UNIVERSITY OF CALIFORNIA SAN DIEGO

Antisense Oligonucleotide Treatment for Focal Malformations of Cortical Development

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Swapnil Mittal is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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LIST OF ABBREVIATIONS

ASO	Antisense oligonucleotides
CSF	Cerebrospinal fluid
DNM	*De-novo* mutations
FMCD	Focal malformations of cortical development
FCD	Focal cortical dysplasia
GFP	Green fluorescent protein
HME	Hemimegaloencephaly
IUE	*In-utero* electroporation
MCD	Malformations of cortical development
PBS	Phosphate buffered saline
RFP	Red florescent protein
SNP	Single nucleotide Polymorphisms
TSC	Tuberous sclerosis complex

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PUBLICATIONS

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Joseph Gerard Gleeson, Chair
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Focal Malformations of Cortical Development (FMCD) are neurological developmental disorders that are a major cause of drug-resistant pediatric epilepsy. Along with seizures, patients display disrupted cortical architecture and neuronal organization. Current treatment involves invasive surgical resection of the epileptic focus, which may only show partial benefit. Recent studies have identified several categories of post-zygotic somatic mutations that cause FMCD, however this effort has not yet been utilized to generate therapies. Antisense oligonucleotides (ASOs) are safe, stable and programmable molecules that can be

designed to target specific mutations such as those that cause FMCD. To demonstrate the therapeutic potential of ASOs against FMCD, we introduced AKT3^{E17K}, an FMCD causing variant in mice brain and subsequently treated them with ASOs designed to silence the toxic gene. The ASO treatment was able to rescue cortical architecture. Later studies may explore the dependency of treatment on the stage of the disease, the dose of the drug, and the type of FMCD causing mutations. Successful development of ASO therapy can provide safe, effective and non-invasive treatment for FMCD.

INTRODUCTION

FMCDs are neurodevelopmental disorders caused by post-zygotic somatic mutations leading to malformation of cortical structures and often presenting with intractable epilepsy. In extreme cases, FMCD can result in hemimegaloencephaly (HME) that is a debilitating disease characterized by presence of a completely or partially enlarged brain hemisphere. Its milder form can present as focal cortical dysplasia (FCD) that is characterized by the presence of small epileptic foci. The affected brain tissue often displays abnormal neuronal localization, dyslamination and morphology (Palmini et al. 2004). About to 50% of treatment resistant childhood epilepsies are FMCD related, these patients often suffer from drug resistant intractable epilepsy, cognitive-behavioral defects and psychomotor disability from early childhood (Oegema et al. 2020). Most common treatment of FMCD is surgical resection of the lesion to treat epilepsy, however less than half of cases result in total seizure freedom (Martinez-Lizana et al. 2018). Therefore, the discovery and research of non-invasive treatment options is necessary to provide patients with better outcomes.

The search for such treatments has prompted extensive research into characterizing the FMCD causing mutations. Recent studies have found mutations in genes associated with the mTOR pathway, such as *TSC1, TSC2* and, *AKT3*, as the main drivers of FMCD. To this affect, mTOR inhibitors like rapamycin and everolimus have been explored as potential treatment. In fact, rapamycin treatment has been shown to be effective in *DEPDC5, PTEN* and *AKT3* related FMCD models in mice (Ljungberg et al. 2009; Yuskaitis et al. 2019; Baek et al. 2015). Several clinical trials for everolimus are being conducted as a treatment for FMCDs. However rapamycin and everolimus have limited potential as treatment, since they reduce cell proliferation at high doses that causes severe side effects such as reduced platelet count, anemia and leukopenia

(Blagosklonny 2019). Further, mTOR inhibitors can only treat mTOR related FMCDs, which excludes additional pathways such as synapsis and calcium signally which have recently been implicated in FMCDs (Chung et al. 2022).

ASO therapies offer solutions to many of the limitations of mTOR inhibitors. ASOs are a rapidly emerging class of drugs that are capable of modulating the transcriptome to change the expression profile of cells. In a manner similar to RNAi, ASOs can bind to pre-mRNA in the nucleus and target it for RNAseH mediated decay (Liang et al. 2017). Since many FMCDs are caused by gain-of-function variants, ASOs against specific variants can be designed to knock down toxic gene products in an allele specific manner. This allows ASOs to targets the cause of the disease without impacting healthy functionality of cells and thus avoid the side effects which may be caused by global inhibition of, say the mTOR pathway. ASOs can also be formulated against any gene, therefore they may be used to treat non-mTOR related FCDs aswell.

To investigate the potential of ASOs to treat FMCD, we modeled $AKT3^{E17K}$ related FCD in mice, using in-utero electroporation. The electroporated DNA construct contains the binding site for the targeting ASO upstream of $AKT3^{E17K}$ gene. In the future we aim to use ASOs designed to directly target $AKT3^{E17K}$, however these molecules are under production at this time.

RESULTS

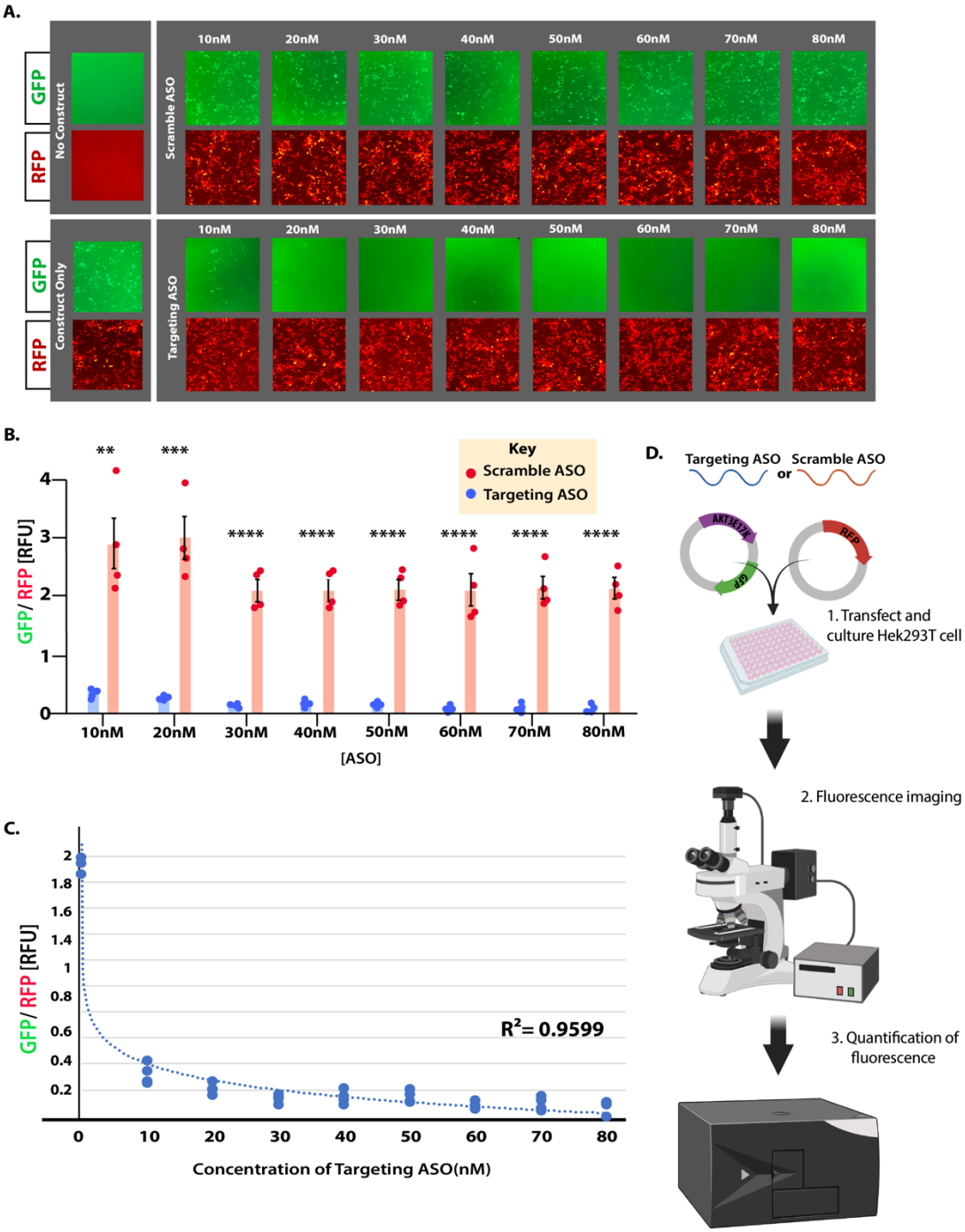

Figure 1: Targeting ASO selectively knocks down expression in Hek293Tcells.(A) Florescence microscopy images of Hek293T cells transiently transfected with pCAG-MALAT-AKT3^{E17K}-IRES-GFP and pCAG-RFP, with targeting or scrambled ASO added to the media at final concentration of 10nM to 80nM. (B) Relative florescence (RFU) of GFP (488nm) over RFP (594nm) of Hek293Tcells after addition of either scramble ASO (red) or targeting ASO (blue). (C) RFU of GFP over RFP as a function of targeting ASO concentration showing a logarithmic trend (R2=0.9599). (D) Schematic showing of the experimental design, following transfection and ASO administration in Hek293Tcells, florescence imaging and lastly quantification.

To validate the ability of ASOs to knock down transiently transfected gene, Hek293T cells were transfected with the following constructs pCAG-MALAT1AS-AKT3^{E17K} and pCAG-RFP (see methods) followed by administration of either targeting or scramble ASOs directly into the media (Fig 1D). 48hrs post transfection florescence level RFP florescence levels were not significantly different between the cells either the scramble ASO treated or targeting ASO treated cells (Fig 1A). Addition of targeting ASO lead to a 96% decrease GFP expression, while there was no significant reduction in GFP florescence observed with the addition of scrambled ASO (pValue = 1.3505E-05) (Fig1B). We also observed a logarithmic dose dependent reduction of expression with increasing ASO concentration. Where 10nM Targeting ASO demonstrated 88.7% reduction in florescence compared to scramble and 80nM Targeting ASO demonstrated 97% reduction compared to scrambled (pValue = 0.0017) (Fig1C). Thus the targeting ASOs was able to selectively knock down pCAG-MALAT1AS-AKT3^{E17K}-GFP and not pCAG-RFP, while the scrambled ASO had no significant effect.

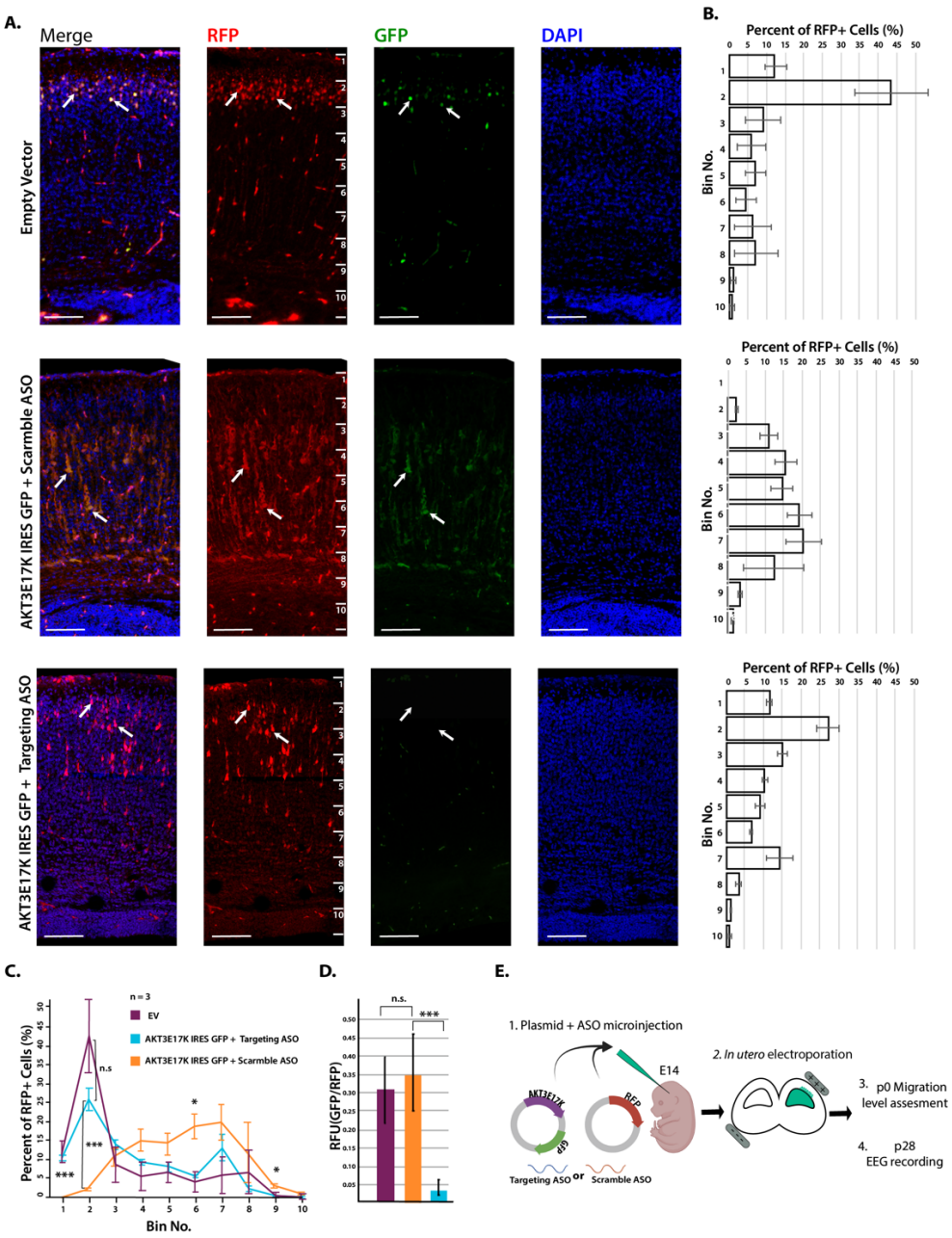

Figure 2: Targeting ASO rescues neuronal migration defect.(A) Florescence imaging of 20µm cryosections of p0 mice, stained with antiGFP and antiRFP antibodies with DAPI nuclear stain. Scale bar is 100µm. White arrows point towards electroporated neurons (B) Percent of RFP positive cells present in each of the 10 equal width bins between the ventricle and cortical surface. (C) % RFP cells in each bin of empty vector (magenta), pCAG-MALAT1AS-AKT3^{E17K}-IRES-GFP + scramble ASO (blue) pCAG-MALAT1AS-AKT3^{E17K}-IRES-GFP + targeting ASO (Orange). * shows two tailed t-test between p-values between scrambled ASO and targeting ASO treated brains. (D) Relative florescence (488nm/594nm) of electroporated cells in either empty vector (magenta), pCAG-MALAT1AS-AKT3^{E17K}-IRES-GFP + scramble ASO (blue) and pCAG-MALAT1AS-AKT3^{E17K}-IRES-GFP + targeting ASO (Orange). p-values = 2.29912E-28. (E) Schematic of the experimental design. Mice were electroporated at E14 with either pCIG (empty vector) or pCAG-MALAT1AS-AKT3^{E17K}-IRES-GFP (AKT3^{E17K}-IRES-GFP) followed by injection of targeting or scrambled ASO. Neuronal Migration assessment was performed at p0.

Next, we investigated whether ASOs targeting toxic-mutation can rescue migration defect. To achieve this, we electroporated pCAG-MALAT1AS-AKT3^{E17K}-EGFP or pCIG (empty vector) along with pCAG-RFP in dorsal subventricular zone at mouse embryonic day 14 (E14). This was followed by injection of either scrambled or targeting ASO. We then harvested tissue at p0 to asses neuronal migration (Fig2C). We found that EGFP-positive cells AKT3^{E17K} with the scramble ASO failed to migrate to the cortical surface, a phenotype previously observed to be associated with AKT3^{E17K} in mice (Baek et al. 2015). However, no EGFP-positive cells were found in the case of AKT3^{E17K} + Targeting ASO (Fig2A, D). To quantify neuronal migration defects, we divided the cortex into 10 equal width bins between the cortical surface and the ventricle and then counted the RFP positive cells in each bin. Most RFP positive cells in the AKT3^{E17K} + scramble ASO were located in bin 6-7 compared to empty vector and AKT3^{E17K} + Targeting ASO, both of which had most of its cells in bin 2 (Fig2B, C).

METHODS

Antisense oligonucleotides

ASOs were designed and synthesized by Ionis Pharmaceuticals. Sequence of targeting ASO is as follows: 3'-eA*eG*eT*ACTATAGCATeC*eT*eG*-5'. The targeting and the scramble ASO are 3-10-3 cEt gapmers.

DNA Construct and cloning

The pCAG-IRES-GFP (pCIG) vector was digested using Xho1 and Xba1enzymes, stuffer was removed using gel electrophoreses. Backbone was extracted from the gel cleaned up using Zymo gel cleanup kit. $AKT3^{E17K}$ insert was PCR amplified using forward 5'-TAG GGA ATT CTC GCC ACC ATG AGC GAT GTT ACC-3' reverse 5'-GGG AGG GAG AGG GGC GGA TCC CGG GTT ATT CTC GTC CAC TTG C-3' to create $AKT3^{E17K}$ insert. The template was from previously used $AKT3^{E17K}$ cDNA. gBlock containing artificial intron sequence 5'-ATC ATT TTG GCA AAA ATT GCT CGA GAC ATT TGC TTC TGA CAC AAC TGT GTT CAC TAG CAA CCT CAA ACA GAC ACC GGC GCG CCG TTG GTA TCA AGG TTA CAA GAC AGG TTT AAG GAG ACC AAT AGA AAC TGG GCA TGT GGA GAC AG-3', and oligo with sequence: 5'-AAC TGG GCA TGT GGA GAC AGC AGA TGC TAT AGT ACT CAG ATG CTA TAG TAC TCA GAT GCT ATA GTA CTA GAA GAC TCT TGG GTT TCT G-3' and 5'-ATG GTG GCG AGA ATT CCC TAA GGG TGG GAA AAT AGA CCA ATA GGC AGA GAG AGT CAG TGC CTA TCA GAA ACC CAA GAG TCT TCT-3' were bought from IDT.com. The backbone, two oligoes, $AKT3^{E17K}$ insert and the gBlock were ligated together using Gibson assembly.

Tissue culture and transfection

Hek293T cells were plated at 0.11E6 cells per well in a 96-well tissue culture plate in 100µl of 10% FBS in Dulbecco's Modified Eagle Medium (DMEM). Cells were dissociated using falcon cell strainer. Bio-rad TC20 Automated Cell Counter was used to count cells before plating. At 70% confluence, Lipofectamine 3000 protocol was used to transfect in 0.2ng of pCAG-MALAT1-AKT3^{E17K}-IRES-GFP or pCIG and pCAG-RFP vector in each well. 3hrs post transfection, scramble or targeting ASOs were added to each well for a final concentration of 10nM to 70nM. 48hrs post transfection culture media was aspirated and gently replaced with 100µl of PBS while avoiding cell detachment immediately before quantification. Expression was quantified by live-cell fluorescence imaging using Tecan infinite M200 pro microplate reader.

Animals

Timed pregnant females (CD-1) were obtained by overnight breeding with CD1 males. The time of pregnancy was noted by presence of a vaginal plug at 9am and the pregnant dame was separated to a new cage, noon after mating was considered embryonic day (E) 0.5. Animals were obtained from Charles River Laboratories, Harlan Laboratories or Jackson Laboratory. The cages were housed in a humidity, temperature and day cycle controlled environment with weekly change of food and bedding according to the Animal care, maintenance and experimental procedures by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (IACUC) standards at the University of California San Diego (protocol S15113).

Immunohistochemistry and staining

Brains were dissected and drop fixed for embryonic or neonatal brain, or perfusion fixed for adult with 4% paraformaldehyde/PBS. After cryoprotection in 30% sucrose/PBS, brain sections were obtained with a sliding microtome at 20μm and stained using anti-GFP Aves GFP-1020, 1:500; anti-RFP Ab65856, 1:500 primary antibody and were used per standard protocol.

In-utero electroporation

In utero electroporation was performed using endotoxin-free plasmids (0.5–1 μg/μl) plus 0.05% Fast Green (Sigma). Pregnant dame was anesthetized using 5% isoflurane in O2 for 5min, then 1.5% isoflurane in O2 for the duration of the surgery. Hair was removed from the belly using hair removal cream and the skin was cleaned completely 70% ethanol and betadine. Sterile surgical tools were used to make a 0.5 inch incision along the midline out of which the uterus was gently pulled out of the incision. The uterus was kept moist and warm using prewarmed (37 C) sterile PBS. 1.25 μl of diluted plasmid were injected into right lateral ventricle of E14.5 embryos using pulled glass needle through the uterine wall. Electroporation was performed by placing the anode on the side of DNA injection and the cathode on the other side of the head to target cortical progenitors. Four pulses of 45 V for 50 ms with 500-ms intervals were used. An additional injection of 1μl of ASO (3-5μg/μl) in 0.05% Fast Green was given in the same ventricle. 5-8 embryos are injected before gently pushing the uterus inside the incision. The peritoneum and skin were separately sutured. Post operation care and analgesia was given according to Institutional Animal Care and Use Committee (IACUC) standards at the University of California San Diego (JoVE Science Education Database, 2022).

Statistical analysis and cell counting

Imaging analysis was performed using ImageJ tool and cell counting was done in a double blinded manner. Statistical analysis was done using Microsoft excel. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$

DISCUSSION

Here we demonstrate that ASO targeting the construct encoding toxic gene product of $AKT3^{E17K}$ was able to successfully knock down expression as reported by GFP florescence in both mammalian cell lines (Hek293T cells) and in mice cortex (Fig 1A, 2A, 2D). This treatment was also able to rescue migration defects caused by $AKT3^{E17K}$ in these mice as shown by quantification of neuronal migration (Fig 2A, B, C). ASOs can be designed against any gain of function mutation which causes FMCD and therefore have the potential to provide a much needed drug therapeutic for this disease. In the future, we aim to investigate the effect of the ASO treatment on electrographic seizures observed in mice as a result of $AKT3^{E17K}$.

The work has just begun with this investigation. The correction of cortical architecture was observed with a co-injection of ASOs and $AKT3^{E17K}$ at embryonic stage E14, which has low clinical relevance. Preliminary results suggest that post-natal injection is not able to rescue the migration defect after 7 days post injection (data not shown). This is consistent with previous attempts to treat $AKT3^{E17K}$ related FMCD in mice using mTOR inhibitor rapamycin (Baek et al. 2015). However, the electrographic phenotype may be still rescued after later treatment. Furthermore, we used ASOs which targeted the sequence upstream of the $AKT3^{E17K}$ and not the gene body itself. This was done to establish a control before attempting the use of variant specific ASOs which target the specific SNPs in $AKT3^{E17K}$ in order to demonstrate clinical applicability. These variant specific ASOs will be used to show knockdown in Hek29T3 cell lines and then used in the in-vivo model.

Along with the promise of personalized and effective treatment of FMCD, ASO therapies come with their own set of challenges. Disease causing variants for high allele fraction FMCDs

such as HME and TSC can easily be detected from the CSF. However variants causing FCD manifest with very low allele fraction which makes it challenging to detect them without invasive surgeries. In such cases where surgical resection is inevitable, ASOs can further help to reduce the occurrence of seizures after detection of toxic DNM from the resected tissue. The greatest challenge is to design and synthesize allele specific ASOs given a specific toxic DNM. Only about one in ten SNPs can be targeted by an allele specific ASO (Mittal, Tang, and Gleeson 2022). In the future, this could be circumvented by finding other SNPs phased with toxic DNM to increase the chances of finding allele specific options. Advancements in personalized drug therapies like ASOs, along with increasing accuracy and strength of sequencing technologies will may alleviate the burden of mutation on brain diseases.

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