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TITLE: An Unbiased Approach to Search for the Cause of the Reduced Osteogenic Differentiation Potential of NF1-Deficient Osteoprogenitors

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14. ABSTRACT

We will use the critical observation that Nf1+/- and Nf1-deficient osteoprogenitors are both characterized by constitutive activation of ERK signaling, but only the latter fail to differentiate, to identify ERK-independent pathway/genes causing the reduced differentiation potential of Nf1-deficient BMSCs, using a nonbiased RNA-Seq approach. The candidate gene signature will be "enriched" in high confidence genes/pathways by selecting differentially expressed genes between genotypes conserved between mouse and human BMSCs. The second part of the proposed work will consist in testing, functionally and in vitro, the contribution of selected candidate genes and pathway(s) in "rescue" types of experiments based on the use of Nf1-deficient BMSCs and osteoblast differentiation assays as primary readout.

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1. INTRODUCTION

Unilateral bowing of the tibia, fracture and recalcitrant healing (pseudarthrosis) represents, along with NF1 dystrophic scoliosis, the most challenging orthopedic conditions to manage in children with NF1. The etiology of these skeletal dysplasiae remains unclear, thus limiting treatment options. Candidate approaches have failed to identify such targets, hence an alternative unbiased exploratory strategy is proposed. *The hypothesis of this work is that inhibition of the MAPK/ERK pathway, as currently focused on for other NF1 manifestations, is not going to be beneficial to improve the management of NF1 pseudarthrosis, and that this condition stems from an ERK-independent anomaly*. In Aim 1, we will search for differentially expressed genes between both mouse and human-derived bone marrow stromal cells (BMSCs) heterozygous and homozygous for *NF1* mutations or *Nf1* flox recombination, respectively. In Aim 2, we will use gain or loss-of-function experiments to functionally determine the contribution of selected candidate genes to the reduced osteogenic potential of *NF1*-/- BMSCs.

2. KEYWORDS

NF1, Neurofibromatosis type 1, bone non-union, pseudarthrosis, osteoblast, differentiation, RNAseq, ERK

3. ACCOMPLISHMENTS

- What were the major goals of the project?

Specific Aim 1: Identify differentially expressed genes between mouse and human-derived BMSCs heterozygous and homozygous for *NF1* mutations/ *Nf1* flox recombination, respectively.		
Major Task 1: Prepare mouse and human-derived BMSCs		
Subtask 1: Generation of BMSCs from mice.	1-2	Completed

Mice used: *Nf1* flox/flox (10), flox/+ (10) and +/+ (10) to generate cells. Cell used: Mouse BMSCs from *Nf1* floxed mice infected with Ad-cre [primary cells].		
Subtask 2: FAC-sorting of *NF1*+/- and *NF1* "-/-" human BMSCs prepared from 2 (deidentified) patients with NF1 pseudarthrosis (already available). Cell used: human BMSCs [primary cells].	1-2	Completed
Subtask 3: Immortalization of human BMSCs. Cell used: human BMSCs [primary cells].	2-5	Not completed due to senescence in KO cells
Subtask 4: Sequencing of *NF1* mutations in FAC-sorted human BMSCs. Cell used: human BMSCs [primary cells]	5-6	Completed for patient 1
Major Task 2: RNAseq analyses		
Subtask 1: RNA extraction and QCs.	2-6	Completed
Subtask 2: RNAseq assay.	6-7	Not completed
Subtask 3: RNAseq analyses.	7-9	Not completed
Milestone(s) Achieved: identification of specific ERK-independent gene targets of NF1 in BMSCs	9	
Specific Aim 2: Use gain or loss-of-function experiments to functionally determine the contribution of selected candidate genes to the reduced osteogenic potential of NF1-/- BMSCs.		Not completed
Major Task 1: Validate lead target genes		
Subtask 1: RT-qPCR measurements. Cell used: mouse and human BMSCs [primary cells].	10-11	Not completed
Subtask 2: Gain or loss of function experiments with gene expression and *in vitro* functional assays as readout. Mice used: *Nf1* flox/flox (30), flox/+ (30) and +/+ (30) to generate cells. Cell used: mouse and human BMSCs [primary cells].	12-24	Not completed
Milestone(s) Achieved: Identification of genes(s) and pathways whose blockade or stimulation improves the differentiation of mouse and human BMSCs deficient for NF1. Publication of 1-2 peer reviewed papers and presentation at conferences, including the CTF, ASBMR annual meetings.	24	Not completed

- What was accomplished under these goals?

We have successfully prepared bone marrow stroml cells (BMSCs)ef from all three genotypes, infected them with Ad-GFP and Ad-Cre adenoviruses and grown these cultures for two weeks in osteogenic conditions. RNA was collected and purified. In the course of these experiments, we noticed that cKO cultures were containing a high proportion of F4/80+ macrophages compared to WT cultures (**Figure 1A**). This is a potential issue because Ad-cre infection of these cultures will not only generate KO BMSCs but also KO macrophages, which could make interpretation of the findings difficult. To avoid this technical issue and the use of adenoviruses to induce *Nf1* recombination, we thus turned to the alternative of extracting WT, Nf1+/- and Nf1-/- BMSCs from the *Nf1*$_{Osx}$-*Cre* mouse line, in which *Nf1* is recombined in osteoprogenitors. We have grown BMSCs and periosteal cell cultures for one week in osteogenic conditions. RNA was collected and purified. In both cKO cultures, *Nf1* expression was reduced as expected, but validation for genes whose expression in WT and cKO BMSCs is known was more consistent in periosteal-derived cultures. Our current effort aims at further validating these cultures to make sure we take the cell preparations for the RNAseq assay that are the best adapted and validated.

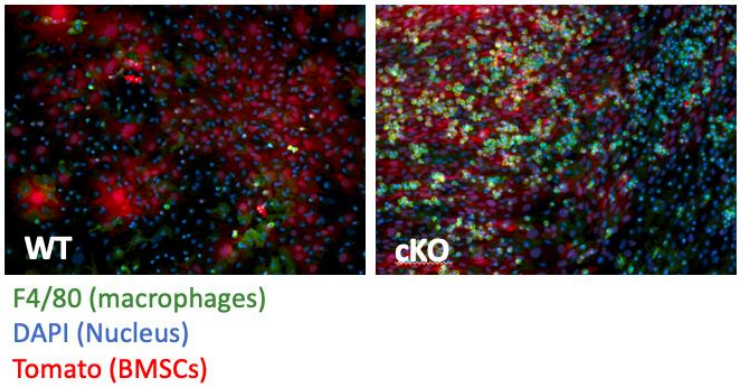

Figure 1: Presence of contaminating F4/80 macrophages in Nf1 cKO BMSC cultures.

Cells from two patients were sorted and the EREG$_{high}$ and EREG$_{low}$ fractions were frozen. During the necessary expansion of these cells, we discovered that the EREG$_{high}$ fractions fell behind in term of doubling time and showed signs of senescence, as shown by increased cell size, spreading and SA b-Gal activity higher than in EREG$_{low}$ *NF1*$_{+/-}$ cells. This phenotype limits our ability of obtaining enough sorted cells and RNA for RNAseq and immortalization. Sequencing of these EREG$_{high}$ cultures also revealed that less than 6% of cells had somatic mutations in these cultures, which is low to detect significant changes in gene expression. Based on these new results, we decided that single cell RNAseq instead of bulk RNAseq will be the best way to detect differences between genotypes and related disrupted pathways that could lead us to the identification of new targets. This approach, although a bit more costly, will allow us to cluster cells based on their overall expression profiles and know traits of cells with double hit somatic mutations will allow us to identify the cluster representing these cells. This dataset will then be compared to the mouse bulk RNAseq dataset to identify differentially expressed genes (DEGs) conserved between species. Because of the sensitivity of this scRNA approach and its higher cost, we plan to use cells from one patient, and to confirm DEGs in the other second sample of human cells by qPCR. We should be able to obtain this scRNAseq dataset over the summer.

- **What opportunities for training and professional development has the project provided?**

Dr. Efi Cuko (Post-doctoral fellow) in the laboratory was trained in primary bone cell culture, RNA preparation and quantitative gene expression analyses.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Once RNAs from mouse cell cultures are validated, they will be shipped for library preparation and sequencing. Data will then be analyzed in collaboration with Dr. Coarfa and top differentially expressed genes will be validated by qPCR.

Regarding human cells, scRNAseq will allow us to examine the sequence information from individual cells with optimized next-generation sequencing technologies, thus providing a higher resolution of cellular differences and a better understanding of the behavior of individual cells in the context of these mosaic cultures than bulk RNAseq. A frozen stock of EREG$_{high}$ cells will be thawed in July and RNA will be collected, QC'ed and sent for scRNA sequencing in August.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

Although not directly related to this project and its aims, the finding that *Nf1* deficiency in BMSCs leads to senescence is a finding that is novel and the focus of new efforts in the laboratory.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

BMSCs and periosteal progenitor cells directly isolated from WT, cHet and cKO mice are compared to *Nf1*$^{flox/flox}$ BMSCs infected in vitro with Ad-GFP or Ad-Cre because of issues with cKO macrophage contamination in cKO cultures. This is minor technical change.

Human mesenchymal bone cells with somatic *NF1* mutations show signs of senescence compared to their NF1+/- counterpart, which limits possibility to expand these cultures and immortalize them. We will thus use single cell RNAseq instead of bulk RNAseq to define the transcriptional landscape of KO cells versus Het cells in the same mosaic cultures.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The transition to the use of BMSCs directly isolated from WT, cHet and cKO mice created some delay as new breeding had to be used to generate all three genotypes and collect BMSCs, which had to be validated too. During this process, we had to stop all breeding, reduce our mouse colonies to a minimum and were not able to access the laboratory due to the COVID19 pandemic. We are now (07/2020) back to phase 1 reopening with about 50% of laboratory access compared to normal conditions. Mouse breeding was prioritized for this experiment which is ongoing. We thus lost about 4-5 months due to the COVID pandemic.

- **Changes that had a significant impact on expenditures**

COVID-related delays will impact the timing of the proposed work. NCE might be requested at the end of the award.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

N/A

- **Significant changes in use or care of human subjects**

N/A

- **Significant changes in use or care of vertebrate animals.**

N/A

- **Significant changes in use of biohazards and/or select agents**

N/A

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Nothing to Report.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Florent Elefteriou*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2 calendar months
Contribution to Project:	*Design and data analysis*
Funding Support:	DOD NF180077 NIH R01 AG055394-01

Name:	*Efrosini Cuko*
Project Role:	*Personnel*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	2.4 calendar months
Contribution to Project:	*Generation of mice, cell extraction and culture, gene expression analyses and validation.*
Funding Support:	DOD NF190061

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

"Nothing to Report."

- **What other organizations were involved as partners?**

"Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES

N/A