CRISPR-Cas9 GENE EDITING OF CDK5RAP2 IN HUMAN PLURIPOTENT STEM CELLS, DERIVATION OF GENETICALLY STABLE CLONAL LINES AND FORMATION OF CEREBRAL ORGANOIDS USING THE STEMdiff™ HUMAN CEREBRAL ORGANOID CULTURE SYSTEM

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Introduction

Modeling human disease using human pluripotent stem cells (hPSCs), in combination with CRISPR-Cas9 gene editing, are emerging as important strategies for studying mechanisms of pathogenesis. We used the ArciTect™ CRISPR-Cas9 system to generate clones harboring a C-terminus truncation of CDK5 regulatory subunit-associated protein 2 (CDK5RAP2), a gene associated with the development of primary microcephaly (Lancaster et al, Nature 2013). We successfully generated stable clones which we further characterized for cell quality attributes (karyotype, pluripotency, morphology, and marker expression) prior to differentiation into cerebral organoids using STEMdiff™ Human Cerebral Organoid Kit.

Methods

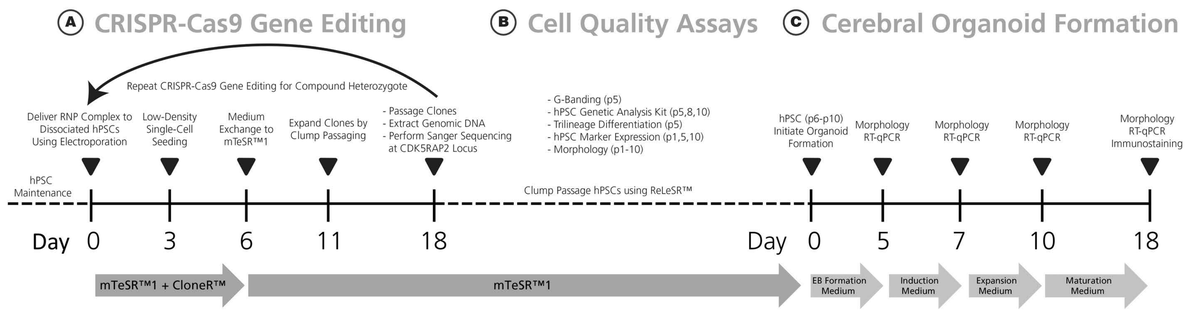

FIGURE 1. ArciTect™ CRISPR-Cas9 Gene Editing and Cerebral Organoid Workflow

A) ArciTect™ CRISPR-Cas9 ribonucleoprotein, in complex with a guide RNA targetting CDK5RAP2, was formed and introduced into a blood-derived hPSC line (STiPS-B004) using electroporation. Transfected cells were incubated at 37°C for 3 days in mTeSR™1 + CloneR™. After single-cell dissociation, cells were seeded at a density of 400 cells per 10 cm^2 dish in mTeSR™1 + CloneR™ and incubated at 37°C for 3 days. A full medium change was performed with mTeSR™1, then cells were incubated for an additional 5 days. Single clones were then selected and expanded in mTeSR™1 in 24-well plates for 7 days. At the first passage, genomic DNA was purified and the CDK5RAP2 locus was amplified by polymerase chain reaction (PCR); product was sent for Sanger sequencing to determine whether editing occurred. Out of approximately 24 clones selected, 2 - 3 clones were chosen that harbored a heterozygous frameshift mutation that led to a premature stop codon. A second round of CRISPR-Cas9 gene editing was initiated (indicated by black arrow) with these heterozygous clones to generate a compound heterozygous clone harboring a mutation in both CDK5RAP2 alleles that led to a premature stop codon. **B)** All clones were dissociated into clumps using ReLeSR™ and expanded in mTeSR™1. Clones were also further characterized by the following phenotypic and functional assays: low-resolution karyotype by G-Banding (p5), screening for high-resolution karyotypic abnomalities at 9 loci using hPSC Genetic Analysis Kit (p5, p8, and p10), differentiation potential using STEMdiff™ Trilineage Differentiation Kit (p5), marker expression for OCT4 (p1, p5, and p10) and cell morphology assessment. Stable genetic clones harboring the CDK5RAP2 C-terminus truncation were used between p6 and p10 to generate human cerebral organoids. **C)** Cerebral organoids were generated using STEMdiff™ Cerebral Organoid Kit. RNA was extracted on Day 5, 7, 10, and 18 and assayed by RT-qPCR for neuronal markers (DCX and TUJ1), and neural progenitor markers (PAX6 and SOX2). Cryosectioning and immunostaining were also performed on Day 18 organoids for PAX6 and TUJ1.

Results

FIGURE 2. ArciTect™ CRISPR-Cas9 Gene Editing of CDK5RAP2 Generates an Early Stop Codon

A) Primary sequence of CDK5RAP2. Disease mutations associated with primary microcephaly are shown in (black), protein-protein interacting regions (grey) and centrosome-binding domain (green) is found in the C-terminus. Box: Designed guide RNA primary sequence is highlighted in orange. **B)** Off-target gene editing was determined by Sanger sequencing at loci predicted as possible off-target sites for the designed guide RNA targetting CDK5RAP2. Results confirmed that no off-target editing occurred at the loci assayed. Mismatched bases are highlighted in red. **C)** DNA and amino acid sequences obtained from Sanger sequencing of the control, heterozygous, and compound heterozygous cell lines reveal a 2 base-pair (Heterozygote) and 4 base-pair (Heterozygote/Compound Heterozygote) deletion leading to a frameshift and premature stop codon.

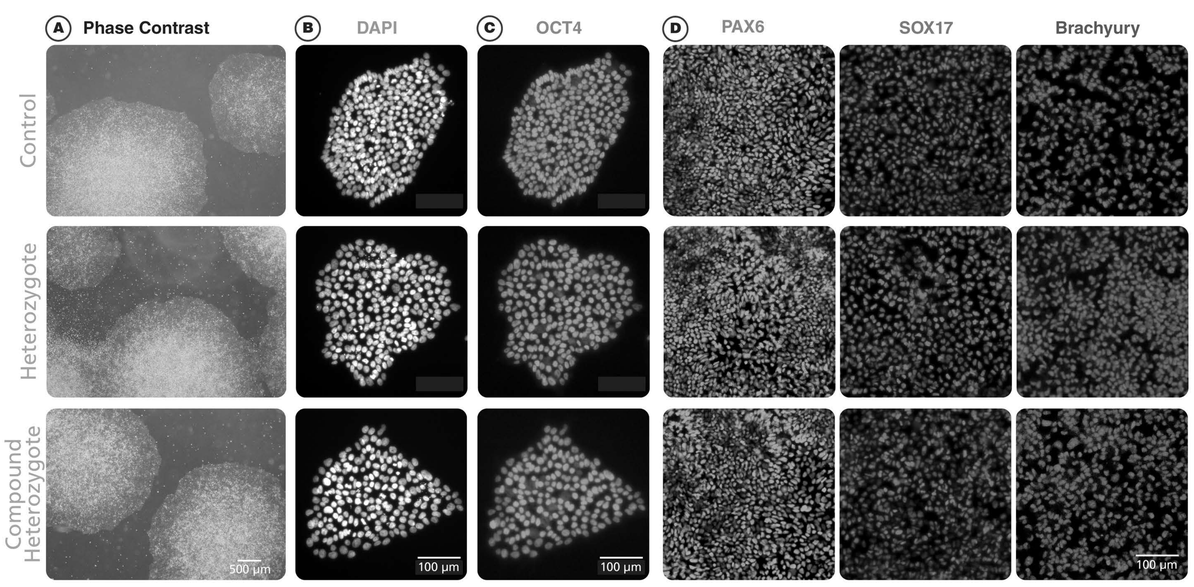

FIGURE 3. Gene-Edited CDK5RAP2 Clones Exhibit hPSC Morphology and Pluripotency

A) Representative phase contrast morphology of stable hPSC clones exhibit multi-layering and dense packing similar to the control cell line. **B)** The nuclei of hPSC colonies are marked by DAPI (grey) **C)** Clonal cell lines expressed the undifferentiated cell marker OCT4(OCT3) (green). **D)** Differentiation potential for each clonal cell line was assessed using STEMdiff™ Trilineage Differentiation Kit. All clonal cell lines exhibited high differentiation potential to ectoderm (PAX6), endoderm (SOX17) and mesoderm (Brachyury).

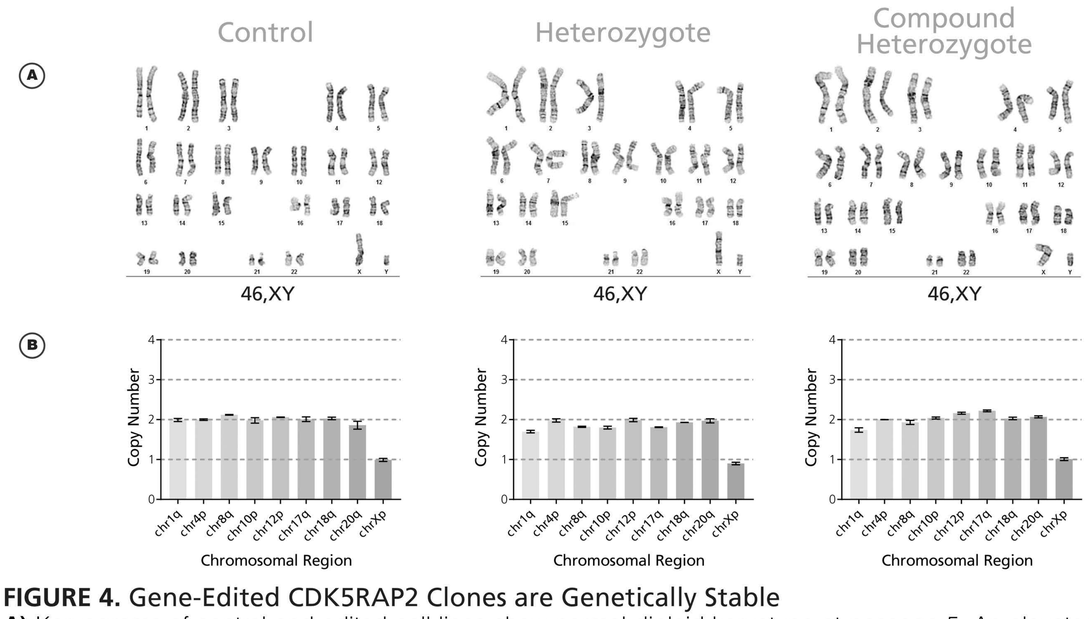

FIGURE 4. Gene-Edited CDK5RAP2 Clones are Genetically Stable

A) Karyograms of control and edited cell lines show normal diploid karyotype at passage 5. An elevated level of polyploidy was observed in both the heterozygote (~20%) and compound heterozygote (~33%) cell lines compared to typical background levels in hPSCs (<10%). This may indicate a potential error in chromosome segregation at metaphase or in cytokinesis. **B)** Edited cell lines were screened for recurrent karyotypic abnormalities at 9 loci, all cell lines displayed normal diploid copy number at p5 and p10.

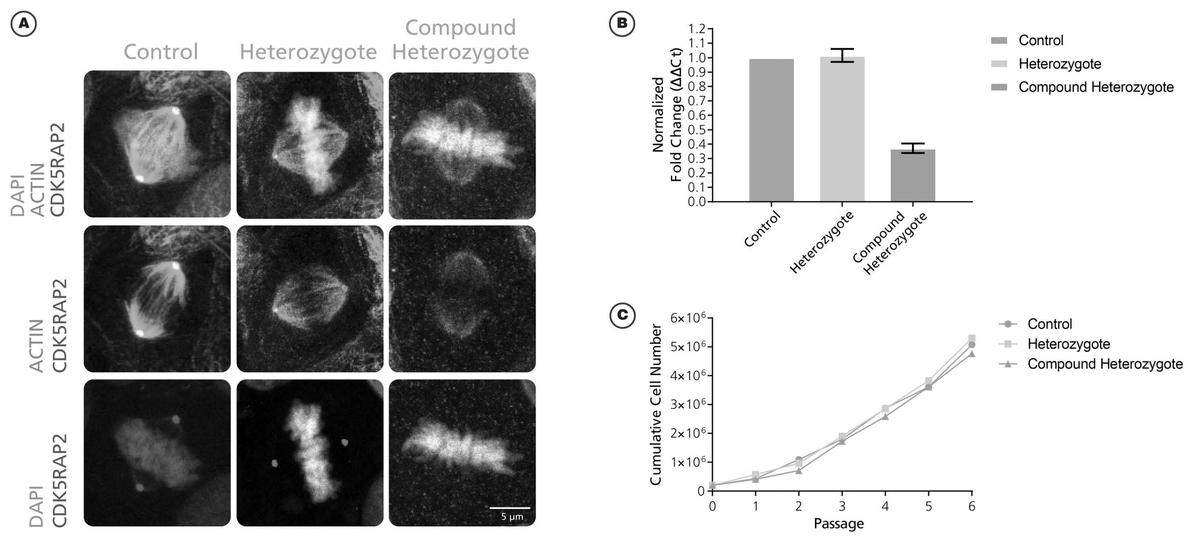

FIGURE 5. CDK5RAP2 is Mislocalized in the Compound Heterozygote Clone During Cell Division but Does Not Impact Growth Rates of hPSCs

A) Representative images of hPSCs undergoing metaphase show distinct localization of CDK5RAP2 (purple) at each pole of the mitotic spindle marked by ACTIN (green) in both the control and heterozygote cells. Nuclei are marked by DAPI (grey). No localization of CDK5RAP2 (purple) to the mitotic spindle is observed in the compound heterozygote. **B)** RT-qPCR analysis of CDK5RAP2 transcript in hPSCs shows decreased transcript expression in the compound heterozygote compared to the control and heterozygote (n=3 timepoints per data point, average +/- SEM, normalized to Control; $P \leq 0.0001$ when comparing compound heterozygote to heterozygote) **C)** The growth rates of hPSCs in all clonal cell lines were not significantly different, (n=3 technical replicates per data point, average +/- SEM; $P > 0.05$).

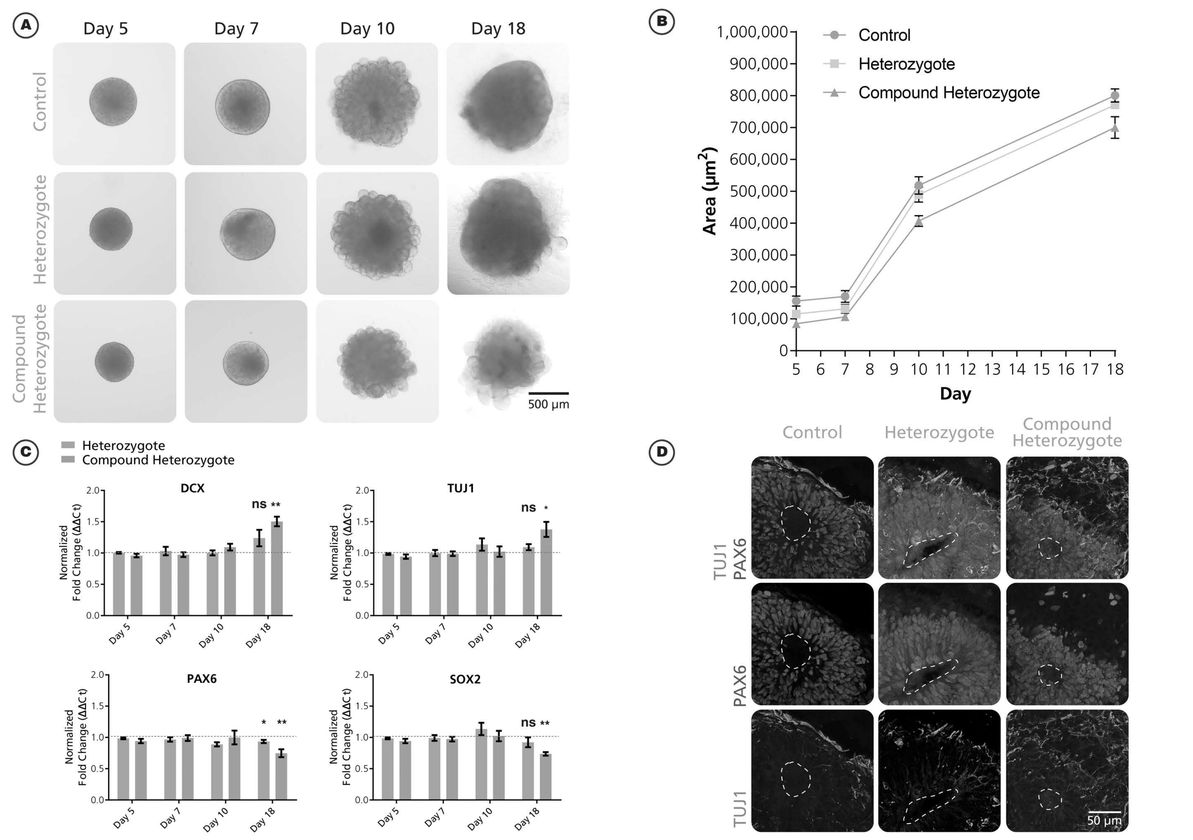

FIGURE 6. CDK5RAP2 Decreases Cerebral Organoid Size and Impacts Marker Expression at Day 18

A) Representative phase contrast image of organoids generated from hPSCs at each stage of organoid formation. **B)** Area measurements (μm^2) at each stage of organoid formation show a decreased size in the CDK5RAP2 compound heterozygote mutant at all stages compared to the control (n=4, 12-16 organoids per data point, average +/- SEM; $P \leq 0.05$ when comparing control to compound heterozygote). **C)** RT-qPCR analysis of organoids at each stage of cerebral organoid formation reveals a difference in neural progenitor marker expression (SOX2 and PAX6) and neuronal marker expression (DCX and TUJ1) in the compound heterozygote compared to control at Day 18. (n=4, 12-16 organoids per data point, average +/- SEM, normalized to Control; ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$). **D)** Immunostaining of Day 18 organoids reveals an increase in the neuronal marker TUJ1 (green) and decrease in the neural progenitor marker PAX6 (purple) in cortical layer regions, in compound heterozygote clones compared to control and heterozygote clonal cell lines. Ventricular zone-like regions are marked by a white dashed line.

Summary

- Established a work flow for gene editing, cell quality, and generation of stable gene-edited clones using the ArciTect™ CRISPR-Cas9 system
- CDK5RAP2 truncation leads to mislocalization of CDK5RAP2 from mitotic spindles during mitosis
- CDK5RAP2 C-Terminus truncation clones generate smaller cerebral organoids compared to a control, and exhibit increased neuronal marker expression and decreased neural progenitor marker expression, similar to data from Lancaster et al.

References:
Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA. Cerebral organoids model human brain development and microcephaly. Nature. 2013 Sep 19;501(7467):373-9.