Pharmacologically targeting beta-catenin for NF1 associated deficiencies in fracture repair

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Abstract

Patients with Neurofibromatosis type 1 display delayed fracture healing and the increased deposition of fibrous tissue at the fracture site. Severe cases can lead to non-union and even congenital pseudarthrosis. Neurofibromatosis type 1 is caused by a mutation in the *NF1* gene and mice lacking the *Nf1* gene show a fracture repair phenotype similar to that seen in patients. Tissue from the fracture site of patients with Neurofibromatosis type 1 and from mice deficient in the *Nf1* gene both show elevated levels of β -catenin protein and activation of β -catenin mediated signaling. Constitutively elevated β -catenin leads to a delayed and fibrous fracture repair process, and (*RS*)-5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine (Nefopam, a centrally-acting, non-narcotic analgesic agent) inhibits β -catenin mediated signaling during skin wound repair. Here we investigate Nefopam's potential as a modulator of bone repair in mice deficient in *Nf1*. Mice were treated with Nefopam and investigated for bone fracture repair. Bone marrow stromal cells flushed from the long bones of unfractured mice were treated with Nefopam and investigated for osteogenic potential. Treatment with Nefopam was able to lower the β -catenin level and the *Axin2* transcript level in the fracture calluses of *Nf1* deficient mice. Cultures from the bone marrow of *Nf1*^{-/-} mice had significantly lower osteoblastic colonies and mineralized nodules, which was increased when cells were cultured in the presence of Nefopam. Fracture calluses were harvested and analyzed 14 days and 21 days after injury. *Nf1*^{-/-} calluses had less bone, less cartilage, and higher fibrous tissue content than control calluses. Treatment with Nefopam increased the bone and cartilage content and decreased the fibrous tissue content in *Nf1*^{-/-} calluses. These findings present a potential treatment for patients with Neurofibromatosis 1 in the context of bone repair. Since Nefopam is already in use in patient care, it could be rapidly translated to the clinical setting.

Keywords: Neurofibromatosis type 1, fracture repair, osteoblast differentiation, Nefopam

1.0 Introduction

Neurofibromin is a protein encoded by the *NF1* gene on chromosome 17 in humans[1, 2]. Mutations in neurofibromin cause Neurofibromatosis type 1 (NF1)[3, 4], a disorder that is autosomal dominant and affects approximately 1 in 3000[5]. Approximately half of all cases of NF1 occur from spontaneous mutations of the *NF1* gene[6]. Patients with NF1 present with café au lait macules[7], freckles[7], optic pathway gliomas[8], learning disabilities[9], short stature, low bone mineral density, susceptibility to fracture and pseudarthrosis, and deposition of fibrous tissue at the fracture site[10, 11].

In some cases, a fracture in a NF1 patient will result in pseudarthrosis. Treatments for such fractures in NF1 patients involve stabilization and fixation of the area and/or grafting of bone and osteoid tissue onto the site[12-14]. In addition to these surgical procedures, pharmaceutical-based treatments have been attempted to aid bone tissue regeneration such as the use of bisphosphonates, bone morphogenetic protein 2, and/or bone morphogenetic protein 7[15-18]. The efficacy of these treatments is variable and can lead to unresolved pseudarthrosis, in some cases even requiring amputation of the limb[10].

In Neurofibromatosis type 1, GTPase function is ablated leading to unsuppressed activation of the Ras/MAPK signaling pathway[19], which can lead to enhanced Wnt/ β -catenin signaling through quenching GSK-3 β 's inhibitory effect on Wnt/ β -catenin signaling[20]. Recent work confirmed that Wnt/ β -catenin signaling is indeed increased in NF1 patients[21]. Several studies[22-25] show that during mesenchymal stem cell differentiation to osteochondral progenitor cells, β -catenin levels must be tightly regulated to ensure proper commitment to the osteochondral lineage. Increased levels of β -catenin in undifferentiated mesenchymal stem cells leads to commitment towards a fibrous phenotype. Accordingly, we hypothesize that the elevated levels of β -catenin signaling observed in NF1 patients[26, 27] likely directs progenitor cells, either during bone homeostasis or during the early stages of bone fracture repair, away from an osteochondral lineage and towards a fibrous phenotype.

Here we investigate a therapeutic intervention to improve bone repair in NF1, by targeting the Wnt/ β -catenin signaling pathway using (*RS*)-5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine (Nefopam). Nefopam is an analgesic agent which also is a modulator of β -catenin signaling[28].

2.0 Materials & Methods

2.1 Transgenic mice – Animal protocols were approved by the animal care committee of the Toronto Centre for Phenogenomics. *NF1^{fl/fl}* mice contained loxP sites flanking exons 31-32 of the *NF1* gene[29]. Upon excision of the flanked region by Cre-recombinase, neurofibromin activity is ablated (*Nf1^{-/-}*). Recombination was verified using DNA and protein analysis. Mice were 4-months of age at time of fracture or bone marrow aspiration. Aged male mice were of the C57BL/6J strain and 18 months of age at time of fracture or bone marrow aspiration.

2.2 Cell Culture – Tibiae of unfractured 4-month old *NF1^{fl/fl}* mice were isolated and contents of the medullary cavity (bone marrow) was flushed using AMEM culture medium (Wisent). Cell suspensions were passed through a 18G needle to dissociate cell aggregates, and cells were plated at a density of $250 \times 10^3/\text{cm}^2$ in plating medium (AMEM, 10% FBS, 100 U/ml Penn/Strep) for seven days. Cells were allowed to adhere to tissue culture plastic for four days, cells were transfected overnight with Adenovirus containing either GFP+Cre-recombinase or GFP alone (multiplicity of infections of 250). To determine the colony forming units-fibroblastic; wells were then washed with PBS, fixed with 10% formalin, and stained with 0.25% crystal violet. For osteoblastic differentiation; cells were cultured in osteogenic media (AMEM, 10%FBS, 100U/ml Penn/Strep, 30 μM ascorbic acid, 10^{-8} M dexamethasone, 8 mM sodium phosphate) after seven days in plating media. After 15 days in differentiation media, wells were washed with PBS, fixed using 10% formalin, and stained for alkaline phosphatase using FastRed (Sigma Inc.) or for mineral using 2.5% silver nitrate solution (Von Kossa) on a light box. Colony forming units were determined from three groups of triplicate experiments. For Nefopam-treated culture experiments, 0.2mM of Nefopam was used in the culture media beginning two days before and two days after differentiation was initiated.

2.3 Fracture surgery – Fractures were generated as previously described[30]. Briefly, 4-month old mice were anesthetised and the surgical area was shaved and disinfected. Following an incision proximal to the knee, the tibia was stabilized by inserting a 0.7mm insect pin into the medullary cavity of the tibia and cut flush to the tibial plateau. A fracture was induced mid-shaft of the tibia using blunt scissors and 2×10^8 pfu of Adenovirus containing either GFP-labelled Cre-recombinase or GFP control was injected into the site of fracture. Cre-mediated recombination was ensured as previously described, by analyzing genomic DNA[31]. The incision was then closed using sutures and wound clips. Nefopam was administered via an intraperitoneal injection (30mg/kg/day) on the day before, the day of, and the day after fracture surgery. Dosing analysis has previously been performed[28].

2.4 Evaluation of Fracture Healing – Radiographs of healing fractures were obtained using a Faxitron MX20, with the identical setting used for each sample. Radio-intensity maps of the radiographs were generated to be used as a visual tool to better indicate patterns of tissue density. ImageJ was used to analyze radiographs in a manner similar to previously published[32, 33]. Briefly, the radio-intensity was determined of tissue found at the fracture site and the radio-intensity found at unfractured bone and these values were plotted as a relation. μ CT analyses were performed using a SCANCO μ -CT 40 at a scan resolution of 8 μ m (Scanco Medical). Fracture calluses were scanned 1mm proximal and 1 mm distal from the fracture site and 100 contiguous slices were analyzed for total callus volume (TV) in mm^3 , percent bone volume within the total callus volume (% BV/TV), and bone mineral density (BMD) of the callus in mg HA/mm^3 . Isolated tibiae fracture calluses were fixed in 10% formalin for 7 days. Calluses were demineralized in 20% EDTA (pH 8.0) at room temperature. Samples were then cleared of EDTA and embedded in paraffin, cut into 5 μ m sections, and stained using Safranin-O (to stain proteoglycans/cartilage red) and counter-stained with Fast Green (to stain bone green/blue). After staining, tissue phenotype was used as an indicator of tissue type. A minimum of five sections from a

minimum of five fractured limbs were analyzed by computer assisted histomorphometry analysis and results presented as percent of tissue area deposited relative to the total fracture callus area. A minimum of five replicates of all samples were analyzed.

2.5 Western blot analysis – The fracture calluses of mice treated with saline or Nefopam were harvested on the last day of treatment. Calluses were pulverized in liquid nitrogen and protein lysis buffer (Roche) was used to extract proteins. Protein concentration was measured using the BCA protein quantification method and 25 µg of protein lysate was loaded per well. Indicated antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescence. Anti-β-catenin antibody (AB19022) was purchased from Millipore and anti-β-actin (CP01) was purchased from Calbiochem.

2.6 RT-PCR – Tissue samples were harvested, immersed immediately into liquid nitrogen, and pulverized into powder prior to RNA extraction while cell culture samples were washed twice with PBS prior to RNA extraction. Total RNA was isolated using TRIzol Reagent (Invitrogen Inc.) according to the manufacturer's protocol, purity and quantity of RNA was determined using spectrometric methods. cDNA template was generated using random hexamers. TaqMan primers for *Alp* (Mm00475834_m1), *BSP* (Mm00492555_m1), and *Col1* (Mm00801666_g1) were purchased from Applied Biosystems and samples were investigated using a 7900HT Fast Real-Time PCR System and compared to the transcript of ribosomal protein 18S as a housekeeping control. A minimum of five replicates of all samples were analyzed.

2.7 Statistical Analysis – Data are expressed as mean $\pm$ 95% confidence interval. One-way analysis of variance (ANOVA) was performed followed by Dunnett's test when significant differences were detected. The data was considered to be statistically significant at a confidence level of 95% ($p < 0.05$).

3.0 Results

3.1 Nefopam increases osteoblastic differentiation in $Nf1^{-/-}$ bone marrow cells – Bone marrow from $Nf1^{fl/fl}$ mice was flushed and plated onto tissue culture plastic. Cultures were incubated with either Ad-GFP or Ad-GFP+Cre and expression of GFP-labelled Cre-recombinase was verified by observation of fluorescence (Supplemental Fig 1a). The number of colony forming units-fibroblastic (CFU-F), a surrogate indicator of the number of mesenchymal progenitor cells present, was measured using crystal violet staining. There was no difference in the number of CFU-F colonies between $Nf1^{fl/fl}$ cells incubated with Ad-GFP or Ad-GFP+Cre (Supplemental Fig 1b). A subset of the plated cells were differentiated to osteoblastic cells using osteogenic media. There were significantly lower numbers of osteoblastic colonies (ALP stain - ALP) and significantly lower mineralized nodules (VK stain - VK) in cultures treated with Ad-GFP+Cre than cultures treated with Ad-GFP control (Fig 1c). Treatment with Nefopam was investigated as a method of modulating *in vitro* osteogenic potential of $Nf1^{fl/fl}$ cultures and decreased the protein level of β -catenin, and decrease expression of its transcriptional target gene, *Axin2* (Fig 1a&b). Nefopam treatment resulted in enhanced osteoblastic differentiation (ALP) and enhanced mineralization (VK) of $Nf1^{-/-}$ cultures (Fig 1c&d). Transcript levels of markers for osteoblastic differentiation (*Alp*, *Bsp*, *Col1*) were investigated using RT-PCR. All three genes showed a decrease of transcript levels in $Nf1^{-/-}$ cells relative to controls however treatment with Nefopam was able to reverse this decrease in osteogenic markers, confirming the trend observed from stained osteoblastic cultures (Fig. 1 e). Thus Nefopam treatment of $Nf1^{-/-}$ bone marrow stromal cells during early stages of osteoblastic differentiation resulted in more robust osteoblastic differentiation and mineralization.

3.2 Fracture repair of $Nf1^{-/-}$ calluses is improved by treatment with Nefopam – $Nf1^{fl/fl}$ mice were treated with Nefopam or carrier and subjected to tibial fracture surgery, inducing a stabilized tibial fracture. Fractured mice were either treated with Ad-GFP or Ad-GFP+Cre. Expression of vector-associated protein

was confirmed using Western blot analysis. Immunoblot analysis showed Nefopam treatment decreased β -catenin protein level (Supplementary Fig. 1c). Fracture calluses were harvested and analyzed for their repair phenotype using radiographs and histology at 14- and 21-days post fracture.

At 14-days after fracture, radiographs of fracture calluses from *Nf1*-deficient mice displayed delayed healing. Histological sections from the fracture calluses of *Nf1*-deficient mice stained with Safranin-O/Fast Green indicated a greater amount of fibrous tissue, little to no cartilaginous tissue deposited, and a smaller amount of bone tissue relative to the control (Fig. 2a). Radiographs from *Nf1*^{-/-} fracture calluses of mice treated with Nefopam show improved healing relative to untreated *Nf1*^{-/-} calluses, as measured by bone bridging and radiographic density (Fig 2a). Intensity maps of the fractured tibia further demonstrated increased density of tissue deposited in the fracture callus of Nefopam-treated *Nf1*^{-/-} fracture calluses relative to untreated *Nf1*^{-/-} fracture calluses. Histological sections of *Nf1*^{-/-} fracture calluses stained with Safranin-O/Fast Green indicated decreased fibrous tissue at the site of injury and higher amounts of cartilage and bone than untreated *Nf1*^{-/-} fracture calluses (Fig. 2b). Fracture calluses from *Nf1*^{-/-} mice treated with Nefopam contained 68% more bone, 300% more cartilage, and 49% less fibrous tissue than fracture calluses from untreated mice (Fig. 2b).

At 21-days post fracture radiographs of *Nf1*^{-/-} fracture calluses displayed delayed healing (Fig. 3a). Intensity maps of the fractured tibia further demonstrated increased density of tissue deposited in the fracture callus of Nefopam-treated *Nf1*^{-/-} fracture calluses relative to untreated *Nf1*^{-/-} fracture calluses and the Relative Intensity of the callus tissue within the radiograph was 25% higher in Nefopam-treated *Nf1*^{-/-} fracture calluses than in untreated *Nf1*^{-/-} fracture calluses. μ -CT analysis confirmed that untreated *Nf1*^{-/-} fracture calluses were composed of less tissue, lower bone volume, and had lower bone mineral density than controls (Fig 3c). However, these shortcomings were alleviated upon treatment with Nefopam. Histological sections from these fracture calluses stained with Safranin-O/Fast Green displayed the trend seen at 14-days post fracture for these mice; decreased amounts of cartilage

and bone and greater amounts of fibrous tissue relative to fracture calluses from control mice. Radiographs of 21-day *Nf1*^{-/-} fracture calluses treated with Nefopam indicated bone bridging (Fig. 3a). Histological sections from these fracture calluses stained with Safranin-O/Fast Green indicated 87% more bone tissue and 60% less fibrous tissue in *Nf1*^{-/-} fracture calluses treated with Nefopam than in untreated *Nf1*^{-/-} fracture calluses (Fig 3b). μ analysis definitely showed rescue of the *Nf1*^{-/-} fracture phenotype as Nefopam treatment of fracture *Nf1*^{-/-} mice led to larger fracture calluses, higher bone volume within the fracture callus, and a higher bone mineral density within the fracture callus.

3.3 Nefopam treatment enhances fracture repair and osteoblastic differentiation in aged mice.

Nefopam's ability to decrease β -catenin signaling was further assessed using aged mouse models. β -catenin protein level and signaling increases with age and correcting this increase has been shown to enhance bone repair and osteoblast differentiation[22]. Aged mice were treated with Nefopam or carrier and subjected to tibial fracture. Radiographs and histological sections stained with Safranin-O/Fast Green indicate better healing in aged mice (21 days post fracture) treated with Nefopam (Supp Fig 2a). Histomorphometry confirmed more bone deposition and less fibrous tissue in the fracture calluses of aged mice treated with Nefopam (Supp Fig 2b). Analysis of fracture calluses confirmed a decrease in β -catenin protein and *Axin2* transcript in the fracture calluses of aged mice treated with Nefopam (Supp 2c). BMSC's aspirated from aged mice displayed increased osteoblastic differentiation and increased mineral formation in cultures treated with Nefopam (Supp Fig 2d).

4.0 Discussion

Neurofibromatosis type 1 commonly leads to musculoskeletal complications which translate to a decrease in the quality of life[5]. Neurofibromatosis type 1 patients are predisposed to poor fracture repair and congenital pseudarthrosis, potentially leading to amputation[10]. In this study we identified Nefopam as a modulator of osteoblastic differentiation and bone fracture repair in *Nf1* deficient mice. Bone marrow stromal cells deficient in *Nf1* had decreased osteoblast differentiation capacity and decreased osteoblastic activity (mineralization) relative to *Nf1^{fl/fl}* controls, however treatment of these cells with Nefopam returned the osteogenic potential to a level similar to that of control cells. Induction of an injury in the tibia fracture model resulted in delayed healing in the *Nf1*-deficient fracture calluses associated with decreased amounts of bone and cartilage tissue and increased fibrous tissue at the fracture site relative to control animals. Treatment with Nefopam resulted in fracture calluses that contained higher proportions of bone and lower proportions of fibrous tissue, resulting in a fracture-repair phenotype similar to that seen in control mice. While these findings result from investigating tibial fracture repair in an NF1 mouse model; they lend themselves to understanding long-bone repair, development of pseudarthrosis, and identifications of potential treatment strategies for NF1 patients.

Wnt/ β -catenin signaling is activated upon mutation of *NF1*[21, 26, 27, 34]. The Wnt/ β -catenin pathway plays an important role in bone regeneration and progenitor cell differentiation. β -catenin levels need to be precisely controlled for an undifferentiated mesenchymal cell to differentiate to an osteochondral progenitor. If Wnt/ β -catenin signaling is too high before cellular commitment, then cells tend to differentiate towards a fibroblastic phenotype[23]. This is a phenomenon previously reported in mouse models of age and indeed within this study, we have shown that treatment with Nefopam is able to decrease β -catenin signaling and enhance fracture repair and osteoblastic differentiation[22]. It should be noted that while we have used aged mice in this study as a surrogate of elevated beta-catenin signaling, the biology of aging is more complicated than modulation of this signaling pathway alone.

Taking this limitation into consideration, both *in vitro* and *in vivo* models displayed enhanced cellular differentiation and matrix production however, previous work has indicated Nefopam treatment to lead to decreased cellular proliferation [28]. These observations work in concert with the idea of β -catenin playing a disparate role in bone healing.

In NF1 patients, β -catenin levels are elevated which could lead to progenitor cells involved in bone fracture repair to differentiate to a more fibroblastic lineage, leading to the fibrosis seen at the fracture site of these patients. This would result in lower bone mineral density and increased fibrous tissue deposition in resting bone and in fracture calluses, as seen for NF1 patients. Previous work by us has identified elevated β -catenin signaling to play a deleterious role in fracture healing of Neurofibromatosis[31]. Thus modulators of Wnt/ β -catenin signaling could be of use when treating NF1 patients for fractures.

Current treatments for poor bone repair in NF1 are invasive, requiring surgery and/or installation of an agent locally in the bone. A less invasive approach, perhaps using an orally available agent would have advantages over the current treatments. One possible such approach is a temporal modulation of the Wnt/ β -catenin pathway. To this end Nefopam is a potential therapeutic agent which might serve this purpose which could be delivered orally. Our work here suggests that Nefopam therapy may be a therapeutic approach aiding bone healing in patients with Neurofibromatosis.

5.0 Conclusions

Patients with Neurofibromatosis 1 present with a skeletal phenotype, with high rates of non-union, and the deposition of fibrous tissue at the fracture site. Nefopam is an analgesic agent that has previously been shown to affect β -catenin expression and may be involved in a decrease in fibrous tissue deposition during skin wound repair. In this study we investigated the *Nf1*^{-/-} fracture repair process in a mouse tibial fracture model. *Nf1*^{-/-} fracture calluses contained lower amounts of cartilage and bone tissue and higher amounts of fibrous tissue relative to control animals. Treatment with Nefopam decreased β -catenin protein levels, and enhanced *Nf1*^{-/-} fracture repair. This finding presents a possible systemic treatment for patients with Neurofibromatosis 1 in the context of bone repair. Since Nefopam is already in use in patient care, it could be rapidly translated to the clinical setting.

6.0 Acknowledgements

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7.0 References

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Figure Legends

Figure 1 – Treatment with Nefopam reverses the decrease in osteogenic potential observed by mutation of the *Nf1* gene. Bone marrow stromal cells were aspirated from the tibiae of mice homozygous for a floxed *Nf1* gene, leading to deletion of the gene upon Cre recombination. Cells were plated and investigated for osteogenic potential. Cultures were transfected Ad-GFP+Cre (Cre) during initial stages of plating and a subset of these cultures were treated with 0.2mM Nefopam (Nef) immediately prior to inducing osteogenic differentiation. **a)** Western blot analysis was used to investigate the level of total β -catenin and the level of α -actin. **b)** rt-PCR was used to investigate the relative amount of *Axin2* transcript. **c)** After 15 days in differentiation media cultures were washed, fixed, and stained for alkaline phosphatase (ALP) and mineral (Von Kossa – VK). **d)** Differentiation potential was quantified using by analysing the number of colony forming units (CFU) for ALP (white bars) and VK (black bars). **e)** RNA was extracted from differentiating cultures of *Nf1^{fl/fl}* (white bars), *Nf1^{-/-}* (grey bars), and *Nf1^{-/-}* + Nef (black bars) and transcripts investigated for osteogenic markers. Data are expressed as mean +/- 95% confidence interval. *Statistically significant, $p < 0.05$ (Dunnett's test) (n=5).

Figure 2 – Treatment with Nefopam reverses the shortcomings in fracture repair seen for *Nf1^{-/-}* mice 14 days after injury. Mice homozygous for a floxed *Nf1* gene were investigated for fracture repair potential. Mice were treated with Ad-GFP (*Nf1^{fl/fl}*) or Ad-GFP+Cre (*Nf1^{fl/fl}*, Cre). A subset of these mice were treated with Nefopam (Nef). Fracture calluses were isolated 14-days post fracture, fixed, and investigated for tissue regeneration. **b)** Histomorphometry was used to quantify the amount of bone, cartilage, and fibrous tissue deposited at the site of injury. Data are expressed as mean +/- 95% confidence interval. *Statistically significant, $p < 0.05$ (Dunnett's test).

Figure 3 – Treatment with Nefopam reverses the shortcomings in fracture repair seen for *Nf1^{-/-}* mice 21 days after injury. Mice homozygous for a floxed *Nf1* gene were investigated for fracture repair potential. Mice were treated with Ad-GFP (*Nf1^{fl/fl}*) or Ad-GFP+Cre (*Nf1^{fl/fl}*, Cre). A subset of these mice were treated with Nefopam (Nef). Fracture calluses were isolated 21-days post fracture, fixed, and investigated for tissue regeneration. **a)** Calluses were observed using radiographs (left) and radio-intensity maps (middle), and histological analysis using Safranin-O/Fast Green stain (right). **b)** The relative intensity of fracture calluses was determined from radiographs. **c)** Histomorphometry was used to quantify the amount of bone, cartilage, and fibrous tissue deposited at the site of injury. Data are expressed as mean +/- 95% confidence interval. *Statistically significant, $p < 0.05$ (Dunnett's test).

Figure 1

Figure 2

Figure 3