AWARD NUMBER: W81XWH-19-1-0552

TITLE: Treatment of NF1-Driven Neurofibromas Through VDR-Mediated Stromal Reprograming

PRINCIPAL INVESTIGATOR: Ronald M. Evans

CONTRACTING ORGANIZATION: Salk Institute for Biological Studies, La Jolla, CA

REPORT DATE: January 2024

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
January 2024	Final	15 Sep 2019 - 14 Sep 2023

4. TITLE AND SUBTITLE

Treatment of NF1-Driven Neurofibromas Through VDR-Mediated Stromal Reprograming

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0552

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Evans, Ronald M.
Downes, Michael R.
Truitt, Morgan L.

E-Mail: evans@salk.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Salk Institute for Biological Studies
10010 Torrey Pines
La Jolla, CA 92037-1002

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Virtually every patient diagnosed with neurofibromatosis is faced with the challenge of managing neurofibroma tumor growth. This can range from the emotional difficulties of cosmetically disfiguring dermal neurofibromas to the more painful growth of deep-tissue plexiform neurofibromas. In this proposal we took a new approach to target neurofibromatosis-associated tumors by breaking down their stromal support network. This work demonstrated a potent ability for nerve-associated fibroblasts to support resistance to MEK inhibition in association with activation of the PI3K/Akt pathway, supporting the therapeutic potential of stromal targeting therapies. To pursue this, we tested the ability for a clinically approved class of drugs that activate the vitamin D receptor (VDR) to promote therapeutic responses to MEK inhibition. We found that while VDR agonist was able to suppress markers of fibroblast activation *in vitro*, it had no impacts on tumor growth i*n vivo* either as single agents or in combination with the MEK inhibitor selumetinib. Notably, this work did uncover a putative role for VDR activation in promoting tumor vascularization, a finding that has been associated with enhanced delivery and efficacy of chemotherapeutics in pancreatic cancer and is the focus of ongoing clinical trials. Future studies will be needed to see if the same observations can be extended to the treatment of neurofibromatosis-associated tumors.

15. SUBJECT TERMS
Neurofibromatosis, neurofibroma tumor, stromal support network, vitamin D, VDR stromal remodeling therapy, VDR therapies

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

NF-1 driven neurofibromas are characterized by a strong fibrotic response, where up to 70% of tumor mass can be stromal components. Despite a growing appreciation for the role of the tumor microenvironment in neurofibroma development and growth, the functional contribution of tumor-associated fibroblast populations is largely unknown. Previous work from our group has identified the Vitamin D receptor (VDR) as a type of molecular 'on/off' switch of fibrotic activity. In this proposal, we test how VDR-mediated control of fibrosis impacts the development, progression, and therapeutic response of neurofibromas and MPNSTs to MEK inhibition. By addressing how fibroblasts contribute to neurofibromatosis-related pathologies, this work has the potential to uncover new approaches for understanding and targeting this disease. Notably, VDR therapies are safe, already have FDA approval for other applications, and are currently being tested in clinical trials in pancreatic cancer for their ability to promote chemotherapeutic responses.

2. KEYWORDS:

Vitamin D Receptor; fibrosis; fibroblasts; neurofibroma; NF1; Schwann cell; TGFβ

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1)

- Test VDR loss of function *in vivo* (Milestones)
    - Local IACUC Approval- completed
    - Analysis of Col1a2-CREER; VDRflox/flox SKP transplants- alternative approach attempted
    - Analysis of Col1a2-CREER; VDRflox/flox MPNST transplants- alternative approach attempted
- Test VDR activation *in vivo* (Milestones)
    - Local IACUC Approval- completed April 2021
    - Analysis of calcipotriol treated SKP transplants- technical limitations
    - Analysis of calcipotriol treated MPNST transplants- completed
    - Analysis of VDR responses in fibroblast subpopulations from transplants- completed

Specific Aim 2)

- Potentiating MEK inhibitor responses with VDR agonist (Milestones)
    - Local IACUC Approval- completed
    - Analysis of all in vitro co-culture assays- completed
    - Analysis of combination therapy experiments in SKP transplants- technical limitations
    - Analysis of combination therapy experiments in MPNST transplants- completed

What was accomplished under these goals?

This project has sought to understand how peripheral nerve-associated fibroblasts influence the growth and therapeutic response of neurofibromas and their malignant counterparts MPNSTs. In particular, we have used primary peripheral nerve-associated fibroblasts as well as fibroblasts isolated from patient neurofibromas to test

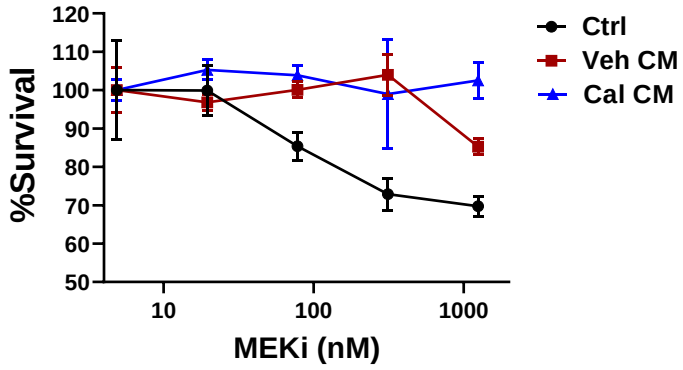

Figure 1. Fibroblasts promote neurofibroma resistance to MEK inhibition in a VDR-independent manner. Results from cell-titer-glo assays showing that fibroblast conditioned media (CM) promotes MEK inhibitor resistance in the human neurofibroma line NF 95.11 regardless of whether CM is from control (Ctrl) or VDR agonist (Cal) treated cells.

how soluble factors from these cells can influence tumor growth and therapeutic responses and explore if VDR agonist therapy can intercept these effects. Using these systems, we have demonstrated that conditioned media from fibroblasts can promote resistance to MEK inhibition in both neurofibroma models (Figure 1) as well as MPNSTs (data not shown). We next sought to test if our observations on the ability of VDR agonist to broadly block fibroblast activation would translate to nerve-associated fibroblasts. Surprisingly, we found that long-term treatment (3 days) of nerve-associated fibroblasts was unable to disrupt their ability to overcome MEK inhibition (Figure 1) despite robustly engaging VDR target genes (Figure 2). This work suggests that soluble factors driving MEK inhibitor resistance in tumor cells are not regulated by VDR. To gain insight into how these soluble factors may be overcoming therapeutic resistance to MEK inhibitors (MEKi), we assessed whether cell survival was associated with restoration of the MAPK signaling pathway or alternative activation of a parallel pathway. Analysis of pERK levels in tumor cells treated with fibroblast conditioned media revealed that MEKi were still effective at blocking MAPK signaling under these conditions (Figure 3A-B), suggesting the activation of an alternative pathway. As both PI3K/AKT and JAK/STAT signaling have been previously implicated in promoting MEKi resistance, we evaluated activation of these parallel signaling pathways. While we found the STAT pathway was not robustly activated or altered under these conditions, our data reveal an increase in pAKT levels upon treatment with fibroblast conditioned media (Figure 3A, C). These data support that PI3K/AKT activation could underlie the effect of fibroblast conditioned media, in which case PI3K/ AKT pathway inhibitors should overcome their protective effects. Preliminary analysis of fibroblasts for expression of soluble mediators capable of supporting tumor cell survival and PI3K/AKT activation has identified known pro-survival/ growth factors, including IGF and EGF, as well as high expression of the ERBB3 and 4 ligand NRG1 (data not shown).

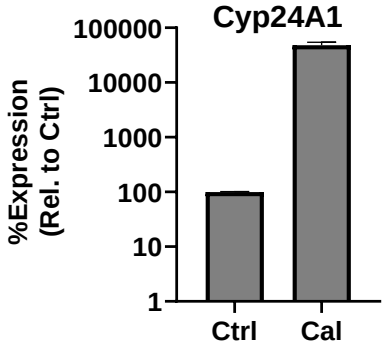

Figure 2. Nerve-associated fibroblasts respond robustly to VDR agonist. qPCR analysis of the canonical VDR target gene CYP24A1, demonstrating that primary fibroblast can respond robustly to VDR agonist (Cal).

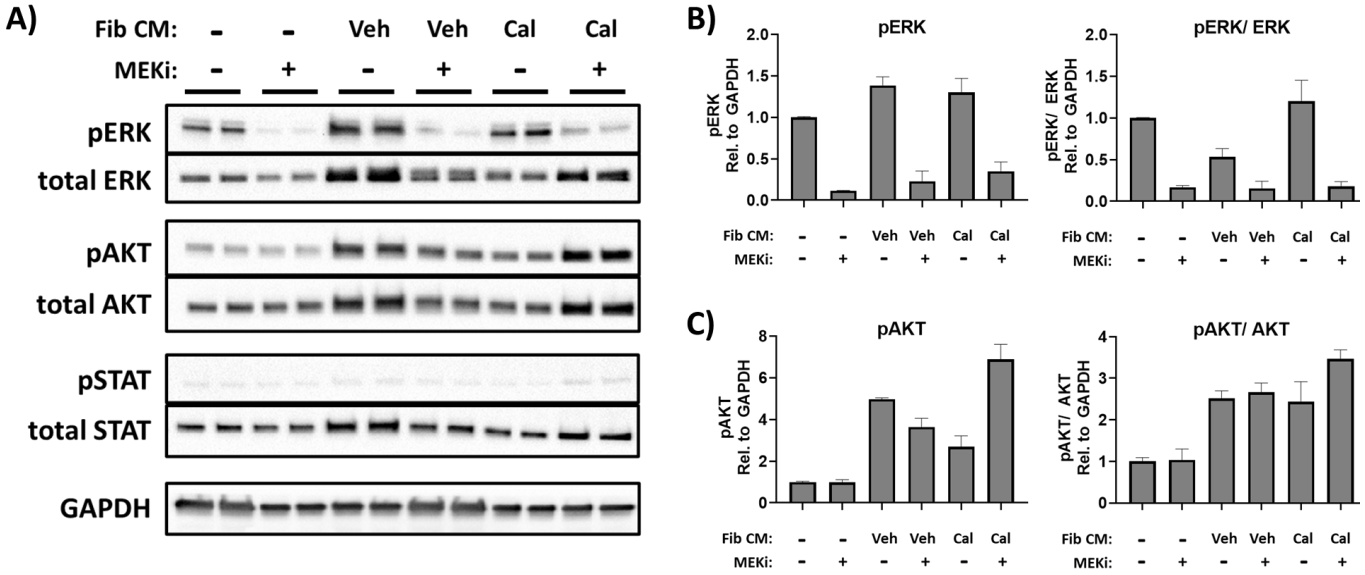

Figure 3. Fibroblasts conditioned mediate promotes activation of the PI3K/AKT pathway. A) Western blot analysis and B), C) quantifications showing that pre-treatment (4hrs) of the NF 95.11 neurofibromas line with fibroblast conditioned media (CM) does not alter MAPK/ ERK signaling or STAT signaling but does promote PI3K/AKT signaling.

Importantly, while the above data demonstrate an inability for VDR agonist to alter soluble pro-survival factors, gene expression analysis in fibroblasts has also revealed that VDR agonist therapy can robustly impact factors that contribute to *in vivo* tumor growth and therapeutic responses, such as the production of extracellular matrix proteins, including Col1a1, Col1a2, Col6a1, and Col6a2 (Figure 4). Based off these findings, we have tested how VDR agonist therapy impacts tumor growth *in vivo*, employing orthotopic transplant models with two different MPNST lines obtained from the Parada lab at the Memorial Sloan Kettering Cancer Center. Importantly, these

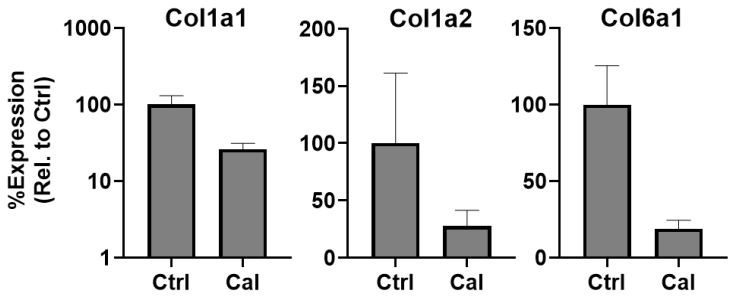

Figure 4. VDR agonist impacts expression of ECM genes in nerve-associated fibroblasts. qPCR analysis demonstrating that VDR agonist (Cal) downregulates expression of multiple collagen components of the extracellular matrix (ECM)

lines can be syngeneically transplanted into immunocompetent mice, maintaining a normal immune microenvironment. Using these models, we demonstrate that while the clinically approved MEK inhibitor selumetinib can significantly reduce MPNST growth, VDR agonist has no effect on tumors either as a single agent or in combination with MEK inhibition (Figure 5). In line with this lack of efficacy, we found find that while VDR agonist was able to suppress fibroblast expression of collagen *in vitro*, tumor collagen levels were seemingly unaffected by treatment with the VDR agonist calcipotriol (data not shown), suggesting that other cell types are substantially contributing to *in vivo* collagen production (such as the transformed Schwann cells themselves) and providing a mechanistic rationale for why VDR agonist therapy failed to show any effect. In addition, while ongoing work in the lab has validated a general role for VDR expression within macrophages, macrophage recruitment to VDR agonist treated MPNSTs was unchanged (data not shown).

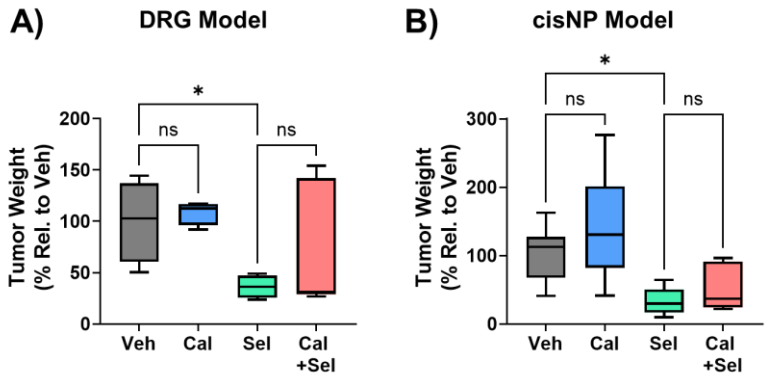

Figure 5. Impact of VDR agonist on MPNST tumor growth and MEK inhibitor response. Relative tumor weights from mice orthotopically transplanted with either A) DRG MPNST line or B) cisNP MPNST line and treated with vehicle (Veh), VDR agonist (Cal), the MEK inhibitor Selumetinib (Sel), or the combination (Cal + Sel) for two weeks following establishment of palpable tumors. (* = p<0.05, ANOVA)

Interestingly, histological analysis of MPNSTs treated with VDR agonist did reveal one potentially therapeutically relevant difference. Expression of SMA, a marker for activated myofibroblast and pericytes, was significantly increased by treatment (Figure 6). Moreover, SMA expression appeared to be largely restricted to pericytes suggesting that pericytes and the vasculature they are associated with is increased in tumors by VDR agonist treatment. This is in line with previous work in pancreatic cancer demonstrating that VDR agonist can promote vascular normalization in pancreatic tumors in association with enhanced delivery and efficacy of chemotherapeutics. Future studies will be needed to validate if increased pericyte presence does truly reflect an increase in functional vasculature and explore if this can promote chemotherapy responses in MPNSTs.

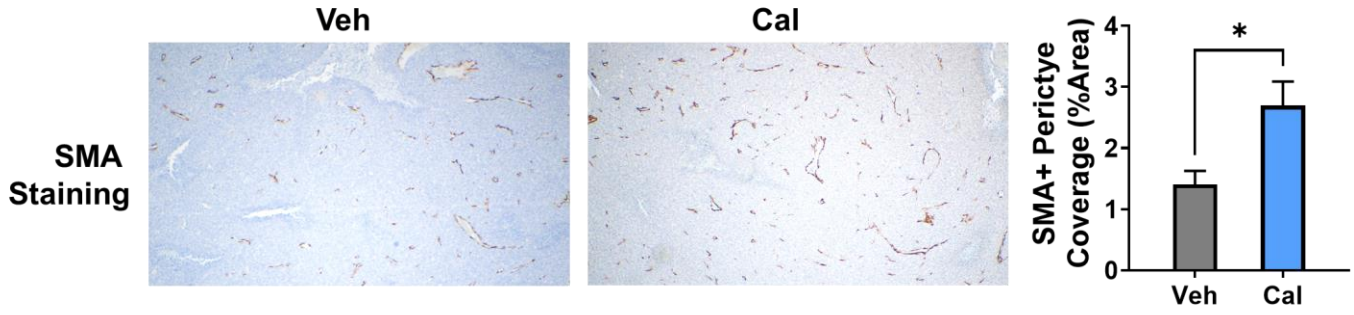

Figure 6. VDR agonist increases pericytes in MPNSTs. Representative immunohistochemistry (40X magnification) and quantification (n = 3 mice per arm) for the pericyte marker SMA demonstrating that VDR agonist (Cal) increases pericyte presence in orthotopic transplants of the DRG MPNST line. (* = p<0.05, t-test)

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?

Nothing to report.

4. IMPACT:

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

A prominent stromal response has long been associated with neurofibroma and MPNST tumors, however, a functional role for stromal fibroblasts in influencing therapeutic responses has never been investigated. This project tested the hypothesis that VDR agonist would promote MEK inhibitor responses in NF1-driven nerve tumors through its ability to suppress fibroblast activation. Although these studies ultimately failed to show any therapeutic benefit from VDR agonist either as single agents or in combination with MEK inhibitors, they nevertheless demonstrated a prominent role for nerve-associated fibroblasts in promoting resistance to MEK inhibitors, suggesting that alternative approaches for targeting the fibroblast compartment should be further explored. Moreover, while VDR agonist did not promote MEK inhibitor responses, our data suggest that VDR agonist treatment can promote vascularization within nerve tumors. This mirrors our findings in pancreatic tumors, where enhanced vascularization was associated with improved delivery and efficacy of chemotherapeutics. Future studies will be needed to address if similar synergy between VDR agonist and chemotherapy can be achieved in NF1-driven nerve tumors.

What was the impact on other disciplines?

These results are likely to translate to other tumor types with strong desmoplastic responses, where fibroblast targeting therapies could similarly promote MEK inhibitor responses. In support of this being more broadly applicable, we have found that conditioned media from skin fibroblasts can also promote tumor cell survival under MEK inhibition.

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

Due to multiple technical difficulties, we had to initiate alternative approaches to address some of our primary research questions. In regards to our proposed experiments to explore the *in vivo* role of VDR using analogous loss-of-function experiments, we have uncovered that while the Col1a2-CREER allele can efficiently recombine our VDR floxed locus *in vitro*, it fails to work robustly *in vivo*. As a result we attempted an alternative approach using co-transplanted fibroblasts where the VDR locus has been deleted *ex vivo*. Initial experiments using primary fibroblasts from mouse sciatic nerves revealed that due to their limited proliferative capacity, we would need to immortalize these cells before transplant. However, these cells have proven remarkably resistant to viral based immortalization techniques and co-transplant experiments have not been feasible.

Finally, we have had significant technical difficulties in establishing the SKP transplant system for studying neurofibroma growth *in vivo*. While we ultimately hope to overcome these issues, we plan to test orthotopically transplanted human neurofibroma lines as an alternative experimental model for studying in vivo VDR impacts on tumor growth in future studies. While this system will require the use of immunodeficient mice, and thereby potentially impact the immune microenvironment, we expect that it should still properly model VDR agonist responses in fibroblasts.

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

In the past, this program faced logistical issues with personnel that delayed experiments but these were ultimately resolved.

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:

Significant changes in use or care of human subjects

N/A

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**
  Nothing to report.
- **Journal publications.**
  Nothing to report.
- **Books or other non-periodical, one-time publications.**
  Nothing to report.
- **Other 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: Ronald M. Evans
Project Role: PI
Researcher Identifier: ORCID ID 0000-0002-9986-5965
Contribution to Project: Project management/ oversight.
Nearest person month worked: 1
Funding Support: NIH, Fondation Leducq, Lustgarten Foundation, Salk Translation Fund, California Institute for Regenerative Medicine (CIRM), Larry L. Hillblom Foundation, Samuel Waxman Cancer Research Foundation

Name: Michael Downes
Project Role: Co-Investigator
Researcher Identifier: ORCID ID 0000-0002-6351-9585
Contribution to Project: Project management/ IACUC protocol.
Nearest person month worked: 1
Funding Support: NIH, California Institute for Regenerative Medicine (CIRM), Lustgarten Foundation

Name: Morgan Truitt
Project Role: Postdoctoral Researcher
Researcher Identifier: ORCID ID 0000-0001-7012-1228
Contribution to Project: Conducting all in vitro and in vivo work for this project.
Nearest person month worked: 5
Funding Support: Lustgarten Foundation

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?

- **Organization Name:** Memorial Sloan Kettering Cancer Center (MSKCC)
- **Location of Organization:** New York, NY
- **Partner's contribution to the project *(identify one or more)***
  - **Other.** The laboratory of Dr. Luis Parada has provided us with MPNST lines suitable for syngeneic orthotopic transplants and consulted with us on their use.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: N/A