ADAR-Mediated RNA Editing of Premature Termination Codon Results in Functional Correction in MECP2 for Rett Syndrome

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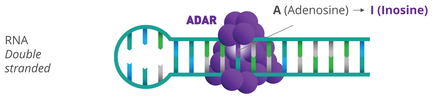
Introduction

Axiomer™ Editing oligonucleotides (EONs) as a potential therapeutic approach

ProQR researchers have developed a therapeutic RNA platform called Axiomer using editing oligonucleotides, or "EONs", learning from ADAR's natural process. EONs are designed to bind a single stranded RNA with high specificity. (**Fig. 1**) This forms a double stranded structure which recruits endogenously expressed ADARs to change an Adenosine (A) to an Inosine (I) in the RNA with high specificity. The Inosine (I) is then translated as a Guanosine (G) – with the potential to correct an RNA with a disease-causing mutation back to a normal (wild type) RNA, or to modulate proteins, so that it will have a new function or wild type like function that helps prevent or treat a disease.

Natural ADAR editing *(A-to-I)*



Editing Oligonucleotide (EON)-directed therapeutic editing *(A-to-I)*

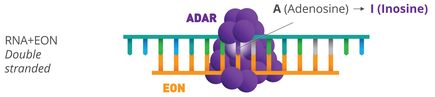


Figure 1. *On the top,* ADAR enzymes naturally act on double stranded RNAs and perform a specific A-to-I editing, changing Adenosine (A) to Inosine (I). *On the bottom,* by learning from this natural process, ProQR has developed editing oligonucleotides (EONs) which consist of short single stranded RNA molecules that are complementary to a target RNA. The target RNA is also a single strand. By binding to it, editing oligonucleotides create a double stranded structure which will attract ADARs and allow the specific A-to-I edit to be performed.

The premature termination codon p.R270X, a commonly occurring pathogenic variant in Rett Syndrome

A potential therapeutic application for the Axiomer platform is the restoration of wild-type like function of MECP2 in Rett Syndrome using AX-2402 program. Rett Syndrome is a severe neurodevelopmental disorder affecting approximately 350,000 people worldwide. It mainly occurs in females and is caused by mutations on the methyl CpG binding protein 2 (*MECP2*) gene on the X-chromosome. This gene codes for the MECP2 protein which is a key regulator of gene expression and is vital for maintaining normal brain function and development. The severity of the disease is determined by the location, mutation type and level of X-chromosome inactivation.

Introduction (continued)

The premature termination codon gives rise to p.R270X which is one of the most commonly occurring pathogenic variants of *MECP2*. Overexpression of MECP2 can result in MECP2 duplication syndrome, a condition linked to toxicity and significant health challenges. (**Fig. 2**) In 2007, Adrian Bird's lab demonstrated that Rett syndrome symptoms are reversible in mice when MECP2 protein are restored to physiological levels[1].

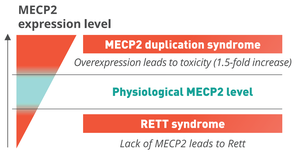


Figure 2. Clinical phenotype according to MECP2 protein expression level, over-expression leading to MECP2 duplication syndrome and lack of MECP2 protein leading to Rett Syndrome. *Figure adapted from Guy J, et al. Science. 2007 Feb 23;315(5815):1143-7.*

Objectives

- **To investigate how Axiomer EONs perform *in vitro* in CNS models and translatability *in vivo***
- **To investigate how Axiomer technology can become a potential therapeutic approach for Rett Syndrome**
- **To assess Axiomer EON editing efficiency *in vitro* and capacity to restore wild-type like function of MECP2, the protein deficient in Rett syndrome.**

Results

Axiomer potential in the brain: translation from human organoids to animal models

To assess CNS-specific editing, Axiomer RNA editing oligonucleotides targeting APP mRNA were evaluated in human spheroids *in vitro* and *in vivo* in rat brain tissue. In the first experiment, human 3D neurospheroids of 20 days were treated. They contained 3 cell types - 90% of neurons with a 70:30 ratio of glutamatergic neurons: GABAergic neurons and 10% astrocytes. (**Fig. 3**) In this model, editing efficiencies reached over 40% in a dose-dependent manner. (**Fig. 4**) In rats *in vivo*, a single ICV dose resulted in broad CNS distribution and sustained editing of up to ~32% across multiple brain regions after two weeks. (**Fig. 5**)

These results confirm the potential of Axiomer editing as a robust and translatable modality, providing foundation for therapeutic development in neurological disorders such as Rett syndrome.

Results (continued)

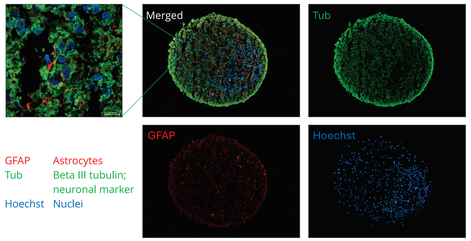


Figure 3. Images of 20 days 3D human neurospheroids containing 3 cell types; 90% neurons with a 70:30 ratio of glutamatergic neurons: GABAergic neurons and 10% astrocytes.

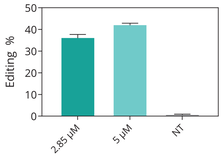


Figure 4. RNA editing of *APP* in human spheroid *in vitro* following treatment with EONs targeting *APP* mRNA. 3 groups of 16 spheroids were treated with 2.8 and 5 µM *APP* EON, gymnotic uptake, 2 weeks treatment, assessment at D21. Data represented are mean ± SEM, and analyzed via ddPCR

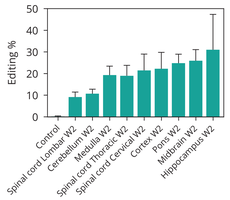
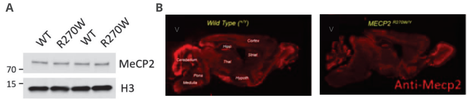


Figure 5. RNA editing of *APP* in the CNS of rats following treatment with EONs targeting *APP* mRNA. Five rats were treated via ICV, 500µg *APP* EON, single dose, assessment at 2 weeks. Data represented are mean ± SD, and analyzed via ddPCR

Axiomer Editing oligonucleotides (EONs) have the potential to restore physiological levels of functional MECP2

In 2023, a transgenic mice model has demonstrated that correcting the mutant to a Tryptophan (p.R270W) Mecp2 protein variant resulted in mice being indistinguishable from healthy wild-type counterparts.[2] (**Fig. 6**)



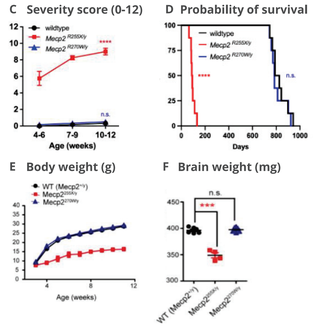


Figure 6. Mecp2 protein expression in mice brain via (**A**) Western blot (**B**) immunostaining and comparison of the WT, *Mecp2* R255X and R270W phenotypes on (**C**) Severity score (0-12) (**D**) Probability of survival (%) (**E**) Body weight (grams) and (**F**) brain weight (milligrams). *Figure adapted from Colvin, S. (2023) thesis. Massachusetts Institute of Technology.*

Axiomer ADAR-mediated RNA editing technology can change the p.X270 stop codon (UGA) to Tryptophan (UGG). This A-to-I>G change allows restoration of physiological level and wild type like function of MECP2 protein. (**Fig. 7**)

Figure 7. (**A**) Rett syndrome caused by nonsense variant in *MEPC2* leads to truncated Exon 4. (**B**) Axiomer technology potential to restore WT like phenotype and exon read through.

EON mediated editing in patient's cells restores protein expression

To assess Axiomer potential in Rett syndrome, patient-derived fibroblasts carrying the *MECP2* R270X mutation were transfected with 100 nM Axiomer EON A for 48 hours (non-treated cells served as controls). EON A treatment achieved up to 80 % editing of the R270X *MECP2*. (**Fig. 8A**)

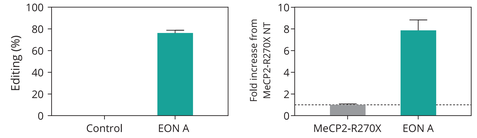


Figure 8. (**A**) RNA editing of the R270X MECP2 following EON A treatment in MECP2 patient fibroblasts (Transfection, 100 nM, N=2, 48h. Data represented are mean ± SEM, and analyzed via dPCR) (**B**) MECP2 protein reporter activity following treatment reported in Fig. 8A. Data represented are mean ± SEM.

Furthermore, editing with Axiomer EON led to restoration of MECP2 protein expression (up to 8-fold) probably due to absent premature termination codon (PTC) induced NMD and increase in *MECP2* mRNA level. (**Fig. 8B**) This dual action - recoding the PTC and stabilizing the mRNA - ensures that the corrected RNA is not only functional but also abundant.

Efficient and Isoform-Independent RNA Editing of *MECP2* Transcripts

Using a dual luciferase reporter system in HEK293 cells, we demonstrate that Axiomer EONs achieve robust and dose-dependent restoration of MECP2 protein levels in the context of the pathogenic R270X mutation. At 100 nM, both EON A and EON B yielded >90% luciferase activity relative to positive control, with comparable EON efficiency across ADAR1 and ADAR2 isoforms.

These findings underscore the versatility and potency of Axiomer RNA editing technology, enabling flexible therapeutic design regardless of ADAR isoform expression in target tissues.

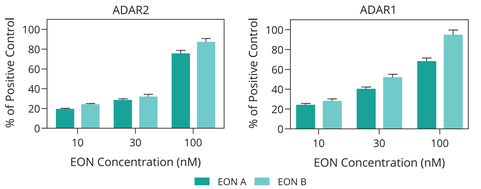


Figure 9. (**A**) MECP2 NanoLuc reporter activity - ADAR2. (**B**) MECP2 NanoLuc reporter activity - ADAR1 in HEK293 cells (Plasmid reverse transfection 5h, 100ng/ml, turbofect, EON forward TF, 10, 30 and 100nM, 72h, RNAiMax, N=4)

Conclusion

- **Axiomer RNA editing demonstrated robust editing efficiency in CNS models both *in vitro* and *in vivo*.**
- **Axiomer EONs have the potential to restore wild-type like function of MECP2 in Rett syndrome by converting a nonsense codon in *MECP2* into a tryptophan (UGG) leading to a protein carrying similar activity as wild-type protein.**
- **Findings in Rett patient-derived fibroblasts - with high editing activity and restoration of MECP2 protein activity - support further development of Axiomer AX-2402 program as a promising therapeutic approach to address the unmet medical needs in Rett syndrome.**

Literature: 1. Guy J, et al. Science. 2007 Feb 23;315(5815):1143-7; 2. Colvin, S. (2023) thesis. Massachusetts Institute of Technology.