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TITLE: Cellular Delivery of Protein Therapeutics for the Treatment of Amyotrophic Lateral Sclerosis Using Endogenous Retroviruses

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14. ABSTRACT
Over 90% of Amyotrophic Lateral Sclerosis (ALS) cases are sporadic without a clear initiating molecular event. However, like in many neurodegenerative diseases, protein aggregation and defects in protein degradation are common features in ALS. In 97% of patients with ALS, these neuronal protein aggregates include a protein called TAR DNA binding protein-43 (TDP-43). Clearance of neuronal TDP-43 aggregates in experimental models of ALS using intracellular protein therapeutics, such as monoclonal antibodies targeting TDP-43 aggregates for degradation, is associated with increased cell survival and reduced motor defects. In this project we are developing a new cell therapy for the targeted degradation of aggregated TDP-43. To date, we have identified the genetic elements required for the efficient delivery of genetically encoded payloads including TDP-43-degrading proteins, stably engineered cells as delivery vehicles and validated this approach using a series of orthogonal in vitro assays. In addition, we have established the required in vivo model and required analytical assays.

15. SUBJECT TERMS
Amyotrophic lateral sclerosis, cell therapy, TDP-43, degradation

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disorder resulting in motoneuron death and a mean duration of survival of only 3-5 years. Few treatments are available for ALS and none substantially increase the patients' life expectancy. Recent failures in early- and late-stage clinical trials further exemplify the urgent need for new approaches. Over 90% of ALS cases are sporadic without a clear initiating molecular event. However, like in many neurodegenerative diseases, protein aggregation and defects in protein degradation are common features in ALS. In 97% of patients with ALS, these neuronal protein aggregates include a protein called TAR DNA binding protein-43 (TDP-43). Clearance of neuronal TDP-43 aggregates in experimental models of ALS using intracellular protein therapeutics, such as monoclonal antibodies targeting TDP-43 aggregates for degradation, is associated with increased cell survival and reduced motor defects. However, to date no efficient approaches exist for the persistent intracellular delivery of such protein therapeutics. In this study, we will determine the efficacy of a novel mRNA-producing adoptive cell therapy (M-PACT) approach for the intracellular delivery of mRNA encoding protein therapeutics to diseased neurons.

2. Keywords

Amyotrophic lateral sclerosis, cell therapy, TDP-43, degradation.

3. Accomplishments

What were the major goals of the project?

Please find below the major tasks as stated in the approved SOW

Major Task 1: Determine targeted degradation of TDP-43 by M-PACT cells in vitro. (months: 1-7; percentage of completion: 50%)

Major Task 2: Determine efficacy of M-PACT approach for the treatment of ALS in transgenic mouse model. (months 8-24; percentage of completion: 10%)

Major Task 3: Determine efficiency of Sleeping Beauty (SB) transposase system for M-PACT manufacturing. (months: 10-14; percentage of completion: 70%)

Major Task 4: Reduce M-PACT genetic payload size using deletion mutants of PEG10, Arc, and packaging UTRs. (months: 15-19; percentage of completion: 100%)

What was accomplished under these goals?

Effect of fusogens on syncytia formation and M-PACT cell expansion (Luetkens lab)

The goal of this project is to develop and validate a cell therapy that is able to transfer therapeutic payloads to ALS cells. To achieve this transfer, we proposed to employ retroviral proteins encoded in the human genome. Specifically, we are focusing on the use of capsid-like and fusogenic proteins. Capsid proteins form a macromolecular complex to protect the therapeutic payload from degradation while fusogens are required to facilitate the fusion of two cell membranes to enable the transfer of payloads to target cells via extracellular vesicles, virus-like particles, or direct cell-cell fusion. The therapeutic agent developed in this project will be an mRNA-producing adoptive cell therapy (M-PACT): engineered cells stably expressing the endogenous retroviral proteins and the respective payload.

In our preliminary data we demonstrated transfer of the fluorescent protein mCherry from M-PACT cells to target cells in a short-term co-culture. However, we observed that some M-PACT cells engineered using 293T cells showed delayed growth compared to wildtype 293T cells. We hypothesized that expression of some fusogens may result in complete cell-cell fusions, resulting in non-viable syncytia. Transient transfection of 293 T cells using different endogenous fusogens indeed revealed overt syncytia formation when using the mSYNA fusogen (Fig. 1A). mSYNA cells showed substantially reduced expansion compared to GFP control transfected cells (Fig. 1B). The widely used vesicular stomatitis virus G protein fusogen, which is used as a non-human control, as well as the human fusogen hSYNA showed a slight decrease in expansion but no overt syncytia formation. These data indicate that use of mSYNA may not be possible in a cell therapy approach due to its detrimental effect on cell expansion.

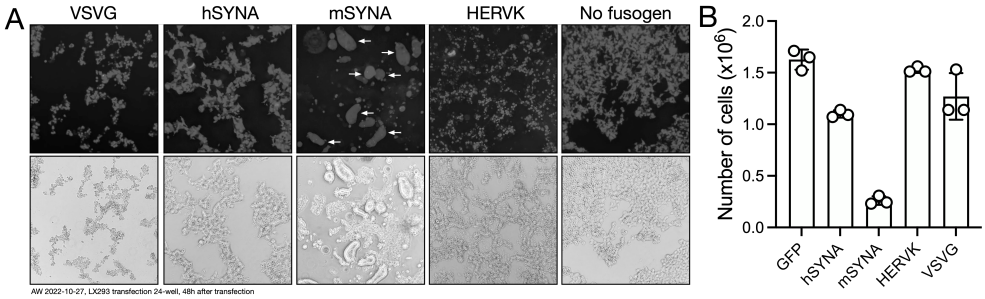

Figure 1: Effect of fusogens on syncytia formation and M-PACT cell expansion. (A) Microscopy images of 293T cells transfected with the indicated fusogen and mCherry. Top: mCherry; bottom: brightfield. **(B)** Number of cells remaining in culture, 48h after transfection as determined by automated cell counting.

Validation of hSYNA expression in engineered human cells (Luetkens lab)

Based on our observation that hSYNA transfection appears to be well-tolerated in human cells, we next set out to determine whether overexpression of hSYNA was indeed achieved in M-PACT cells. However, using various commercially available antibodies, we were unable to detect hSYNA in the transfected cells. hSYNA is a relatively little-studied protein and hSYNA-specific research reagents have not been extensively validated. We therefore generated the full open-reading frame of hSYNA fused to a C-terminal hemagglutinin (HA) tag (hSYNA-HA) and engineered cells to transiently express hSYNA-HA. In these cells, we were able to detect high levels of hSYNA-HA as determined by western blot when probing using a well-validated anti-HA antibody (Fig. 2). Importantly, a commercially available hSYNA antibody, which was used as a control, was not able to detect overexpressed hSYNA. We demonstrate that hSYNA can be overexpressed at high levels using our approach and provide an effective method for the assessment of hSYNA expression in engineered cells.

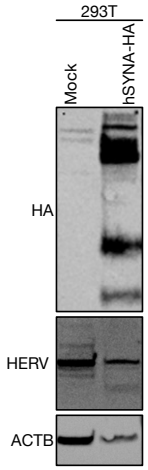

Figure 2: Validation of hSYNA expression in engineered human cells. HSYNA-HA expression as determined by western blot after probing with an anti-HA, anti-TDP-43 or an anti-β-actin (ACTB) antibody in 293T cells transiently transfected with hSYNA-HA expression construct.

Cytosolic payload transfer using M-PACT approach (Luetkens lab)

We had demonstrated in our preliminary data the efficient transfer of the fluorescent reporter mCherry to target cells using the M-PACT approach. However, it is possible that mCherry was taken up by target cells through endocytosis and not via membrane fusion. In the therapeutic setting, payloads would need to be able to access the cytosol or the nucleus to be able to interact with their target proteins and we therefore next developed a novel assay for the quantification of cytosolic payload transfer to determine if this approach would be able to deliver payloads to the correct subcellular space. Specifically, we developed a luciferase-complementation assay using a split NanoLuc pair, with the C-terminal luciferase fragment stably expressed in the M-PACT cells and the N-terminal fragment stably expressed in the target cells' cytosol (Fig. 3A). Upon transfer of the C-terminal fragment from the M-PACT cells co-transfected with a fusogen, both fragments readily assemble in the cytosol of the target cell, generating luminescence upon incubation with the NanoLuc substrate furimazine. Using the luminescence complementation assay, we found that co-culture of hSYNA- and VSVG-based M-PACT cells with target cells resulted in the efficient delivery of the NanoLuc payload to the target cells' cytosol (Fig. 3B). Importantly, this transfer was only slightly enhanced by addition of a gag-like protein indicating that a fusogen only may be sufficient for payload transfer (Fig. 3C). This finding was mirrored by determining fluorescent protein and NanoLuc transfer in the presence of the proposed gag-like proteins, use of payload open-reading frames flanked by untranslated regions derived from gag-like protein mRNAs, and fusogens in 293T cells and J76 cells (data not shown). Although we did observe slightly increased payload transfer when gag-like proteins were added, we decided to go forward with a simple fusogen-only approach due to the lower complexity facilitating the technology's translational development and smaller DNA footprint allowing more efficient M-PACT cell manufacturing.

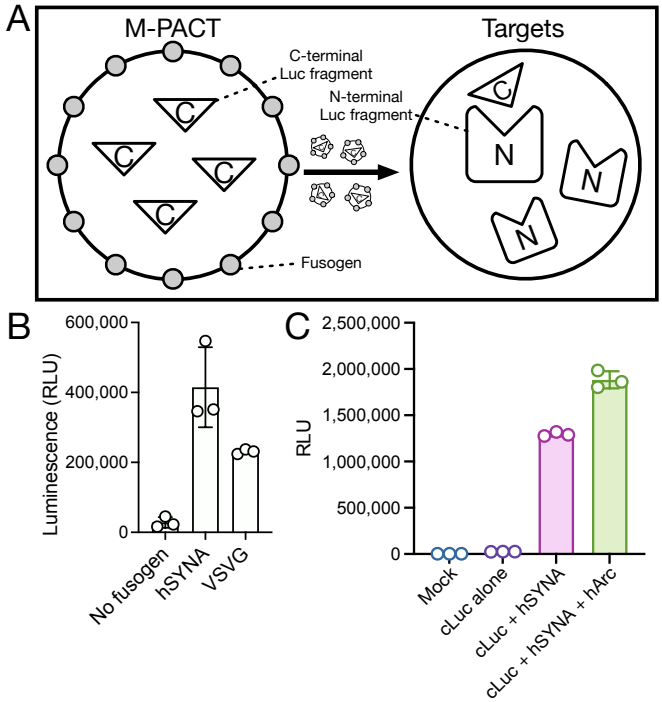

Figure 3: Luciferase complementation confirms cytosolic payload delivery and validates fusogen-only M-PACT approach. (A) Schema of luciferase complementation assay used to determine cytosolic payload delivery. **(B)** Luminescence resulting from luciferase complementation after 24h co-culture of 293T-based M-PACT cells transfected with the indicated fusogen and the C-terminal NanoLuc fragment with target cells expressing the N-terminal NanoLuc fragment. **(C)** Luciferase complementation using hSYNA-based M-PACT cells with or without use of the gag-like protein hArc.

Sleeping Beauty-based stable M-PACT cell generation (Luetkens lab)

A key advantage of the M-PACT approach would be the persistent delivery of therapeutics to diseased cells in order to target chronic disease processes. To determine whether it is feasible to stably engineer cells to continuously and stably express fusogens and payloads, we used a transposase-based approach to stably integrate the required genetic elements in M-PACT cells. Specifically, we transfected 293T cells as well as the T cell line Jurkat with the C-terminal NanoLuc fragment by itself or together with VGVG or hSYNA flanked by inverted tandem repeats. In addition, we transfected the same cells with the enhanced Sleeping Beauty transposase SB100X. The Sleeping Beauty constructs generated for this purpose contained antibiotic resistance genes allowing the subsequent selection of stably engineered cells using antibiotic selection. After 2 weeks, we characterized the cells by flow cytometry analyzing fluorescent reporters contained within the respective constructs. We show that we were able to generate highly enriched stable M-PACT cells using this approach (Fig. 4A).

Subsequently, we determined the ability of stable M-PACT cells to transfer payloads to target cells. To determine whether M-PACT-mediated payload transfer is effective across different cell types we generated a panel of target cell lines derived from different tissues. We found that payload transfer by M-PACT cells based on the hSYNA fusogen was effective in all cell types using both 293T and J76/Jurkat-based M-PACT cells (Fig. 4B), indicating that this approach may be effective across disease settings.

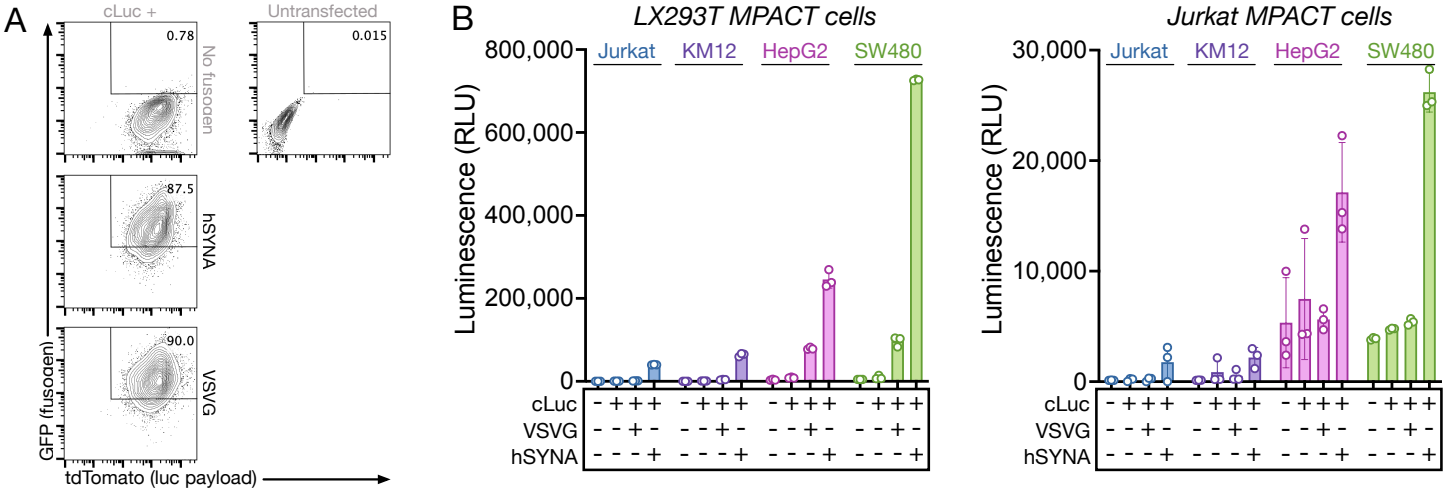

Figure 4: Sleeping Beauty-based stable M-PACT cell generation. (A) Purity of stable 293T-based M-PACT cells generated using Sleeping Beauty transposase system. Expression of fluorescent reporter proteins was determined after 2 weeks in the presence of antibiotics to select for stably transfected cells. **(B)** Luciferase complementation assay using stable 293T or Jurkat-based M-PACT cells using target cells derived from different human tissues.

Cell-to-cell delivery of a DNA-modifying enzyme using hSYNA (Luetkens lab)

In the majority of patients with ALS, the disease occurs spontaneously without known genetic causes. However, in a subset of patients, a set of inherited mutations have been identified and causally linked to the disease phenotype. In those patients with familial ALS, it may be possible to treat the disease by correcting the disease-causing mutations or by downregulating the mutated genes. In fact, the latter has shown promising clinical efficacy when using a small interfering RNA approach. An alternative to the siRNA-based approach may be the correction of the defect on the DNA level, for example using CRISPR/Cas9. In addition, demonstrating that the M-PACT approach is able to alter endogenous target cell behavior is essential to support its clinical development. To determine if M-PACT cells based on hSYNA are able to deliver DNA-modifying enzymes to target cells, we created a Cre-LoxP based reporter system. Specifically, we engineered target cells to carry a LoxP-flanked DsRed fluorescent reporter including a stop codon followed by an eGFP reporter (Fig. 5A). Following Cre-mediated excision of the LoxP-flanked cassette, target cells switch from DsRed to eGFP. We engineered M-PACT cells to express hSYNA and tamoxifen-inducible Cre. We found that M-PACT cells engineered to express Cre and hSYNA efficiently converted target cells to eGFP (Fig. 5B). These data demonstrate that M-PACT is effective at delivering a DNA-modifying enzyme to target cells and validates M-PACT as an effective approach to alter target cell function.

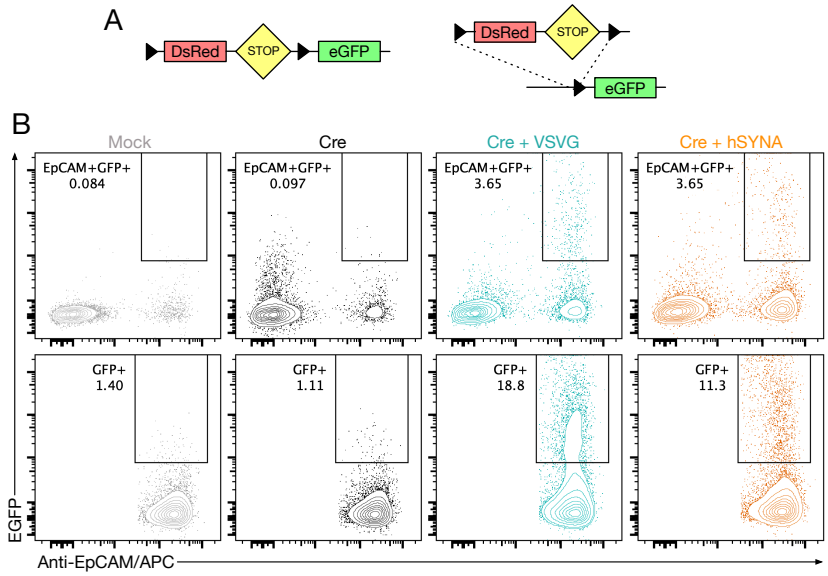

Figure 5: Cell-to-cell delivery of a DNA-modifying enzyme using hSYNA. (A) Schema of construct used to detect Cre activity in target cells in the absence of Cre (left) and after Cre-mediated excision (right). Black triangles indicate LoxP sites. **(B)** Conversion of EpCAM+ SW480 cells to eGFP following Cre-mediated excision of LoxP cassette after 48 h co-culture with M-PACT$_{Cre}$ cells in the presence of tamoxifen as determined by flow cytometry. Top panels show all cells, bottom panels show only EpCAM+ SW480 cells.

Targeted TDP-43 degradation using TDP-43-specific single-chain variable fragments (Luetkens lab)

ALS is characterized by the formation of intracellular protein aggregates in motoneurons leading to cell death. One of the major components of these aggregates is the protein TDP-43 and it has been shown that mutations in TDP-43 that lead to protein aggregation can cause ALS. Previously, two single chain variable fragments have been developed that can cause degradation specifically of TDP-43 aggregates. To determine whether M-PACT is able to deliver such TDP-43-targeting scFvs and reduce TDP-43 levels in target cells, we established a model system to test this approach. We generated tetracycline-inducible constructs for the conditional expression of wildtype TDP-43 or TDP-$43_{C173S/C175S}$, a spontaneously aggregating form of TDP-43, fused to GFP. 293T cells engineered to carry these constructs using lentiviral gene transfer and exposed to doxycycline, show substantial levels of the respective TDP-43-GFP transgenes (Fig. 6). Transfection of the same cells with the two aggregated TDP-43-specific scFvs resulted in successful degradation of mutant TDP-43 but not wildtype TDP-43 demonstrating the potential utility of these constructs in removing aggregated TDP-43.

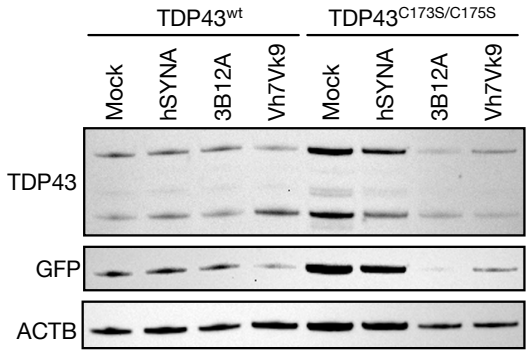

Figure 6: Targeted TDP-43 degradation using TDP-43-specific single-chain variable fragments. 293T cells stably engineered to express wildtype TDP-43 (TDP-43_{wt}) or mutant TDP-43 (TDP-$43_{C173S/C175S}$) fused to GFP under control of a tetracycline-inducible promoter were transfected with expression plasmids encoding hSYNA or one of two TDP-43 targeting scFvs, 3B12A and Vh7Vk9. 24h after transfection, tetracycline was added to cells. Cells were collected and analyzed by western blot for levels of TDP-43 and GFP.

Degradation of mutant TDP-43 using M-PACT cells (Luetkens lab)

M-PACT technology relies on the stable production and transfer of therapeutic payloads to target cells. We therefore generated 293T cells and Jurkat cells using Sleeping Beauty transposase to stably express hSYNA with or without the two TDP-43-targeting scFvs. When co-cultured with 293T-based M-PACT cells, we observed efficient reduction in mutant TDP-43 in tetracycline-treated target cells expressing TDP-43 variants fused to GFP as determined by western blot using M-PACT cells. Specifically, we observed efficient degradation when using the TDP-43-specific scFv Vh7Vk9. These data indicate that M-PACT cells are able to cause the efficient degradation of high levels of mutant TDP-43 in target cells.

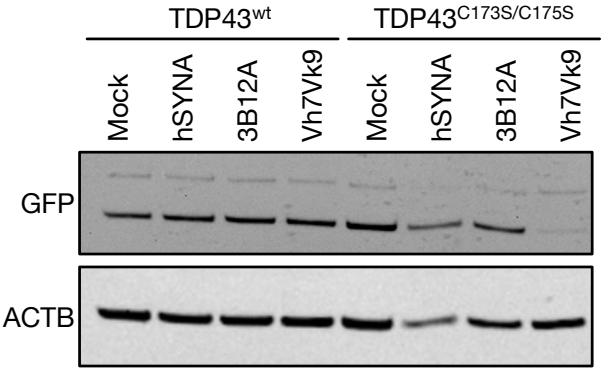

Figure 7: Degradation of mutant TDP-43 using M-PACT cells. 293T cells stably engineered to express wildtype TDP-43 (TDP-43_{wt}) or mutant TDP-43 (TDP-$43_{C173S/C175S}$) fused to GFP under control of a tetracycline-inducible promoter were incubated with 293T cells expressing hSYNA alone or together with a TDP-43-specific single-chain variable fragment (3B12A or Vh7Vk9). 24 h after initiating the co-culture, tetracycline was added to the co-culture. After 48h, target cells were collected by fluorescence-activates cell sorting and lysates prepared for western blot to determine levels of levels of TDP-43 and GFP.

Establishment and validation of UBQLN2-transgenic mouse model (Monteiro lab)

To determine the therapeutic activity of the M-PACT approach in ALS, we have established UBQLN2-transgenic mouse colonies and validated their previously described phenotype. In our validation experiments, we observed earlier disease onset in untreated animals than previously described and revised the experiment

timeline slightly to perform molecular/pathology analyses at month 7 (November 2024) and month 10 (February 2025), and complete the phenotypic assessment by month 10. In addition, we have validated the various proposed phenotypic and molecular assays to assess disease severity and in vivo TDP-43 degradation. Having successfully generated the required therapeutic cell products and established the mouse model, we anticipate initiation of M-PACT treatment by the end of this month (April 2024).

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?
Having engineered and validated a robust cell-based platform for the delivery of protein therapeutics to various target cell types and having demonstrated the targeted degradation of TDP-43 in engineered target cells, we will next determine the effect of M-PACT treatment in relevant models of ALS. Specifically, we will determine degradation of TDP-43 in vitro using primary motoneurons expressing wildtype or ALS-associated mutant TDP-43 (*Luetkens lab*). In addition, we will perform the proposed in vivo experiments using a transgenic mouse model of ALS and determine the effect of M-PACT treatment on ALS progression as well as cellular and molecular correlates of ALS (*Monteiro lab*).

4. Impact
What was the impact on the development of the principal discipline(s) of the project?
The main goal of this project is the development of a cellular therapy that is able to deliver protein therapeutics to the inside of a diseased cell using only human proteins. To date, we have been able to show for the first time that such an approach is in fact feasible, which genetic elements are necessary and sufficient to engineer such cells, that it is possible to engineer cells stably producing and transferring therapeutic payloads, and that it is possible to degrade TDP-43 in target cells using this approach. All of these findings are major advances in the field of cell therapy, especially for the treatment of ALS. We anticipate that publication of our findings will enable other researchers to use and adapt this technology to develop therapeutics for ALS and other chronic diseases.

What was the impact on other disciplines?
As part of our validation strategy, we were able to demonstrate that the technology developed as part of this project is able to deliver DNA-modifying enzymes to target cells in order to correct genetic defects. While this project focuses on a therapeutic strategy for the persistent degradation of aggregated TDP-43, in familial cases of ALS such as those resulting from mutations in SOD1, delivery of DNA-modifying enzymes could be a promising alternative to current siRNA-based treatments. In addition, the technology may ultimately be used for the treatment of diseases resulting from other genetic defects, for example to correct mutations in the CFTR gene leading to cystic fibrosis.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
This project is inherently translational, aiming to develop a novel platform technology for the delivery of therapeutics to diseased cells, specifically for patients with ALS. Our work so far has resulted in a successful preclinical proof-of-concept of the technology proposed in our application. While additional work is needed to further validate the approach, our initial findings are a key step for this technology to eventually enter clinical practice and improve the lives of patients with ALS.

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
Due to the need for the development of additional validation assays and generation of DNA constructs without ribosomal skip sequences needed for the efficient manufacturing of stable M-PACT cells, we experienced a

slight delay in the initiation of assays involving the use of motoneurons and our in vivo studies. However, we recently completed manufacturing of the respective M-PACT cell products needed for these studies and will now be able to initiate the proposed in vivo studies. In vitro assays involving the use of motoneurons will be carried out concurrently to the in vivo studies. In addition, we were able to complete most of the work related to Sleeping Beauty-mediated M-PACT cell generation and reduction in genetic payload size that was proposed for year 2, within the first year. We therefore anticipate that all of the proposed work will be completed within the award period.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

6. Products
Publications, conference papers, and presentations
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
Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
We have generated a comprehensive set of preclinical in vitro data demonstrating the development of a cellular therapy that is able to deliver protein therapeutics to the inside of a diseased cell using only human proteins. In addition, we have generated a bank of well-characterized stable M-PACT cell lines and various target cell lines used for validation experiments that can be shared readily with other ALS researchers upon completion of this project. To date, we have been able to show for the first time that such an approach is in fact feasible, which genetic elements are necessary and sufficient to engineer such cells, that it is possible to engineer cells stably producing and transferring therapeutic payloads, and that it is possible to degrade TDP-43 in target cells using this approach. All of these findings are major advances in the field of cell therapy, especially for the treatment of ALS. We anticipate that publication of our findings will enable other researchers to use and adapt this technology to develop therapeutics for ALS and other chronic diseases.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name: Tim Luetkens
Project Role: PI
Researcher Identifier: ORCID ID 0000-0002-7085-9027
Nearest person month worked: 2.4
Contribution to Project: Dr. Luetkens has coordinated and overseen all work related to the development of M-PACT technology including construct design, data analysis, and troubleshooting.

Name: Alexander Wang
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6
Contribution to Project: Mr. Wang has generated the described M-PACT and target cell lines, cloned expression constructs and carried out in vitro validation experiments under supervision of Dr. Luetkens.

Name: Mervyn Monteiro
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Dr. Monteiro coordinates and oversees all proposed in vivo work, including ensuring regulatory compliance, validation of the model system, and performance of the proposed in vivo experiments.

Name: Peyton Fuller
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 5
Contribution to Project: Ms. Fuller established, maintains, and validates the required mouse colonies and performs the proposed in vivo experiments under supervision of Dr. Monteiro.

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.

8. Special Reporting Requirements

Collaborative awards: This report includes a description of all completed and proposed work performed in the context of this project. Specifically, it includes all work performed in the Luetkens (PI) and the Monteiro (Co-I) labs at University of Maryland Baltimore and indicates in which lab the respective work was carried out.

9. Appendices

None.