iPSC & CRISPR/Cas9 Technologies Enable Precise & Controlled Physiologically Relevant Disease Modeling for Basic & Applied Research

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INTRODUCTION

- Human induced pluripotent stem cell (iPSC) technology has provided unique ways to understand and potentially treat human diseases using cells from individual patients.
- iPS cells are amenable to genomic modifications using site-specific gene editing technologies such as CRISPR/Cas9, by which we can correct/ introduce precise disease causing mutations in patient or healthy iPSCs, respectively.
- Genome edited iPSCs and their isogenic control (or parental line) offer highly-controlled experimental models for reliable comparison of results.
- These isogenic cell lines can be further differentiated into target cell types that are relevant to the disease, and then used to interrogate disease phenotypes or to screen novel therapeutic agents.
- Here, we describe this process from (1) iPSC generation from a somatic cell source, (2) engineering disease models with precise genomic modifications using CRISPR/Cas9; (3) differentiation to neural lineages; and (4) the usability of these iPSC-differentiated neurons as reliable *in vitro* models for drug or neurotoxicity screening .

Experimental Design

iPSC Cell Line Models to Mimic Human Diseases
iPSC Generation (healthy/ patient lines)
iPSC Gene Editing (healthy/ patient lines)

→

iPSC Differentiation to Neural Lineage Cells
Neurons
Astrocytes

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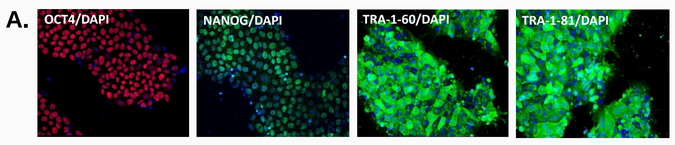
Cell-based assays for phenotype & drug screening
Biomarker expression
Electrophysiology

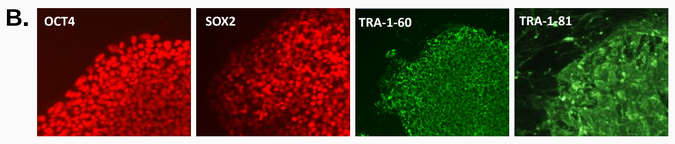
Integration-Free iPSC Reprogramming from Somatic Cells

iPSCs from Control/ Healthy CD34+ Cord Blood Cells:



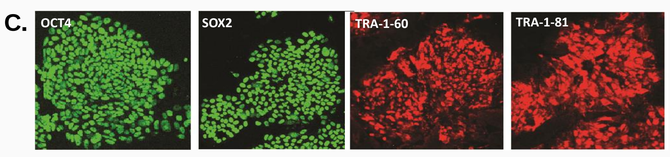
Human iPSCs were generated from CD34+ cord blood cells from a healthy patient using episomal vector-based reprogramming methods. HiPSC lines were generated and maintained using feeder-free conditions. Immunohistochemistry for pluripotency marker: OCT4, NANOG, TRA-1-60, and TRA-1-81; nuclear staining: DAPI (blue)

iPSCs from Healthy PBMCs:



Human iPSCs were reprogrammed from peripheral blood mononuclear cells (PBMCs) from a healthy patient using sendai viral-based methods and feeder-free culture conditions. Immunohistochemistry for pluripotency marker: OCT4, SOX2, TRA-1-60, and TRA-1-81; nuclear staining: DAPI (not shown)

iPSCs Fibroblasts of Patient with a Neurological Disorder:

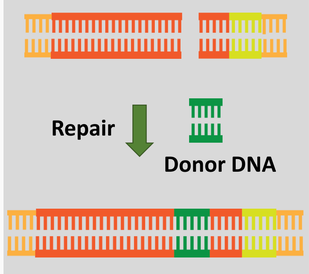


Human iPSCs were generated from dermal fibroblasts from a patient with a neurological disorder. The hiPSCs were generated using episomal vectors in feeder-free culture conditions. Immunohistochemistry for pluripotency marker: OCT4, SOX2, TRA-1-60, and TRA-1-81; nuclear staining: DAPI (not shown)

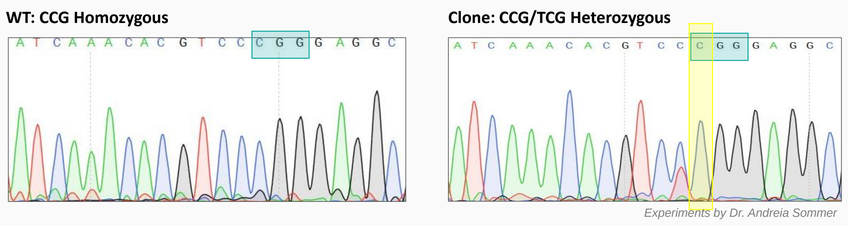
Genome Editing in iPSCs to Model Human Diseases

CRISPR/Cas9

Gene knock-out
Point mutation
Small DNA insertion



Targeted Heterozygous Point Mutation Without Silent Mutation:



Experiments by Dr. Andreia Sommer

Sequence chromatogram showing **heterozygous point mutation**, **CCG > TCG** (green box) in a human iPSC line derived from control/ healthy fibroblasts. The ssODN was designed **without silent mutation** and precisely modified the gene to yield heterozygous clones.

Bi-Allelic Knockout of a Gene Associated with Parkinson's Disease:

Allele 1: deletion of 10 bp

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WT sequence:    GCCATGGTTTCCCAGTGGAGGTCGATTCT
Allele 1:       GCCATGGTTT-----T-----TCAATTCT
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Allele 2: deletion of 4 bp

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WT sequence:    GCCATGGTTTCCCAG----TGGAGGTCGA
Allele 2:       GCCATGGTTTCCCAGTCAGTGGAGGTCGA
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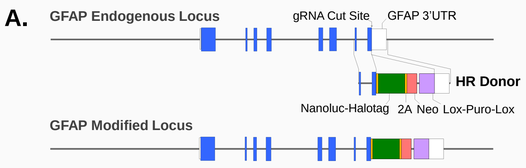
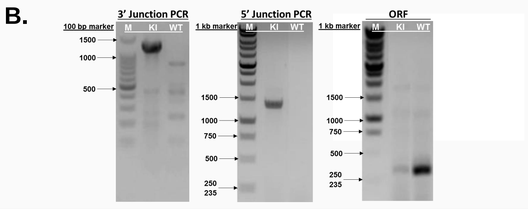
Sequence alignment between control/ parental iPSC line (WT; wildtype) and a representative knockout clone. The homozygous knockout clone shows a 10 bp deletion in allele 1 and a 4 bp deletion in allele 2 as compared to wild type sequence in parental iPSC line..

Generation of an Astrocyte-Specific Reporter Line:



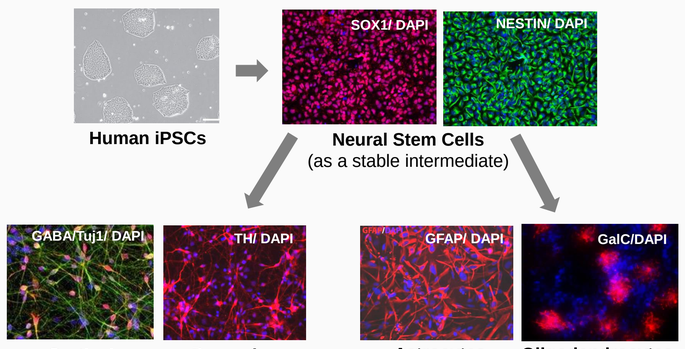
A. Schematic representation of a reporter gene (Nanoluc-Halotag) knock-in into an endogenous locus of an astrocyte-specific gene.



B. Junction PCR to confirm the insertion of the reporter gene to the locus of choice in a control iPSC line. Three sets of primers were used to verify insertion of the reporter gene: 3' Junction PCR for right arm integration; 5' Junction PCR for left arm integration; open reading frame (ORF) PCR to confirm insertion at the correct locus. The insertion of reporter gene was compared against wild type (WT) fragment.

Differentiation of iPSCs to Neural Lineage Cells

Differentiation of Control Human iPSCs to Isogenic Neural Stem Cells and Neural Lineage Cells:

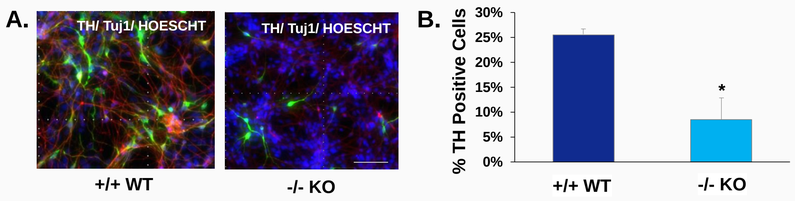


Immunohistochemistry to characterize neural lineage cells differentiated from human iPSCs. Control human iPSCs were first differentiated into neural stem cells (NSC) as a stable precursor: SOX1 (red) and NESTIN (green).

These NSCs were further differentiated into functionally viable neurons: > 90% cortical/ mixed neurons (GABA: red; Tuj1: green) or > 30% dopaminergic (dopamine) neurons (TH: red). The NSCs were also differentiated into glial cell lineages: > 85% astrocytes (GFAP: red) and about 50% oligodendrocytes (GalC: red). Nucleus marker: DAPI, blue.

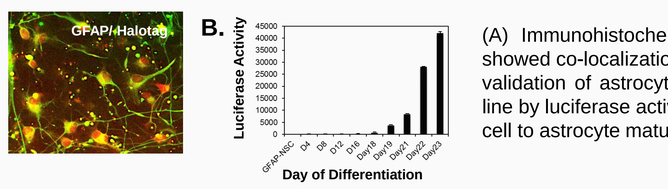
Differentiation of the Parkinson's Gene Knockout iPSCs into Dopamine Neurons:

Dopamine neurons differentiated from gene knockout iPSCs recapitulate the disease phenotype seen in patient-derived dopamine neurons



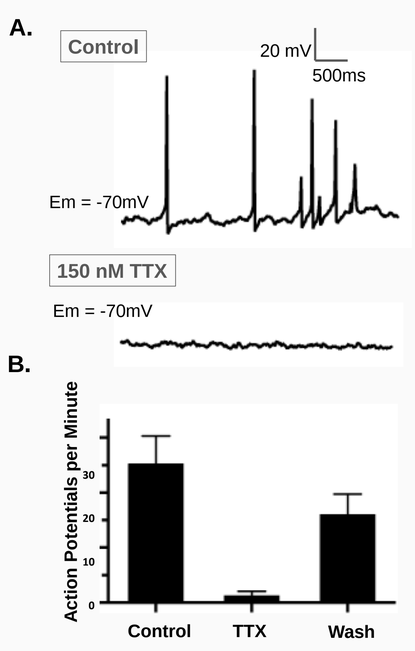
(A) Immunocytochemical for dopamine neuron marker, TH; green) showed reduced TH+ staining in gene knockout (-/- KO) iPSC-derived dopamine neurons as compared to parental/ control (+/+ WT) iPSC-derived neurons. Neuronal marker: Tuj1; red was used to stain total population of neurons. (B)The number of TH+ dopamine neurons derived from Park2 -/- iPSCs was also significantly lower as compared to control (+/+ WT) cell line.

Differentiation of Astrocyte-Specific Reporter iPSC Line to Astrocytes:



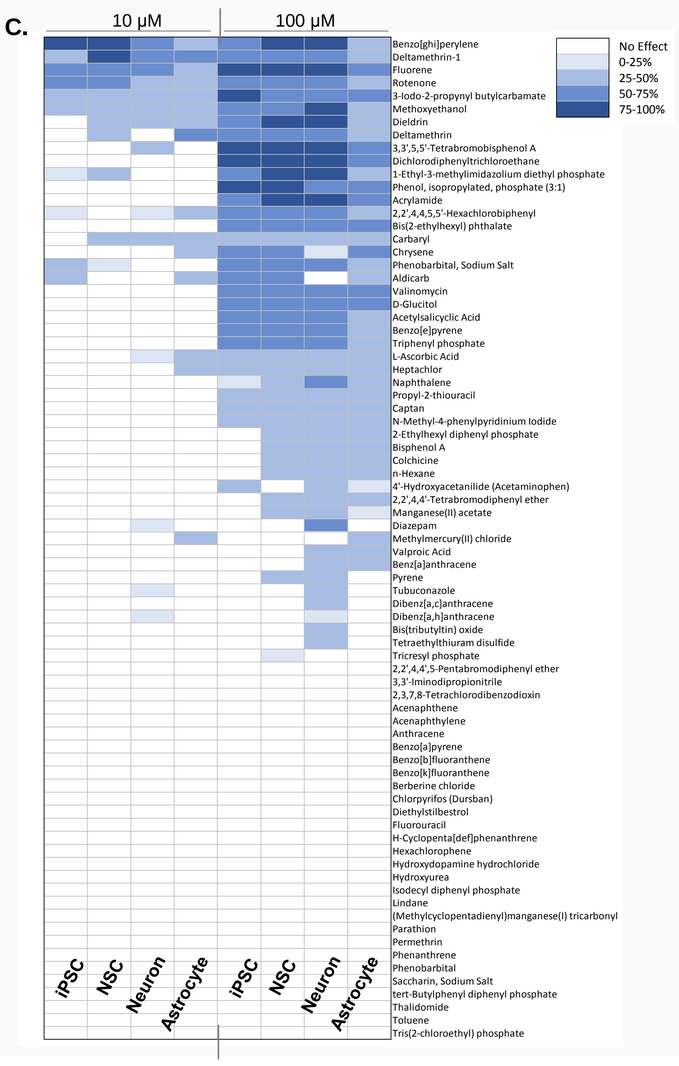
(A) Immunohistochemical staining for GFAP (green) and Halotag (red) showed co-localization (yellow) of reporter gene in astrocytes. (B) Functional validation of astrocytes differentiated from astrocyte-specific reporter iPSC line by luciferase activity assay measured over 23 days from the neural stem cell to astrocyte maturation.

Screening Drugs for Neurotoxicity



Inhibition of action potential by Tetrodotoxin (TTX) in iPSC-derived Neurons. A. The recordings show action potentials in the absence (top; control) and presence (bottom) of 150nM TTX. B. The histogram shows the mean (±SEM) number of action potentials per minute (y-axis) in the absence, presence and following washout of TTX.

C. 77 compounds from the Tox21 library were screened for toxicity in iPSCs, neural stem cells (NSCs), neurons, and astrocytes using an MTT assay in 96-well plate format. Two doses of each compound were used.



CONCLUSIONS

- iPSCs offer a novel solution for generate predictive *in vitro* models of human diseases that are renewable, easily available and .reliable, especially for hard-to-source or difficult to model human diseases.
- iPSCs can be genetically engineered using gene editing technologies such as CRISPR/Cas9 to precisely introduce mutations to mimic various human diseases, or to correct disease mutations in patient lines for drug discovery or cell therapy research.
- Genetically engineered iPSCs and their isogenic parental lines provide a controlled experimental platform.
- iPSCs can be differentiated into functional and somatic lineages in an isogenic background for studying genetic functionality and effect of drugs across different tissues.
- We have shown that iPSC-derived neuronal and glial cells can be used for modeling neurodegenerative diseases as well as for neurotoxicological and neuroprotective drug screening.