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CONTRACTING ORGANIZATION: Northwestern University

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14. ABSTRACT

Normally cells continually synthesize new proteins and dispose of old and damaged proteins. In ALS, damaged proteins are not properly eliminated, leading to their accumulation and this compromises cell health. Major therapeutic efforts are underway world-wide to devise new strategies for enhancing elimination of damaged proteins. Several labs have shown that reducing the abundance of a key regulatory protein called RAD23 has a broad action of enhancing elimination of damaged proteins. In a variety of disease models this benefits neuronal healthy and survival. To translate these observations into a human, we will test the efficacy of a therapeutic technology called anti-sense oligonucleotides (ASO). The goal of this project is to determine whether ASO that target the destruction of RAD23 confers benefits on a mouse model of ALS.

15. SUBJECT TERMS

Amyotrophic Lateral Sclerosis, protein homeostasis

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Introduction

In a *C. elegans* genetic screen we determined that a null mutation in a gene called *rad23* suppressed TDP43 toxicity. Mammals have two *rad23* genes (*rad23a* and *rad23b*) and follow up studies in rat spinal cord cultures showed that knockdown of either *rad23a* or *rad23b* protected motor neurons from TDP43 toxicity. To study the *in vivo* relevance of these observations we reduced the abundance of *rad23a* or *rad23b* in a mouse model of TDP43'opathy. With the support of the Medical Research and Development Command, US Army, we undertook behavioral, biochemical and histological studies and establish that *rad23a* is an important modifier of TDP43 toxicity.

Keywords

TDP43	Tar DNA binding protein of 43 kDa molecular weight
Rad23	gene name
ASO	Antisense oligonucleotide
TAR4	Transgenic mice expressing WT human TARDBP ($TDP43^{WT}$)
ICV	intracerebroventricular
P0	postnatal day zero
KD	Knockdown
µg	microgram
GFAP	glial fibrillary astrocytic protein
Iba1	ionized calcium binding adapter molecule 1
IHC	Immunohistochemistry

Accomplishments

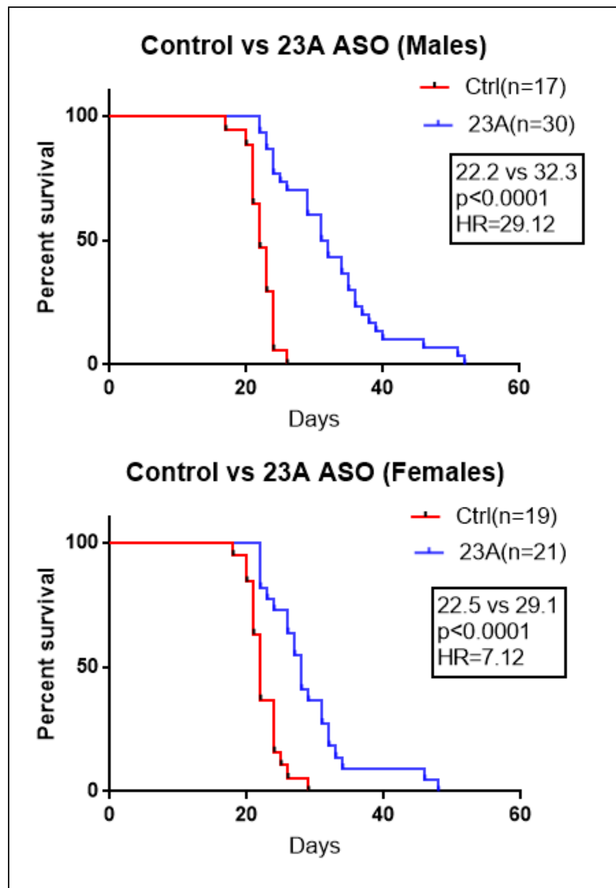
Major goals of the project

The major goals of this project were to determine if reduction of RAD23A and/or RAD23B abundance blunted disease phenotypes in the TAR4/TAR4 mouse model of TDP43'opathy. If these was achieved, our second goal was to get mechanistic insight into why reducing the abundance of these proteins was beneficial.

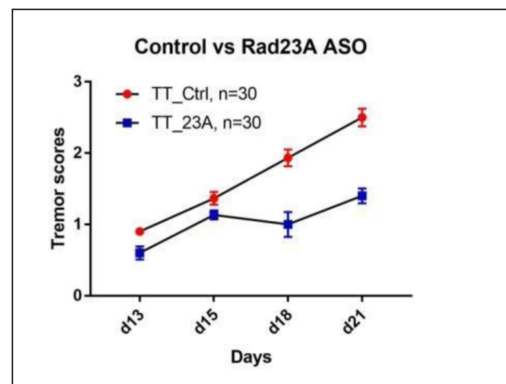
What was accomplished under these goals

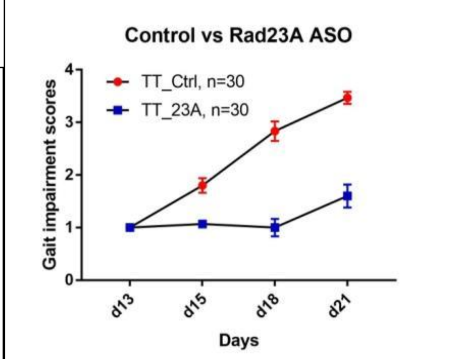
To reduce the abundance of RAD23A, two different methods were employed. First, we injected antisense oligonucleotides (ASOs) that target the rad23a or rad23b mRNA intracerebroventricularly (ICV) at postnatal day zero (P0). We established that a single ICV injection of 50-60 µg of ASO targeting *rad23a* led to a 84% reduction of the target when assayed by western blot at postnatal day 14. There was no reproducible effect on the abundance of *rad23b*. The ASO mediated KD of *rad23a* led to the following effects in the TAR4/TAR4 mouse:

1. In a double blinded manner, we determined that male TAR4/TAR4 mice with rad23a KD lived 10 number days longer than mice that received a scramble sequence ASO. Female TAR4/TAR4 mice with rad23a KD lived 7 number days longer than mice that received a scramble sequence ASO. We repeated this analysis with a second independent cohort of mice and found again that ASO targeting *rad23a*, but not a scrambled sequence ASO, led to a 7-10 day longer life span. We undertook two different motor function behavior tasks – tremor score and gait impairment score. TAR4/TAR4 with *rad23a* KD fared better in these assays in comparison with TAR4/TAR4 animals who received the scramble sequence ASO by ICV. I conclude that reducing the abundance of rad23a extends life and slows decay in motor function of the TAR4/TAR4 mouse.



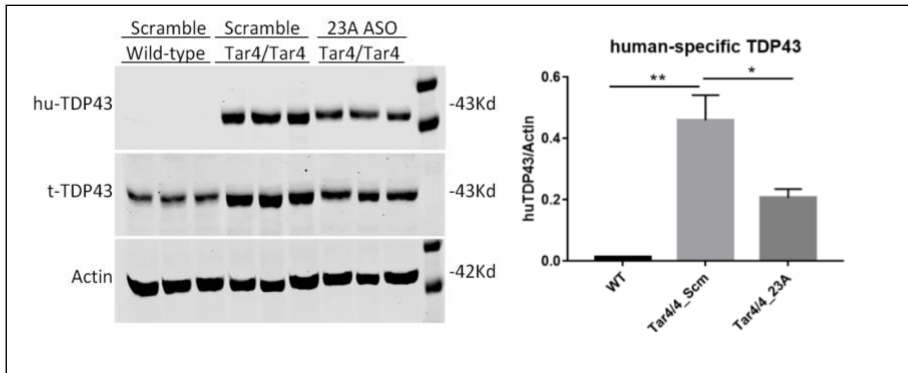
Rad23A targeting ASOs extend lifespan of Tar4/4 mice. Rad23A ASOs (n=51) or scramble controls (n=36) were intracerebroventricular (ICV) injected to Tar4/4 mice at P0. Mice were sacrificed at a gait impairment score of 4.0 in which the animals fell over and were unable to right themselves within 30 sec on all 3 of 3 trials. Extended lifespan was observed after Rad23A ASOs administration compared to scramble-treated littermate controls (from 22.4 to 31 days). This improvement applied to both males (n=40) and females (n=47).



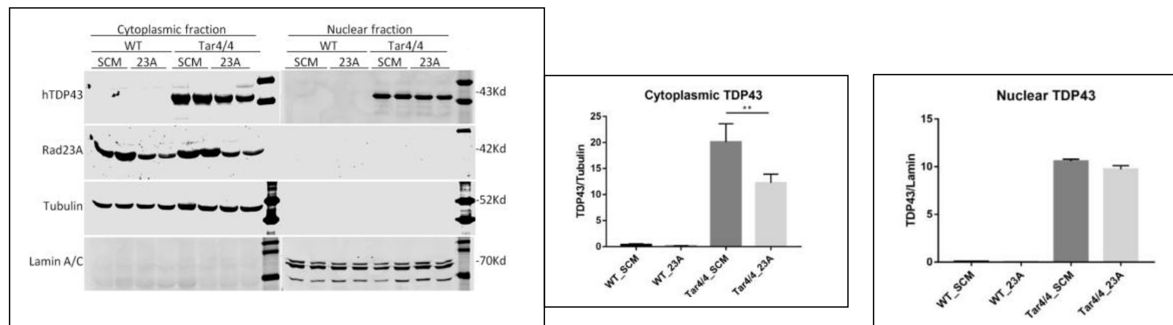


Effects of Rad23A ASOs treatment improved locamotive behaviors in Tar4/4 mice. Phenotype scoring was performed in Rad23A ASO or mock treated animals blindly. **(A)** The gait impairment score progressed fast with age increasing in Tar4/4 mice. This was attenuated by administration of Rad23A ASOs. **(B)** Similarly, tremor score increased in control-treated ALS mice, which was also decreased by Rad23A ASOs.

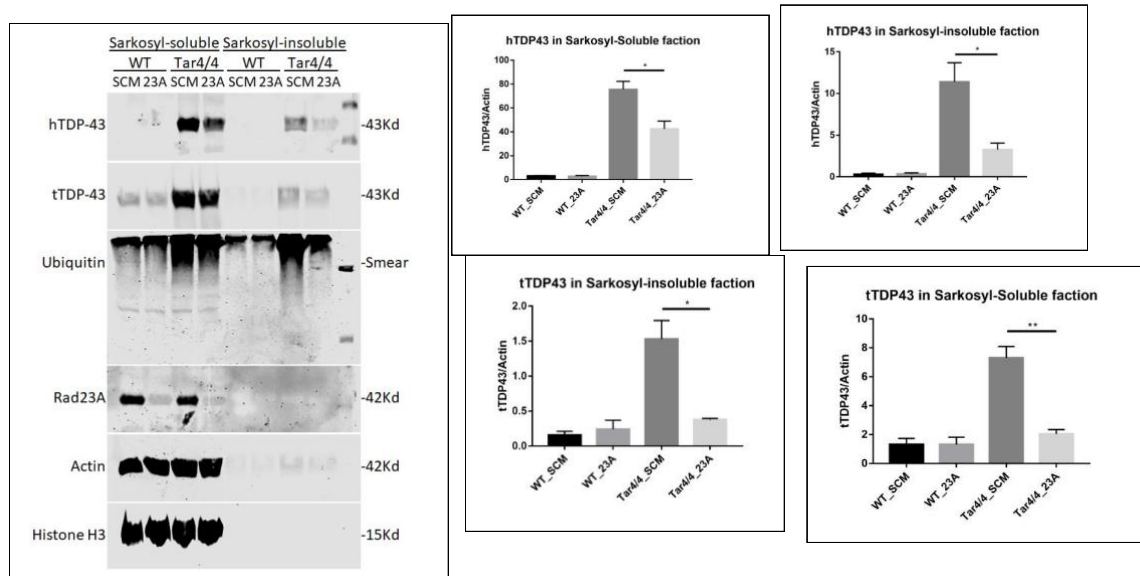
2. Biochemical interrogations reveal that knockdown of *rad23a* (in comparison with scramble sequence ASO) in the TAR4/TAR4 animal leads to a reduction in the total abundance of TDP43, a reduction of the sarcosyl insoluble fraction of TDFP43, a reduction of cytoplasmic but not nuclear TDP43 abundance and a reduction in the intensity of the smear of total ubiquitinated proteins. There is no difference in the mRNA level of human TDP43 in the TAR4/TAR4 animals receiving the ASO to *rad23a* versus scramble sequence. I conclude that many of the biochemical abnormalities associated with expression of WT human TDP43 and believed to be pathophysiological drivers in the TAR4/TAR4 mouse are blunted by reduction in the abundance of *rad23a.*



Rad23A ASOs treatment influenced TDP43 at protein level but had no effects at mRNA levels in Tar4/4 mice. Mice were administrated at P0 with Rad23 A ASOs or scramble controls. Cortex and spinal cord tissues were harvested at P21 for qRT-PCR. (**A, B**) Significant amount of TDP43 protein was detected in Tar4/4 mice as compared to WT controls, which was downregulated by administration of Rad23A ASOs. (**C**) TDP43 mRNA was elevated in Tar4/4 mice as compared to WT controls, which was maintained regardless of Rad23 ASOs treatment in both cortex and spinal cord tissues. *: $p<0.05$; **: $p<0.01$.

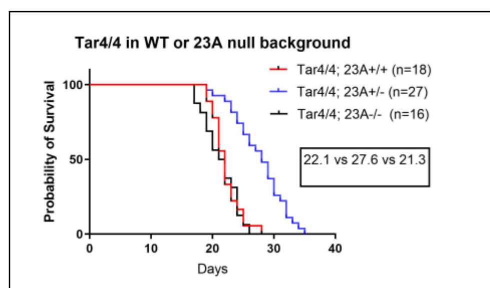


Effects of Rad23A ASOs on TDP43 cellular distributions. Mice were administrated at P0 with Rad23A ASOs or scramble controls. Cortex tissues were harvested at P21. Cytoplasmic and nuclear fractions were separated for western blot. TDP43 protein was heavily detected in both cytoplasmic and nuclear fractions. Administration of Rad23A ASOs significantly reduced amount of TDP43 in cytoplasm by 38.6% but remained unchanged in nucleus. *: $p<0.05$; **: $p<0.01$.



Effects of Rad23A ASOs on TDP43 solubility to 2% Sarkosyl. Mice were administrated at P0 with Rad23A ASOs or scramble controls. Cortex tissues were harvested at P21. Sarkosyl-soluble and -insoluble fractions were separated for western blot. TDP43 protein was heavily detected in Tar4/4 mice in both Sarkosyl supernatant and pellet, which was significantly reduced upon administration of Rad23A ASOs. In addition, elevated Ubiquitin in Tar4/4 mice was also reduced after Rad23A ASOs treatment

3. We used CRISPR Cas9 to generate mice with a null allele of *rad23a*. Homozygous null animals are viable, fertile and have no obvious phenotype as reported 20 years ago the original KO mice (no longer available). By western blot, no RAD23A protein is found in the *rad23a*$^{-/-}$ mice and an approximately 50% reduction in the abundance of RAD23A protein is found in the *rad23a*$^{-/+}$ mice. We generated mice with the following genotypes TAR4/TAR4;*rad23a*$^{-/-}$ or TAR4/TAR4;*rad23a*$^{-/+}$ and compared them to TAR4/TAR4;*rad3a*$^{+/+}$. Interestingly complete ablation of rad23a does not confer a survival advantage to the TAR4/TAR4 mice while haploinsufficiency with an approximately 50% reduction of RAD23A does extend life.

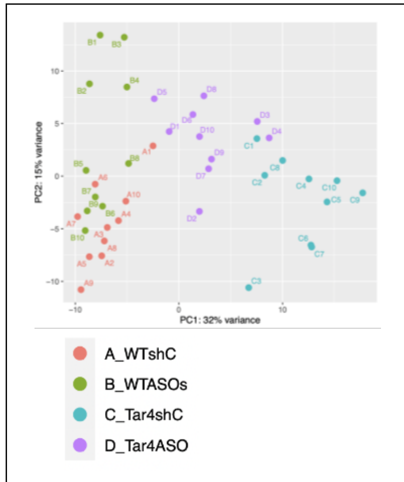


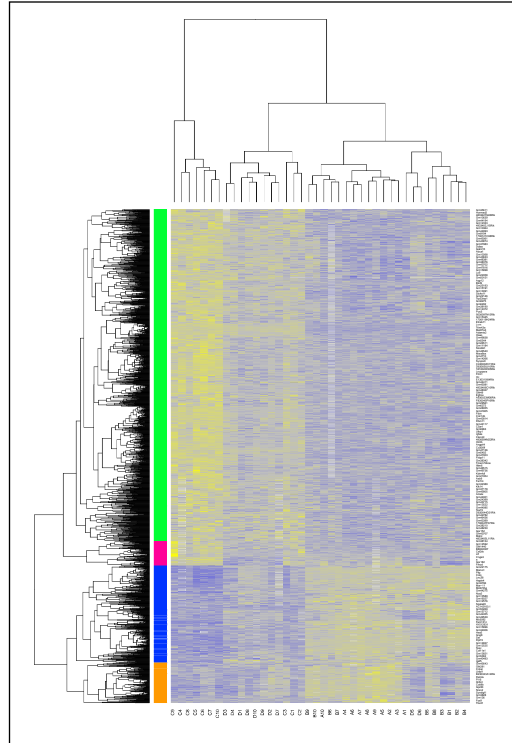
Genetic partial reduction of rad23a promotes survival of TAR4/TAR4 mouse. Lifespan of the three genotypes is displayed and while complete loss of rad23a has no effect on lifespan of the TAR4/TAR4 mouse (red and black lines), haplo-insufficiency adds 5 days to lifespan (blue line). Number of animals per group in parentheses.

4. We undertook RNAseq analysis of the P21 motor cortex from 4 experimental groups:
   a. TAR4/TAR4; scramble sequence ASO administered ICV
   b. TAR4/TAR4; rad23a targeting ASO administered ICV
   c. WT mice; scramble sequence ASO administered ICV

d. WT mice; rad23a targeting ASO administered ICV

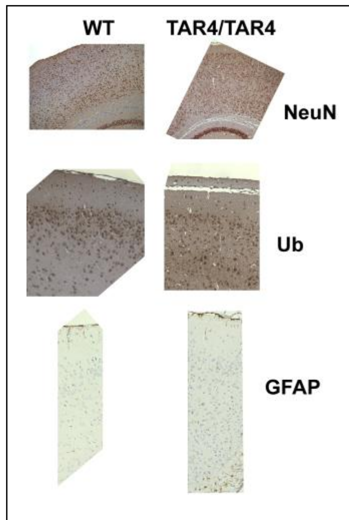
In this experiment the motor cortex from half the brain was used for western blot and the motor cortex from the other half of the brain was used for isolation of RNA. We only used samples that showed rad23A KD when the rad23a targeting ASO administered ICV and only used that showed no rad23A KD when the scrambled sequence ASO administered ICV. This work was undertaken in collaboration with the laboratory of Jemeen Sreedharan (King College London). A principal component analysis reveals that WE find X number of differentially expressed genes that distinguish mice (a) from mice (b).





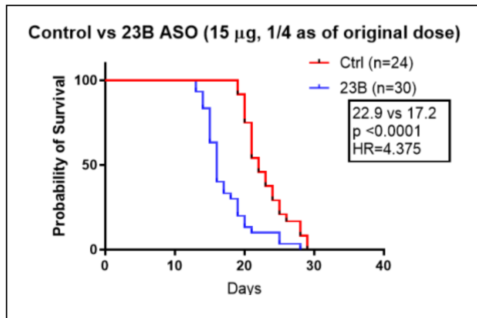
In Tar4/Tar4 mice, knocking down Rad23a shifts the transcriptome towards the Wild Type. We analyzed gene expression patterns using two unsupervised statistical methods, PCA and Hierarchical clustering. The study involved forty samples that represented four different conditions. These conditions were: 1) Wild Type treated with scramble shC (A); 2) Wild Type treated with ASOs to knockdown Rad23a (B); 3) Tar4/Tar4 treated with scramble shC (C); and 4) Tar4/Tar4 treated with ASOs to knockdown Rad23a (D). The analyses show that Tar4/Tar4 mice have transcriptomes that are different from the other three groups. However, when the Rad23a gene is silenced in Tar4/Tar4 mice, there is a shift in the transcriptome that makes it more similar to the WT group. A) PCA analysis includes all the genes expressed in the forty samples, and the Dseq2 Package was used to perform PCA. B) Hierarchical clustering analysis shows two major clusters: a cluster of Tar4/Tar4 and another cluster of the other three groups. Hierarchical clustering analysis was carried out on 1893 differentially expressed genes between Tar4/Tar4(C) and WT(A); and Tar4/Tar4 (D) and Tar4/Tar4(C). The Hierarchical clustering was performed using the R-Cran package.

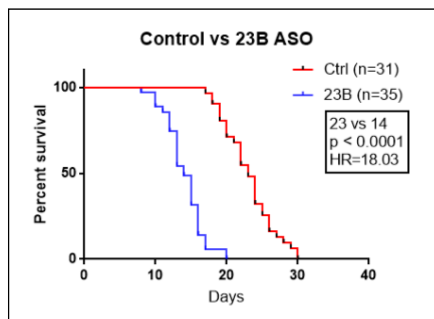
5. Immunohistochemistry (IHC) for NeuN, human TDP43, ubiquitin, p62, GFAP and Iba1 was performed on WT and TAR4/TAR4 animals. Preliminary experiments demonstrate the feasibility to perform antigen retrieval on paraffin embedded 5 um sections and obtain reason able staining



Immunocytochemical staining of WT and TAR4/TAR4 cortex with anti-NeuN, ubiquitin and GFAP

To reduce the abundance of *rad23b* we undertook ICV injection of 50-60 ug of ASO targeting *rad23b*. When administered to the TAR4/TAR4 animals, there was a significant acceleration of disease with animals dying on average 9 days earlier than TAR4/TAR4 animals receiving the scramble control ASO. We considered the possibility that the ASO to *rad23b* was toxic and so looked at the effects of less ASO. We found that even 15 ug of ASO to *rad23b* led to a detectable decrement in the abundance of the RAD23B protein but this till accelerated the disease in the TAR4/TAR4 animals (average 5 days earlier than control). I conclude that in contrast with reduction of *rad23a*, reduction in rad23b adversely affects the pathophysiology of the TAR4/TAR4 mouse. The precise differences in cellular biochemistry evoked by loss of *rad23a* versus *rad23b* are currently under investigation.





Rad23B targeting ASOs does not extend lifespan of Tar4/4 mice. Rad23B ASOs (n=30) or scramble controls (n=24) were intracerebroventricular (ICV) injected to Tar4/4 mice at P0. Mice were sacrificed at a gait impairment score of 4.0 in which the animals fell over and were unable to right themselves within 30 sec on all 3 of 3 trials. Reduced lifespan was observed after Rad23B ASOs administration compared to scramble-treated littermate controls (from 22.9 to 17.2 days).
Right: Standard dose (50-60 ug of ASO); 23 days vs 14 days
Left: low dose (15 ug ASO) ; 22.9 days vs 17.2 days

What opportunities for training and profession development has the project provided

Not part of this proposal, although the postdoctoral fellow on the project has mastered all the skills required to acquire the above data.

How were the results disseminated to the communities of interest

We are nearly finished with the data acquisition and then a manuscript will be assembled and submitted

What do you plan to do during the next reporting period

This is the terminal report for this grant however there are a number of experiments and analyses that need to be performed to complete this project.

1. Perform western blots of TAR4/TAR4 animals with and without rad23a knock down or ablation for p62, activated caspase, Iba1, GFAP and potentially other reporters of neuronal function and health
2. The RNA seq analysis is inchoate and needs more attention. The RNAseq data can be aligned with other databases (such as Cmap and Clue) to find pathways of particular interest that can be interrogated in the future. In addition, an analysis of splicing changes in the TAR4/TAR4 mice with and without rad23a knock down or ablation needs to be done
3. The IHC is very preliminary. In addition to mastering all of the antigens we want to follow, we need to develop quantitative methods for group comparisons.
4. No mechanistic studies were proposed as part of this grant. However we have been pursuing mechanism in several different ways.

Impact

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

The vast majority of individuals with sporadic ALS have mislocalized and insoluble TDP43. Abundant work implicates the mislocalized and insoluble TDP43 is a key pathophysiology driver. We have shown that reducing the abundance of *rad23a* lessens the toxicity of human TDP43 in a mouse model of disease. Together these observations suggest that targeting *rad23a* in humans with sporadic ALS could be a powerful new therapeutic target.

What was the impact on other disciplines?

TDP43 pathology is seen in Frontotemporal dementia and approximately half of individuals with Alzheimer's disease. Thus targeting *rad23a* may have applicability to numerous neurodegenerative diseases

What was the impact on technology transfer?

IONIS Pharmaceuticals made ASO in this project and I believe they have ownership of the intellectual property.

What was the impact on society beyond science and technology?

None

CHANGES/PROBLEMS:

Changes in approach and reasons for change

None

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

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS

Publications, conference papers, and presentations

We have a relatively small amount of further work to do before submitting a manuscript on the effects of rad23 on TDP43 pathology in mice.

Journal publications.

In process

Books or other non-periodical, one-time publications

None

Other publications, conference papers, and presentations.

None

Website(s) or other Internet site(s)

None

Technologies or techniques

All of the primary RNAseq data will be placed in a public repository when the manuscript is accepted for publication

Inventions, patent applications, and/or licenses

None

Other Products

None

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Guo Xue shui*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Xueshui performed all the studies in this body of work*
Funding Support:	*This award*

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

No

What other organizations were involved as partners?

No

- ○

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

None

QUAD CHARTS

N/A

APPENDICES

None