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CONTRACTING ORGANIZATION: **Johns Hopkins University, Baltimore, MD**

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14. ABSTRACT

Osteosarcoma (OS) is the most common primary skeleton malignancy, and inordinately affects children and young adults. Despite improved outcomes with current multimodal care, the prognosis for patients with metastatic disease is grim with an overall 5 year survival rate <20%. Our research group has examined a novel osteogenic differentiation factor, NELL-1, and its potential applications in tissue engineering. Recently, our group has shown that NELL-1 is significantly upregulated across multiple OS cell lines and over 50 human high grade OS samples. Despite many years of research, the precise cell surface receptor which transduces NELL-1 signaling remained elusive. Recently, we have determined that NELL-1 binds to Cntnap4 (Contactin-associated protein-like 4) which propagates downstream signaling pathways responsible for its cellular effects. Collectively, the NELL-1 / Cntnap4 signaling axis has emerged as a novel and targetable pathways in human osteosarcoma. In a comprehensive, *in vitro / in vivo* proposal, we will now interrogate the role of NELL-1 / Cntnap4 signaling in the positive regulation of osteosarcoma disease progression.

15. SUBJECT TERMS
Cancer in children, adolescents, and young adults and rare cancer.

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TABLE OF CONTENTS

	Page
1. Introduction	3
2. Keywords	3
3. Accomplishments	3
4. Impact	8
5. Changes/Problems	10
6. Products	11
7. Participants & Other Collaborating Organizations	11
8. Special Reporting Requirements	15
9. Appendices	15

1. INTRODUCTION:

Our objective is to define the role of NELL-1 (NEL-like molecule-1) / Cntnap4 (Contactin-associated protein-like 4) / MAPK signaling axis in osteosarcoma disease progression. Our overall hypothesis is that NELL-1 enhances osteosarcoma (OS) cell proliferation, attachment, migration, invasion, and ultimately disease progression, via Cntnap4 binding and downstream MAPK / FAK signaling activation. Furthermore, *Cntnap4* knockout will inhibit OS cell attachment, invasion and disease progression.

2. KEYWORDS:

Cntnap4, Nell1, osteosarcoma, sarcoma

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1 – Regulatory and study preparatory tasks (100% complete)
Subtask 1 – Local IACUC approvals (100% complete)
Subtask 2 – ACURO approval (100% complete)
Subtask 3 – HRPO review and approval (100% complete)

Major Task 2 – *In vitro* studies (65% complete)
Subtask 1 – Perform Cntnap4 KO in 143B, Saos2 and primary OS cells (50% complete)
Subtask 2 – Perform proliferation, migration, attachment and invasion assays (80% complete)
Subtask 3 – Perform in vitro assays with or without NELL-1 treatment (25% complete)

Major Task 3 – *In vivo* studies (15% complete)
Subtask 1 – Perform xenografts using 143B and primary OS cells (15% complete)
Subtask 2 – Perform radiographic imaging and analysis of tumor xenografts (15% complete)
Subtask 3 – Histologic and immunohistochemical analysis of tumor xenografts (15% complete)

What was accomplished under these goals?

Major Task 1 – Regulatory and study preparatory tasks (100% complete)
All ACUC, ACURO, IRB and HRPO approval were obtained.

Major Task 2 – *In vitro* studies (65% complete)
Subtask 1 – Perform *Cntnap4* KO in 143B, Saos2 and primary OS cells (50% complete)
Studies have been performed demonstrating the effects of *Cntnap4* gene deletion in 143B OS tumor cells and Saos2 cells. As shown below by T7 endonuclease I assay (Fig. 1A) and qPCR (Fig. 1B), 4 single cell clones of 143B cells with CRISPR-mediated knockout of *Cntnap4* were generated. 2 of 4 clones showed complete absence of *Cntnap4* expression by qPCR. Polyclonal populations of 143B cells or Saos2 cells demonstrated reduced proliferation and attachment with *Cntnap4* KO in comparison to vector control (VC) (Fig. 2). Moreover, single cell clones of 143B OS cells with or without *Cntnap4* KO showed a similar phenotype (Fig. 3), with reduced cellular proliferation, attachment, migration and invasion potential as compared to VC.

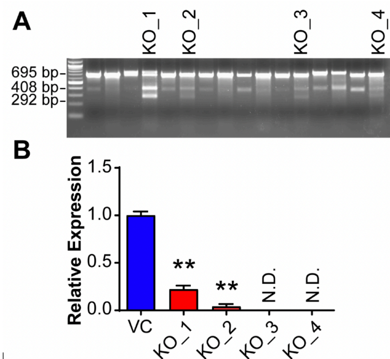

Fig 1: Validation of *Cntnap4* gene deletion in human 143B OS cells, by (A) T7 endonuclease I assay, and (B) qPCR.

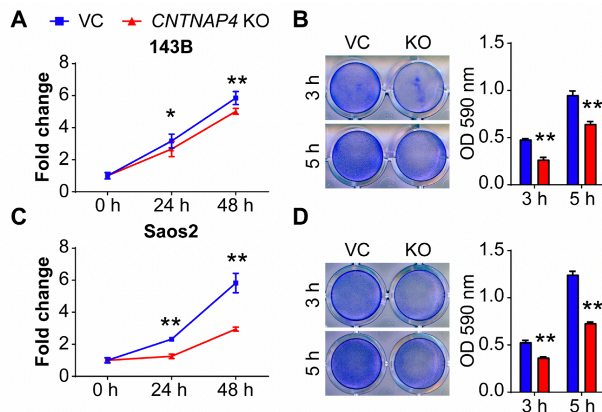

Fig 2: Cellular phenotype of polyclonal osteosarcoma cell lines with *Cntnap4* gene deletion, including (A,B) 143B OS cells and (A,C) Saos2 cells. (A,C) MTS assays to assess proliferation. (B,D) Attachment assays with whole-well images (left) and quantification (right). *P<0.05; **P<0.01.

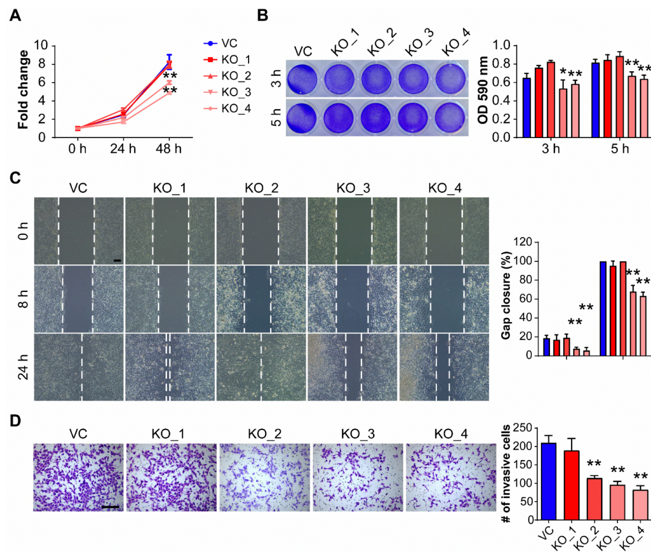

Fig 3: Cellular phenotype of single cell clones of 143B osteosarcoma cell lines with *Cntnap4* gene deletion, including (A) MTS assays to assess proliferation, (B) Attachment assays with whole-well images (left) and quantification (right), (C) migration by scratch wound healing assays at 8 and 24 h (images on left with quantitative assessment on right), and (D) invasion assay (images on left with quantitative assessment on right). *P<0.05; **P<0.01 in relation to VC at the corresponding timepoint.

In conclusion, *Cntnap4* expression is required to maintain the proliferative, attachment and invasive potential of human OS cell lines. These effects strongly phenocopy those findings obtained from *Nell1* gene deletion (separate work under consideration for publication). Ongoing work is examining the effects of *Cntnap4* gene deletion on primary osteosarcoma cells which have already been obtained in our laboratory. In addition, we are performing experiments with recombinant NELL-1 in the context of *Cntnap4* gene deletion.

Major Task 3 – *In vivo* studies (15% complete)

Major task 3 examines the effects of *Cntnap4* and *Nell1* gene deletion (singly or combined) in an orthotopic OS xenograft model. Results thus far have been obtained with Nell1 gene deletion in relation to vector control in 143B OS cells (Fig. 4). Here, NELL1 KO led to a significant reduction in tumor size by caliper measurement (Fig. 4B) and MR imaging (Fig. 4C). NELL1 loss was confirmed by immunohistochemistry (Fig. 4D). Immunofluorescent staining demonstrated a reduction in tumor cell proliferation (Fig. 4E) and tumor associated blood vessel numbers (Fig. 4F). A notable absence in pulmonary metastasis was found among NELL1 KO xenograft mice (Fig. 4G,H).

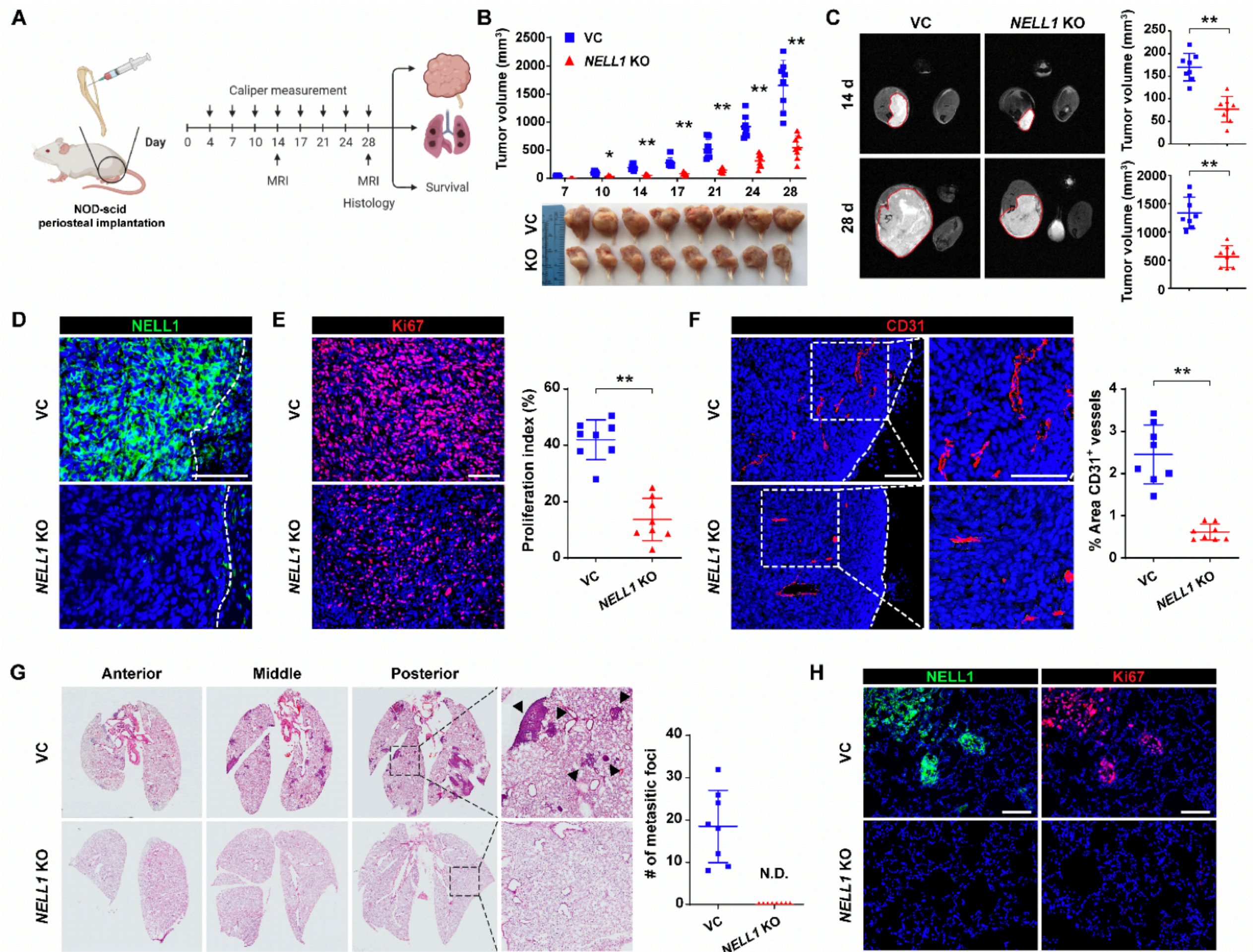

Fig 3: *NELL1* knockout mitigates OS disease progression in 143B xenograft model. Orthotopic implantation of *NELL1* KO or vector control clonal 143B cells within the proximal tibia of NOD-Scid mice (n=8 mice per group). (A) Schematic diagram of study design. (B) Tumor volume, calculated by caliper measurements twice weekly until 28 d post-injection (above) and gross pathology of all tumors (below). (C) Representative MRI imaging at 14 and 28 d post-injection (left) and tumor volume (right). (D) Confirmation of *NELL1* KO in xenograft tumors by immunostaining. Dashed line indicates edge of tumor. (E) Ki67 immunostaining (left) and quantification (right). (F) CD31 immunostaining (left) and quantification (right). Dashed line indicates edge of tumor. (G) Cross-sections of pulmonary fields (left) and quantification of metastatic foci (right). (H) Representative NELL1 and Ki67 immunostaining in lung metastatic foci.

In conclusion, Nell1 expression is required to maintain normal disease progression among 143B cells in a xenograft model. Ongoing work is examining the effects of Cntnap4 gene deletion and double deletion in the same model system.

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

Nothing to report.

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?

Additional in vitro studies are being performed including the use of primary OS cells for *Cntnap4* gene deletion, gene expression analysis for downstream signaling changes, and use of recombinant NELL-1 treatment in the context of *Cntnap4* deletion in OS cell lines and primary cells (Aim 1). Additional *in vivo* studies are being performed including assessment of tumor growth and metastasis with *Cntnap4* deletion or *Cntnap4/Nell1* double deletion (Aim 2).

4. IMPACT:

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

Nothing to report.

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

Nothing to report.

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

The COVID19 pandemic has led to significant delays in hiring and in some cases supply purchasing. Despite this, we are on track to accomplish the stated Aims by next year. As the pandemic course is unpredictable, further slowdowns may occur.

Changes that had a significant impact on expenditures

As above, some delays in personnel hiring and supply purchasing have been encountered due to the COVID19 pandemic. Our hopes is that this does not occur in year 2.

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

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

None.

Books or other non-periodical, one-time publications.

None.

Other publications, conference papers and presentations.

None.

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

None.

- **Technologies or techniques**

None.

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

None.

- **Other Products**

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Aaron James
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0002-2002-622X
Nearest person month worked: 1.8
Contribution to Project: Dr. James has overseen all aspects of the project.
Funding Support: NIH, DOD, ACS, Maryland TEDCO

Name: Qizhi Qin
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12
Contribution to Project: Dr. Qin has performed work all work in gene editing, cell culture and assessments in Aim 1. He has worked on all in vivo work for Aim 2.

Name: Yiyun Wang
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6
Contribution to Project: Dr. Wang has assisted with all regulatory paperwork, as well as gene editing methods for Aim 1 studies and immunohistochemistry for Aim 2 studies.
Funding Support: NIH

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

R21 DE027922 - ENDED
K08 AR068316 - ENDED
MTF Biologics - ENDED
2019-MSCRFD-5074 - ENDED
W81XWH-18-1-0121 - ENDED

W81XWH-20-1-0795 - BEGAN
P01 AG066603 - BEGAN
R01AR079171 - BEGAN
2021-MSCRFD-5641 - BEGAN

What other organizations were involved as partners?

None.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: