Joint Programming Neurodegenerative Disease:

European research projects on neurodegenerative diseases: risk and protective factors, longitudinal cohort approaches and advanced experimental models

Acronym:

Title of the transnational collaborative project:

Report year: 2017 (January to August)

Project Coordinator (name, full address and e-mail):

Partners (subproject leader, affiliation and e-mail):
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1. Short progress summary of the collaboration, major achievements

2. Short progress summary of the respective subprojects

WP1: Generation of iPSCs and isogenic controls from various Parkinson's disease patients

To enable generation of 3D midbrain cultures for idiopathic forms of PD, we obtained fibroblasts from 3 idiopathic PD patients and from 3 age- and sex-matched control individuals from the Integrated BioBank of Luxembourg. For non-integrative reprogramming, we made use of the T7-VEE-OKS-iG plasmid (Addgene #58974) containing the venezuelan equine encephalitis (VEE) virus RNA replicon 3´ORF encoding OCT4, KLF4, SOX2 and GLIS1 (Yoshioka et al. 2013).

Fig. 1: Graphical representation of the plasmid containing the *Venezuelan equine encephalitis virus* RNA replicon encoding OCT4, KLF4, SOX2 and GLIS1 (adapted from Addgene).

After *in vitro* transcription and optimization of RNA transfection in regard to cell density, the amount of transfected RNA, and the duration of puromycin selection, we successfully obtained several iPSC clones from fibroblasts from the 3 idiopathic PD patients and the 3 control individuals. After several passages, we verified that that iPSCs were free of transgenes by performing PCR on total RNA from iPSCs for three sequences, which are specific for the RNA replicon.

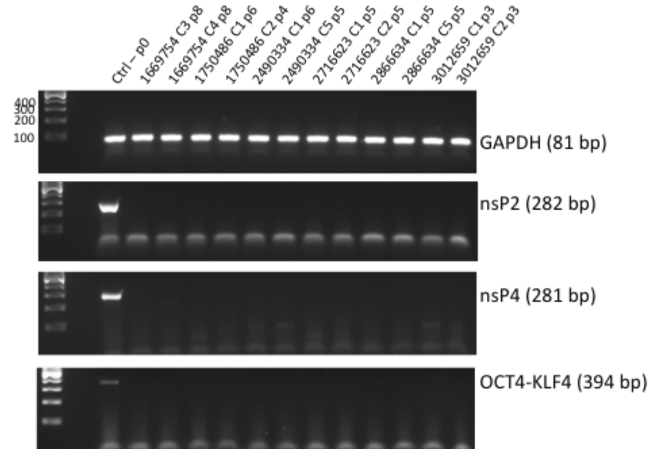

Fig. 2: PCR-based analysis for persistent VEE RNA replicon in iPSC clones derived from iPD patient fibroblasts and control fibroblasts. Total RNA was prepared from fibroblasts after transfection (Ctrl) and from iPSCs after three to eight passages and PCRs were conducted for the non-structural proteins nsP2 and nsP4 as well as for the OCT4-T2A-KLF4 region of the replicon and for GAPDH as loading control.

We subsequently analyzed iPSCs for expression of stem cell markers by immunocytochemical staining. All clones showed strong staining of the pluripotency markers OCT4, NANOG, SSEA4 and TRA-1-60.

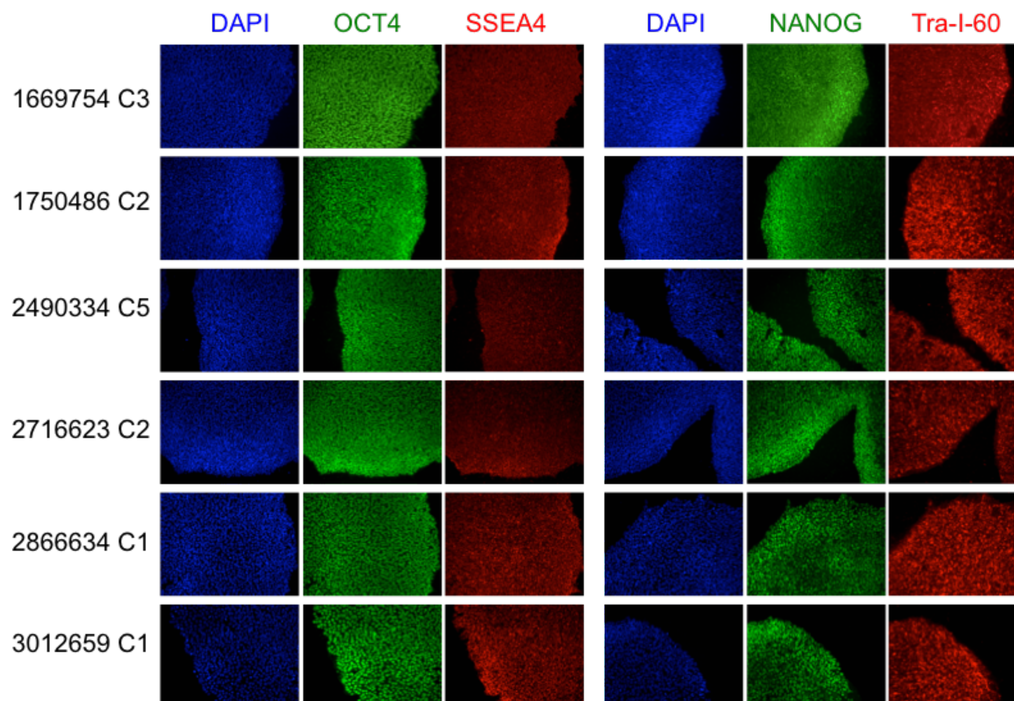

Fig. 3: Immunocytochemical staining for the indicated stem cell markers in iPSC clones.

To further characterize the iPSC clones, we performed qRT-PCR for the expression of hES cell marker genes. Consistent with expression levels in human H9 embryonic stem cells, iPSCs reprogrammed using T7-VEE-OKS-iG expressed robust levels of OCT4, SOX2, cMYC, NANOG and REX1 in contrast to low levels in the fibroblasts from which the iPSCs were derived from.

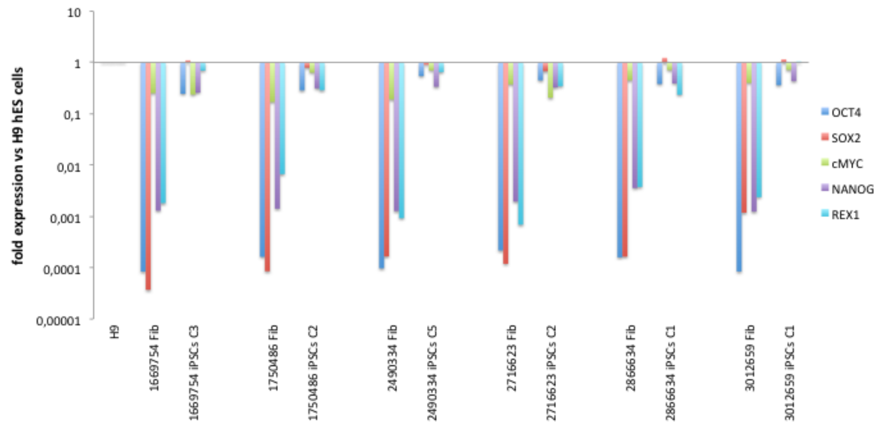

Fig. 4: Expression of indicated pluripotent marker genes in iPSC clones normalized to H9 hES cells as evaluated by qRT-PCR.

Next, we tested the pluripotent differentiation potential of each iPSC clone via embryoid body-mediated differentiation. Positive immunostaining for AFP (endoderm), SMA (mesoderm) and TUBBIII (ectoderm) showed that all iPSC clones could be differentiated into representative cell types of all three germ layers.

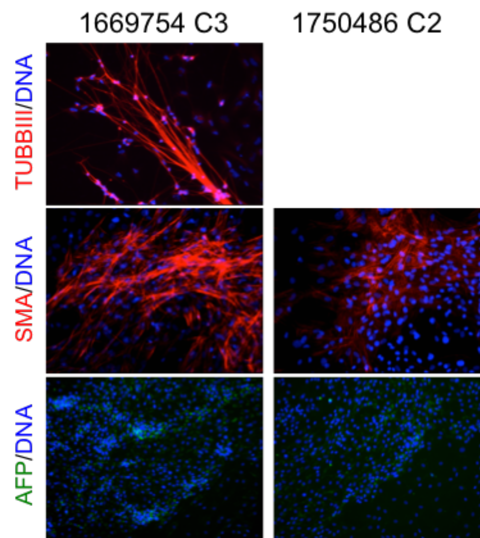

Fig. 5: Immunocytochemical staining of EB-mediated differentiation of iPSC clones into cells of all three germ layers. TUBBIII was used as marker for ectoderm, SMA as marker for mesoderm and AFP as marker for endoderm.

Furthermore, iPSCs were karyotyped and all clones were found to contain normal diploid karyotypes.

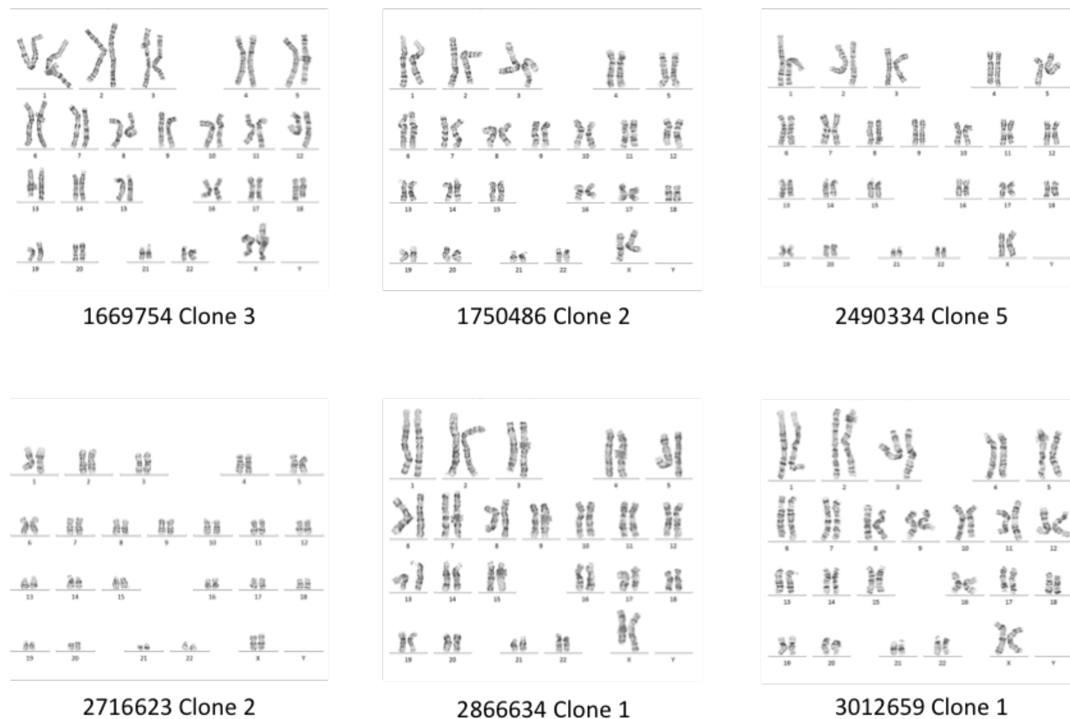

Fig. 6: Karyotype analysis of iPSC clones.

For 3D midbrain models of genetic forms of PD, we have begun with the generation of isogenic control lines that will allow us to dissect the effects of the disease-causing mutation from the modifying effects of the genetic background of the affected individual. Using the CRISPR/Cas9 system for genome editing, we have designed sgRNAs targeting the mutant FBXO7 (R494X; T22M, IVS7 +1G/T) and SYNJ1 (R258Q) genomic sites and cloned them into vectors harboring either Cas9 or mutant Cas9 D10A nickase (Cong et al. 2013). We subsequently verified the functionality of sgRNAs in T7E1 assays. After transfection of iPSCs with the sgRNA/Cas9 containing plasmid and a repair template, however, we did not obtain any gene-corrected clones, but only clones in which the targeted genes were knocked out. These findings were most likely due to the low efficiency of homology directed repair, which is required for gene-correction, and the lacking ability to select for gene-corrected cells. Therefore, we changed our approach to the piggyback transposon-based method described by Eggenschwiler *et al.* (2016), which allows for dual-fluorescence-based selection of bi-allelic targeted cells and for thymidine kinase-based negative selection after excision of the selection cassettes with an excision-only piggyback transposase. The plasmids containing the selection cassettes (AAT-PB-CG2ApΔtk and AAT-PB-CD2ApΔtk) were a kind gift from Dr. Tobias Cantz (Hannover Medical School, Hannover, Germany). We performed PCR on gDNA from Gibco® human episomal iPSCs to generate homology arms for gene correction of patient-derived iPSCs homozygous for $SYNJ1^{R258Q}$ (49iC3, 49iC10, 50iC4, 50iC5) and on gDNA from 50iC5 to generate homology arms for insertion of the $SYNJ1^{R258Q}$ mutation into Gibco® human episomal iPSCs. The homology arms were subsequently inserted into the donor plasmids after the homology arms for AAT had been removed by restriction enzyme digestion. iPSCs were subsequently co-transfected with the sgRNAs/Cas9D10A nickase containing plasmid and the donor constructs harboring both the long flanking arms and the piggyBac site-flanked puroΔtk selection/counter-selection cassette. Following puromycin selection, we obtained GFP^+, $dsRed^+$ and GFP^+/$dsRed^+$ cells, which were isolated by FACS and analyzed for integration of the selection cassette and simultaneous correction/insertion of SYNJ1 R258Q. We successfully obtained clones in which the mutation was inserted on both alleles of the Gibco® human episomal iPSC line.

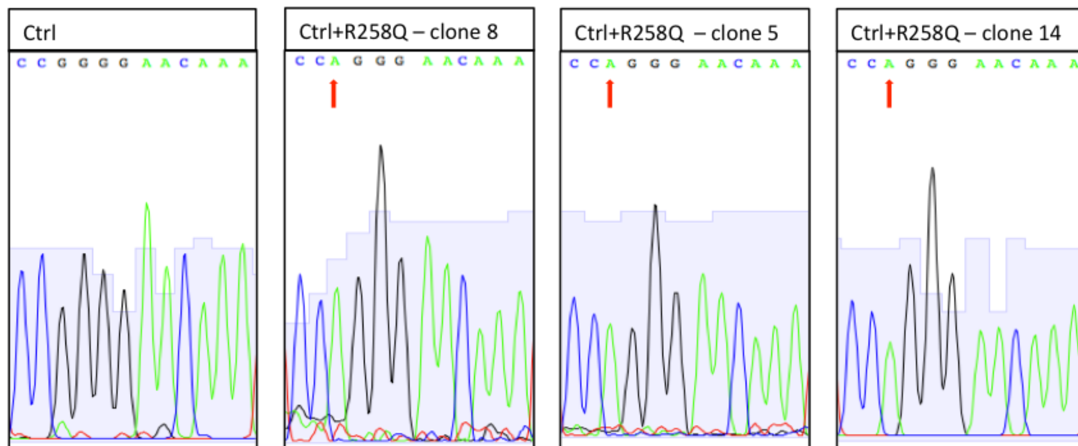

Fig. 7: Sequencing of SYNJ1^{R258Q} mutant Gibco® human episomal iPSC line.

We also obtained clones in which the mutation was corrected in both alleles of the Parkinson patient-derived iPSC lines 49iC3 and 50iC4.

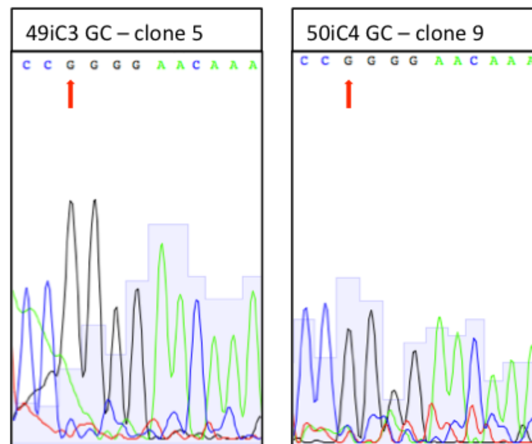

Fig. 8: Sequencing of gene-corrected Parkinson patient-derived iPSCs.

Furthermore, transfection of the Parkinson patient-derived iPSC line 49iC10 yielded several clones in which the PCR product, that spans the genomic region of both homology arms, is absent, indicating integration of the selection cassette and simultaneous gene correction. Sequencing of these clones is currently underway.
We next transfected the corrected/mutated iPSCs with a plasmid coding for an excision-only piggyBac transposase to remove the selection cassette from the genome. However, no cells survived negative selection with fialuridine (FIAU), indicating that the selection cassette has not been removed from the genome.

Efficient generation of human iPSCs by a synthetic self-replicative RNA. Yoshioka N, Gros E, Li HR, Kumar S, Deacon DC, Maron C, Muotri AR, Chi NC, Fu XD, Yu BD, Dowdy SF. *Cell Stem Cell. 2013 Aug 1;13(2):246-54. doi: 10.1016/j.stem.2013.06.001.* 10.1016/j.stem.2013.06.001

Multiplex Genome Engineering Using CRISPR/Cas Systems*. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Science. 2013 Jan 3. 10.1126/science.1231143* PubMed 23287718

Improved bi-allelic modification of a transcriptionally silent locus in patient-derived iPSC by Cas9 nickase. Eggenschwiler, R., Moslem, M., Fráguas, M.S., Galla, M., Papp, O., Naujock, M., Fonfara, I., Gensch, I., Wähner, A., Beh-Pajooh, A., Mussolino, C., Tauscher, M., Steinemann, D., Wegner, F., Petri, S., Schambach, A., Charpentier, E., Cathomen, T. and Cantz, T. (2016), *Scientific Reports*, 6, p. 38198. doi: 10.1038/srep38198.

3. Amendments to the original work plan (if applicable) and its rationale

4. Problems and their solutions (if applicable)

We are currently facing problems in excising the piggyBac-site flanked selection cassettes that were inserted into iPSCs during gene correction. Following transfection of gene-corrected iPSCs with the plasmid encoding the piggyback excision-only transposase, no cells survived when treated with 0.5 μM FIAU, indicating that the selection cassette, which contains the puroΔtk fusion construct, had not been removed from the genome. Lowering the concentration of FIAU to 0.2 μM had no positive effect on the survival of iPSCs after transfection. As even lower FIAU concentration were not sufficient in killing non-transfected control cells, the FIAU concentration appeared not to be the cause of the problem. Potential approaches to solve the problem are testing other promoters to drive expression of the piggyback excision-only transposase or inserting a fluorescent protein into the plasmid encoding the transposase to examine transfection efficiency.

5. Publications (please state only direct outcome of the funded project)

6. Patents, PhD thesis and other outcomes (if applicable)

7. Exchange of researchers, students etc. (if applicable)

Please complete each section. If any topic is not applicable to your project, please state this explicitly.
Please submit by **January 31st** of the subsequent year to:

JPND Joint Call Secretariat
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E-Mail: sabrina.voss@dlr.de