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 2023

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
January 2023	Final	15Sep2019 - 14Sep2022

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 North Torrey Pines Road
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. 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	11	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
    - Analysis of Col1a2-CREER; VDRflox/flox SKP transplants- alternative approach in progress
    - Analysis of Col1a2-CREER; VDRflox/flox MPNST transplants- alternative approach in progress
- 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- in progress
    - Analysis of VDR responses in fibroblast subpopulations from transplants- in progress

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- in progress

What was accomplished under these goals?

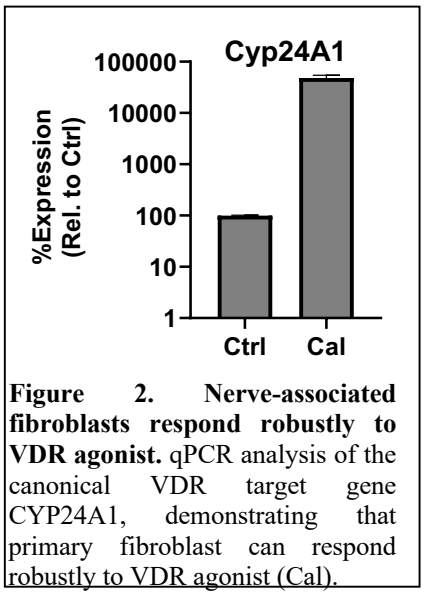

Figure 2. Nerve-associated fibroblasts respond robustly to VDR agonist. qPCR analysis of the canonical VDR target gene CYP24A1, demonstrating that primary fibroblast can respond robustly to VDR agonist (Cal).

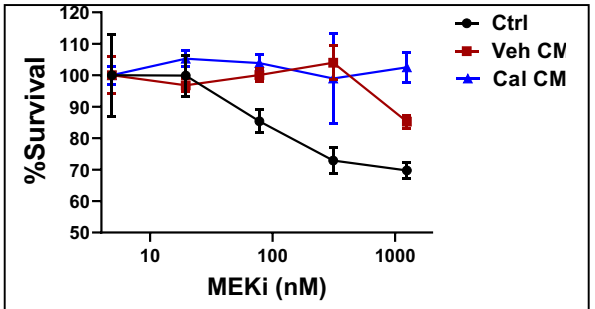

Figure 1. Fibroblasts promote neurofibroma resistance to MEK inhibition in a VDR-independent manner. Results from cell-titer-glo assays showing that fibroblast conditioned media (CM) promotes MEK inhibitor resistance in the human neurofibroma line NF 95.11 regardless of whether CM is from control (Ctrl)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

While this project has encountered numerous logistical and technical challenges, we have made significant progress in the last year towards our efforts 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 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 have assessed whether 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. While we are currently testing this hypothesis, we have also begun to survey fibroblasts for the production of soluble mediators capable of supporting tumor cell survival and PI3K/AKT activation. Preliminary analysis for known pro-survival/ growth factors, including IGF and EGF, has uncovered high level expression of the ERBB3 and 4 ligand NRG1 as one possibility (data not shown).

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). Currently, we are conducting bulk RNA-Seq analysis of fibroblast gene expression programs in the presence/ absence of VDR agonist to elucidate the full

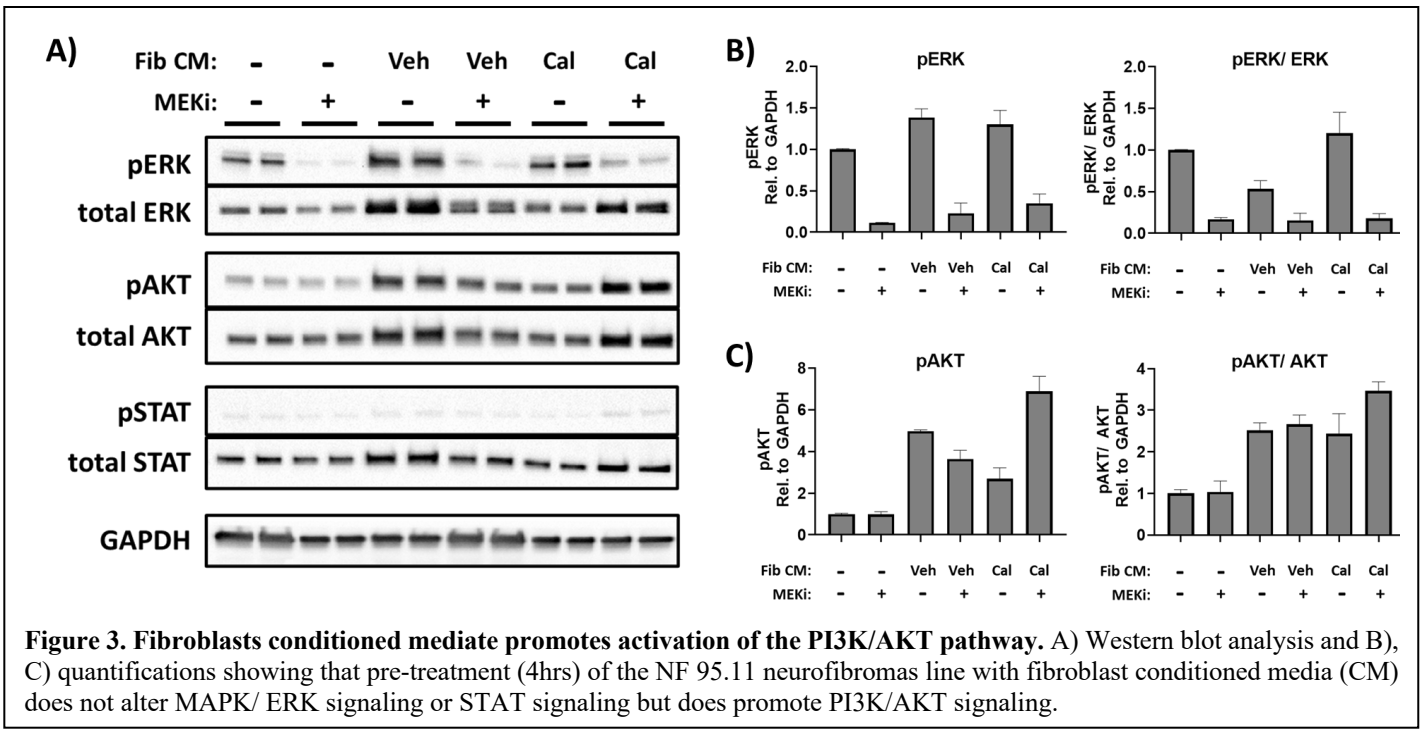

Figure 3. Fibroblasts conditioned mediate promotes activation of the PI3K/AKT pathway. A) Western blot analysis and B), C) quantifications showing that pre-treatment (4hrs) of the NF 95.11 neurofibromas line with fibroblast conditioned media (CM) does not alter MAPK/ ERK signaling or STAT signaling but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

genome-wide impacts of VDR signaling. In addition, to explore how VDR agonist therapy impacts tumor growth *in vivo*, we are currently testing the ability of VDR agonist to impact the growth and response of orthotopically transplanted MPNST lines. These MPNST lines were recently acquired from the Parada lab at the Memorial Sloan Kettering Cancer Center and represent two different MPNST models that allow for syngeneic transplant into immunocompetent mice. This ongoing work will be completed in the next several months under what we hope will be a formal no-cost extension to this funding opportunity. Analysis of these tumors and their associated stromal response will provide critical insight into the ability of VDR agonist to rewire the tumor microenvironment and uncover further therapeutic opportunities for VDR agonist combination therapies.

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*. Currently we are attempting an alternative approach using co-transplanted fibroblasts where the VDR locus has been deleted *ex vivo*. To this end, we have made significant progress on routinely isolating and establishing primary fibroblasts from mouse sciatic nerves. However, due to their limited proliferative capacity, we will need to immortalize these cells, something that has been technically challenging for us to execute with our standard immortalization lentiviral vectors (expressing Large T antigen). While we expect to overcome these issues, it should be noted that similar fibroblast and tumor co-transplant experiments in other tissues has revealed that transplanted fibroblasts are often rapidly replaced by endogenous fibroblasts, something we will need to assess carefully in our nerve co-transplants. In addition, although outside

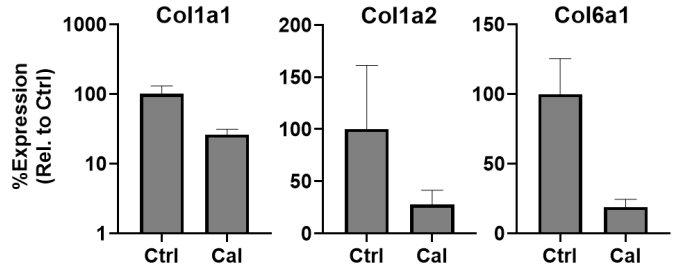

Figure 4. VDR agonist impacts expression of ECM genes in nerve-associated fibroblasts. qPCR analysis demonstrating that VDR agonist (Cal) downregulates expression of multiple collagen components of the extracellular matrix (ECM).

of the original goals of this proposal, we plan to follow up on our observations that VDR is a potent immune cell regulator (see last report). We will do this by transplanting MPNST cells into mice with VDR loss in the immune compartment (using Vav-Cre). Notably, this system can be used to dissect the relative contribution of hematopoietic VDR signaling to any responses we see from VDR agonist therapy treatment of MPNSTs.

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 are currently planning to test orthotopically transplanted human neurofibroma lines as an alternative experimental model for studying in vivo VDR impacts on tumor growth. 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.

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?

We ask for a 6 month no cost extension to finalize our ongoing analysis of *in vivo* MPNST responses to VDR agonist therapy as well as our genome-wide analyses of VDR agonist responses in fibroblasts. Outside of this no cost extension, we plan to continue our work using co-transplanted fibroblasts as an alternative approach to studying VDR loss in the fibroblast compartment. In addition, we hope to begin experiments exploring how VDR signaling within the immune compartment impacts neurofibroma and MPNST outcomes.

4. IMPACT:

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

While a prominent stromal response has long been associated with neurofibroma and MPNST tumors, this work highlights a critical functional role for fibroblast in the stromal in influencing tumor therapeutic responses. Although it remains unclear if VDR agonist therapy will ultimately prove a benefit in vivo, our findings nevertheless demonstrate the potential for stromal targeted therapies to improve patient outcomes. It strongly suggests that alternative approaches for targeting the fibroblast compartment should be further explored in these NF1-driven diseases.

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 have initiated alternative approaches to address our primary research questions. These include the use of fibroblast co-transplants, where VDR can be deleted *ex vivo*, to overcome the poor deletion seen *in vivo* with the Col1a2-CREER allele. In addition, while outside this proposal, due to technical challenges in establishing SKP cultures and transplants, we propose to use orthotopically transplanted human neurofibroma xenografts in future experiments studying the impact of VDR agonist on *in vivo* neurofibroma growth and therapeutic responses.

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

While significant progress was made, there were some logistical issues with personnel that delayed ongoing experiments, including *in vivo* analysis of MPNST responses and genome-wide transcriptional analysis of VDR responses in fibroblasts. For this reason, we are requesting a 6 month extension to complete these ongoing experiments. Of note, we believe these experiments do not face any significant technical challenges that should preclude them from being completed.

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

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: 1
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?

Yes.

Evans, Ronald M.

Completed:
Fondation Leducq, 16 CVD 01 (C. Glass, PI), Epigenetic targeting of monocytes and macrophages to reprogram cardiovascular disease, 01/01/2017 - 12/31/2022, total costs, 5% effort. The major goal of this project is to characterize the relationship of epigenetic states in human monocytes and macrophages with atherosclerotic risk, identify underlying regulatory mechanisms, and assess novel interventional approaches to control disease

National Institute of Environmental Health Sciences, P42 ES010337 (R. Tukey, PI), Toxic Responses Mediated by Nuclear Xenobiotic Receptors, 07/01/2017 - 03/31/2022, total costs, 10% effort. The major goal of Project 2, Nuclear Receptor Mediated Epigenetic and Immune Cell Changes in Liver Fibrosis Resulting from Toxicant Exposure, is to advance our understanding of TASH, a physiological consequence of Superfund toxicant exposure. The identification of putative biomarkers and epigenetic and immune therapeutic targets has the potential to impact both the prevention and remediation of toxicity attributed to hazardous substances.

National Institute of Diabetes and Digestive and Kidney Diseases, R01 DK120480 (R. Evans, PI), Engineering human islet-like organoids for transplantation, 02/01/2019 - 05/31/2022,otal costs, 20% effort. The major goal of this project is to develop the next generation of human islet-like organoids (HILOs) from stem cells for efficient and immune evasive transplantation.

Office of Naval Research, N00014-16-1-3159 (T. Broderick, PI), Epigenomic signatures of human physical performance, 02/01/2019 - 08/31/2022, 5% effort. The major goal of the Evans component, Epigenomic Signatures of Human Physical Performance, is to functionally associate exercise-induced epigenetic and gene expression changes in human skeletal muscle biopsies and cells with measurable improvements in performance.

The Mark Foundation for Cancer Research, ASPIRE (R. Evans, PI), Aberrant glycosylation and altered FXR activity converge to drive PDA progression, 09/30/2018 - 10/31/2022, , 2% effort. The major goal of this project is to explore our hypothesis based on preliminary data that dysregulated FXR activity and aberrant glycosylation converge to drive PDA progression in pancreatic cancer.

David C. Copley Foundation (R. Evans, PI), Decoding Immunotherapy Responses in Pancreatic Cancer Patients, 09/15/2019 - 11/30/2022, , 0.1 % effort. The major goal of this project is to employ cutting-edge technologies (including digital spatial profiling and single-cell genomics) to determine the cellular and molecular features that differentiate responders from non-responders.

New:
Salk Translation Fund – Cancer, 397 (R. Evans, PI), Evolution of intestinal-specific FXR agonists for the treatment of Colorectal Cancer, 04/01/2022 - 03/31/2023, total costs, .01% effort. The major goal of this project is to develop therapies for the prevention and treatment of CRC.

Samuel Waxman Cancer Research Foundation, SWCRF Institute Without Walls (R. Evans, PI), FXR as a novel therapeutic target in Colitis-induced Colorectal Cancer, 01/01/2022 - 12/31/2024, total costs, 0.1% effort. The major goal of this project is to continue to advance our work in the following areas: Elucidating the FXR signaling pathway in intestinal immune cells, leveraging FXR agonism to enhance current therapies for colitis and decreasing long-term risk for CRC, and survey and identify additional receptors for endogenous or microbiome-produced bile acids that could potentially serve as novel therapeutic targets against CRC.

California Institute for Regenerative Medicine , DISC2-13213 (R. Evans, PI), Extending Immune-Evasive Human Islet-Like Organoids (HILOs) Survival and Function as a Cure for T1D, 04/01/2022 - 12/31/2024, total costs, 20% effort. The major goal of this project is to optimize the generation and viability of an unlimited, reproducible source of human engineered islets for transplantation.

Larry L. Hillblom Foundation, 2021-D-001-NET (R. Evans, PI), Adipose PDE4D: a new druggable pathway to overcome insulin resistance, 01/01/2022 - 12/31/2025, total costs, 10% effort. The major goal of this project is to establish that the activation of phosphodiesterase PDE4 by FGF1 functions as an alternative anti-lipolytic regulatory pathway, and that this functionality is maintained in the diabetic state.

Downes, Michael R.

Completed:
Fondation Leducq, 16 CVD 01 (C. Glass, PI), Epigenetic targeting of monocytes and macrophages to reprogram cardiovascular disease, 01/01/2017 - 12/31/2022, total costs, 5% effort. The major goals of this project are to characterize the relationship of epigenetic states in human monocytes and macrophages with atherosclerotic risk, identify underlying regulatory mechanisms, and assess novel interventional approaches to control disease

National Institute of Environmental Health Sciences, P42 ES010337-20 (R. Tukey, PI), Toxic Responses Mediated by Nuclear Xenobiotic Receptors, 07/01/2017 - 03/31/2022, total costs, 10% effort. The major goal of Project 2, Nuclear Receptor Mediated Epigenetic and Immune Cell Changes in Liver Fibrosis Resulting from Toxicant Exposure, is to advance our understanding of TASH, a physiological consequence of Superfund toxicant exposure. The identification of putative biomarkers and epigenetic and immune therapeutic targets has the potential to impact both the prevention and remediation of toxicity attributed to hazardous substances.

National Institute of Diabetes and Digestive and Kidney Diseases, R01 DK120480 (R. Evans, PI), Engineering human islet-like organoids for transplantation, 02/01/2019 - 05/31/2022, total costs, 20% effort. The major goal of this project is to develop the next generation of human islet-like organoids (HILOs) from stem cells for efficient and immune evasive transplantation.

New:
Salk Translation Fund – Cancer, 397 (R. Evans, PI), Evolution of intestinal-specific FXR agonists for the treatment of Colorectal Cancer, 04/01/2022 - 03/31/2023, total costs, .01% effort. The major goal of this project is to develop therapies for the prevention and treatment of CRC.

California Institute for Regenerative Medicine , DISC2-13213 (R. Evans, PI), Extending Immune-Evasive Human Islet-Like Organoids (HILOs) Survival and Function as a Cure for T1D, 04/01/2022 - 12/31/2024, total costs, 20% effort. The major goal of this project is to optimize the generation and viability of an unlimited, reproducible source of human engineered islets for transplantation.

Truitt, Morgan

Completed:
None.

New:
None.

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