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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 translatable molecular 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. Mice used: *Nf1* flox/flox (10), flox/+ (10) and +/+ (10) to generate cells.	1-2	Completed

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 feasible 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
Major Task 2: RNAseq analyses		
Subtask 1: RNA extraction and QCs.	2-6	Completed
Subtask 2: RNAseq assay.	6-7	Completed
Subtask 3: RNAseq analyses.	7-9	Completed
Milestone(s) Achieved: identification of specific ERK-independent gene targets of NF1 in BMSCs	9	Completed
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.		
Major Task 1: Validate lead target genes		
Subtask 1: RT-qPCR measurements. Cell used: mouse and human BMSCs [primary cells].	10-11	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, ongoing
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	No manuscript published yet but NIH R01 grant secured to follow up on these findings and directly test effect of senolytics in vivo in a model of NF1 pseudarthrosis.

- **What was accomplished under these goals?**

2019-2020 (year 1):

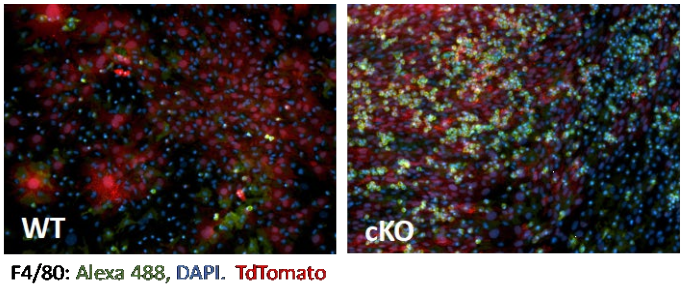

Figure 1: Presence of contaminating F4/80 macrophages in *Nf1* cKO BMSC cultures.

Mouse cells: We have successfully prepared bone marrow stromal cells (BMSCs) from *Nf1*$^{flox/flox}$ and *Nf1*$^{flox/+}$ mice, infected them with Ad-GFP and Ad-Cre adenoviruses to generate all three genotypes (WT, cHet and cKO), and grown these cultures for two weeks in osteogenic conditions. RNA was purified and used to confirm *Nf1* knock-down and proper osteogenic differentiation of WT cells. In the course of these experiments, we noticed that cKO cultures contained a high proportion of F4/80+ macrophages compared to WT cultures (**Fig. 1**). This is a potential issue because Ad-cre infection of these cultures will not only generate *Nf1* cKO BMSCs but also cKO macrophages, which could confound interpretation of the findings. To avoid this 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; Nf1*$^{f/f}$ mouse line, in which *Nf1* is recombined in Cre+ osteoprogenitors upon removal of doxycycline from the diet. We have grown BMSCs and periosteal cell cultures for one week in osteogenic conditions. RNA was collected and purified. In both cultures, *Nf1* expression in *Nf1* cKO cells was reduced compared to cHet and cKO cultures, as expected from our previous work.

Human cells: 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, known to harbor *NF1* second hit mutations, fell behind in term of doubling time, to the point that expansion was not possible. This phenotype limited our ability of obtaining enough sorted cells and RNA for RNAseq analyses 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 via bulk RNAseq analyses. These preliminary results suggested that human bone marrow stromal cells with *NF1* loss-of-function were impaired in their ability to proliferate or survive after digestion from pseudarthrotic bone tissues. We thus decided that single cell RNAseq instead of bulk RNAseq would 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 more costly, will allow us to cluster cells based on their overall expression profiles and know traits of cells with double hit *NF1* somatic mutations and 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 later on confirm DEGs in other samples of human cells by qPCR or adapted experiments.

2020-2021 (Year 2)

Mouse cells: WT, *Nf1* cHet and *Nf1* cKO BMSC cultures from DOX-treated *Nf1*$_{Osx}$*-Cre; Nf1*$^{+/+}$, *Nf1*$_{Osx}$*-Cre; Nf1*$^{f/+}$ and *Nf1*$_{Osx}$*-Cre; Nf1*$^{f/f}$ mice were generated and DOX removed from the culture at time of confluence (day -3) to induce *Nf1* gene recombination. RNAs were collected from undifferentiated cells (day 0) and differentiated cells (day 7) following culture in osteogenic medium. Gene expression assays by qPCR analyses validated the downregulation of *Nf1* in cKO BMSCs and the increase in osteogenic markers upon differentiation (d7 versus d0) in WT cells. RNA quality was verified to be RIN>7. RNA sequencing was performed by Genewiz. Following base calling, quality score evaluation, filtering of low quality reads, alignment to reference transcripts from mm10 genomes and generation of expected read counts for each transcript, differential gene expression analysis was performed by DeSeq2. Gene Set Enrichment Analysis (GSEA) was performed using all expressed genes and analyzed for enrichment of all curated pathways in the Molecular Signatures Database (MSigDB). The dataset was analyzed by Gene ontology enrichment (GO), KEGG database, REACTOME database and Ingenuity (IPA) pathway analyses to compare DEGs between WT, cHet and cKO BMSCs.

GeneSets	NF1.KO_over_WT_Day7	NF1.KO_over_Het.Day7
GO_DEFENSE_RESPONSE	4.22	5.94
GO_IMMUNE_EFFECTOR_PROCESS	5.14	5.82
GO_CELL_ACTIVATION	5.41	5.81
GO_ADAPTIVE_IMMUNE_RESPONSE	3.54	5.31
GO_REGULATION_OF_IMMUNE_SYSTEM_PROCESS	2.85	5.29
GO_INFLAMMATORY_RESPONSE	3.35	5.28
GO_REGULATION_OF_IMMUNE_RESPONSE	3.02	5.28
GO_CYTOKINE_PRODUCTION	3.16	5.10
GO_CYTOKINE_MEDIATED_SIGNALING_PATHWAY	3.55	4.94
GO_MYELOID_LEUKOCYTE_ACTIVATION	5.53	4.92
GO_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE	5.18	4.87
GO_RESPONSE_TO_CYTOKINE	3.10	4.79
GO_LEUKOCYTE_MEDIATED_IMMUNITY	5.12	4.76
GO_POSITIVE_REGULATION_OF_IMMUNE_SYSTEM_PROCESS	2.51	4.67
GO_LYMPHOCYTE_ACTIVATION	3.34	4.67
GO_LEUKOCYTE_MIGRATION	3.25	4.64
GO_REGULATION_OF_CELL_ACTIVATION	2.92	4.53
GO_RESPONSE_TO_BIOTIC_STIMULUS	3.11	4.48
GO_INNATE_IMMUNE_RESPONSE	3.36	4.47
GO_POSITIVE_REGULATION_OF_IMMUNE_RESPONSE	2.70	4.43
GO_SECRETION	3.93	4.43
GO_RESPONSE_TO_BACTERIUM	2.91	4.22
GO_REGULATION_OF_IMMUNE_EFFECTOR_PROCESS	2.34	4.20
GO_CELL_CHEMOTAXIS	3.10	4.19
GO_DEFENSE_RESPONSE_TO_OTHER_ORGANISM	3.75	4.16
GO_BIOLOGICAL_ADHESION	-5.46	4.13
GO_REGULATION_OF_SIGNALING_RECEPTOR_ACTIVITY	3.22	4.10
GO_LEUKOCYTE_PROLIFERATION	3.29	4.09
GO_MYELOID_LEUKOCYTE_MEDIATED_IMMUNITY	5.59	4.07
GO_NEGATIVE_REGULATION_OF_CYTOKINE_PRODUCTION	2.59	4.03

GeneSets	NF1.KO_over_WT_Day7	NF1.KO_over_Het.Day7
GO_TUBE_DEVELOPMENT	-6.08	-2.46
GO_ANIMAL_ORGAN_MORPHOGENESIS	-5.90	-3.64
GO_EMBRYONIC_MORPHOGENESIS	-5.79	-3.11
GO_TUBE_MORPHOGENESIS	-5.62	-2.43
GO_BIOLOGICAL_ADHESION	-5.46	4.13
GO_EMBRYO_DEVELOPMENT	-5.31	-2.36
GO_CELL_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES	-5.30	-2.20
GO_CIRCULATORY_SYSTEM_DEVELOPMENT	-5.23	-2.23
GO_POSITIVE_REGULATION_OF_RNA_BIOSYNTHETIC_PROCESS	-5.23	0.00
GO_CARDIOVASCULAR_SYSTEM_DEVELOPMENT	-5.17	-2.52
GO_ANATOMICAL_STRUCTURE_FORMATION_INVOLVED_IN_MORPHOGENESIS	-5.14	-2.66
GO_POSITIVE_REGULATION_OF_GENE_EXPRESSION	-5.14	-1.82
GO_SKELETAL_SYSTEM_DEVELOPMENT	-5.10	-4.00
GO_OSSIFICATION	-5.01	-3.84
GO_SENSORY_ORGAN_DEVELOPMENT	-4.94	-3.55

GO_PATTERN_SPECIFICATION_PROCESS	-4.87	-2.97
GO_CENTRAL_NERVOUS_SYSTEM_DEVELOPMENT	-4.78	-2.73
GO_SKELETAL_SYSTEM_MORPHOGENESIS	-4.73	-3.12
GO_NEGATIVE_REGULATION_OF_RNA_BIOSYNTHETIC_PROCESS	-4.72	-2.47
GO_NEUROGENESIS	-4.72	-2.42
GO_CELL_FATE_COMMITMENT	-4.71	-2.77
GO_CONNECTIVE_TISSUE_DEVELOPMENT	-4.65	-2.96
GO_HOMOPHILIC_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES	-4.64	-1.77
GO_EXTRACELLULAR_STRUCTURE_ORGANIZATION	-4.63	-3.31
GO_POSITIVE_REGULATION_OF_TRANSCRIPTION_BY_RNA_POLYMERASE_II	-4.62	0.00

GeneSets	**NF1.KO_over_WT_Day7**	**NF1.KO_over_Het.Day7**
HALLMARK_INTERFERON_GAMMA_RESPONSE	4.91	5.89
HALLMARK_INTERFERON_ALPHA_RESPONSE	4.85	5.55
HALLMARK_INFLAMMATORY_RESPONSE	3.36	4.94
HALLMARK_ALLOGRAFT_REJECTION	3.49	4.65
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.98	4.08
HALLMARK_IL2_STAT5_SIGNALING	2.26	3.50
HALLMARK_KRAS_SIGNALING_UP	2.88	3.46
HALLMARK_IL6_JAK_STAT3_SIGNALING	2.28	3.45
HALLMARK_OXIDATIVE_PHOSPHORYLATION	5.69	3.03
HALLMARK_APOPTOSIS	2.00	2.96
HALLMARK_P53_PATHWAY	2.00	2.88

GeneSets	**NF1.KO_over_WT_Day7**	**NF1.KO_over_Het.Day7**
REACTOME_INNATE_IMMUNE_SYSTEM	5.75	4.70
REACTOME_GPCR_LIGAND_BINDING	-4.27	3.94
REACTOME_CLASS_A_1_RHODOPSIN_LIKE_RECEPTORS	3.29	3.86
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	3.02	3.81
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	3.30	3.75
REACTOME_CELLULAR_RESPONSES_TO_EXTERNAL_STIMULI	3.84	3.36
REACTOME_NEUTROPHIL_DEGRANULATION	5.32	3.30
REACTOME_SIGNALING_BY_GPCR	-4.20	3.27
REACTOME_INTERLEUKIN_4_AND_INTERLEUKIN_13_SIGNALING	2.40	3.26
REACTOME_SIGNALING_BY_INTERLEUKINS	2.30	3.24
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	2.85	3.17
REACTOME_CHROMATIN_ORGANIZATION	-3.17	3.03
REACTOME_TOLL_LIKE_RECEPTOR_CASCADES	2.51	2.86
REACTOME_TRANSCRIPTIONAL_REGULATION_BY_TP53	3.04	2.83
REACTOME_LDL_CLEARANCE	1.80	2.80
REACTOME_INTERFERON_SIGNALING	2.43	2.79
REACTOME_CELLULAR_RESPONSES_TO_STRESS	3.45	2.76
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	-2.42	2.75
REACTOME_METABOLISM_OF_AMINE_DERIVED_HORMONES	0.00	2.73
REACTOME_INFLAMMASOMES	1.86	2.71
REACTOME_AUTOPHAGY	2.30	2.63
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	3.07	2.60
REACTOME_TOLL_LIKE_RECEPTOR_4_TLR4_CASCADE	2.22	2.58
REACTOME_INTERLEUKIN_18_SIGNALING	2.02	2.58

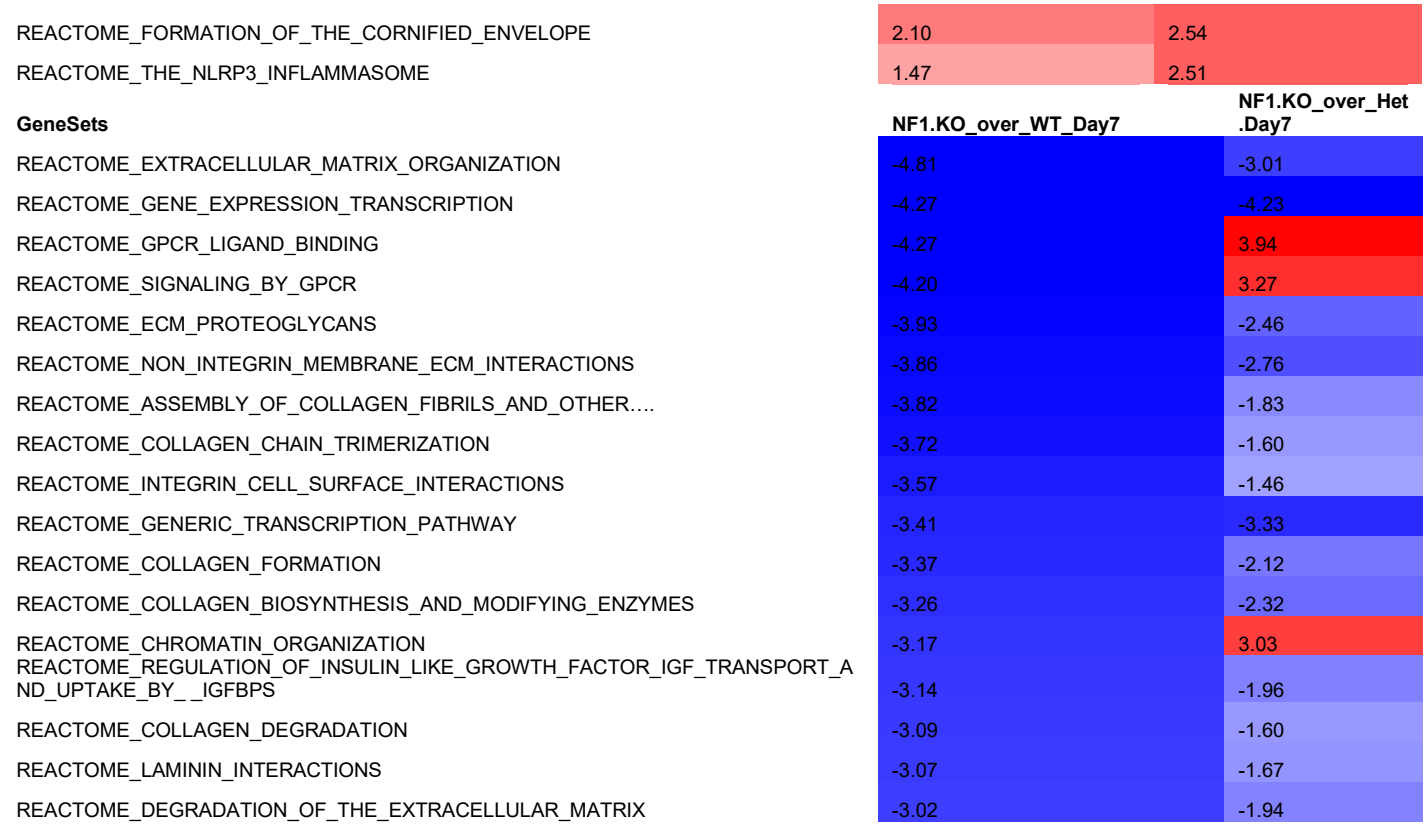

Figure 2. Molecular signature database analysis of the RNAseq dataset from WT and *Nf1* cKO BMSC cultures after 7 days of osteogenic differentiation.

The most obvious category of processes affected by *Nf1* deficiency in BMSCs, comparing KO cultures versus cHet (and WT) cultures were related to activation of immune cell process and inflammation (whereas processes related to ossification, skeletal system morphogenesis and ECM/connective tissues processes were downregulated, as expected from the reduced osteogenic potential of these cultures). Hallmark terms also showed clear immune-related changes with Interferon response observed as top dysregulated Hallmark in cKO cultures, consistent with the accumulation of macrophages in cKO cultures. Reactome ranking revealed a predominance of terms related to altered cell signaling and cytokines as upregulated in cKO BMSCs, and a reduction in genes related to ECM synthesis and degradation. The immune process and cytokine profiles detected through this dataset are in line with our observation that macrophages accumulate in cKO cultures (**Fig. 1**) and suggest that *Nf1* cKO BMSCs secrete a set of cytokines involved in immune cell recruitment, function or survival. This is potentially important as immune cells are involved at the early stage of bone repair. We are continuing to analyze this RNAseq dataset with the goal of identifying major common traits between mouse and human BMSCs characterized by neurofibromin loss of function.

Human cells:

Cells previously isolated and frozen from the pseudarthrosis site of one patient (GIAN2) were grown and RNA was submitted for scRNA sequencing after confirmation of RNA quality and NGS to demonstrate presence of cells with *NF1* loss-of-function (c.2033dupC; p.Ile679Aspfs* in exon 18; pathogenic truncating variant, heterozygous ; c.5839C>T; p.Arg1947* in exon 40; somatic pathogenic variant, stop codon).

Single-cell transcriptome sequencing was performed with the 3' v3.1 library preparation protocol using the 10X Genomics Chromium platform. Single-cell libraries were subjected to Illumina sequencing targeting 20,000 reads per cell. Sequence reads were mapped to the human reference genome (GRCh38) using the 10X Genomics CellRanger platform, which identified 8,906 cells with a median 6,015 genes sequenced per cell **(Fig. 3A-C)**. Following quality filtering to remove predicted cell doublets, a total 5,902 (66%) of cells were retained for analysis **(Fig. 3D-F)**.

Following normalization and removal of genes whose expression was not detected across all cells, t-SNE and k-means analysis identified two distinct cell clusters **(Fig. 3G)**. To identify biologic-meaningful differences between the two clusters, we performed differential expression analysis. ANOVA analysis detected 806 significantly differentially-expressed genes (DEGs) between the two cell clusters **(Fig. 3H)**. Enrichment analysis of DEGs demonstrated that genes regulating cell cycle processes were significantly ($p=1.71e^{-13}$) differentially expressed between the two cell clusters **(Fig. 3I)**. We continue to analyze this scRNAseq dataset for further identification of somatically-mutated *NF1*$^{-/-}$ cell and detection of molecular processes dysregulated between co-cultured *NF1*$^{-/-}$ and *NF1*$^{+/-}$ cells.

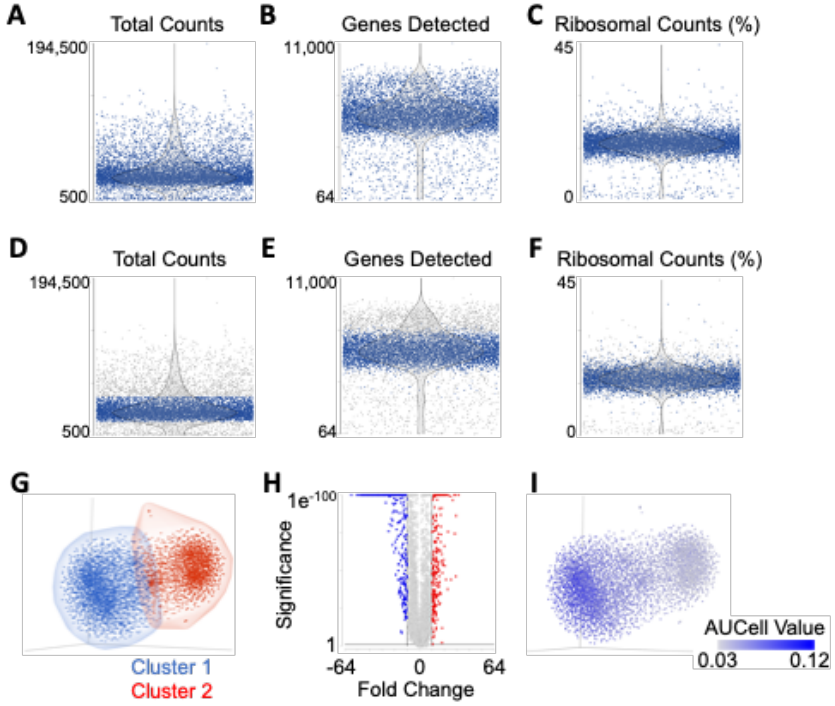

Figure 3. NF1 pseudarthrosis scRNAseq analysis. Results from scRNAseq quality control analyses (**A-C**) prior to and (**D-F**) after quality filtering. (**G**) t-SNE plot demonstrating two cell populations identified by K-means analysis. (**H**) Volcano plot showing results from differentially gene expression analysis between the two cell clusters. (**I**) t-SNE plot of single cells depicting differential expression of genes involved in cell cycle regulation. Expression values were summarized using AUCell area under the curve analysis.

In the course of these studies, we consistently observed that freshly prepared cultures of *Nf1*-deficient mouse BMSCs expanded faster than WT BMSCs initially, consistent with RAS/ERK activation caused by the loss of *Nf1*, only to lag behind after passaging these cultures, in line with the scRNAseq data from human BMSCs that pointed to changes in cell cycle genes as a characteristic of one of the two clusters. We also observed that cells in cKO cultures were larger and more flat than WT controls (**Fig. 4A**), that these cultures had a significant increase in the number of cells in G2/M (**Fig. 4B**) and in the proportion of b-gal+ cells, typical of senescent cells (**Fig. 4C**). To further explore the hypothesis that loss of *Nf1* causes senescence of BMSCs, we measured expression of senescence markers in mouse *Nf1*+/- and *Nf1*-/- BMSCs prepared as above and from *Nf1*$^{flox/flox}$ BMSCs infected with Ad-GFP (WT control) or Ad-Cre (cKO). We found in both sets of cKO BMSCs an increased expression of p16 (but not p21) and of genes related to senescence-associated secretory phenotype (SASP) (**Fig. 4D**).

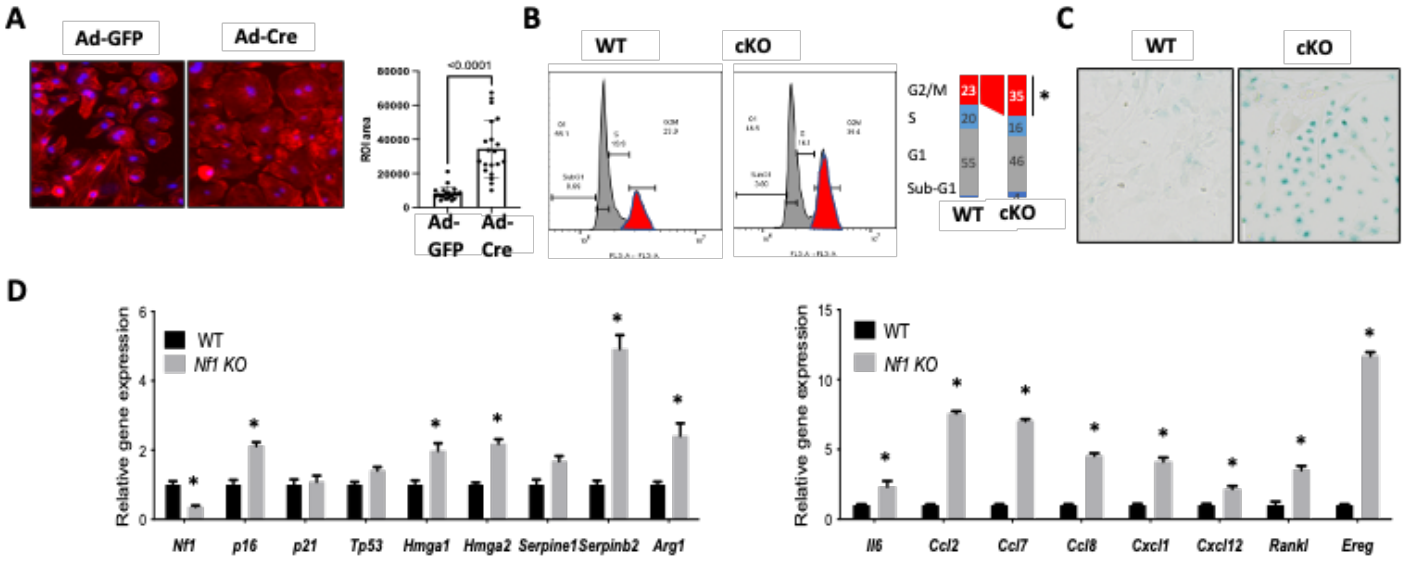

Figure 4. Evidence of senescence in *Nf1* cKO BMSCs. A. Increased cell size in *Nf1* cKO (Ad-Cre) BMSC cultures. **B**. Cell cycle FACS analysis showing higher number of cells in G2/M in cKO BMSC cultures. **C.** Increased proportion of b-Gal+ cells in *Nf1* cKO BMSC cultures. **D.** Gene expression (qPCR) between WT and *Nf1* cKO BMSC cultures after 14 days of differentiation in osteogenic medium (n=3). *:p<0.05.

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

Dr. Efrosini Cuko was trained in primary bone cell extraction from mouse and human tissues, culture and differentiation, RNA preparation, quantitative gene expression analyses and RNAseq analyses, FACS analyses and cell tracing. She was also trained in calcified tissue histology.

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

Scientific Zoom presentations were given by Drs. Cuko and Elefteriou.

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

N/A

4. IMPACT:

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

These results are important for several reasons. First and foremost, they attribute to skeletal osteoprogenitors with *Nf1* loss-of-function a characteristic that could make them "targetable", as senescent cells are sensitive to senolytics, which are drugs that can clear senescent cells. This opens up the possibility of clearing the bones of patients with NF1 from skeletal osteoprogenitors deficient for *NF1* pharmacologically, prior to bowing and fracture or after fracture to promote bone repair and reduce the high incidence of recalcitrant bone healing seen in these patients. These results also point to the SASP as the causal determinant of the impaired bone healing in NF1, as many of these factors promote a state of chronic inflammation that may have an inhibitory impact on several steps of the bone repair process. In that regard, senomorphics may be of potential use as they could reduce the SASP and thereby promote bone healing in NF1. Therefore, the most logical continuation of these studies is to determine the effect of senolytics and senomorphics in the context of NF1 pseudarthrosis and the functional effect of the SASP generated by *Nf1* cKO BMSCs on all cells required for proper bone repair, which is ongoing.

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

If results are confirmed, findings and implications could be relevant to other conditions associated with NF1.

- **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**

2019:

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.

2020:

Based on the results of the study, we believe targeting senescent *Nf1*-deficient cells with senolytics or senomorphics will be more efficient and clinically feasible than targeting specific molecules of the SASP. Hence the rescue-type experiments originally proposed are no longer adapted and are replaced by effort to assess the efficacy of clearing *Nf1*-deficient cells from the bone environment and measuring impact on bone development and repair.

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

2020: 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. Back to phase 1 reopening with about 50% of laboratory access occurred in July 2020. Mouse breeding was prioritized for these experiments. We thus lost about 4-5 months due to the COVID pandemic.

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

N/A

- **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**

- Nov. 2019 Skeletal dysplasia in NF1, one bad apple may spoil the whole barrel, BCM Molecular and Human Genetic Seminar, Houston, TX.
- Dec. 2020 Skeletal Dysplasia in Neurofibromatosis Type 1, Penn Center for Musculoskeletal Disorders professorship seminar series, UPenn School of Medicine, Philadelphia, PA (Zoom talk).

- **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**

Part of these results were used in support to a NIH R01 grant application to follow up on the role of senescence in NF1 CPT. This grant has been funded starting summer of 2021.

- *Etiology of musculoskeletal maladies in NF1*

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PI: Elefteriou
04/01/2021- 03/31/2026

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 NIH R01 AR077949

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?**

New grant: NIH R01 AR077949

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

"Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES

N/A