iPSC: Basic Science and Pre-Clinical Studies

ZTTK-iPSC: A Framework For The Rapid Generation Of Patient-Specific Rare Disease Cell Models

Rachel Koenigsberg[1, 2] , Colleen Richards[1], Intisar Turner[1], Chad C. MacArthur[1], Terry J. Bichell[3], Erin E. Ahn[4], Uma Lakshmipathy[1]

[1]Pharma Services Group, Thermo Fisher Scientific, San Diego, CA, United States; [2]Biological Sciences, California Polytechnic State University, San Luis Obispo, CA, United States; [3]COMBINEDBrain, Brentwood, TN, United States; [4]Pathology, The University of Alabama at Birmingham Heersink School of Medicine, Birmingham, AL, United States

Introduction

Rare diseases have low prevalence and are often complex and genetic in origin, making them particularly difficult to diagnose, treat or cure. Zhu-Tokita-Takenouchi-Kim Syndrome (ZTTK) is one such rare disease caused by a de novo heterozygous mutation in the *SON* gene, which is essential for correct RNA splicing of genes critical for brain development, neuronal migration and metabolism. This mutation leads to haploinsufficiency and loss-of-function characterized by moderate to severe intellectual disability and developmental delays affecting multiple systems. Finding treatment for rare diseases is a complex and resource-intensive process and despite progress in patient advocacy, medical research and technology, therapeutic strategies are still in early stages due to limited scientific understanding and insufficient models. Induced pluripotent stem cell (iPSC) technology holds significant advantages as patient-specific, genetically accurate models of disease for the study and screening of potential drugs for rare diseases. In this study, we report the first successful iPSC generation of ZTTK cells from a 2-year-old with a 4 bp (TTAG) deletion in the *SON* gene resulting in a frameshift mutation leading to a premature stop codon and nonsense mediated mRNA decay. Additionally, normal iPSCs were precisely gene edited to introduce the 4 bp mutation to create syngeneic lines.

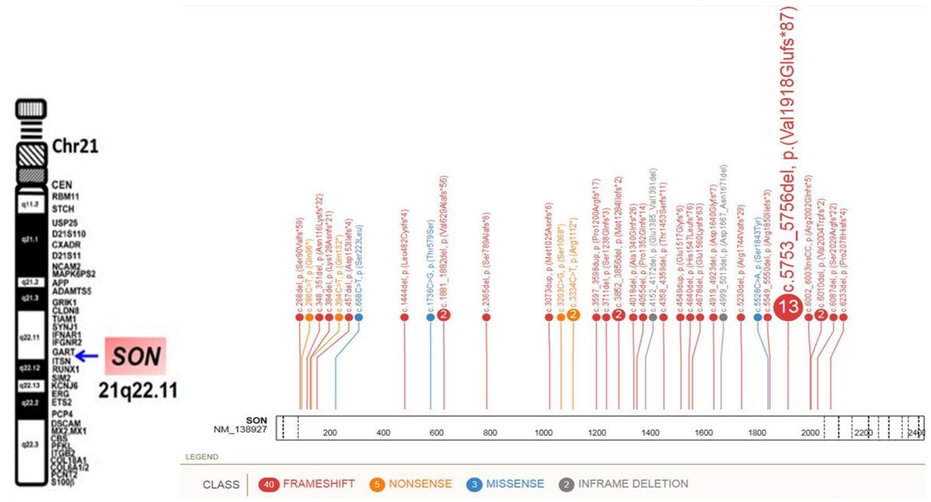

Figure 1. *SON* gene and variants. *SON* is located on 21q22.11 and encodes a ubiquitously expressed RNA and DNA binding protein . It functions as a splicing cofactor, promoting efficient splicing of transcripts with weak and alternative splice sites. All known variants but one are found in exon 3, attributable to the size of the exon (87% of the coding region). 25% of all known patients carry a 4bp (TTAG) deletion causing a frameshift: p.(Val1918Glufs*87).

Materials and Methods

IPSC Generation

Peripheral blood from an infant with ZTTK syndrome and from a healthy adult human donor was processed and used for somatic reprogramming using the CytoTune-iPS 2.0 Sendai Reprogramming Kit™. Since the *SON* gene is known to play a crucial role in regulating the pluripotency network, a contingency plan was built to conditionally express *SON* in the event that reprogramming failed to yield colonies. However, the latter approach was not necessary and iPSCs were successfully generated and banked.

Gene Editing

Three different approaches using Cas9, Cas12a, or TALENs were evaluated for optimal target recognition and high indel formation. iPSCs were transfected using the Neon™ Transfection System. Small molecules such as HDR enhancers and NHEJ inhibitors were assessed for increasing cell survival and HDR efficiency. Editing efficiency was determined using Synthego's ICE Analysis tool with input Sanger sequencing traces.

Single Cell Sorting

A streamlined process for clonal isolation and expansion of iPSC and edited cells was fully established. Manual dilution and the Nodexus NX One were compared for optimal growth. ROCK inhibitors were evaluated for increasing cell survivability upon singularization.

Characterization

Successfully generated ZTTK-iPSC were characterized to confirm pluripotency, normal karyotype and the presence of mutation. Edited WT-iPSC with the 4bp edit will undergo the same process once clonality is determined.

Method	Format	gRNA/TALEN 1	gRNA/TALEN 2	Type of Cut	Distance from Mutation (bp)
Cas9	single	Cas9 guide 1	-	blunt	5
	single	Cas9 guide 2	-	blunt	15
	dual	Cas9 guide 3UP	Cas9 guide 3DN	blunt	145/21
	dual	Cas9 guide 4UP	Cas9 guide 4DN	blunt	440/28
Cas12a	single	Cas12a guide 1	-	staggered	32
	dual	Cas12a guide 2UP	Cas12a guide 2DN	staggered	36/92
TALEN	paired	TALEN 1F	TALEN 1R	staggered	2
	paired	TALEN 2F	TALEN 2R	staggered	5

Table 1. Summary of gene editing designs. CRISPR methods were designed as either single cuts or dual-cuts (excisions) and chosen based on high gRNA computational scores. TALENs were designed as pairs flanking the mutation site using the Invitrogen TrueDesign™ Genome Editor.

Figure 2. Sanger sequencing traces distinguish mixed edited populations from non-edited control
Sanger sequencing traces aligned to reference from PCR amplicons generated by amplifying the region around the 4bp DEL. Non-edited (wild-type) iPSCs serve as negative control. Mixed peaks after the cut site indicate expected nuclease activity due to modification and are seen in the Cas12a sample. TALENs did not generate the expected mixed peaks after modification.

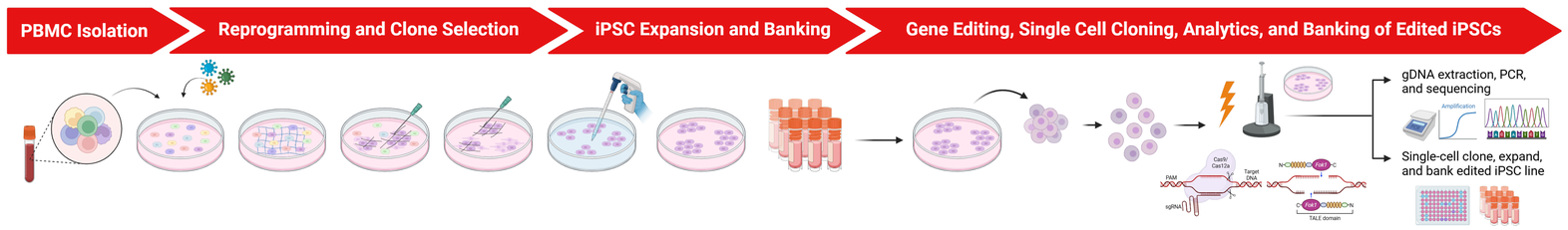

Figure 3. Project workflow from iPSC generation to clonal selection. PBMCs were isolated and reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit. After expansion and banking, healthy iPSCs were transfected, pooled, and editing efficiency was determined via ICE analysis. The pool with the highest editing efficiency was used for enrichment of the mutation via single cell cloning at 3 cells per well. Sub-pools were screened for the mutation, and positive sub-pools were sequenced. Single cell cloning of the sub-pool with the highest percentage of edited cells was completed, and clones were screened for the correct zygosity.

Results

Reprogramming of Patient Cells

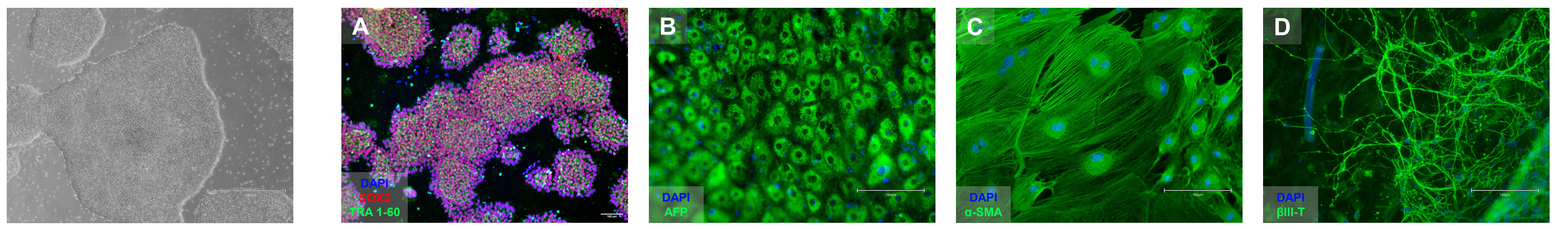

Figure 4. iPSC Morphology ZTTK iPSCs were observed 10+ passages after reprogramming, and proper morphology was confirmed via microscopy, at 4X magnification. Scale bar is 1560 µm.

Figure 5. Immunocytochemistry staining of ZTTK patient derived iPSCs showing pluripotency markers for iPSCs and differentiation markers for embryoid bodies generated. Representative images of iPSCs and subsequent embryoid bodies generated from ZTTK patient cells. Cells show expression of pluripotency markers in the iPSC stage (A), including SOX2 (red) and TRA-1-60 (green), with DAPI (blue) counterstaining nuclei. Expression of markers for all three germ layers was also observed after embryoid bodies were cultured under differentiation conditions for 21 days and stained for lineage specific markers (B-D), including alpha-fetoprotein (AFP, green), a definitive endoderm marker, alpha-smooth muscle actin (α-SMA, green), indicative of mesodermal commitment, and beta-III tubulin (βIII-T, green), an ectoderm marker. Similarly, nuclei are counterstained with DAPI (blue) to visualize tissue architecture. Scale bars are 100uM or 150uM.

Gene Editing Optimization

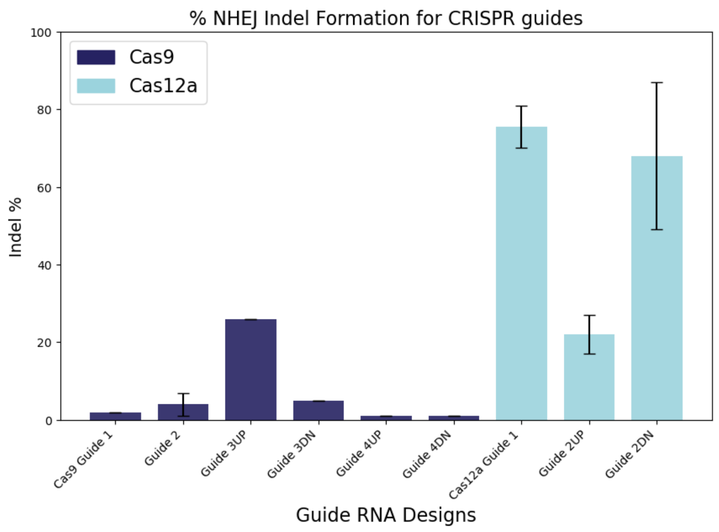

Figure 6. ICE analysis of transfections with no donor DNA template show Cas12a guide 1 as optimal approach. IPSCs were transfected with each RNP complex, without donor DNA, to determine the cutting efficiency of each guide. Indel % was determined via ICE analysis.

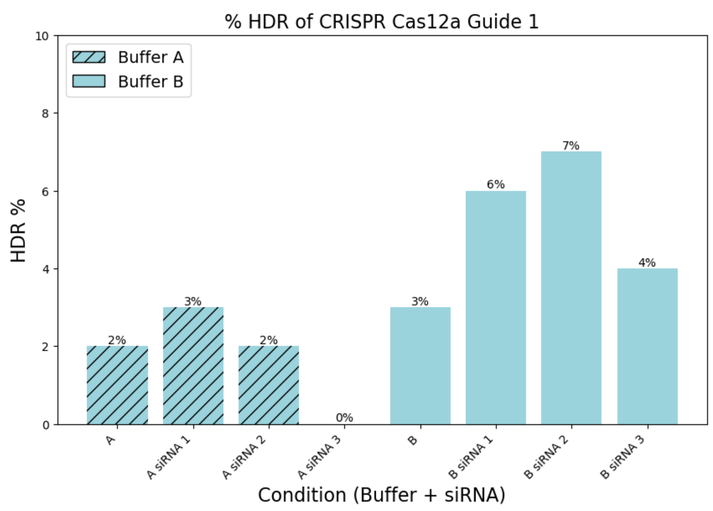

Figure 7. ICE analysis of transfections using Cas12a guide 1 with donor DNA demonstrates optimal siRNA concentration and buffer. iPSCs were transfected with Cas12a guide 1 RNP with donor DNA containing the 4bp deletion and increasing concentrations of p53 siRNA in different buffers. HDR % was determined via ICE analysis.

Single Cell Cloning Optimization

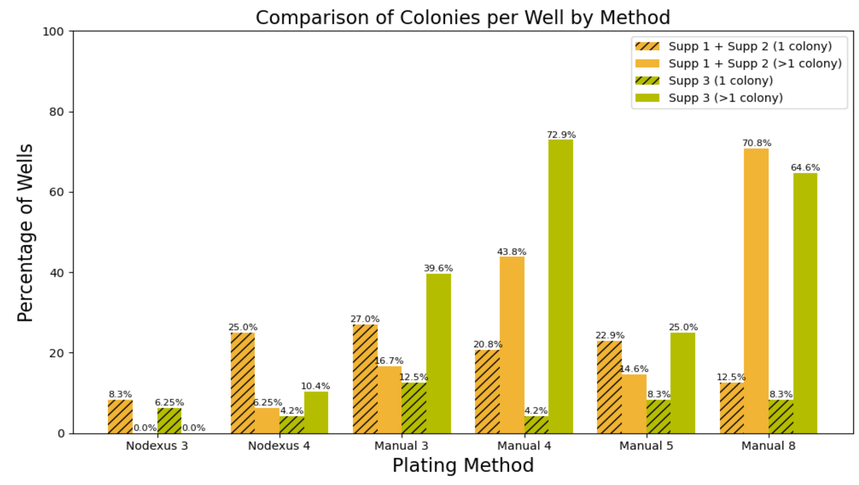

Figure 8. Comparison of plating method and media supplementation for single cell cloning. Wild-type iPSCs were plated in 96-well plates at 3, 4, 5, or 8 cells per well using either the Nodexus NX One or manual dilution. Media was supplemented with either Supplement 1 and Supplement 2 or Supplement 3 alone. Plates were imaged daily via the Sartorius Incucyte S3. At day 10, the number of wells with one colony and more than one colony were counted. The optimal plating method is one that maximizes the number of wells with one colony and minimizes those with more than one colony.

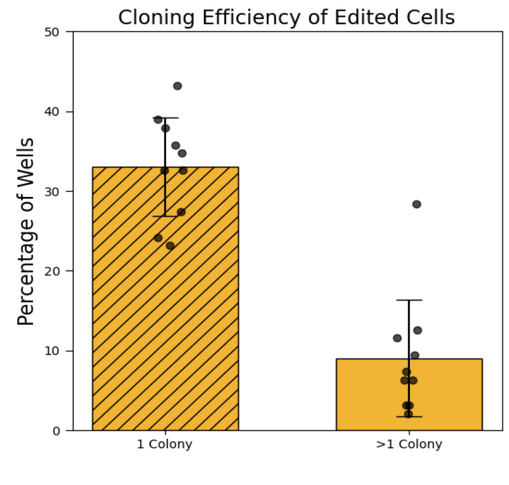

Figure 9. Efficiency of single cell cloning of edited cells using the Nodexus NX One. Edited cells were plated at 3 cells per well in ten 96-well plates in optimal media conditions (Supp 1 and Supp 2). After 10 days, Incucyte images were used to distinguish wells with colony outgrowth. On average, 33.1% of wells had one colony, and 9.1% had more than one colony.

Conclusion

Cells from a ZTTK patient were successfully reprogrammed to iPSCs and characterized for pluripotency and differentiation potential. Additionally, wild-type iPSCs were modified to introduce the desired 4bp deletion in the *SON* gene. Cas12a was identified as the optimal approach in these studies, however Cas9 and TALENs were also tested and may be valuable approaches for other studies. Optimal single cell cloning and gene editing parameters were established and can be implemented for future experiments.

Upon confirmation of clonality, the modified iPSC clones from gene editing experiments will be characterized, cryopreserved, and deposited in a cell bank for researchers to use as a valuable model system for ZTTK syndrome research.

Future Work

Edited iPSCs will continue to be characterized and expanded to generate the wild-type to mutant iPSC lines. An additional iPSC disease-correction model will be generated by repairing the mutation in the patient iPSCs, and clonal populations will be characterized, expanded, and made available for use by researchers.

Acknowledgments

Thank you to all members of the Thermo Fisher Scientific, Patheon Translational Services team for their contributions and support and to the Cal Poly, San Luis Obispo CIRM Bridges program for providing funding for this internship and project. Also, thanks to the ZTTK *SON*-Shine Foundation, to Nathan and Ada Guo, and to Tim Yu from Boston Children's Hospital for their oversight, comments, and suggestions.