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Award Number: W81XWH-13-1-0101

TITLE: Gene Therapy for Childhood Neurofibromatosis

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REPORT DATE: May 2014

TYPE OF REPORT: Final

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

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE May 2014		2. REPORT TYPE Final		3. DATES COVERED 1 Jun 2013 – 31 May 2014	
4. TITLE AND SUBTITLE Gene Therapy for Childhood Neurofibromatosis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0101	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Segal, David J. E-Mail: djsegal@ucdavis.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, Davis Davis, California 95616				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 This high-risk, high-reward project was based on the observation that plexiform neurofibromas require a tumor microenvironment consisting of cells heterozygous for the neurofibromin (NF1) gene. Cells with two functional alleles of NF1 did not support tumor growth. The treatment objective was therefore to increase the level of expression from the one active copy of NF1 to complement the haploinsufficiency in the cells of the tumor microenvironment. This was to be accomplished by designing artificial transcription factors (ATFs). The ATFs were to be expressed from non-pathogenic, tumor-colonizing bacteria, which would introduce the ATF into the cells of the microenvironment. The hypothesis was that this treatment would ultimately halt or regress the tumors. Unfortunately the development of an active bacterial delivery vector was more difficult than we anticipated. While we were not able to complete all of the objectives outlined in the statement of work (SOW) within the one-year funding period, our data do indicate partial success in creating an ATF-bacterial delivery system.					
15. SUBJECT TERMS Neurofibromatosis, tumor microenvironment, neurofibromin gene (NF1), artificial transcription factor, TALE DNA-binding protein, bacterial delivery vector					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

The objective of this project was to develop an innovative therapy for neurofibromatosis. Neurofibromatosis type 1 (NF1) is one of the most common genetic disorders (1). It is initiated by the inherited or de novo inactivation of one allele of the neurofibromin gene (i.e., NF1+/-). Neurofibromas arise when a second hit occurs in the Schwann cells that provide the myelin sheath for peripheral neurons, converting these cells to NF1-/- . A critical observation from mouse models of NF1 was that Nf1-/- Schwann cells are necessary but not sufficient for tumor formation (2). Neurofibromas require interactions between Nf1-/- Schwann cells and Nf1+/- cells in the tumor microenvironment. In particular, Nf1-/- Schwann cells secrete high levels of stem cell factor (SCF), the ligand for the c-kit receptor on Nf1+/- mast cells. This recruits mast cells to the neurofibromas, where they promote tumor growth. Importantly, only Nf1+/- mast cells mobilize to the neurofibromas. Mouse plexiform neurofibromas fail to develop if the Nf1-/- Schwann cells occur in Nf1+/+ mice, or if Nf1+/- bone marrow is replaced with Nf1+/+ bone marrow (2). We therefore proposed to therapeutically up-regulate expression of one remaining wild type NF1 allele in the tumor microenvironment, thus compensating for the Nf1+/- haploinsufficiency. We proposed to accomplish this by designing a type of protein drug called artificial transcription factors (ATFs). Our laboratory has extensive expertise in designing engineered zinc finger or transcription activator-like effector (TALE) DNA-binding proteins (3). These proteins can be designed to recognize specific DNA sequences in the promoters of target genes. Attachment of a transcriptional activator domain creates an ATF that can activate expression of its target gene. Another important aspect of our approach was tumor targeting. We proposed to use non-pathogenic bacteria to deliver our ATFs to the cells of the neurofibroma microenvironment. It has been known for more than a century that bacteria will grow preferentially in vivo in tumors (4). This targeting capability has been exploited in recent years to use bacteria as delivery vehicles for therapeutic agents. One means by which bacteria transfer proteins from their own cytoplasm into eukaryotic cells is through a needle-like structure known as a type III secretion system (T3SS). In nature, bacteria use a T3SS to secrete TALE proteins into their host cells (5). We proposed to co-opt this natural system, engineering bacteria to inject our TALE-ATFs into cells in the neurofibroma microenvironment. Our methods might therefore have additional broad implications for treatment of other solid tumors in cancer. Unfortunately, we were not able to complete all of the objectives outlined in the statement of work (SOW) within the one-year funding period. However, our data do indicate the important partial success in creating an ATF-bacterial delivery system.

BODY

Our approved SOW described the following aim and subaims:

Aim 1. Test the delivery and activity of TALE-ATFs in a mouse cell model of NF1 (months 1-12)

1a. Construction of five TALE-ATFs (months 1-6)

1b. Construction of bacterial delivery system (months 1-6)

1c. Establish mouse mast cell culture system in lab (months 3-6)

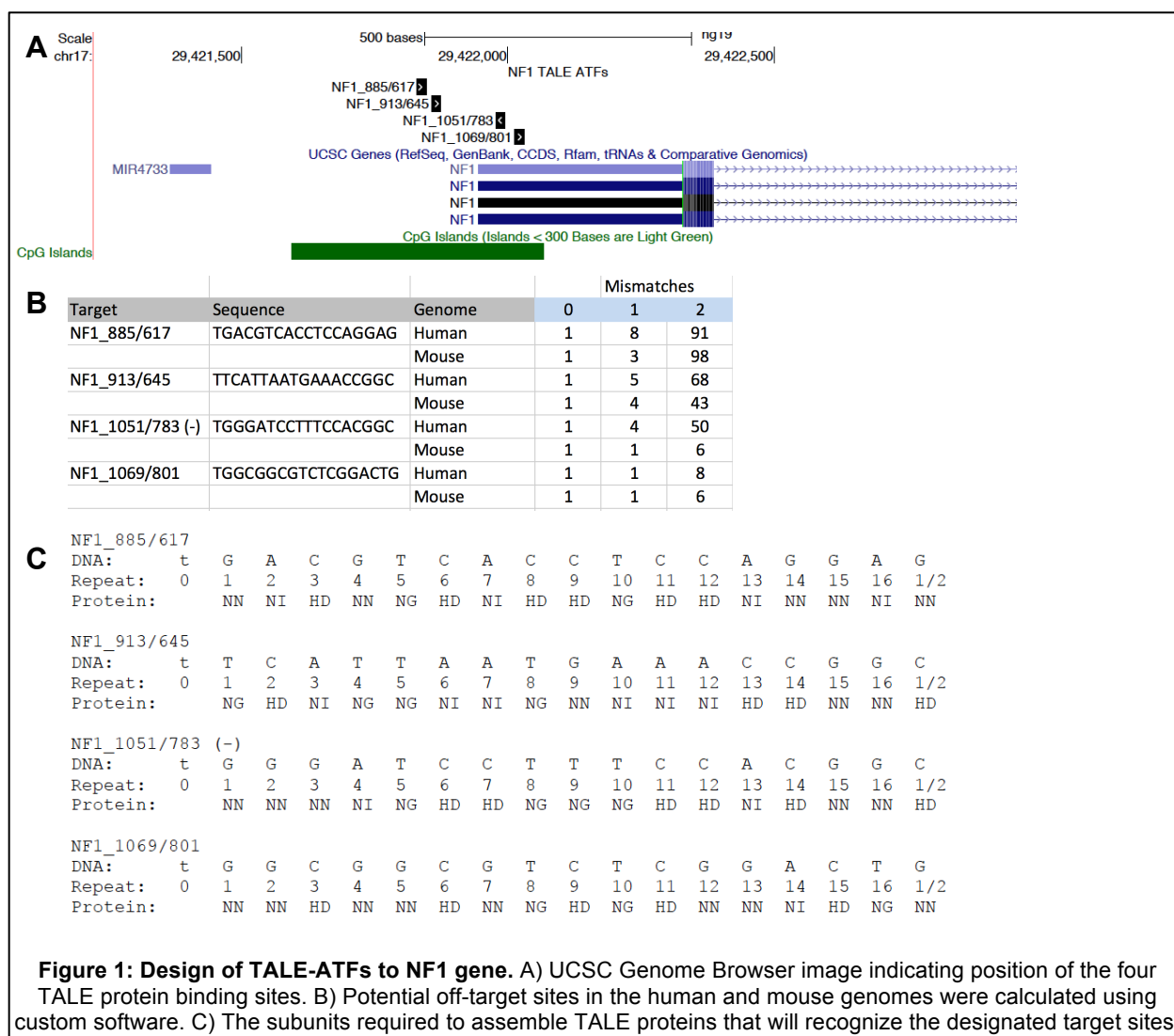
1d. Screen five TALE-ATFs in mouse mast cells (months 6-9)

1e. Test if TALE-ATF treatment produces *Nf1*+/+ phenotype in *Nf1*+/- mast cells (months 7-12)

Here we describe our progress on each of these subaims, indicating clearly our rationales for deviations.

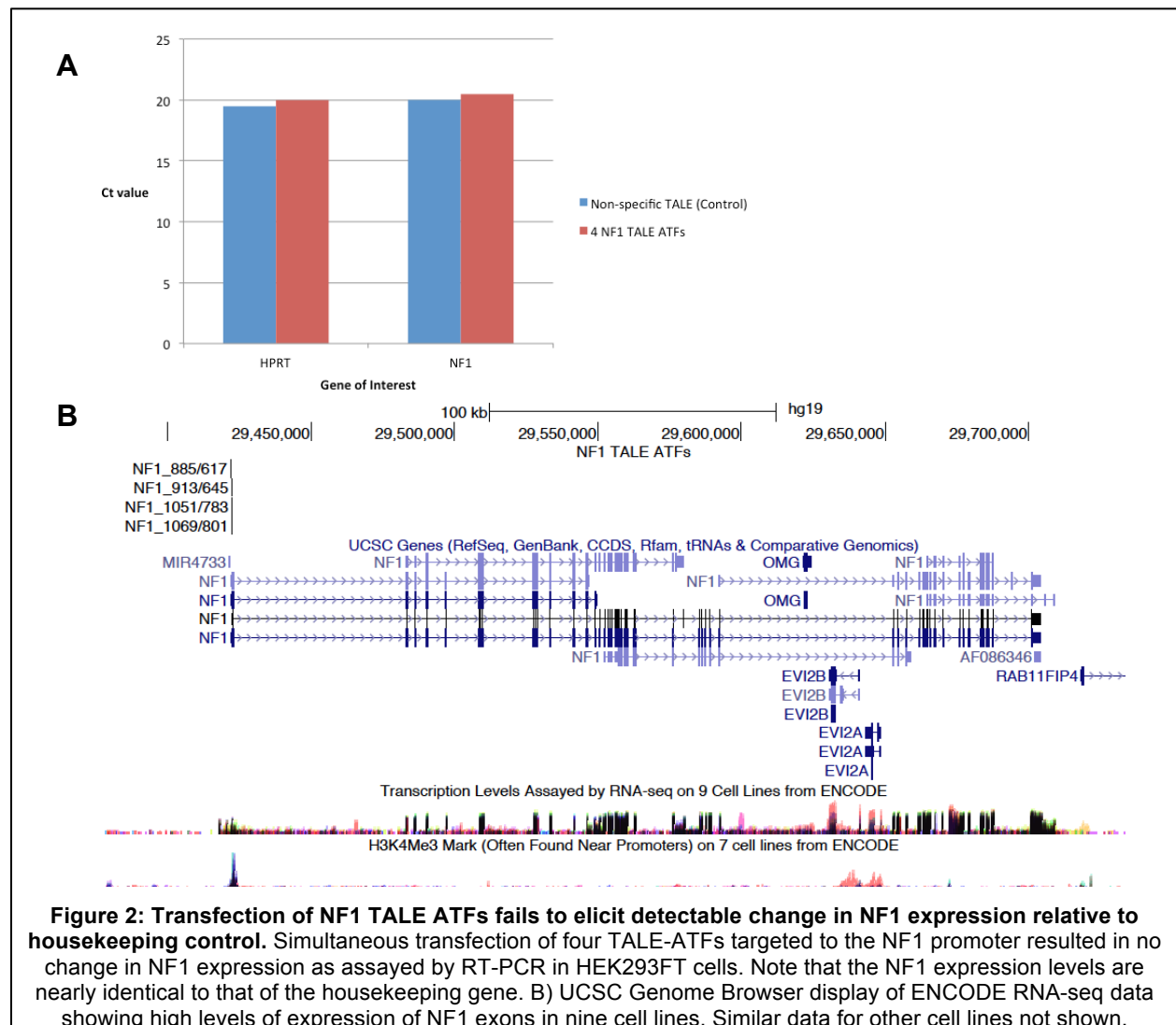
1a. Construction of five TALE-ATFs (months 1-6)

Four TALE-ATFs were designed and constructed to bind near the transcriptional start site (TSS) of the neurofibromin gene (NF1). Four were made instead of five because it was more convenient for the process of construction. Two were designed to bind upstream of the TSS, and two downstream (**Figure 1A**). The sites were chosen to occur both in mouse and human, therefore allowing the factors to be evaluated in both species. The genomes of both species (hg19 and mm9) were searched for sites that differed by 0, 1, or 2 mismatches to the intended target site (**Figure 1B**). This analysis showed that all 18-bp sites were unique in these genomes, with relatively few predicted off-target sites. Generally, TALE proteins designed to sites that have this profile can be expected to be highly specific for their target site and demonstrate potent activity at the target site. The TALE DNA-binding regions were assembled using the standard “Golden Gate” cloning kit (TALEN Kit #1000000024, Addgene)(**Figure 1C**).



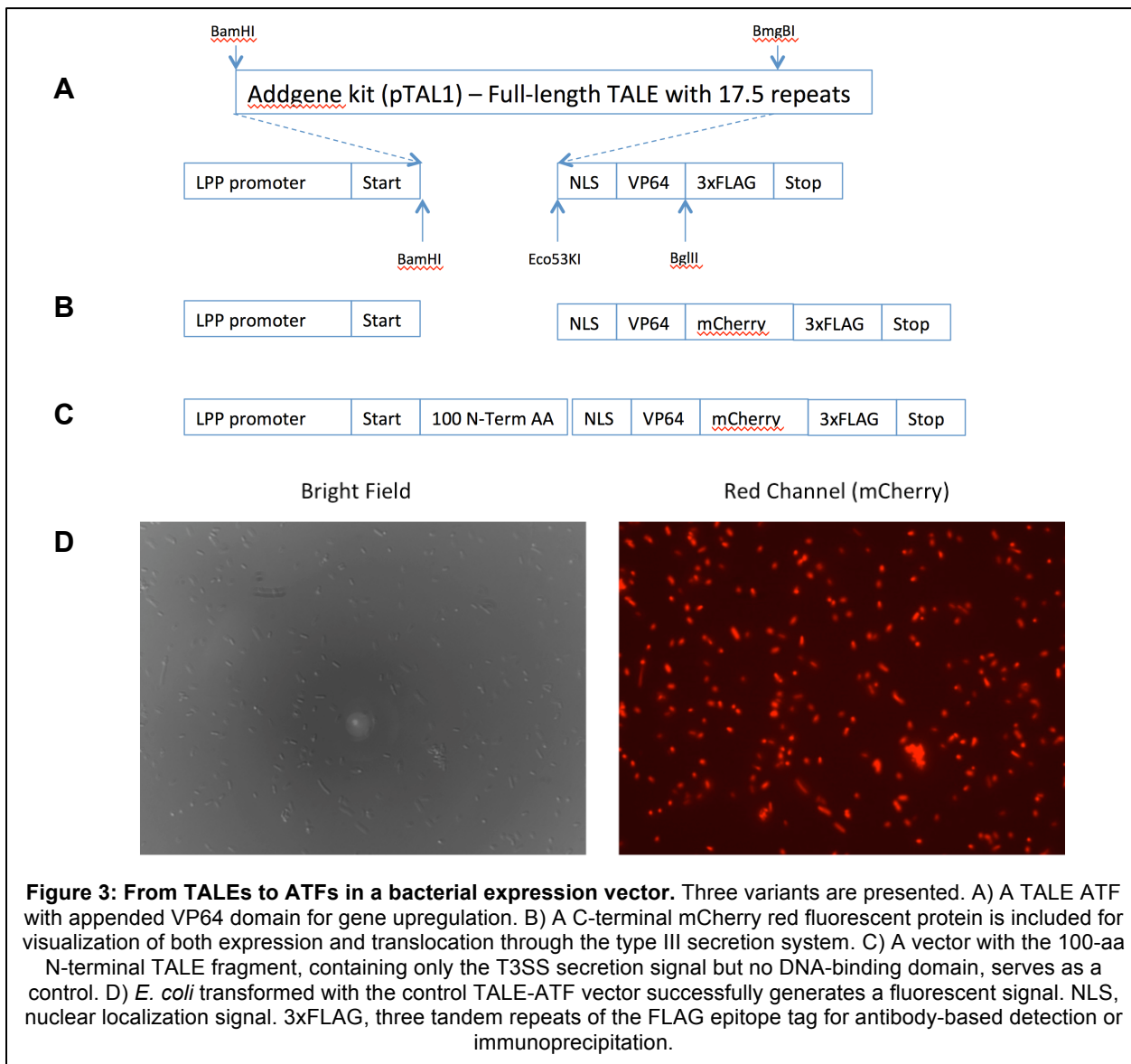
To demonstrate the ability of these proteins to activate expression of the NF1 gene, the TALE DNA-binding proteins were cloned in frame with a C-terminal VP64 transcriptional activation domain, creating artificial transcription factors (ATFs) in a mammalian expression vector (3).

These TALE-ATF constructs were transfected into HEK293FT cells, a human cell line in common use because it is easy to transfect. Previous studies have demonstrated that multiple ATFs targeting the promoter of the same gene produce a synergistic activation effect that is much stronger than any of the four factors alone (6). For this reason, all four NF1 TALE-ATFs were co-transfected together. RT-PCR analysis was performed to examine the expression of the NF1 gene using a housekeeping gene, HPRT, as a control. Unfortunately, the four NF1 TALE-ATFs failed to activate the NF1 gene more than a control TALE-ATF that was not expected to activate any gene in a human cell (**Figure 2A**). However, we noticed the expression level of NF1 was similar to the level of the HPRT housekeeping gene, suggesting NF1 might be already fully active at both alleles and incapable of further activation. Indeed, further investigation of expression data already available from the UCSC Genome Browser suggested that NF1 was actively expressed in most cell types and established cell lines (**Figure 2B**). Therefore, evaluation of the ability of the four NF1 TALE-ATFs to activate NF1 expression might require cells in which NF1 expression was known to be reduced, such as the NF1^{+/-} mast cells from collaborator Dr. D. Wade Clapp. However, since those primary cells require some effort to obtain from live mice, and need to be used immediately without substantial time in culture, we decided to wait until there was evidence the bacterial delivery system was working before requesting these cells.



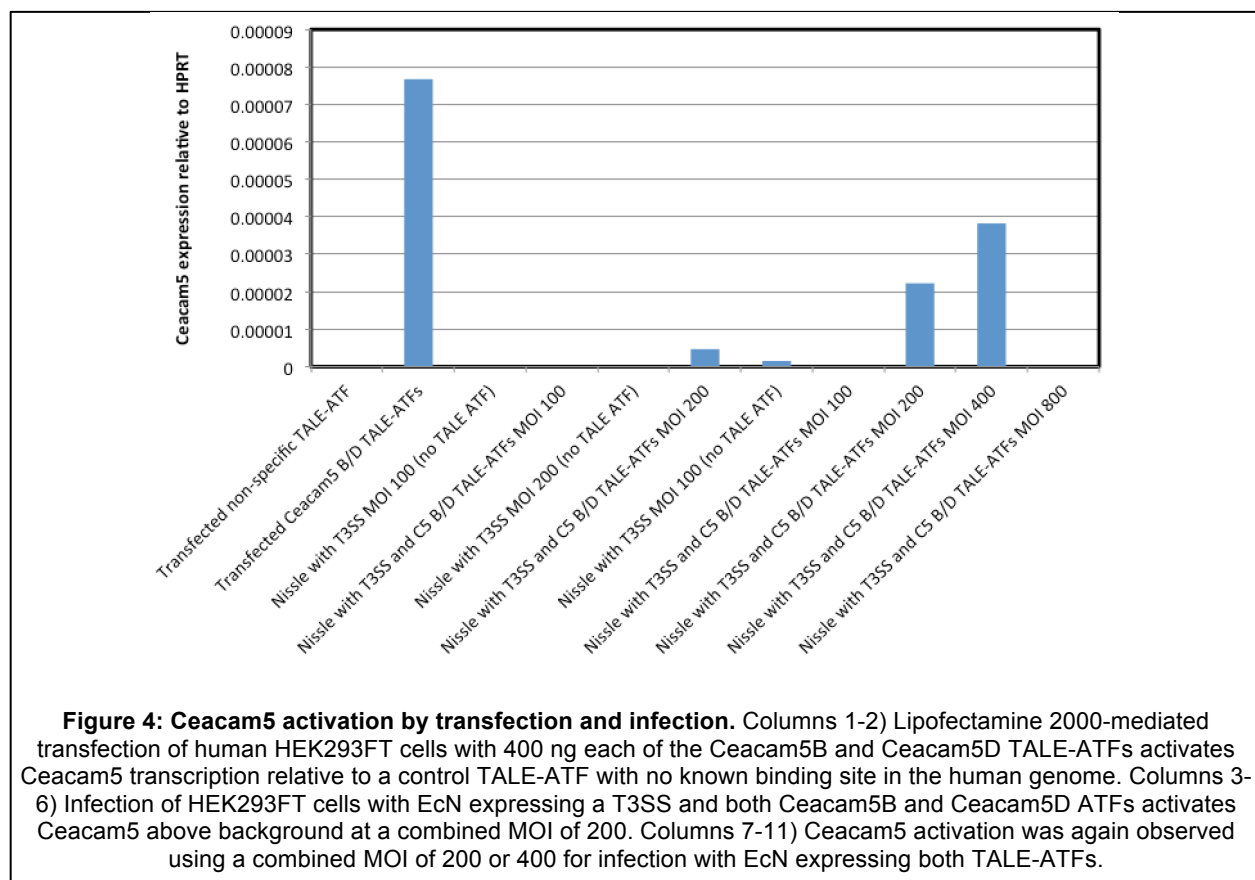
1b. Construction of bacterial delivery system (months 1-6)

To create ATFs that can translocate into mammalian cells through the type III secretion system (T3SS), three bacterial expression constructs were designed for the TALE DNA binding proteins (**Figure 3A-C**). All vectors contained a LPP promoter for strong constitutive expression in bacteria, as well as a VP64 domain for strong transcriptional activation of the target gene. Some vectors additionally contained an mCherry red fluorescent protein to visualize expression and translocation through the T3SS. The N-terminal 100 aa of the TALE protein contains the secretion signal for the T3SS. When this TALE-ATF vector was transformed into *E. coli* bacteria, a clear red fluorescent signal was observed (**Figure 3D**). These results suggest that the TALE-ATF vectors are able to express their contents in bacteria.



However, by now it had become clear that it would be difficult to demonstrate the efficacy of a bacterial expression system on NF1 gene activation using our human cell lines that already

express high levels of NF1. We therefore instead chose to test our system to activate the gene Ceacam5, which has very low expression HEK293FT cells. TALE-ATFs that could activate this gene have been previously described by others (6). These TALEs were cloned into the bacterial expression vectors described in Figure 3. These cosmids, which also expressing the T3SS (7), were introduced into the non-pathogenic bacteria strain *E. coli* Nissle 1917 (EcN, Ardeypharm GmbH). The transformed EcN bacteria were applied to human HEK293FT cells for 3 hours at 37°C, and then infection was cleared by adding media containing Ciprofloxacin. Ceacam5 expression was analyzed by RT-PCR 12 hours after infection. After many failed attempts and a significant amount of protocol optimization, EcN expressing the T3SS and TALE-ATFs demonstrated activation of Ceacam5 when applied at multiplicities of infection (MOI) of 200 – 400 (**Figure 4**). In these preliminary experiments, the level of activation was about half of that obtained by plasmids expressing the TALE-ATFs that were transfected into the human cells using Lipofectamine 2000 (Life Technologies). Nonetheless, these results indicate that EcN can deliver TALE-ATFs though a T3SS into human cells.



1c. Establish mouse mast cell culture system in lab (months 3-6)

1d. Screen five TALE-ATFs in mouse mast cells (months 6-9)

1e. Test if TALE-ATF treatment produces Nf1^{+/+} phenotype in Nf1^{+/-} mast cells (months 7-12)

These subaims represent the next logical steps in the testing of the EcN-TALE-ATF system to activate Nf1 in mouse Nf1^{+/-} mast cells. Unfortunately, our success in creating an active ATF bacterial delivery system came too late in the project period to justify establishing the mouse mast cell culture system (subaim 1c). Without this source of Nf1^{+/-} cells, we were also unable to

complete subaims 1d and 1e. However, the development of the EcN delivery system was clearly the most complex part of the project, which required the creation of a system that did not exist previously. The remaining subaims only involved testing of the created system using methods that are already established. Thus progress on the testing subaims would likely have been faster than for the development phase. This project was originally envisioned to require two years, and in retrospect it was probably a strategic error to have restructured the effort to only one year. However, with the critical component now in hand, completion of the testing and eventual publication of the data can occur quickly once sufficient funding is secured.

KEY RESEARCH ACCOMPLISHMENTS

- Constructed four TALE-ATFs to activate the NF1 gene in both mouse and human.
- Developed a novel bacterial TALE-ATF delivery system that was able to activate an endogenous gene in human cells.

REPORTABLE OUTCOMES

No manuscripts or published results have been generated yet from this project, although we anticipate an eventual publication of the system that was developed and perhaps a patent. We plan to apply for additional funding to complete this work started under this award to enable publication. The PhD degree that Joshua Meckler anticipates to obtain next year will have been partially supported by this award. The bacterial TALE-ATF delivery system developed from this award will likely find future use by us and other investigators.

CONCLUSIONS

There are currently no medical treatments for plexiform neurofibromas. Several drugs are in clinical trial, but none affect the underlying genetic deficiency. This project represents the first therapeutic approach to restore normal levels of NF1 gene expression in the cells that support the tumor. ATFs can be considered a new paradigm in therapeutic development. ATFs work on a genetic level and can thus target "undruggable" proteins like NF1. We are also proposing to use tumor-infiltrating bacteria to continuously produce the ATFs at the tumor site until the tumor is gone. ATFs have never been delivered this way before. The ATFs are based on transcription activator like effector (TALE) transcription factors, which in nature are injected from bacteria into eukaryotic target cells. Therefore this project usurps the natural delivery system of these proteins to treat plexiform neurofibromas. Although we were not able to complete the testing objectives of this project, we were successful in developing the core enabling technology. This approach still represents a plausible and very different way to treat childhood neurofibromatosis, as well as other solid tumors.

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APPENDICES

None.