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Mechanical Signaling in *NF1* Osteoblast Cells

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Abstract

Neurofibromatosis Type I (*NF1*) syndrome is characterized by neurofibromas and neural tumors but is also associated with skeletal abnormalities. The cellular pathophysiology of skeletal abnormalities in *NF1* is not understood. These abnormalities result from constitutive active RAS and its downstream effectors, RASERK pathway, due to mutation of *NF1* gene which converts active RAS-GTP into inactive RAS-GDP. In osteoblast cells, RAS-ERK pathway is involved in cell proliferation and differentiation and is also involved in mechanical signals transduction.

In this study, we propose that *Nf1* mutation in osteoblast cells will affect the response to mechanical stimulation through the RAS pathway. The Flexcell tension system was used to mechanically stimulate calvarial osteoblast precursor from conditional knockout mice, *Nf1*(ob-/-), and wild type calvarial osteoblast precursor cells, (WT. The protocol of cyclic mechanical strain was 2% to 4% elongation at 0.16 Hz (10 cycles per minute) for 24 h. Mechanically stimulated cells showed lower expression levels of the osteoblast marker gene, *RUNX2*, measured at 4 h and 8 h post-stretch. Mineralized matrix deposition, assessed by Alizarin red staining, was decreased in *Nf1*(ob-/-) compared to (WT) cells following mechanical stimulation. the *Nf1*(ob-/-) and WT osteoblast precursor cells were then treated with RAS inhibitor (FTI-277), for 4 h and 8 h. *RUNX2* expression level was increased in *Nf1*(ob-/-) cells compared to non-treated cells. However, the opposite result was seen in (WT) cells. The FTI-277 treatment resulted in lower *RUNX2* expression level and lower mineralized matrix deposition.

This response of (WT) cells was normal. However, the *Nf1*(ob-/-) response showed that these cells although they have hyper-active RAS, but when it is exposed to stress, it loses its ability to express osteoblast markers or lay down mineralized matrix. Our results indicate that, the hyper-active RAS in *NF1* mutant osteoblast will result in cells being stuck in proliferative state and unable to differentiate.

Keywords: Neurofibromatosis; Osteoblasts; Bone; Mechanical signaling; Craniofacial

Introduction

Neurofibromatosis type 1 (*NF1*) is an autosomal dominant disorder caused by loss of function mutations in the *NF1* gene with an incidence of approximately 1 in 3000, making it one of the most common genetic disorders [1,2]. *NF1* syndrome is primarily characterized by subcutaneous neurofibromas and neural tumors. In addition, *NF1* is associated with several skeletal abnormalities including, scoliosis, tibial bowing and sphenoid wing dysplasia [3,4]. Unfortunately, the cellular pathophysiology of the *NF1* skeletal dysplasia is still not fully understood [5]. The main functional domain of *NF1* gene is known to be located between exon 27 and 34, known as RAS-GAP domain which gives the *NF1* gene it's tumor suppressor property [6,7]. The *NF1* gene encodes neurofibromin, a RAS GTPase-activating protein (GAP) that promotes the conversion of an active RAS-GTP-bound form to an inactive RAS-GDP form and functions to negatively regulate the activity of RAS effectors, including the RAF–MEK–ERK signaling pathway [8,9]. Thus, *NF1* mutations results in activation of canonical mitogen-activated protein kinase (MAPK) signaling [10,11]. Of relevance to skeletal development, *NF1* expression has been reported in hypertrophic chondrocytes, which are an important intermediate step during endochondral ossification, and also in adult osteoblast and osteoclasts [12,13] either of which might explain the skeletal involvement in *NF1*. It is established that skeletal tissue can sense mechanical loading which induces bone remodeling activity, resulting in structural changes through different cellular pathways. Several studies have shown that, RAS-MAPK-ERK pathway is the main contributor in mechanical signaling response in osteoblast cells [14-17]. The role of MAPK signaling components have been shown to favor osteoblastic cell proliferation and differentiation. In particular, ERK1/2 is involved in cell proliferation, differentiation and the survival of several cell types, including osteoblasts [18,19]. ERK1/2 signals can promote the proliferation and anabolism of osteoblasts in order to facilitate bone turnover, thereby contributing to the homeostasis of bone tissue [20,21].

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Knowing that neurofibromin is expressed in bone cells and acting on RAS signaling pathway and that bone cells can adapt to mechanical stimulation through activation of the RAS signaling pathway, we hypothesize that the response of *NF1* mutant osteoblast to mechanical stress is defective which contributes to the skeletal tissue abnormalities in *NF1* patients.

Signaling in *NF1* Osteoblast Cells

Here we conduct multiple experiments to determine how *NF1* mutant osteoblast cells respond to mechanical stress in terms of bone formation and differentiation. We tested if antagonizing the hyper-active RAS in *NF1* osteoblast cells would reverse the effect of the mutation. In the mouse model employed (herein called *NF1*(ob-/-) mice), ablation of *NF1* occurs at the pre-osteoblast stage and is restricted to bone forming cells [22-24]. The global *Nf1* knock-out mouse models, such as *Nf1*(-/-) is prenatal lethal due to cardiomyopathies and the *Nf1* heterozygotic mouse *Nf1*(-/+) mice does not develop any skeletal abnormalities. *Nf1*(ob-/-) mice allows us to study the effect of *Nf1* mutation in osteoblast. In this model, the *Nf1* gene has been knocked-out using the Cre-LoxP system under the control of 2.3 kb collagen1-alpha1 promoter (Col11), which is primarily expressed in osteoblast [23,25]. Our current view of the *NF1* bone pathology, based on the analysis of osteoblast function, whether the imbalance of bone homeostasis and improper response of osteoblast cell to mechanical stress cause the *NF1* skeletal manifestations [26-35].

The *NF1* dystrophic skeletal pathologies, including the progressive sphenoid wing dysplasia (SWD), are associated with functional disability and changes in bone shape for which treatment or prevention is not available [1,36,37]. This highlights the dearth of knowledge related to *NF1* pathophysiology in bone. We conducted this study to understand how ablation of *NF1* in osteoblasts will affect their function in response to mechanical stress. Several *Nf1* mouse models have been developed to study cancer and bone abnormalities [38]. Initially, studies focused on generation of mice with a targeted mutation in *Nf1* gene. Then the next generation models focused on exon-specific knock-out mice. However recently tissue-specific *Nf1* gene knock-out has shown the function of neurofibromin in specific cell types. Here we isolated osteoblasts from mice in which *Nf1* gene has been deleted using 2.3 collagen1-alpha1 promoter (Col1), which is expressed mainly in osteoblasts [23,25]. Unlike ablation of *Nf1* in osteo-chondroprogenitor, these cells allowed us to study the effect on osteoblast function. In regard of RAS-GAP and *Nf1*, constitutively active RAS and its downstream kinase ERK1/2, are thought to underlie *NF1* skeletal manifestations [23,24,39]. In the experiments presented here, osteoblast cells from *Nf1*(ob-/-) produce little mineralized matrix when growing in an osteoinductive medium (OIM) compared to *NF1*(WT) cells.

Results and Discussion

This result supports the previous finding of the lack of *NF1* in osteoblast resulting in reduced bone mineral density (BMD) and reduced mechanical properties [40]. Blockade of RAS protein post-translation modification is shown to prevent its targeting to the cell membrane and abrogation of its downstream effects. FTI-277 is an anti-RAS treatment which prevents RAS isoprenylation [35,41]. We hypothesize that if we inhibit hyper-active RAS using FTI-277, then the *NF1* mutant osteoblast cell should be able to differentiate normally. We find that, when treated with 5 µM of FTI-277 in OIM culture medium *Nf1*(ob-/-) cells were able to produce more mineralized matrix. However, the *Nf1* (WT) osteoblast cells treated with 5 µM of FTI-277 produced less mineralized matrix when compared to untreated cells. This indicates that the level of RAS signaling is critical not only for osteoblast proliferation and differentiation but for normal function and matrix deposition. This is confirmed by the level of *RUNX2* expression, which is known to be phosphorylated by ERK1/2 during matrix deposition [42,43].

Since we know that RAS-ERK pathway is involved in mechanical signal transduction [28,44]. We asked what is the role of *NF1* in osteoblast during mechanical stress. Although different systems have been used to strain osteoblast cells, it is very difficult to compare *in-vitro* applied strains with those applied *in-vivo* because the characteristics of the strains are different, i.e. the 3D configuration and presence of interstitial fluid *in-vivo*. The Flexcell system has been a good platform to study the effect of mechano stimulation in many different contexts [45,46]. This system applies an equibiaxial strain to the cells using a flexible silicone bottom plate connected to a computer-controlled vacuum device. In the literature a wide variety of parameters have been used (i.e. the magnitude of stretch, the frequency of stretch and the duration of stretch, to mechanically stimulate different cell types). In terms of bone cells, there is a general concordance that the frequency is more important than the magnitude of stretch applied and 2% to 4% elongation is found to be enough to initiate osteoblast cellular response. At higher magnitudes (i.e. 10% to 12%) it is reported to be lethal to the cell [20,45]. In this study, we used the Flexcell system to apply a mechanical strain to *Nf1* (ob-/-) and *Nf1*(WT) at (10 cycles per minute (0.16 Hz) for 24 h and 3% elongation). We found, in terms of protein expression that *RUNX2* showed lower expression level in *Nf1*(ob-/-) upon mechanical stretching compared to non-stretched control cells. However, the opposite was found in *Nf1*(WT) cells with *RUNX2* expression level increased with stretching in *Nf1*(WT) cells. Also we tested the downstream effects of stretching on mineralized matrix formation. It has been shown that mechanical stimulation leads to increased matrix deposition [32,47].

Conclusion

Our results showed, *Nf1*(ob-/-) cells upon mechanical stimulation were unable to responded normally and unable to form more matrix compared to *Nf1*(WT). *Nf1*(ob-/-) cells

deposit less matrix when mechanically stimulated compared to non-stressed *Nf1*(ob-/-) cells. This potentially explains why *NF1* patients have defective bone healing process and also why the skeletal lesions show a progressive nature. Bone tissue is continually being remodeled depending on the mechanical environment [21], therefore the defective mechanical transduction signals *NF1* osteoblast cells produced upon mechanical stress are responsible for the abnormal response to stress. Coupling between bone formation and resorption and involvement of osteoclasts in this process (*in vivo*) may direct future studies to determine the response of osteoclast cells in *NF1* to mechanical stimulation as this might bridge the gap and help to understand the cellular events leading to skeletal abnormalities in *NF1* patients [48].

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