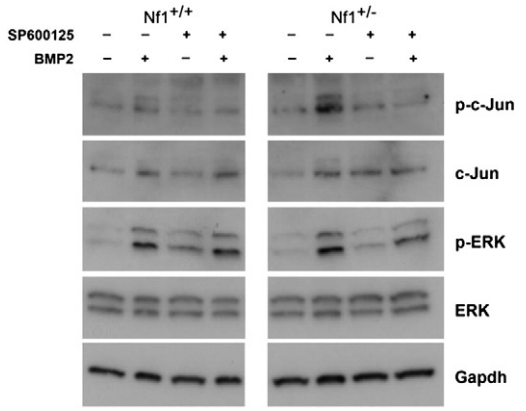


Fig. 4. MAPK pathway activation in wild-type (*Nf1*$^{+/+}$) and *Nf1*$^{+/-}$ BMSCs treated with SP600125 (10 μM) and BMP2 (200 ng/ml).

Ras downstream signaling factors, including the MAPK pathway, have been previously reported to be stimulated by rhBMP-2 [17], and ERK1/2 phosphorylation was dramatically elevated following rhBMP-2 treatment (Fig. 4). In contrast, phospho-c-Jun (a marker of JNK activity) was relatively unaffected in wild type cells following rhBMP-2 treatment. However, in *Nf1*$^{+/-}$ cells, rhBMP-2 led to a rapid activation of phospho-c-Jun. Based on densitometry outcomes, phospho-c-Jun was increased by 5-fold in *Nf1*$^{+/-}$ cells.

Pre-treatment of samples with 10 μM SP600125 for 1 h prior to rhBMP-2 activation prevented the over-activation of phospho-c-Jun in *Nf1*$^{+/-}$ BMSCs. In both wild type cells and *Nf1*$^{+/-}$ cells, ERK1/2 activity was not affected. Overall levels of total ERK and c-Jun were not significantly different between the wild type cells and *Nf1*$^{+/-}$ BMSCs.

JNK inhibition increases osteogenesis in Nf1$^{+/-}$ *BMSCs*

Unlike C2C12s, BMSCs are able to undergo osteogenic differentiation in media supplemented with ascorbic acid and β-glycerophosphate in the absence rhBMP-2. Wild-type and *Nf1*$^{+/-}$ BMSCs were cultured for a period of 14 days with rhBMP-2 and/or SP600125. Individually, rhBMP-2 and SP600125 increased the production of the early bone marker ALP in both wild-type and NF1-deficient cells. When used in combination these compounds further increased ALP production (Fig. 5).

Gene expression of early markers of osteogenesis (*Alp* and *Osx*) and a late marker (*Ocn*) were examined at day 7 and day 14 post-treatment respectively. Treatment with either SP600125 or rhBMP-2 alone was sufficient to increase all three markers (Fig. 6). The response of *Nf1*-deficient cells to rhBMP-2 was reduced compared to wild type cells for all markers. For control wild type cells, the combination of rhBMP-2 and SP600125 had no beneficial effect on early markers, suggesting rhBMP-2 alone was capable of maximal osteogenic stimulation. The late marker *Ocn* was reduced by the combination in wild type cells. In contrast, in *Nf1*-deficient cells the combination of rhBMP-2 and SP600125 led to the maximal expression of early and late osteogenic gene markers (Fig. 6).

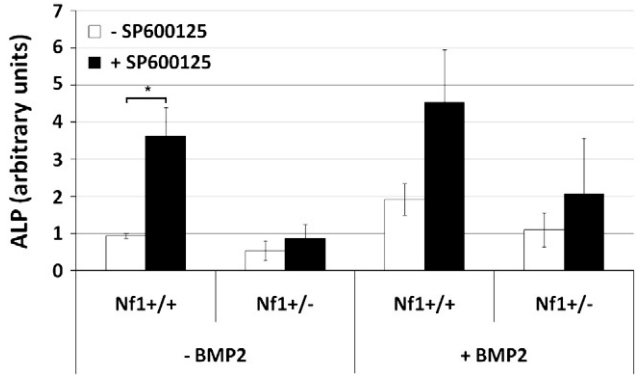


Fig. 5. *Nf1*$^{+/+}$ (WT) and *Nf1*$^{+/-}$ (Nf1) BMSCs show improved alkaline phosphatase production when treated with BMP2 (200 ng/ml) and SP600125. *p<0.05.

Total c-Jun is increased in Nf1^{null} *calvarial cells*

To examine whether the effects of total NF1 ablation on c-Jun phosphorylation were more severe an alternative model was used where *Nf1*$^{flox/flox}$ calvarial cells are transduced with an Adeno-cre virus to produce *Nf1*^{null} osteoblasts. As previously described, western blots were performed to examine the activation and total protein of c-Jun and ERK and the results analyzed quantitatively. From densitometry readings, it was calculated that *Nf1*^{null} osteoblasts had a 4-fold increase in total c-Jun and a 0.5-fold reduction in total ERK, normalized to Gapdh (Fig. 8). Relative kinase phosphorylation was unchanged, but due to increases in total ERK and c-Jun, phospho-ERK and phospho-c-Jun were both elevated in *Nf1*^{null} osteoblasts. These increases were seen in both basal and rhBMP-2 induced samples compared to Adeno-GFP treated control cells with normal NF1 function.

Pre-treatment with SP600125 reduced phospho-c-Jun levels in *Nf1*^{null} calvarial cells by approximately 2-fold, to levels not significantly different from Adeno-GFP treated control cells.

JNK inhibitor induces osteogenesis in Nf1^{null} *calvarial cells*

Nf1$^{flox/flox}$ calvarial cultures were again treated with Adeno-Cre virus to induce an *Nf1*^{null} genotype *in vitro* to examine markers of osteoblast differentiation and function. Following transduction the cells were treated with rhBMP-2 and/or SP600125 for 10 days with mineralization measured at day 14.

RhBMP-2 alone was not sufficient to induce osteogenesis in GFP control or *Nf1*-null cells. RhBMP-2 and SP600125 acted synergistically to increase matrix mineralization in GFP-control cells (Fig. 7), while combined use of these compounds was required to induce mineralization in *Nf1*^{null} cells.

Discussion

For NF1 patients, deficient bone healing and the recalcitrance of pseudarthrosis to standard interventions remain a significant treatment challenge. The underlying cellular causes of poor bone healing remain unclear, however it is likely that NF1 can affect changes in osteoblast, osteoclast, fibroblast, and/or vascular cell proliferation, differentiation, and function [18]. The molecular basis of these cellular changes has been attributed to excessive Ras signaling in NF1-deficient cells. However, increased Ras activity can influence different cell types in different ways such as impairing pre-osteoblast differentiation [2,19], augmenting mature osteoblast function [20], and increasing the sensitivity of hematopoietic progenitors to pro-osteoclastic signals [2]. While canonical Ras-MAPK signaling has repeatedly been shown to be upregulated in NF1-deficient cells [5,19,21–25], the role of other non-canonical pathways is less well understood. A Ras-PI3K-MAPK axis and subsequently a role for Rac signaling have been shown to be important in mediating the effects on osteoclast differentiation [2,26]. The implication of a JNK signaling axis in *Nf1*$^{+/-}$ microglia led us to investigate the role of this pathway in bone cell differentiation [8].

Based on the microglial studies, we hypothesized that JNK signaling could be similarly increased in NF1-deficient osteoprogenitors. While both wild type and *Nf1*$^{+/-}$ BMSCs responded to rhBMP2 by increasing activity of the canonical Ras-ERK pathway [27], only the *Nf1*$^{+/-}$ BMSCs exhibited significantly increased c-Jun phosphorylation. In *Nf1*^{null} cells the total c-Jun levels were increased, resulting in an absolute increase in c-Jun phosphorylation, although the ratio of phospho-c-Jun to total c-Jun remained constant. The significance of

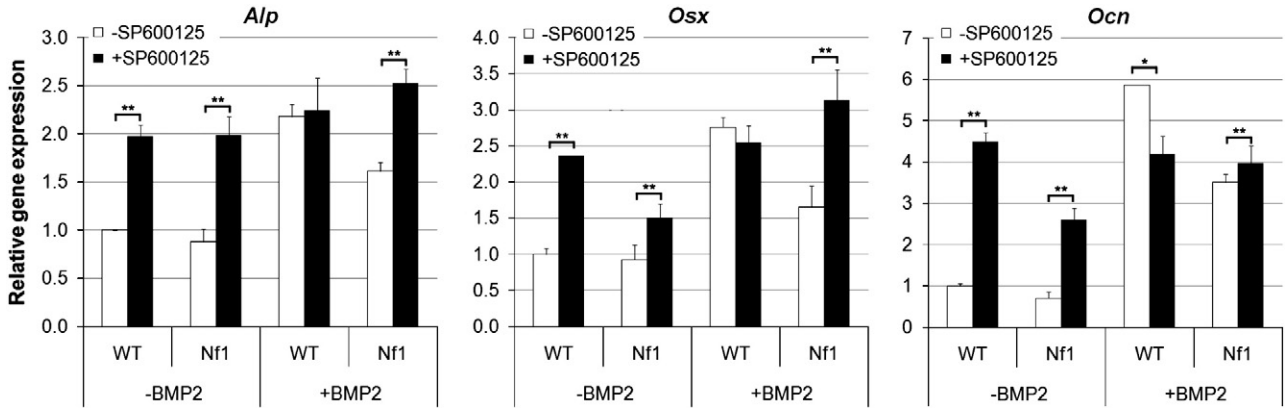


Fig. 6. *Alp*, *Osx*, and *Ocn* gene expression of $Nf1^{+/+}$ (WT) and $Nf1^{+/-}$ (Nf1) BMSCs treated with BMP2 (200 ng/ml) and SP600125. *p<0.05 **p<0.01.

this apparent mechanistic difference between $Nf1^{+/-}$ and $Nf1^{null}$ cells is unclear.

Next, we hypothesized that excessive JNK signaling may contribute to the capacity of NF1-deficient progenitors to undergo normal osteogenic differentiation. Cultured cells from mice featuring NF1-deficiency and knockout confirmed prior findings that NF1 has a key role in osteoblast differentiation [3], and the effects of NF1 deficiency could be rescued using SP600125 treatment. Functional and molecular studies showed augmentation of both early- and late- markers of osteogenesis. The specificity of the inhibitor was validated by no change in phospho-ERK1/2 with 10 μM SP600125.

Notably, work with cultured C2C12 myoblasts and wild-type primary BMSCs showed SP600125 treatment could increase osteogenic markers in the absence of any NF1 deficiency. We hypothesized that JNK may be important in osteogenic commitment and thus examined uncommitted osteoprogenitors. When inhibition with SP600125 was delayed until after osteogenic commitment, there was a blunting of the positive effects seen with early dosing, but the overall effect on osteogenesis remained positive. These data directly contradict the results of Matsuguchi et al. and Liu et al., who recently reported that SP600125 decreased osteogenic potential in MC3T3-E1 cells [16,28]. It is difficult to reconcile the findings of this study with those of Matsuguchi and Liu, although one potential explanation was their use of MC3T3-E1 cells of unreported passage number; MC3T3-E1 cells can show reduced BMP-responsiveness at higher passages [29]. Further work with animal models will be required to resolve the *in vivo* effects of JNK inhibition on bone.

The skeletal defects associated with NF1-deficiency are potentially caused by a combination of complete knockout of NF1 in cells and NF1-deficient cells. $Nf1^{null}$ cells have been identified in pseudarthrotic tissue of NF1 patients [30], however their role in the development of congenital tibial dysplasia remains unclear as they have not been identified in all focal skeletal defects associated with NF1. We have previously shown these cells to be less responsive to bone-inducing signals than both wild-type and $Nf1^{+/-}$ cells (El-Hoss et al., in press). $Nf1^{null}$ calvarial osteoblasts isolated and grown *in vitro* were found to have increased total c-Jun protein and overall c-Jun activation, although no spike in JNK activity was seen in direct response to rhBMP-2 (unlike the $Nf1^{+/-}$ BMSCs).

In conclusion, these experiments present new evidence for the activation of the Ras-JNK pathway in NF1-deficient osteoblasts. The Ras-MAPK pathway has been historically cited as the mechanism underlying deficient NF1 osteogenesis, however it is possible that other kinases downstream of Ras, such as JNK, may also mediate the bone phenotype. Further experiments will be required to validate the *in vivo* utility of JNK-based approaches for intervention in bone healing in NF1.

Acknowledgments

Dr. Schindeler has received support from the Children's Tumor Foundation (New York, USA). Jad El-Hoss receives a scholarship from the University of Sydney. This project has received funding support from the National Health & Medical Research Council (Project Grant 1003478) and the Neurofibromatosis Association of Australia. We would like to thank Lauren Peacock and Kathy Mikulec for assistance animal studies, and Renjing Liu and Alyson Morse for technical assistance.

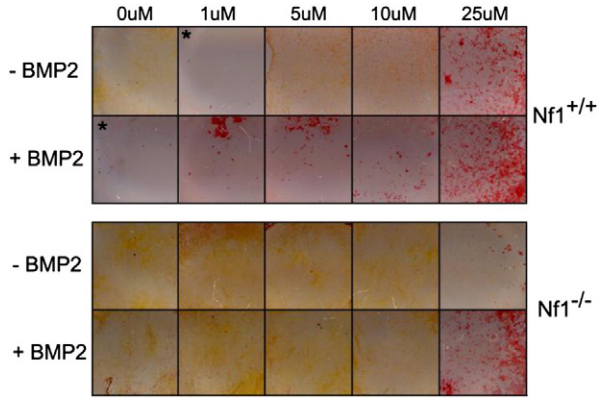


Fig. 7. Increased matrix mineralization of Adeno-GFP ($Nf1^{+/+}$) and Adeno-Cre treated ($Nf1^{-/-}$) mature osteoblasts treated with SP600125 and BMP2. *cell layer lifted.

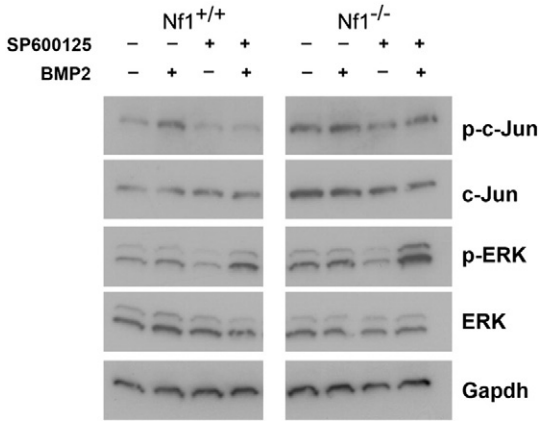


Fig. 8. MAPK pathway activation in Adeno-GFP ($Nf1^{+/+}$) and Adeno-Cre treated ($Nf1^{-/-}$) mature osteoblasts treated with SP600125 (10 μM) and BMP2 (200 ng/ml).

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