NEXT-GENERATION NEUROLOGICAL DISEASE MODELS

ISOGENIC TOOLS FOR INVESTIGATING FRONTOTEMPORAL DEMENTIA, ALZHEIMER'S & PARKINSON'S DISEASES

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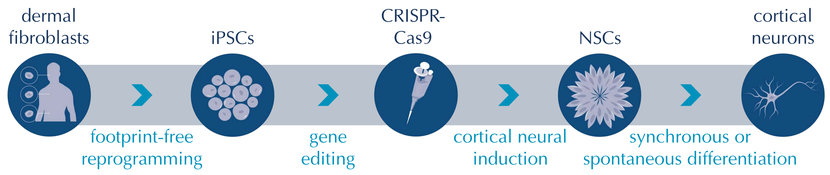
Introduction

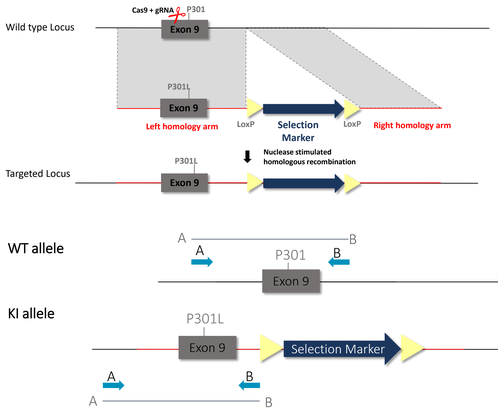
- Neurodegenerative diseases, such as **frontotemporal dementia (FTD), Parkinson's and Alzheimer's diseases** are incurable and debilitating conditions with strong links to age.
- Currently, there are a lack of human cell-based models available in which to further investigate disease mechanisms and determine the efficacy of novel drug compounds.
- Combining the powerful tools of **iPSC genome editing using CRISPR-Cas9** and directed differentiation we have generated human iPSC-derived neural stem cell (NSC) disease models carrying heterozygous and homozygous combinations of neurodegenerative disease-associated missense mutations
  - **P301L**, **V337M** and **R406W** in microtubule-associated protein tau (**MAPT**)
  - **G2019S** in leucine-rich repeat kinase 2 (**LRRK2**)
- Our **axolGEM (genetically edited model) iPSC-Derived Neural Stem Cells** offer a virtually unlimited source of physiologically relevant isogenic lines for use in both disease modelling and drug discovery.

Materials and methods



Footprint-free reprogramming Dermal fibroblasts of a healthy 64-year-old female donor were reprogrammed using defined factors OCT3/4, KLF4, SOX2 and c-MYC to generate iPSCs.

iPSC gene editing using CRISPR-Cas9 iPSCs were transfected with plasmids expressing Cas9 nuclease (either wild-type or nickase), a validated gRNA and a donor template containing a selection cassette. Single cell clones were derived and screened for the incorporation of the mutation of interest. Karyotype analysis of 20 G-banded metaphase spreads was conducted on the resultant iPSC clone and genotype was validated by Sanger sequencing and digital droplet PCR.



Schematic representation of a donor template vector used for engineering the different mutations (e.g. MAPT P301L). The protospacer adjacent motif (PAM) site was disrupted in all plasmid donor templates to prevent re-cutting by the gRNA.

Schematic representation of genotyping primers used to validate mutant lines.

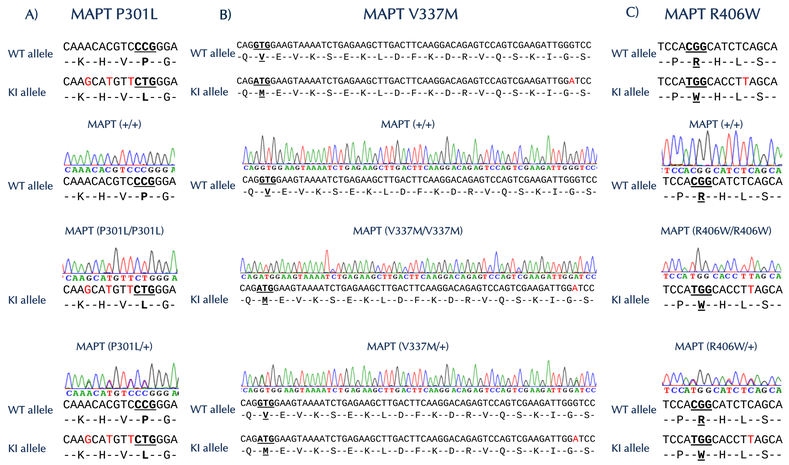
Cortical neural induction and differentiation Fully-defined, cortical neural induction and synchronous/spontaneous differentiation protocols (Shi *et al.*, 2012) were used to generate NSCs and cortical neurons, respectively. Immunocytochemistry was used to confirm the expression of typical NSC markers (nestin, Ki67, FOXG1, PAX6, NCAD), cerebral cortical neuron markers (TBR1, TUJ1, MAP2, CTIP2) and LRRK2. Cells were fixed in 4 % PFA for 15 min at room temperature, permeabilised with 0.3 % Triton X-100 and blocked for 1 hr. Primary antibody was incubated overnight at 4 °C, washed and incubated with secondary antibody for 1 hr, at room temperature, washed and counterstained with DAPI.

1. Target validation

Mutation	% modified clones*	% modified clones sequenced	% KI/KI$	% KI/WT$	% KI/KO$
MAPT P301L	87.5	63	33	4	43
MAPT V337M	50	64	16	29	0
MAPT R406W	83	48	23	14	34
LRRK2 G2019S	48	67	6	19	13

Targeting efficiencies of all desired genotypes. *Modified clones refers to clones identified by PCR that have incorporated a selection cassette at the right locus, $ Calculated relative to the number of modified clones sequenced.

2. Genotype validation

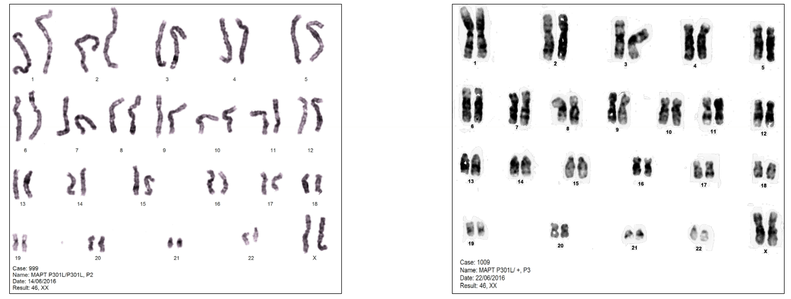


Sanger sequencing data indicated that eight genotypes were achieved, including **A)** MAPT (P301L/P301L) and MAPT (P301L/+), **B)** MAPT (V337M/V337M) and MAPT (V337M/+), and **C)** MAPT (R406W/R406W) and MAPT (R406W/+), **D)** LRRK2 (G2019S/G2019S) and LRRK2 (G2019S/+). Silent mutations added to disrupt binding of the gRNA to the donor sequences are highlighted in red.

Line	Gene copy number	MAPT L301 SNP frequency
Parental iPSCs	1.98	0.05%
MAPT P301L HOM	2.04	99.95%
MAPT P301L HET	1.90	57.40%
Line	**Gene copy number**	**MAPT M337 SNP frequency**
Parental iPSCs	1.98	0.04%
MAPT V337M HOM	1.88	99.72%
MAPT V337M HET	1.94	49.90%
Line	**Gene copy number**	**MAPT W406 SNP frequency**
Parental iPSCs	1.98	0.05%
MAPT R406W HOM	2.03	99.62%
MAPT R406W HET	1.97	49.20%
Line	**Gene copy number**	**LRRK2 S2019 SNP frequency**
Parental iPSCs	1.85	0.04%
LRRK2 G2019S HOM	1.87	99.93%
LRRK2 G2019S HET	1.80	48.90%

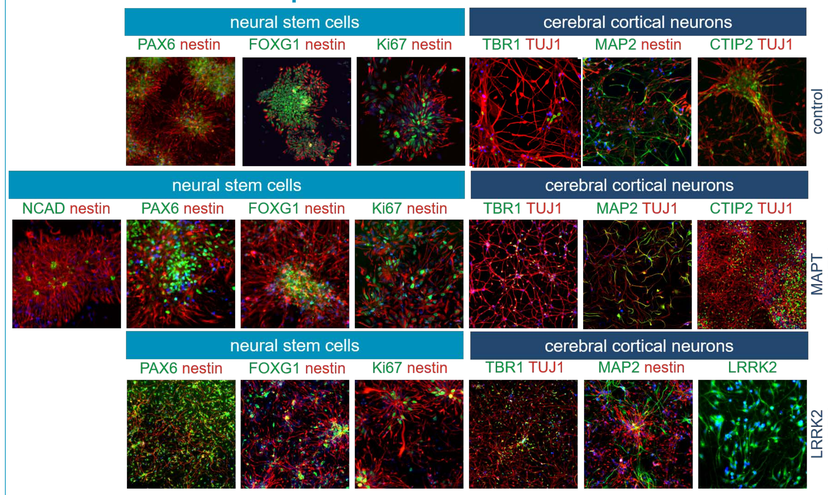
Copy number variation and SNP frequency analysis by digital droplet PCR revealed no off-target integration of the plasmid donor template in any of the validated clones. Clones have approximately 100% and 50% representation of the KI allele which is consistent with the respective homozygous and heterozygous knockin genotypes at a 2N locus.

3. Karyotyping



Chromosome analysis of the fixed axolGEM cell suspension showed an apparently normal female karyotype (46, XX) in 20 cells examined. The preparations obtained were of sufficient quality to detect numerical and large structural abnormalities. Image (left) shows MAPT P301L HOM at P2. Image (right) shows MAPT P301L HET at P3. Karyotype (46, XX) was confirmed in all homozygous and heterozygous knockin lines data not shown).

4. Neural marker expression



Cortical neural differentiation of gene-edited iPSCs and controls. The axolGEM iPSC-Derived NSCs express typical markers (PAX6, Ki67, nestin, NCAD and FOXG1) and spontaneously form polarized neural tube-like rosette structures when plated as a monolayer culture. Additionally, axolGEM iPSC-Derived NSCs are capable of generating a spectrum of cortical neurons that typically express TBR1, CTIP2, TUJ1and MAP2 after synchronous/spontaneous differentiation. LRRK2 was confirmed in appropriate axolGEM lines. All cells counterstained with DAPI.

Conclusions

- Homozygous and heterozygous genotypes were confirmed in all axolGEMs and the cells express typical markers indicative of a neural phenotype.
- The combined technology platforms of both **Horizon Discovery** and **Axol Bioscience** offer a unique opportunity to derive an unlimited amount human iPSC-derived NSCs carrying neurodegenerative disease-associated mutations in a disease-relevant cell type.
- axolGEMs offer a platform for:
  - investigating LRRK2/MAPT variant effect against an isogenic control
  - analysis of gene function and the underlying role in disease establishment or progression
  - drug discovery (phenotypic screening, cytotoxicity, compound screening and target validation)
- The axolGEMs might be used for functional studies that investigate electrical activity, synaptic and network formation in monolayer, 3D and co-culture.

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