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: October 2021

TYPE OF REPORT: Annual

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 *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2021	Annual	15Sep2020 – 14Sep2021

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.

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 & Development Command
Fort Detrick, MD, 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. Here we propose to take a radically new approach to target to neurofibromatosis-associated tumors by breaking down their stromal support network with a clinically approved class of drugs that target the vitamin D receptor (VDR). We will test if this VDR stromal remodeling therapy is sufficient to impact tumor growth on its own and explore its potential to work in combination with clinically approved MEK inhibitors. Of particular relevance for patients, VDR therapies are relatively safe for long-term treatment and are already available in the clinic, allowing this work to immediately translate into clinical trials.

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

16. SECURITY CLASSIFICATION OF: U			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	12	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 using the combination of a novel VDR floxed mouse and clinically relevant VDR agonists. 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 and already have FDA approval for other applications, facilitating the potential translation of these results to the clinic.

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 April 2021
    - Analysis of Col1a2-CREER; VDRflox/flox SKP transplants- to be done
    - Analysis of Col1a2-CREER; VDRflox/flox MPNST transplants- in progress
- Test VDR activation *in vivo* (Milestones)
    - Local IACUC Approval- completed April 2021
    - Analysis of calcipotriol treated SKP transplants- to be done
    - Analysis of calcipotriol treated MPNST transplants- in progress
    - Analysis of VDR responses in fibroblast subpopulations from Col1a2-CreER; R26-LSL-TdT SKP and MPNST transplants- to be done

Specific Aim 2)

- Potentiating MEK inhibitor responses with VDR agonist (Milestones)
    - Local IACUC Approval- completed April 2021
    - Analysis of all *in vitro* co-culture assays- in progress
    - Analysis of combination therapy experiments in SKP transplants- to be done
    - Analysis of combination therapy experiments in MPNST transplants- in progress

What was accomplished under these goals?

Following COVID related restrictions and delays, we received IACUC and ACURO approval for our animal experiments. To expedite our studies, we have initially been focusing on concurrently analyzing how loss of function vs. activation of VDR impacts MPNST growth and therapeutic

responses. These studies are underway, and we eagerly anticipate the results from this work. Initially, preliminary results indicate potential limitations for the ability of Col1a2-CREER in driving efficient recombination of the VDR flox allele *in vivo*. We are still deciphering if this is a technical issue, however, if this bears out and cannot be easily addressed (for example by adjustments to the Tamoxifen dosing schedule), it does represent a potential limitation for our studies. In this case, we may be able to partially address this with an alternative approach of co-transplanting tumor cells with fiboblasts that have had the VDR locus deleted *in vitro*.

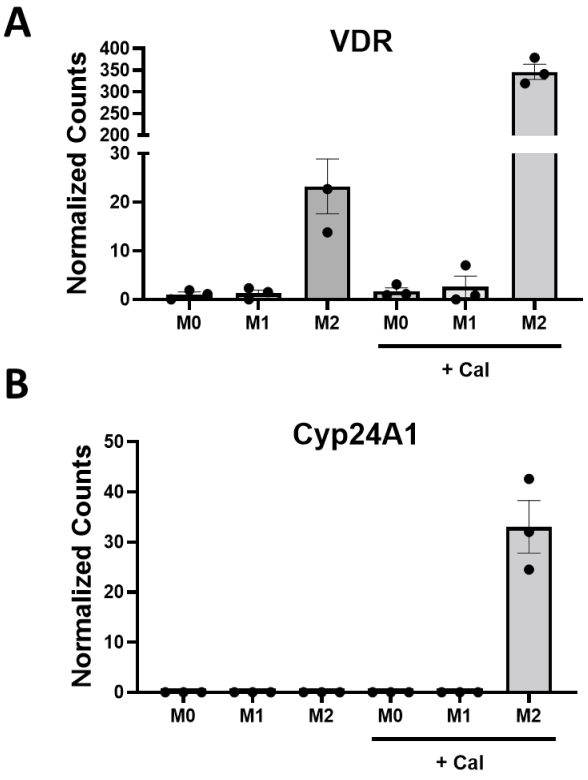

Figure 1. VDR is active in M2 macrophages: Results from RNA-Seq analysis of BMDMs demonstrating that A) VDR is selectively expressed in M2 macrophges and that B) these cells potently induce VDR target genes (e.g., Cyp24A1) in response to VDR agoniost (Cal).

In the past year while waiting for animal protocol approvals, we have also begun to explore in more depth the role of VDR in the immune compartment, which could have important implications for interpreting our *in vivo* VDR agonist treatment studies. Using bone marrow derived macrophages as a model system, we have comprehensively profiled the macrophage response to VDR agonist treatment *in vitro* under M0 (non-activated), M1 (LPS and IFNg activated), and M2 (IL4 and IL13 activated) conditions. Importantly, this work has uncovered that while VDR is nearly undetectable in M0 and M1 macrophages, it is expressed and highly responsive to VDR agonist in M2 macrophages, which are thought to be similar to tumor-associated macrophages (Figure 1). Surprisingly, we found that VDR agonist treatment of M2 macrophages actively upregulated genes that are associated with fibrosis (e.g., PDGFa, FN1), EGFR activation (e.g., HBEGF) and inflammatory cell recruitment (e.g., CCL2 and CCL4) (Figure 2). While M2 activated BMDMs are not a perfect mirror of tumor-associated macrophages, this work nevertheless suggests that VDR activation in macrophages may have detrimental impacts on neurofibroma and MPNST outcomes that could counteract benefits from targeting VDR in fibroblasts. Importantly, we are actively working on developing antibody-targeted nanoparticles

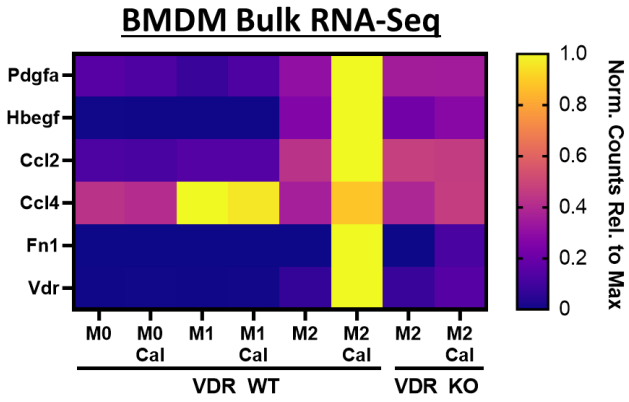

Figure 2. VDR agonist induces the expression of pro-fibrotic, pro-tumorigenic, and pro-inflammatory genes. RNA-Seq analysis reveals that VDR agonist (Cal) induces genes associated with dentrimental outcomes in M2 activated BMDMs.

for achieving cell-selective drug delivery and this platform could be readily adapted for use with VDR agonists. In addition, this work highlights the potential importance of testing the impact of VDR deletion in the immune compartment (for example by using Vav-Cre) on tumor outcomes and therapeutic response.

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

The postdoctoral fellow working on this project attended the annual NF conference this past June, providing an important opportunity for us to stay abreast of current developments in the field. While this conference was only held virtually this year, it nevertheless provided important opportunities for scientific feedback and professional networking.

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?

During the next reporting period, as part of a no-cost extension to this grant, we plan to finish all stated project goals. In the event that we confirm technical limitations for Col1a2-CREER in deleting the VDR flox allele *in vivo*, we will make amendments to our IACUC and ACURO approved protocols to include co-transplant of VDR deleted fibrolbasts. In addition, as our results implicate a role for VDR in the immune compartment, we will also begin exploring this role in MPNTs by selectively delete VDR broadly in immune cells (using Vav-CRE) or more specifically in myeloid cells (using LysM-CRE).

4. IMPACT:

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

While experimental progress has been made, there is nothing significant to report at this time.

What was the impact on other disciplines?

Our findings that VDR agonist can induce pro-tumorigenic and pro-fibrotic gene expression programs in alternatively activated macrophages has far reaching implications for other diseases where this drug is being employed/ tested as a stromal remodeling therapy, including pancreatic cancer, pancreatitis, and liver fibrosis. In this regard, we will plan to formally explore this possibility in other diseases.

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

As previously described, we encounter significant delays on this project due to COVID related shutdowns and restrictions. We have requested a no-cost extension to allow us to complete the proposed work now that these restrictions have been lifted.

Changes that had a significant impact on expenditures

Nothing to report.

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

Aside from COVID related delays, there have been no significant deviations, unexpected outcomes, or changes in approved protocols.

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
Nearest Person Month: 1
Contribution to Project: Project management/oversight.
Funding Support: NIH, Department of Defense, Office of Naval Research, Fondation Leducq, Samuel Waxman Cancer Research Foundation

Name: Michael Downes
Project Role: Co-Investigator
Researcher Identifier: ORCID ID 0000-0002-6351-9585
Nearest Person Month: 1
Contribution to Project: Project management/IACUC protocol.
Funding Support: NIH

Name: Morgan Truitt
Project Role: Postdoctoral Researcher
Researcher Identifier: ORCID ID 0000-0001-7012-1228
Nearest Person Month: 4
Contribution to Project: Conducting all *in vitro* and *in vivo* work for this project.
Funding Support: Life Sciences Research Foundation Fellowship

Name: Hanna Dhiyebi
Project Role: Technician
Researcher Identifier: None
Nearest Person Month: 2
Contribution to Project: Ms. Dhiyebi assists with cell culture, mouse, histology, and molecular biology experiments under the supervision of Dr. Truitt.

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

Yes.

Changes in Other Support

Evans, Ronald M.

Completed
NIH/NHLBI 5R01HL105278-27 (Evans PI, Salk Institute), Spatial Regulation of Developmental Gene Expression, 04/15/2016-09/30/2020, annual direct, 4% effort. The major goal of this project is to investigate the molecular mechanisms of PPARdelta and REV-ERBalpha action in T helper cell subsets, and explore their therapeutic potential in T cell function to ameliorate airway inflammation and asthma.

NIH/NIDDK 5R37DK057978-41 (Evans PI, Salk Institute), Hormonal Regulation of Mammalian Gene Expression, 04/01/2015-03/31/2020; annual direct, 15.5% effort. The major goal of this project is to define how oxidative nuclear receptor ligands and cofactor complexes influence physiology and disease by controlling patterns of gene expression.

DOD/USSOCOM (Evans PI, Salk Institute), Developing the Performance Enhancer MTB1, 07/27/2018-07/26/2021, annual direct, 4% effort. The major goal of this project is to evaluate the potential of a novel drug MTB-1 in enhancing physical performance in individuals from the Special Operations Forces.

American Association for Cancer Research (Von Hoff PI, TGen), Targeting VDR to make pancreatic cancer competent for immunotherapy, 05/01/2017-04/30/2020, annual direct, 8% effort. The major goal of this project is to test if targeting Vitamin D Receptor (VDR) can unlock the potential of immunotherapies to promote tumor cell killing in pancreatic cancer and extend patient survival.

California Institute for Regenerative Medicine DISC2-11175 (Evans PI, Salk Institute), Therapeutic immune tolerant human islet-like organoids (HILOs) for Type 1 Diabetes, 11/01/2018-10/31/2020, annual direct, 20% effort. The major goal of this project is to demonstrate the efficacy and safety of immune tolerant HILOs.

Howard Hughes Medical Institute (Evans PI, Salk Institute), 09/01/2018-08/31/2019, annual direct, effort N/A. The major goals of this project are to isolate, clone and characterize

orphan nuclear receptors and their obligate heterodimer partner, the retinoid X receptor family (RXR), to investigate the role of nuclear receptors in Drosophila development, to investigate cross-regulation of nuclear receptors by orphan receptors, to identify ligands for orphan receptors, and to develop links between orphan receptors, lipid metabolism and fat-related disease.

Lustgarten Foundation for Pancreatic Cancer Research, Distinguished Scholar Award (Evans PI, Salk Institute), 07/01/2014-06/30/2019; annual direct, 8% effort. The major goal of this project is to identify and develop small molecule based drugs that reverse and/or inhibit pathologic epigenomic changes in cancer and/or stromal cells as novel approaches for pancreatic cancer.

Samuel Waxman Cancer Research Foundation (Evans PI, Salk Institute), Cancer Metabolism, Aging and Cancer, 07/01/2016-06/30/2019, annual direct, 2% effort. The major goal of this project is to epigenetically reprogram normal and malignant tissues.

Samuel Waxman Cancer Research Foundation (Evans PI, Salk Institute), FXR as a novel therapeutic target in Colitis-induced Colorectal Cancer, 07/01/2019-06/30/2021, annual direct, 1% effort. The major goal of this project is to determine how inflammation-induced epithelial changes contribute to the initiation and progression of colitis-associated colon cancer, focusing on the causative roles of bile acid dysregulation and aberrant cytokine signaling, and how these disease drivers compromise the protective role of intestinal goblet cells.

New
NIH/NHLBI 1P01HL147835-01A1 (Glass PI, UCSD), A Cardiovascular-NASH disease nexus: Common Mechanisms and Treatments?, 09/21/2020-07/31/2025, annual direct costs, 20% effort. The major goal of Project 2, Tissue-specific roles of FXR in CVD and NASH (R. Evans, PI), is to dissect the tissue-specific activities of the farnesoid X receptor (FXR) in the context of integrative hepatovascular pathophysiology. [Funds shared by two investigators.]

NIH/NIDDK 2R01DK057978-42A1 (Evans PI, Salk Institute), Hormonal Regulation of Mammalian Gene Expression, 05/05/2021-03/31/2025, annual direct costs, 16.67% effort. The major goal of this project is to understand how nuclear hormone receptors influence development and physiology by controlling patterns of gene expression via ligand-directed chromatin modifications, with a focus on actions mediated by their co-repressors.

Lustgarten Foundation for Pancreatic Cancer Research (Shaw PI, Salk Institute), Decoding New Targets for Pancreatic Cancer Therapeutics, 09/01/2021-08/31/2026, annual direct, 5% effort. The major goal of this project is to identify new therapeutic targets and biomarkers for early detection and leverage our findings on how these programs are interconnected in order to develop novel combinatorial strategies. Project 2, Identifying novel glycan vulnerabilities in pancreatic cancer (Evans PI), will identify and develop novel therapeutic approaches targeting the diverse cell types that convergently drive tumor progression and therapeutic resistance.

Mark Foundation for Cancer Research, ASPIRE Award (Evans PI, Salk Institute), Aberrant glycosylation and altered FXR activity converges to drive pancreatic ductal adenocarcinoma progression, 11/01/2021-10/31/2022, annual direct, 2% effort. The major goal of this project is to establish the importance of FXR modulation in PDA and delineate the potential

convergence between aberrant glycosylation and BA signaling.

Changes in Other Support

Downes, Michael R.

Completed
NIH/NHLBI 5R01HL105278-27 (Evans PI, Salk Institute), Spatial Regulation of Developmental Gene Expression, 04/15/2016-09/30/2020, annual direct, 4% effort. The major goal of this project is to investigate the molecular mechanisms of PPARdelta and REV-ERBalpha action in T helper cell subsets and explore their therapeutic potential in T cell function to ameliorate airway inflammation and asthma.

NIH/NIDDK 5R37DK057978-41 (Evans PI, Salk Institute), Hormonal Regulation of Mammalian Gene Expression, 04/01/2015-03/31/2020; annual direct, 4.5% effort. The major goal of this project is to define how oxidative nuclear receptor ligands and cofactor complexes influence physiology and disease by controlling patterns of gene expression.

California Institute for Regenerative Medicine DISC2-11175 (Evans PI, Salk Institute), Therapeutic immune tolerant human islet-like organoids (HILOs) for Type 1 Diabetes, 11/01/2018-10/31/2020, annual direct, 20% effort. The major goal of this project is to demonstrate the efficacy and safety of immune tolerant HILOs.

Lustgarten Foundation for Pancreatic Cancer Research, Distinguished Scholar Award (Evans PI, Salk Institute), 07/01/2014-06/30/2019; annual direct, 2.5% effort. The major goal of this project is to identify and develop small molecule based drugs that reverse and/or inhibit pathologic epigenomic changes in cancer and/or stromal cells as novel approaches for pancreatic cancer.

New
NIH/NHLBI 1P01HL147835-01A1 (Glass PI, UCSD), A Cardiovascular-NASH disease nexus: Common Mechanisms and Treatments?, 09/21/2020-07/31/2025,0, 20% effort. The major goal of Project 2, Tissue-specific roles of FXR in CVD and NASH (Evans PI, annual direct shared by two investigators), is to dissect the tissue-specific activities of the farnesoid X receptor (FXR) in the context of integrative hepatovascular pathophysiology. The major goal of Core A, Phenotyping (Downes PI, annual direct), is to run the Luminex Bio-Plex analyses to measure cytokines and lipids in serum samples generated by the program. The major goal of Core B, Genomics Bioinformatics (Downes PI, annual direct), is to generate custom sequencing libraries and bioformatics support for down-stream analysis.

Lustgarten Foundation for Pancreatic Cancer Research (Shaw PI, Salk Institute). Decoding New Targets for Pancreatic Cancer Therapeutics, 09/01/2021-08/31/2026, annual direct, 10% effort. The major goal of this project is to identify new therapeutic targets and biomarkers for early detection and leverage our findings on how these programs are interconnected in order to develop novel combinatorial strategies. Core 1, Target Validation Core (Downes PI), will provide

advanced and innovative technologies for transcriptomic, epigenomic, and secretomic discovery, and delivery scalable and reproducible approaches for single-cell analyses to the projects.

Samyang Biopharmaceuticals Corporation (Downes PI, Salk Institute), Investigation on the mechanisms of anti-tumoral effects of NQO1 inhibition in cancer, 07/01/2020-12/31/2021, annual direct, 10% effort. The major goal of this collaborative project is to investigate the underlying mechanisms of anti-tumoral effects of small molecule inhibitors of NADH Quinone Oxidoreductase 1 (NQO1) in colorectal and pancreatic cancer.

Changes in Other Support

Truitt, Morgan

Completed
Life Sciences Research Foundation/Howard Hughes Medical Institute (Truitt, PI), VDR-driven reprogramming of immune cell fate and function: a novel strategy for revoking immune privilege in pancreatic cancer, 08/01/2017-07/31/2020; 100% effort, annual direct. The major goal of this project is to demonstrate that VDR agonists will rewire transcriptional and epigenetic networks of key intra-tumoral immune cell populations, driving changes in cell fate and function that revoke the immune privilege of pancreatic cancer.

New
None.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report.

QUAD CHARTS: Nothing to report.

9. APPENDICES: Nothing to report.