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 targeting CDK5RAP2, was introduced into a DNA-loaded electroporation cuvette. Transfected cells were incubated at 37°C for 3 days in mTeSR™ + CloneR™. After single-cell dissociation, cells were seeded at a density of 400 cells per 10 cm dish in mTeSR™ + CloneR™ and incubated at 37°C for 3 days. A full medium change was performed at 3 dpi, then cells were incubated for an additional 5 days. Single clones were then selected and expanded in mTeSR™ 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 sequencing.

**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 of the protein. B) Designed guide RNA primary sequence is highlighted in orange. C) Off-target gene editing was determined by Sanger sequencing at loci predicted as off-target sites for the designed gene targetting CDK5RAP2. Results confirmed that no off-target editing occurred at the loci assayed. Mismatched bases are highlighted in red. D) DNA and amino acid sequences obtained from Sanger sequencing of the control, heterozygous, and compound heterozygous cell lines reveal 2 base-pair (Heterozygous) and 4 base-pair (Heterozygous/Compound Heterozygous) deletions 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 phase line. B) The nuclei of入股 colonies are marked by DAPI (grey). C) Clonal cell lines expressed the undifferentiated cell marker OCT4 (red) and neural marker SOX17 (purple). D) Immunostaining for pluripotent cell line was assessed using STEMdiff™ Trilineage Differentiation Kit. All clonal cell lines exhibited high differentiation potential to endoderm (PAN6), endoderm (SOX17) and mesoderm (Brachyury).

**Figure 4.** Gene-Edited CDK5RAP2 Clones are Genetically Stable

A) All clones were dissociated into clumps using ReLeSR™ and expanded in mTeSR™1. Clones were then selected and expanded in mTeSR™1 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.

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

A) Representative immunofluorescent 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 cell lines. Nuclei are marked by DAPI (grey). No localization of CDK5RAP2 (purple) to the mitotic spindle is observed in the compound heterozygous cell line. B) RT-PCR analyses of CDK5RAP2 transcript levels showed decreased transcript expression in the compound heterozygous compared to the control and heterozygote (n=3 timepoints per data point, average +/-SEM, normalized to Control). 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 (μm²) at each stage of organoid formation show a decrease in size of the CDK5RAP2 compound heterozygote mutant at all stages compared to the control (n=4; 12-16 organoids per data point, average +/-SEM, P < 0.05; when comparing control to compound heterozygote). C) RT-PCR analysis of organoids at each stage of cerebral organoid formation reveals a difference in neural progenitor marker expression (SOCO and PAX6) and neuronal marker expression (SOX2 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: P > 0.05; **P < 0.05; ***P < 0.01). D) Immunostaining of Day 18 organoids reveals an increase in the neural marker TuJ1 (green) and decrease in the neuronal marker progenitor marker PAX6 (purple) in cortical layer regions, in compound heterozygote clones compared to control and heterozygote clonal lines. Ventricular zone-like regions are marked by a white dashed line.

**Summary**

- Established a workflow 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 clone, and exhibit increased neuronal marker expression and decreased neuronal progenitor marker expression, similar to data from Lancaster et al.

References: