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TITLE: Skeletal Complications in Neurofibromatosis Type 1: The Role of Neurofibromin Haploinsufficiency in Defective Skeletal Remodeling and Bone Healing in NF1

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### Abstract

A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including scoliosis and pseudoarthrosis, which are compounded by osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. The cell types and pathway by which neurofibromin haploinsufficiency (Nf1 +/-) leads to dysregulation of bone remodeling and healing are unknown. The aim of this study is to identify the cell types expressing Nf1 in normal bone cell physiology and fracture healing. We demonstrate that in normal mouse bones, neurofibromin is primarily expressed by cells of the osteoblast lineage. Neurofibromin expression was also induced during osteoblastic differentiation of MC3T3 cells. We found that during fracture repair, neurofibromin expression increased in the early stage and was seen at sites of primary bone formation. Taken together these observations indicate that neurofibromin expression is primarily associated with bone-adherent osteoblast lineage cells with minimal expression in other cell types. In addition, neurofibromin expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing. Neurofibromin haploinsufficiency may keep osteoblasts in an immature state. Immature osteoblasts produce large quantities of the osteoclastogenic cytokine RANKL. A high RANKL environment would shift the balance of bone metabolism in favor of bone resorption and may result in the bone-healing defect seen in NF1 patients.

### Subject Terms

- Neurofibromatosis, NF1, fracture, osteoporosis, orthopedic

### Security Classification

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Introduction:
A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including scoliosis and pseudoarthrosis, which are compounded by osteoporosis and poor bone healing. Corrective orthopedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. The cell types and pathway by which neurofibromin haploinsufficiency (Nf1 +/-) leads to dysregulation of bone remodeling and healing are unknown.

Normal bone development, bone healing, and bone metabolism require the concerted actions of bone forming osteoblasts and bone resorbing osteoclasts. Osteoblast differentiation is regulated by intrinsic and local factors while the formation and function of osteoclasts is regulated primarily by osteoblasts through their expression of pro- and anti-osteoclastic factors.

We hypothesize that neurofibromin haploinsufficiency results in dysregulated bone cell differentiation and/or function. Dysregulation occurs through direct effects on osteoblasts or their precursors, through osteoblast-mediated osteoclast formation, or alternatively, through direct effects in the osteoclast itself.

Objectives:
Aim 1. This aim will test the hypothesis that the abnormal bone remodeling associated with NF1 is related to dysregulated osteoblast and/or osteoclast differentiation and activity. We will identify the target cells (osteoblasts or osteoclasts) mediating dysregulated bone cell formation and function in Nf1 +/- mouse cells in culture and assess the role of NF1 in normal bone cell function.

Aim 2. This aim will test the hypothesis that the neurofibromin gene plays an essential role in fracture healing. We will characterize neurofibromin function in bone cells in a fracture repair model.

Aim 3. This aim will test the hypothesis that the tissue and cellular pattern of distribution and functional role of the Nf1 gene identified in the haploinsufficient mouse model will be predictive of the role and distribution of this gene in patients with NF1. We will characterize bone cells in bone samples from Orthopedic NF1 patients.

Beyond providing insights into the molecular pathogenesis of skeletal disease and bone resorption in NF1, this study will determine if current methods of pharmacological blockade can inhibit osteoclast formation in cell culture and inhibit osteolysis in an animal model. If inhibition of osteoclast formation or function is found to be successful in Nf1 +/- mouse osteoclasts, these results may be directly applicable to foster bone healing in orthopedic NF1 patients. We believe that valuable information can be gained using well-established models of bone cell formation and function. Results from the proposed studies may quickly be translated into therapies for orthopedic NF1 patients.
**Body:**

In the first reporting period of the grant (Months 1-12) we have made significant progress toward the specific aims of this project. One setback was an unforeseen delay in obtaining the NF1 +/- mice and then establishing a breeding colony as described in *Task 1*. While this task is underway, we have established procedures, optimized conditions, and determined baseline measurements for the required assays in wildtype mice and cells. Establishment of procedures and conditions will allow us to rapidly analyze animals once they are available.

**Task 1. Procure Nf1+-/ mice and establish a breeding colony, Months 1 – 36:**

This task is underway but the animals required for experimental analysis are not yet available.

**Task 2. Characterize osteoblast differentiation and function from Nf1+/- mouse cells in culture, Months 3 – 18:**

a. Isolation of primary osteoblasts.
b. Perform cell culture and analysis of the time course of osteoprogenitor proliferation and apoptosis.
c. Perform cell culture and analysis of the time course of maturation and mineralization.
d. Isolate RNA, assay osteoblast gene expression by quantitative PCR over the time course of differentiation.

We have isolated primary osteoblast cells from wildtype animals and established conditions for growth and differentiation. These are important controls for Task 2; parts a & b. Using primary wildtype cells and the MC3T3 cell line we have determined conditions and procedures for the differentiation time course, RNA isolation, as well as the QPCR conditions for measuring neurofibromin expression in these cells. These results relate directly to Task 2; parts c &d, and are presented in *Figure 2* below (see appended abstracts 1 and 2). For this task we designed primers and tested for neurofibromin amplification, established amplification conditions, and determined QPCR primer efficiency.

**Task 3. Characterize osteoclast formation and function from Nf1+/- mouse cells in culture, Months 9 – 24:**

a. Time course of osteoclast formation in whole marrow cultures.
b. Characterize osteoclast formation and function in cocultures of Nf1+/- and wild type mouse osteoblasts and marrow macrophage.
c. Characterize osteoclast formation and function from marrow macrophage +RANKL.
d. Isolate RNA, assay gene expression by quantitative PCR over the time course of differentiation.

While we routinely generate osteoclast cells from marrow macrophages +RANKL (Task 3; part c), in Months 9-12 we have determined conditions and protocols for the whole marrow cultures and cocultures, described in Task 3; parts a & b, using wild type mice.

**Task 4. Characterize fracture repair in Nf1+/- mice, Months 12 – 36:**

a. Production of simple transverse fractures in Nf1+/- and wild type control mice.
b. Establish time course of repair by micro-CT analysis.
c. Histology over time course of fracture repair in Nf1+/- mice.
d. Immunohistochemical staining for targets identified in Tasks 2 and 3.
e. Isolate RNA, assay gene expression by quantitative PCR over the time course of fracture repair in Nf1+/- mice and wild type controls.
f. Mechanical testing of unfractured and repaired bones from Nf1+/- mice and wild type controls.
In the absence of NF1 +/- test animals for Tasks 2 & 3, we moved ahead to Task 4 and established the fracture model, for wild type mice. With the assistance of our collaborator, Louis Gerstenfeld, we were taught procedures for the mouse fracture model, as described in Task 4; part a. In addition, we purified RNA over the time course of fracture healing (as in Task 4; part e) in wildtype animals and measured neurofibromin expression by QPCR (see Figure 3 below, and appended abstracts 1 and 2). In addition to establishing these protocols in our hands, we have established the time course of neurofibromin expression over fracture healing.

In order to characterize the cell typed expressing neurofibromin in normal bone and in fracture healing, we have fixed, decalcified, and sectioned samples of mouse bone fracture over the course of healing. In addition, we have optimized conditions for immunohistochemical detection of neurofibromin, including conditions for antigen retrieval, primary antibody concentration, secondary antibody concentration, wash and development conditions. As shown in Figure 1 for normal wildtype bone, and in Figure 4 in the fracture callus, day 7 (see appended abstracts 1 and 2). These procedures are all pertinent to Task 4; parts c & d.

Task 5. Characterize the bone cells and molecules present in revision samples from Orthopaedic NF1 patients, Months 1 – 36:
   a. Ongoing Human NF1 bone sample collection.
   b. Histological analysis of NF1 bone samples.
   c. Immunohistochemical analysis of NF1 bone samples.

Our protocols for human subjects have not been cleared by all applicable IRBs and as such we cannot begin collecting tissues. Protocols for all concerned entities have been written and are under review or have been reviewed and accepted or are in revision. We anticipate that with few changes all IRBs will be satisfied and tissue collection can begin in the immediate future.
Figure 1) Immunohistochemical detection of neurofibromin in osteoblast cells. Tibiae from wild type mice were fixed in 4% paraformaldehyde (PFA) and decalcified with EDTA (0.5M for 3 days). Serial sections (5mm) were prepared and probed with antibodies against neurofibromin (sc-67; SantaCruz) or Cbfa1 (sc-10758; SantaCruz). After washing, antibody was detected with HRP- conjugated secondary antibody and counter stained with hematoxylin. Controls with no primary or no secondary antibody were clean (not shown). In the figure, bone tissue is identified by "B". All bone-adherent cells (osteoblasts and lining cells) stained positive for neurofibromin (left panel). The osteoblast transcription factor Cbfa1 displayed nuclear distribution in the differentiating sub-population of osteoblasts. Therefore, bone-adherent stromal cells, pre-osteoblast cells, and committed osteoblast cells (Cbfa1 positive cells) all express neurofibromin.

Figure 2) Quantitative RT-PCR for neurofibromin in the early osteoblastic differentiation of MC3T3 cells. Mineralizing MC3T3 cells (clone e1) were grown to confluence in 6 well plates and medium was changed to mineralization medium (DMEM, 10% FBS, containing 0.1mM L-ascorbic acid and 1mM b-glycerol phosphate). RNA was purified from triplicate cultures on the days indicated and neurofibromin message steady-state levels were measured by real-time RT-QPCR using Sybr green dye incorporation. Data were analyzed using the $\Delta\Delta$Ct method (Pfaffl et al., 2002) with normalization to the housekeeping gene Hydroxymethylbilane synthase (HMBS). Neurofibromin mRNA steady-state levels were seen to increase in early osteoblastic differentiation. Experiments covering the entire time course of MC3T3 differentiation to mineral nodule formation (day 21) are in progress.
RNA was purified from fracture calluses, on the days indicated (post-fracture). Neurofibromin message steady-state levels were measured by real-time RT-QPCR using Sybr green dye incorporation. Data were analyzed using the DDCt method as described in Figure 2. Neurofibromin mRNA steady-state levels increased in early fracture healing, consistent with the endochondral/primary bone formation stage described by L.C. Gerstenfeld et al (2003).

4) Immunohistochemical detection of neurofibromin in fracture healing of mouse bones. Femora of wild type mice (C57Bl/6) were fractured and bones were harvested after 5, 7, 10 or 14 days. Bones were fixed in 4% PFA and decalcified with EDTA (0.5M for 3 days). Serial sections (5mm) were prepared and probed with antibodies against neurofibromin (sc-67; SantaCruz) as described in Figure 1. After washing, the antibody was detected with HRP conjugated secondary antibody and counterstained with hematoxylin (right panels). Adjacent sections were stained with safarin O and fast green (left panels). Using safarin O and fast green staining, cartilage is stained bright red and bone is stained light blue. Primary bone formation occurs with the resorption of cartilage while secondary bone formation and remodeling to lamellar bone occurs from the edge of the fracture callus. In the day 7 samples shown, neurofibromin expression was detected at sites of 1<sup>o</sup> bone formation and in bone lining cells. Little neurofibromin expression was seen in the cartilagenous fracture callus. Due to the poor preservation of cellular morphology of bone-adherent cells we were unable to identify osteoclasts or chondroclasts in these samples.
KEY RESEARCH ACCOMPLISHMENTS:

Results

1. Establishment and optimization of assay conditions (primer sequences, primer efficiency, amplification conditions, etc.) for real-time RT-PCR amplification of mouse neurofibromin gene product.

2. Time course of neurofibromin mRNA steady-state expression levels over osteoblastic differentiation of MC3T3e1 cells.

3. Establishment of the mouse fracture repair model and preparation of samples for days 5, 7, 10, and 14 for wild type animals.

4. Measurement of neurofibromin mRNA steady-state expression levels over the course of bone fracture healing in a mouse model.

5. Immunohistochemical detection of neurofibromin in normal mouse bone and in a mouse model of fracture healing.

Personnel

We were fortunate to recruit Tania N. Crotti, Ph.D. to work as a postdoctoral fellow on the project. Dr. Crotti comes to us from the University of Adelaide, South Australia. In her first postdoctoral position was awarded a C.J. Martin Fellowship from the Australian Government. Dr. Crotti has broad experience in skeletal disease research. She brings to the project her extensive experience in immunohistochemical detection techniques, especially in skeletal cells and tissues (1, 4, 5). Dr. Crotti trained in molecular techniques in my laboratory, which has resulted in several publications as well as a Novartis Young Investigator Award from the 2005 ECTS/IBMS and an ASBMR Harold Frost Young Investigator Award (2, 3).

REPORTABLE OUTCOMES:

Publications

Abstracts Presented:


CONCLUSION:

Cells expressing Nf1 protein in vivo were identified by immunohistochemistry (IHC). IHC for Cbfa-1 was used to identify osteoblast-lineage cells and TRAP staining identified osteoclast-like cells. Nf1-positive cells were abundant within the primary spongiosa. TRAP positive osteoclast-like cells on the bone surface were weakly positive for Nf1. Bone-adherent osteoblast lineage cells, irrespective of Cbfa-1 expression, uniformly expressed Nf1. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express Nf1 in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism. To verify gene expression of Nf1 in bone lineage cells we measured Nf1 expression in MC3T3-E1 cells over the course of differentiation. Using RT-QPCR, we find that Nf1 mRNA expression is initially low and increases over the course of early osteoblastic differentiation of MC3T3 cells.

We next examined the expression of neurofibromin across the time course of bone fracture healing using quantitative RT-PCR with RNA samples collected from a mouse model of fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification, with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation. Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. In the mouse model of fracture healing neurofibromin mRNA expression was increased by four-fold 5 days post-fracture, with expression declining by day 14 and reduced to background levels by 21 days post-fracture. This time course of neurofibromin mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. Elevated rates of bone resorption are typically observed in the second to fourth weeks post-fracture, during primary and secondary bone formation. Therefore, osteoclastic bone resorption coincides with the decline in neurofibromin expression. Taken together these observations indicate that neurofibromin expression is associated with bone-adherent osteoblast-lineage cells with minimal expression in osteoclasts. In addition neurofibromin expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing.
REFERENCES


NEUROFIBROMIN EXPRESSION IN MOUSE BONE AND IN FRACTURE HEALING

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A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including alterations in bone size and shape, the presence of scoliosis, and a tendency to develop pseudoarthrosis. These skeletal manifestations of NF1 are further compounded by generalized skeletal osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. Normal bone remodeling and healing require the concerted actions of bone forming osteoblasts and bone resorbing osteoclasts. However, the cell type and pathway by which neurofibromin haploinsufficiency leads to dysregulation of normal bone remodeling and healing are unknown.

To better understand the function of neurofibromin in normal bone cell physiology and fracture healing, we used immunohistochemistry (IHC) to identify cells expressing neurofibromin protein in vivo. In addition, IHC for Cbfa-1 was used to identify osteoblast-lineage cells and histochemical staining for tartrate-resistant acid phosphatase (TRAP) identified osteoclasts. In tibiae isolated from young C57Bl6/J mice, neurofibromin-positive cells were abundant within the primary spongiosa, located below the growth plate, which is an active site for resorption of calcified cartilage and formation of trabecular bone. TRAP positive osteoclasts throughout the bone were weakly positive for neurofibromin. Neurofibromin was uniformly expressed by bone-adherent osteoblast lineage cells regardless of their status for Cbfa-1 staining. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express neurofibromin in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism.

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In future experiments characterization of the cellular and molecular events associated with bone healing in the Nf1+/- mouse fracture model will provide a model in which to test new strategies aimed at improving bone quality and healing, leading to improved outcomes and reducing the negative economic, social, and physical impact of this disorder in the primarily pediatric orthopaedic NF1 population.
Neurofibromin Expression in Bone Fracture Healing

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Cells expressing Nf1 protein in vivo were identified by immunohistochemistry (IHC) on tibiae from young mice. IHC for Cbfa-1 was used to identify osteoblast-lineage cells and TRAP staining identified osteoclast-like cells. Nf1-positive cells were abundant within the primary spongiosa. TRAP positive osteoclast-like cells on the bone surface were weakly positive for Nf1. Bone-adherent osteoblast lineage cells, irrespective of Cbfa-1 expression, uniformly expressed Nf1. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express Nf1 in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to the bone surface to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism. To verify gene expression of Nf1 in bone lineage cells we measured Nf1 expression in MC3T3-E1 cells over the course of differentiation. Using RT-QPCR, we find that Nf1 mRNA expression is initially low and increases over the course of early osteoblastic differentiation of MC3T3 cells.

We next examined the expression of Nf1 in a mouse model of fracture healing. mRNA expression was measured by RT-QPCR in samples from the time course of bone fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation. Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. We find that Nf1 mRNA expression is increased by four fold 5 days post-fracture, with expression declining by day 14 to background levels by 21 days post-fracture. This time course of Nf1 mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. Nf1 expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing.