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## Abstract

Protocol collection for the method section for the publication:

"TNF-NF-kB-p53 axis restricts in vivo survival of hPSC-derived dopamine neurons"

from Kim et al. Cell 2024

The list consists of 13 different protocols.

The full publication can be found here:

DOI: [10.1016/j.cell.2024.05.030](https://doi.org/10.1016/j.cell.2024.05.030)

## Transplantation of hPSC-derived mDA neurons into nu/nu rat and NSG mice

- 1 6-OHDA lesioning at 6-8 weeks old rats and cell transplantation were performed as described in Kriks et al. (2011).  
  
For cell transplantation, cells (450 000 cells/rat, 150 000/ml) were stereotaxic injected into right striatum at two deposit sites (1.5 ml/site) (AP: +1.0, ML: 2.5mm; VL: 4.7 and 4.4 mm; toothbar set at 2.5) of rat. Sham group received vehicle solution instead. For mouse studies, 6-8 weeks old NSG (NOD-SCID IL2Rgc / ) mice (Jackson Laboratory) were used, and a total of 2 mL cells (200,000/mouse) were injected at the speed of 0.5 ml/min into the dorsal striatum (AP +0.5, ML 1.8, DV 3.4 from dura) with the aid of stereotactic apparatus and electrical pump (Boston Scientific) to drive the syringe.

## Amphetamine-induced rotation test

- 2 Amphetamine-induced rotation test were performed before transplantation and once in a month after transplantation until 5 months post grafting.  
  
The rats were injected intraperitoneally of D-Amphetamine (Sigma, 5mg/kg). After 10 minutes, the rotation behavior was recorded 40 minutes and the total rotates were automatically counted by Ethovision XT 11.5 (Noldus Information Technology Inc., USA).  
  
The data were presented as (ipsilateral-contralateral) rotates per minute.

## Generation of a NR4A2 (NURR1)-driven GFP expressing hESC line

- 3 The NR4A2 gene was targeted with CAS9 (Addgene) and gRNA (ATTATTTGTCCAACTGTTGGGG in gRNA expression vector (Addgene)).  
  
The donor plasmid was cloned as 5'arm-P2A-H2B-EGFP-PgkPuro-3'arm in a pug19 vector (Clontech) designed as an EGFP expression cassette that can replace the stop codon of NR4A2 by gene targeting.  
5' arm and 3' arm sequences are provided in Table S2.  
  
H9 hESCs were transfected with 1 mg Cas9 plasmid, 1 mg gRNA and/or 2 mg DNA donor plasmid by Nucleofection (Lonza) as described (Mali et al., 2013).

1 mg/ml Puromycin (Thermo) was used for selection during 15 days after transfection. At day 15, individual colonies were isolated and cultured as sub-clones.

## Generation of TNFa knockout hPSC line

- 4 For TNFa knockout in NURR1::GFP hPSC, CACCGCCTCTTCTCCTTCCTGATCG and AAACCGATCAGGAAGGAGAAGAGGC oligos for the generation of TNFa sgRNA were annealed and cloned into a CAG-Cas9-2A-GFP U6-sgRNA vector (Addgene, px458).

5mg of plasmid were transfected to the hPSC using Nucleofector (Lonza, B-016 program), and 2 days later, GFP expressing hPSC was sorted using BD Aria III FACS at MSKCC flow cytometry core facility.

GFP sorted hPSCs were clonally grown and expanded.

TNFa knockout hPSC was validated by sanger sequencing using PCR from the genomic DNA. PCR primers for this are 5-CAGGTTCTCTTCCTCTCACATAC-3 and 5-CCTCTCTTGCGTCTCCATTT-3.

## 6-OHDA mouse model

- 5 Adult female and male NSG mice, (Jackson Laboratory: IMSR\_JAX:005557) (6–12 weeks) were anesthetized with 1%–2% isoflurane mixed in oxygen.

1 mL 6-OHDA (3 mg/mL, in saline with 1% ascorbic acid) was directly injected into the right side of substantia nigra (anterior-posterior [AP] = - 2.9 mm, lateral [ML] = 1.1 mm, vertical [DV] = 4.5 mm, from dura) with rate of 0.5 – 1 mL per minute to generate unilateral toxin Parkinsonian mouse model.

Animals with amphetamine-induced rotation at > 6 rotations per min were selected for cell transplantation 4 weeks after 6-OHDA-lesion surgery.

Animals were randomly grouped and transplanted with CD sorted neurons vs. CD sorted neurons + adalimumab vs. PBS (sham surgery control).

## Stereological analysis

- 6 For short-term survival studies, unbiased stereological counts of NURR1::H2B-GFP DA neurons within the striatum (AP +0.5, ML +/-1.8, DV -3.4 to -3.3 from dura) were performed using stereological principles and analyzed with StereoInvestigator software (Microbrightfield, Williston, VT, USA), as previously described.

For long-term behavioral studies, unbiased stereological counts of TH positive DA neurons within the striatum were performed. The tissue was embedded in O.C.T. or Neg-50 and sections are sliced at 30  $\mu$ m.

Optical fractionator sampling was carried out with an Olympus BX61 microscope equipped with a motorized stage and Olympus 40x objective lens objective. Graft region was outlined on the basis of NURR1::GFP immunolabeling, with reference to a coronal atlas of the mouse brain. Every 3rd - 10th section (depending on the total thickness of the graft) from the beginning of the graft to the end of the graft was randomly and systematically selected for analysis.

For each tissue section analyzed, section thickness was assessed in each sampling site and guard zones of 1 mm were used at the top and bottom of each section.

Pilot studies were used to determine suitable counting frame and sampling grid dimensions prior to counting to achieve enough statistical power and low Gundersen coefficient variance.

The following stereological parameters were used in the final study: for optical fractionator probe, 65  $\mu$ m x 65  $\mu$ m optical dissector, 100  $\mu$ m x 100  $\mu$ m (or 10% of ROI) SRS, 20  $\mu$ m optical dissector height and 1  $\mu$ m guard zone; for cavalier estimator probe, 50  $\mu$ m x 50  $\mu$ m grid spacing, 0-degree grid rotation, and 30  $\mu$ m section cut thickness. For analysis, at least 2-8 sections were evaluated for analysis.

Gundersen coefficients of error for all conditions were less than 0.1. Stereological estimations were performed with the same parameters for all experimental conditions, p53 KO vs. WT (NURR1::GFP sort) or TNFa monoclonal antibodies treatment vs. PBS (CD sort).

## Tissue immunohistochemistry (IHC), TUNEL, and H&E stain

- 7 H&E and IHC on tissues from mice was performed on FFPE (formalin fixed paraffin embedded) sections from xenografts.

Mice were anesthetized with pentobarbital and transcardially perfused using heparinized (10U/mL) PBS (pH 7.4), followed by 10% formalin.

The liquid is administered using peristaltic pump to control the rate of the solution delivery to the system.

Tissues were post-fixed in 10% formalin 24-48 hours at room temperature (can stay up to a week in 10% formalin) and directly transferred to 70% ethanol.

Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow.

Samples were embedded in paraffin and sectioned at 4µm.

Immunohistochemistry was performed on a Bond Rx autostainer (Leica Biosystems) with heat-mediated antigen retrieval using Epitope Retrieval Solution 1 (Leica Biosystems).

Primary antibodies used were rabbit polyclonal total NFκB (Cell Signaling, CST8242, 1:300), Cleaved Caspase-3 (Cell signaling, CST9661, 1:300), p53 (CM5P, 1:500), and p-NFκB (GTX55113, 1:5000) followed by anti-rabbit HRP conjugated polymer system.

Bond Polymer Refine Detection (Leica Biosystems) was used according to the manufacturer's protocol.

After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems).

For TUNEL: Standardized conditions using the Promega DeadEnd Colorimetric Detection System (G3250), Enzyme Digestion for 10 minutes, using the Leica Bond Polymer Refine Detection Kit (DS9800).

For H&E, staining was performed on Sakura Autostainer.

Briefly, deparaffinize the slides in 2 changes of xylene, 2 changes of 100% alcohol, 1 change in 95% alcohol, then wash with water.

Place slides in this sequence: hematoxylin, a rinse with water, define solution, a rinse with water, bluing agent solution, rinse with water, 95% alcohol, eosin, and 95% alcohol.

Finish with two changes of 100% alcohol and two with xylene.

## Transcriptome sequencing

- 8 After RiboGreen quantification and quality control by Agilent BioAnalyzer, 0.5-1ng total RNA with RNA integrity numbers ranging from 6.1 to 10 underwent amplification using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech catalog # 63488), with 12 cycles of amplification.

Subsequently, 1.6-10 ng of amplified cDNA was used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) using 8 cycles of PCR.

Samples were barcoded and run on a HiSeq 4000 or NovaSeq 6000 in a PE50 run, using the HiSeq 3000/4000 SBS Kit or NovaSeq 6000 S1 Reagent Kit (100 Cycles) (Illumina).

An average of 56 million paired reads were generated per sample and the percent of mRNA bases per sample ranged from 48% to 76%.

## Single-cell transcriptome sequencing

9 The samples underwent 10X chromium Single Cell 3' v3 processing.

The reads were aligned to human GRCh38 (GENCODE v32/Ensembl 98) using Cell Ranger 5.0.0.

The resulting filtered count matrix was further filtered for cells with i) minimum 1000 UMI counts, ii) 500 % gene counts % 7000, iii) and mitochondrial gene percentage of less than 25%.

Normalization by deconvolution in scan version 1.22.1 was performed and the signal from the gene expression related to the cell cycle was regressed out as directed by Seurat version 4.1.

The default 2000 highly variable genes were selected, and the first 50 principal components were extracted from the cell cycle-regressed matrix.

Subsequently, the shared nearest neighbors were calculated from the principal components using buildSNNGraph of R software scan using the k parameter of 40.

Seven clusters were identified and using the walktrap algorithm, with the function cluster\_walktrap of R implementation of the igraph package version 1.3.5.

The uniform manifold approximation and projection (UMAP) was performed.

Differential gene expression was performed via the Seurat package using MAST.

Pseudotime was conducted with Monocle in R, while velocity was conducted with scVelo in python.

Cluster annotation was performed via clusterProfiler package version 4.2.2, and differential expression visualization using EnhancedVolcano version 1.12.0.

## Cell preparation for survival surgery and intracranial transplantation

- 10 hESCs-derived dopamine neurons that were sorted based on either NURR1::H2B-GFP or CD49e low and CD184 high were resuspended in  $100,000 \pm 10,000$  cells/mL in neurobasal medium with 200 mM L-glutamine and 100 mM ascorbic acid (AA) transplantation medium (without human albumin or kedbumin 25%).

Unless specified, 3–4  $\mu$ L of sorted neurons were injected at the rate of 0.5–1  $\mu$ L per minute (1 mL per deposit) into the striatum of wild-type (unlesioned) 6 to 8-week-old male NSG mice ([AP] +0.5mm, [ML] +/- 1.8mm, [DV] -3.4 to -3.3 mm from dura).

Each surgery did not exceed more than 30 minutes per animal and the entire surgery time was within 8–10 hours post cell preparation.

For the short-term 1-month survival study, p53 WT (-dox) vs. KO (+dox) NURR1::GFP+ dopamine neurons or CD + PBS vs. CD + adalimumab was bilaterally engrafted into the striatum of the same NGS mouse brain to reduce variability between animals.

For the short-term 7 days survival study, NURR1::GFP+ human dopamine neurons were grafted into the 6 to 8-week-old immunocompetent male and female TNFa WT and KO (Jackson laboratory: IMSR\_JAX:005540; 129S mouse strain background) in the presence or absence of adalimumab.

TNFa KO mouse was generously gifted from Dr. Li Gan lab from Weill Cornell Medicine.

TNFa KO human NURR1::GFP+ dopamine neuron in PBS vs. adalimumab was bilaterally implanted into the striatum of the same NGS mouse brain to determine the survival of the graft 7 days post transplantation.

For the allograft setting, mouse dopamine neuron (background for 129S mouse strain) was transplanted into the striatum of the immunocompetent mouse brain that is 6 to 8-week-old non-isogenic C57BL/6 (B6) mouse strain. The survival was validated 7 days post transplantation.

For other studies including time course or behavior studies, cells are engrafted unilaterally. For the time course experiments, the mice were euthanized and used for immunohistochemistry analysis at designated time points (4 hrs, 1 day, 3 days, and 7 days post engraftment).

For in vivo CRISPR screen, a maximum feasible dose of the cells was injected. Cells were suspended at a cell density of  $200,000 \pm 10,000$  cells/ $\mu$ L and a total of 4 mL each was injected into each striatum in order to have sufficient representation of the guide RNAs after in vivo isolation.

## High throughput cell surface marker screen and enrichment of dopamine neuron with using cell surface markers

- 11 Dopamine neuron differentiated cells at day25 from the NURR1::GFP reporter hESC were single cell suspended in flow cytometer staining buffer (PBS containing 2% bovine serum albumin).

The cells were stained with 387 cell surface (CD) markers (0.2M cells per a CD marker) for 30 min on ice in the dark.

After 3 times washing with PBS, cells were co-stained with DAPI.

All staining for the screen was done in 96 well plates.

Data collection using a flow cytometer to identify CD markers to enrich GFP positive population was performed by the MSKCCFlow Cytometry core facility.

For enrichment of dopamine neuron with using CD markers, 49e and 184, day 25 cells were stained with 49e and/or 184, followed by isolation of 49e weak, 49e weak and 174 high, and 49e weak and 184 high expressed cells via FACS at the MSKCC Flow Cytometry core facility.

## Single-strand guide RNA (sgRNA) Design and Cloning

- 12 sgRNA sequences for pool library were identified by Guidescan (MSKCC) and sgRNA oligos were synthesized on-Chip (Agilent).

Synthesized oligos were PCR amplified and amplicons were restriction cloned into SGL40C.EFS.dTomato (Addgene#89395).

Library representation was assessed by NGS (Illumina).

Individual sgRNA for dTomato and p53, designed by web-based tool (<http://crispor.tefor.net>) and using Guidescan (MSKCC) subsequently, were restriction cloned into SGL40C.EFS.dTomato plasmid vector (Addgene#89395).

## sgRNA barcode Sequencing and Analysis to identify targets

- 13 Cell pellets at each desired time point were lysed, and genomic DNA was extracted (Qiagen) and quantified by Qubit (Thermo Scientific).

A quantity of gDNA covering 1000X representation of sgRNAs was PCR amplified to add Illumina adapters and multiplexing barcodes.

Amplicons were quantified by Qubit and Bioanalyzer (Agilent) and sequenced on Illumina HiSeq 2500.

Sequencing reads were aligned to the screened library and counts were obtained for each gRNA at MSKCC Gene Editing and Screening Core Facility.

The resulting single end reads were checked for quality (FastQC v0.11.5) and processed using the Digital Expression Explorer 2 (DEE2) workflow.

Adapter trimming was performed with Skewer (v0.2.2).

Further quality control done with Minion, part of the Kraken package.

Differential gRNA hits were identified using EdgeR, a Bioconductor package, to identify the primary hits.

We used the Trimmed Mean of M-values for normalization and the glmQLFTest / F test for statistic tests.

Additionally, we used the camera analysis function from EdgeR for gene-level analysis as previously described.

To calculate the correlation between the screen samples, quantifications were normalized by the estimate SizeFactorsForMatrix of DESeq2 using only the non-targeting control and safe harbor probes.

Pearson correlation of the pairwise comparison was plotted using an R package pheatmap (<https://CRAN.Rproject.org/package=pheatmap>)