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# *In vitro* field potential monitoring on multi-micro electrode array for the electrophysiological long-term screening of neural stem cell maturation

# Supplementary Information

## Supplementary Methods

### hiPS culture

hiPS were cultured under feeder-free conditions using GFR Matrigel-coating [Corning] and self-made mTeSR ($mTeSR_{self}$) prepared according to the original publication [1]. Human TGFβI was obtained from PeproTech, human FGF-2 was prepared via bacterial overexpression in *E. coli* Rosetta pLysS and tested for activity and functionality in $mTeSR_{self}$-based stem cell culture in comparison to commercially available FGF-2 [ProSpec] (Supplementary Fig. S7). An EDTA splitting procedure was applied for passaging [2]. The applicability of the established $mTeSR_{self}$-based cell culture procedure was qualitatively and quantitatively validated via staining of pluripotency markers over at least 30 passages (Supplementary Fig. S8) compared to $mTeSR^{TM}1$.

### Flow cytometry

Cells were incubated at 4 °C overnight with primary antibodies (Supplementary Tab. S3) diluted in 5 % BSA, 0.25 % Triton in PBS. Afterwards, secondary antibodies (Supplementary Tab. S3) were diluted 1:100 in 5 % BSA, 0.25 % Triton in PBS and applied for 2 hours at room temperature. Cell nuclei were stained for 10 min at room temperature using 7-AAD (2 µl per 500 µl 0.1 % Triton in PBS; BD Pharmingen). For double staining, the above mentioned secondary antibodies were combined with a Cy5 variant (115-176-062 or 111-176-045; Dianova). Cell nuclei were stained with POPO-

3 iodide (1:60,000; Sigma-Aldrich). For analysis of cell populations, the flow cytometer FACSCalibur (BD Bioscience) with the bandpass filters 670 (Cy5, 7-AAD), 530/30 (Cy2) and 585/42 (POPO-3) was used. Per sample 10,000 cells were measured. Cells were gated positive or negative relative to the respective positive/negative controls.

Immunocytochemical analysis

Cells on glass coverslips were fixed directly in the culture medium using formaldehyde at a final concentration of 4 % for 10 min. Fixed cells were stored at 4 °C in Phosphate buffed saline (PBS). For staining, cells were treated with blocking solution (5 % BSA in 0.25 % Triton X-100/PBS) for 45 min and afterwards incubated with primary antibodies (Supplementary Tab. S3) diluted in blocking solution at 4 °C overnight. Secondary antibodies (Supplementary Tab. S3) diluted 1:100 in blocking solution were applied for 2 h at room temperature. Cell nuclei were marked with DAPI (1 µg/ml [AppliChem], in PBS) for 2 min at room temperature. After glycerol gelatin conservation, staining was analyzed using an inverse Nikon Ti microscope with an A1R+ confocal laser scanning module and the Nikon NIS Elements 4.2 imaging software.

Quantification of immunocytochemical staining fluorescence intensities

Images were all generated using the same protocol and documentation software parameter. Mean fluorescence intensities of stained areas of single channel RGBs were analyzed applying the mean grey value function of the Image J software. All values were plotted relative to the respective DAPI fluorescence intensity for cell number normalization or, for MAP-2 costaining, to the MAP-2 fluorescence intensity.

Quantitative real-time (qRT) PCR

Total RNA was isolated using the RNeasy® Protect kit (Qiagen) according to the manufacturer's protocol. 1 µg of total RNA was reverse transcribed applying the Accu Power® CycleScript RT PreMix (dT20) (Bioneer) following distributor's instruction. mRNA samples were diluted in a ratio of 1:30 with RNase free water. For qRT-PCR, 5 µl of mRNA were mixed with 10 µl GoTaq® qPCR Master Mix (Promega), 4 µl DEPC water and 0.5 µl of the primers (sense und antisense) using a pipetting robot (Corbett Robotics). The qRT-PCR was carried out in the Real-Time Thermal Cycler Rotor-Gene RG-3000 (Corbett Life Science). The applied intron-spanning primers are listed in Tab.S4. PCR products were qualitatively analyzed via an agarose gel electrophoresis containing ethidium bromide for cDNA visualization. For quantification, the number of amplification cycles for the fluorescence signal to reach a common threshold value (Ct) was analyzed and fitted into a Ct standard curve of samples with known cDNA copy number. Based thereon, relative gene expression was determined as a percentage of the housekeeping gene GAPDH.

## Supplementary References

1. T. E. Ludwig, V. Bergendahl, M. E. Levenstein, J. Yu, M. D. Probasco and J. A. Thomson, *Nat Methods*, 2006, **3**, 637-646.
2. J. Beers, D. R. Gulbranson, N. George, L. I. Siniscalchi, J. Jones, J. A. Thomson and G. Chen, *Nat Protoc*, 2012, **7**, 2029-2040.

## 2. Supplementary Tables

Tab. S1. hiPS generation characteristics and origin.

| nomen-clature | hiPS line | original cell type | reprogramming factors | reprogramming strategy | distributor |
|---|---|---|---|---|---|
| hiPS1 | IMR90-4 | lung | OCT3/4, SOX2, LIN28, KLF4 | lentiviral | WiCell |
| hiPS2 | IMR90c01 | | OCT3/4, SOX2 | retroviral | I-Stem |
| hiPS3 | 4603c27 | dermal fibroblast | OCT3/4, SOX2, KLF4, C-MYC | | |

Tab. S2. Composition of NDM.

| Component | Concentration |
|---|---|
| DMEM/F12 | 1:1 |
| GlutaMAX™ | 1 % |
| Glucose | 33.3 mM |
| Insulin | 0.0025 % w/v |
| Apo-Transferrin | 0.0095 % w/v |
| HEPES | 5 mM |
| Heparin | 0.0004 % w/v |
| Sodium selenite | 30 nM |
| sodium bicarbonate | 3 mM |
| Putrescine | 60 µM |
| Progesterone | 20 nM |
| cAMP | 0.91 µM |
| Ascorbic acid | 300 µM |

Tab. S3. Antibodies.

| Antibody | clone | distributor | dilution |
|---|---|---|---|
| goat anti-ChAT | AB144P | Millipore | 1:100 |
| rabbit anti-GAD65/67 | AB1511 | Millipore | 1:1000 |
| mouse anti-Nestin | MAB5326 | Millipore | 1:750 |
| rabbit anti-NF200 | N4142 | Sigma-Aldrich | 1:500 |
| mouse anti-NF-L | sc-20012 | Santa Cruz Biotechnology | 1:100 |
| mouse anti-Oct34 | sc-5279 | Santa Cruz Biotechnology | 1:300 |
| rabbit anti-Pax6 | PRB-278P | Covance | 1:300 |
| rabbit anti-S100 | Z0311 | Dako | 1:500 |
| goat anti-Sox2 | sc-17320 | Santa Cruz Biotechnology | 1:100 |
| mouse anti-SSEA4 | MAB4304 | Millipore | 1:750 |
| rabbit anti-TH | AB152 | Millipore | 1:1000 |
| mouse anti-TRA-1-60 | FCMAB115F | Millipore | 1:100 |
| rabbit anti-vGlut1 | 135 303 | SYSY | 1:500 |
| Cy2 IgG (H+L), goat anti-rabbit | 111-225-003 | Dianova | 1:100 |
| Alexa-488 IgG (H+L) goat-anti-mouse | 115-546-062 | Dianova | 1:100 |
| Cy3 IgG (H+L), goat anti-mouse | 115-166-062 | Dianova | 1:100 |
| Cy3 IgG (H+L), donkey anti-goat | 705-165-147 | Dianova | 1:100 |

Tab. S4. Primer sequences.

| Gene | Primer sense | Primer antisense | Prod. length |
|---|---|---|---|
| PAX6 | GGCAGGTATTACGAGACTGG | CCTCATCTGAATCTTCTCCG | 427 bp |
| NES | CAGCGTTGGAACAGAGGTTGG | TGGCACAGGTGTCTCAAGGGTAG | 389 bp |
| OCT4 | CCCGCCGTATGAGTTCTGTG | CTGATCTGCTGCAGTGTGGGT | 591 bp |
| GATA4 | TCCAAACCAGAAAACGGAAG | AAGACCAGGCTGTTCCAAGA | 352 bp |
| GATA6 | TCTACAGCAAGATGAACGGCCTCA | TCTGCGCCATAAGGTGGTAGTTGT | 125 bp |
| MEOX1 | GAAACCAGACTTCCTGGCGA | GAGAGGTCCAGGTTTACCGC | 492 bp |
| GAPDH | GACAGTCAGCCGCATCTTCT | AAATGAGCCCCAGCCTTCTC | 391 bp |
| NEFL | ATGCAGGACACGATCAACAA | CTTCGATCTGCTCCTCTTGG | 322 bp |
| GFAP | TTGAGAGGGACAATCTGGCAC | GGATCTCTTTCAGGGCTGCG | 300 bp |
| OTX2 | ACGACGTTCACTCGGGCGCAG | ACTGCTGCTGGCAATGGTCGG | 312 bp |
| EN1 | CTGGGTGTACTGCACACGTTAT | TACTCGCTCTCGTCTTTGTCCT | 357 bp |
| GBX2 | GGCGGTAACTTCGACAAGG | TCAGATTGTCATCCGAGCTG | 238 bp |
| KROX20 | TTGACCAGATGAACGGAGTG | TGGTTTCTAGGTGCAGAGACG | 121 bp |
| TH | CCCCTGGTTCCCAAGAAAAGT | TCCAGCTGGGGGATATTGTCTTC | 330 bp |
| VGLUT | CGCTACATTATCGCCATCATGAG | GGTGGGGCCCATTTGCTCCA | 416 bp |
| VGAT | CCGTGTCCAACAAGTCCCAG | TCGCCGTCTTCATTCTCC | 490 bp |
| TPH1 | TCTATACCCCAGAGCCAGATACCT | AGTAGCACGTTGCCAGTTTTTG | 150 bp |
| DBH | CCACTGGTGATAGAAGGACGAAA | GGCCATCACTGGCGTGTAC | 120 bp |
| CHAT | ATCGCTGGTACGACAAGTCC | ATCAGCTTCCTGCTGCTCTG | 151 bp |

Tab. S5. Neurotransmitter, applied concentration and distributor.

| Neurotransmitter | Concentration | Chemical | Nr./Distributor |
|---|---|---|---|
| Dopamine | 100 µM | Dopamine hydrochloride | A11136/Th. Geyer |
| Acetylcholine | 100 µM | Acetylcholine chloride | A2661/Sigma-Aldrich |
| Norepinephrine | 200 µM | Norepinephrine-bitrate salt | A0937/Sigma-Aldrich |
| Glutamic acid | 100 µM | L-Glutamic acid monosodium salt monohydrate | 49621/Sigma-Aldrich |
| GABA | 1 mM | Gamma-aminobutyric acid | A5835/Sigma-Aldrich |
| Serotonine | 100 µM | Serotonin hydrochloride | H9523/Sigma-Aldrich |

## 3. Supplementary Figures

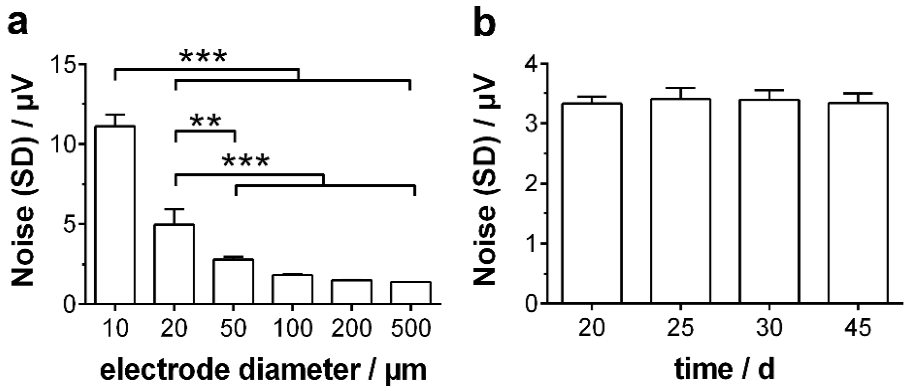


Fig. S4. Characterization of the influence of the electrode diameter on signal noise and long-term stability. (**a**) Analysis of noise standard deviation of recorded field potentials from electrodes with 10 – 500 µm diameter. n = 4 electrodes. (**b**) Analysis of noise standard deviation over prolonged measurement periods (day 20 – 45).

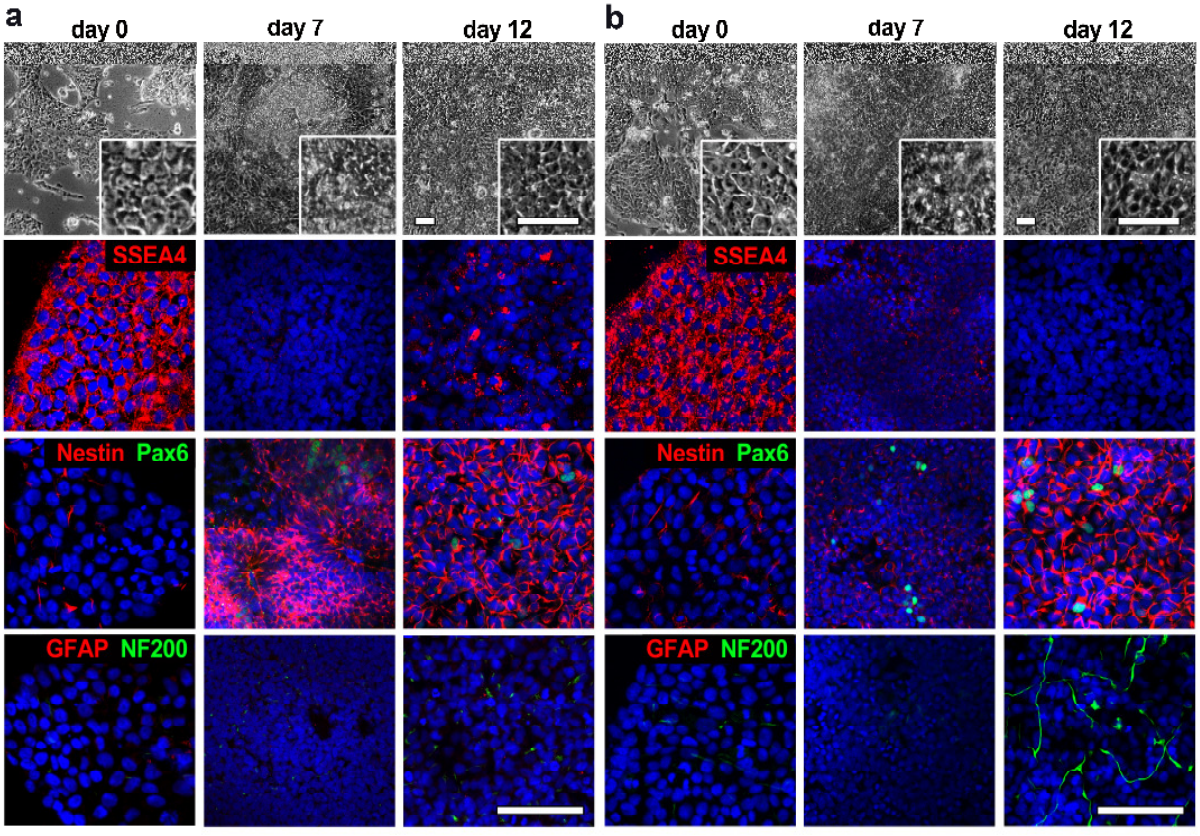


Fig. S2. Morphological as well as marker-specific immunocytochemical analysis of neural induction of the (**a**) hiPS1 and (**b**) hiPS3 line. The NPC marker induction of Nestin and Pax6 as well as the neuronal marker NF200 were analyzed. Furthermore, regulation of stem cell marker SSEA4 was monitored. Scale bar 100 µm.

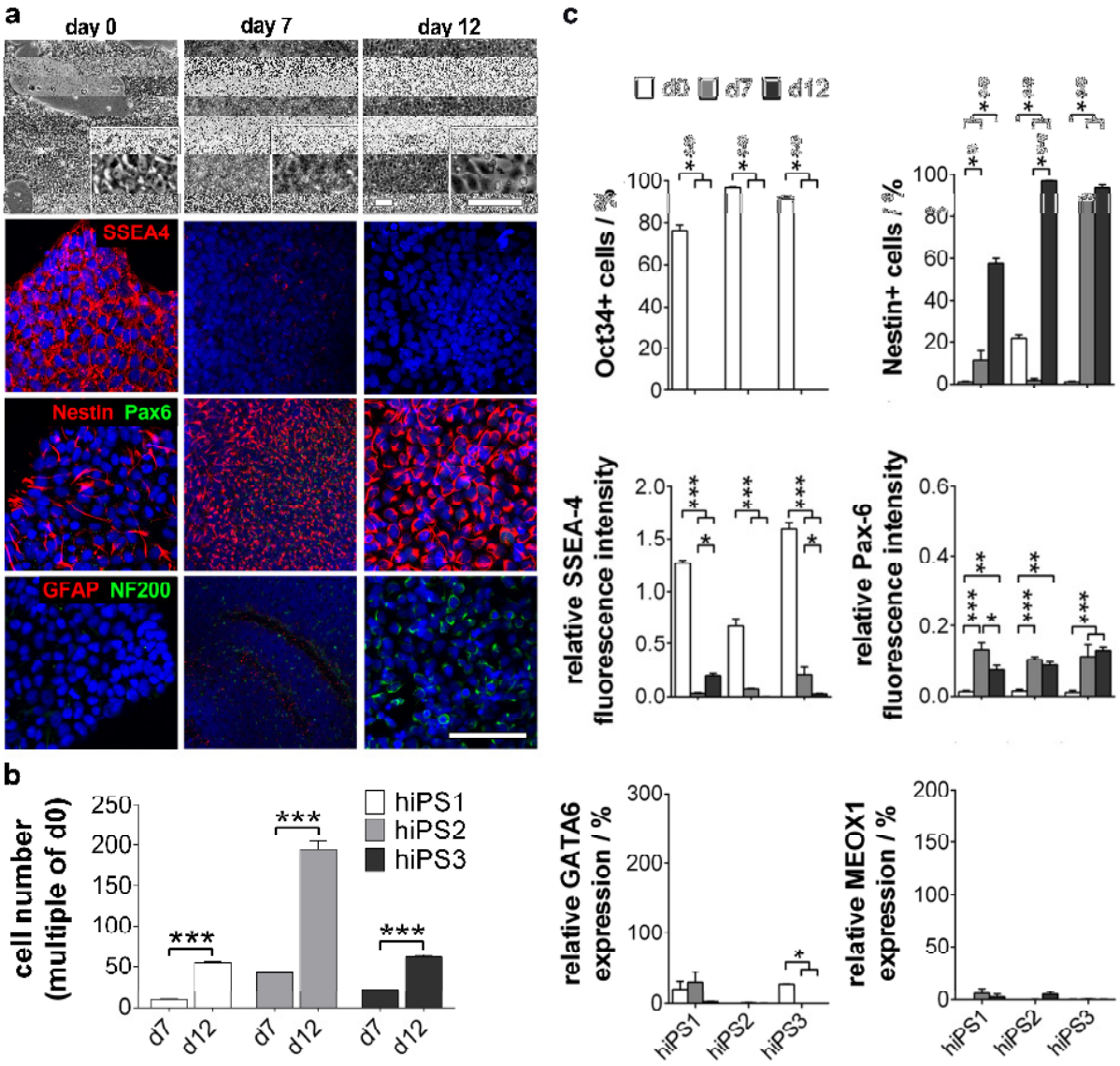


Fig. S3. Characterization of hiPS-dependent NPC generation process. (**a**) Morphological as well as marker-specific immunocytochemical analysis of neural induction of the hiPS2 line. The NPC marker induction of Nestin and Pax6 as well as the neuronal marker NF200 were analyzed. Furthermore, regulation of stem cell marker SSEA4 was monitored. Scale bar 100 µm. (**b**) Quantitative analysis of cell number increase during NPC generation. n = 3 experiments; mean ± s.e.m; *p < 0.05, **p < 0.01, ***p < 0.001. (**c**) Analysis of the stem cell markers Oct34 (flow cytometry) and SSEA-4 (relative immunofluorescence intensity), the early ectodermal marker Nestin (flow cytometry) and the neural progenitor marker Pax-6 (relative immunofluorescence intensity) and mesendodermal determination via GATA6 and MEOX1 (relative mRNA amount) during the NPC generation process. n = 3 experiments; mean ± s.e.m; *p < 0.05, **p < 0.01, ***p < 0.001.

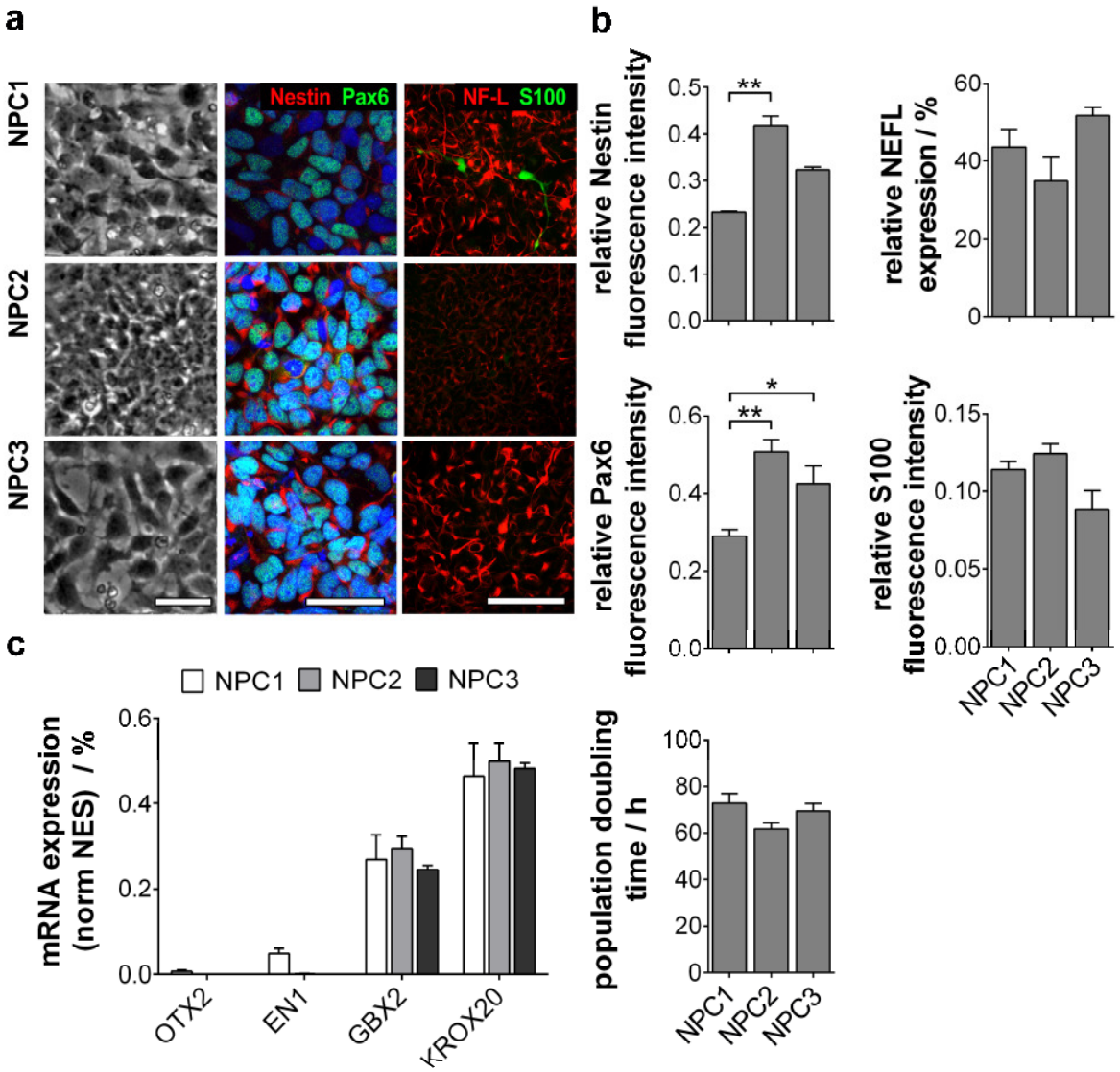


Fig. S4. Characterization of the stable NPC state. (**a**) Morphology as well as localization of ectodermal (Nestin) and early NPC marker (Pax6) as well as neural marker NF-L (developing neurons) and S100 (astrocytes) in hiPS-derived stable NPCs. Scale 50 µm. (**b**) Quantification of progenitor (Nestin, Pax6) and neural (NF-L, S100) gene/protein marker expression (n = 3 experiments) as well as population doubling time of the three established NPC populations. Quantification of population doubling time (bottom; n = 14 experiments). mean ± s.e.m; *p < 0.05, **p < 0.01, ***p < 0.001. (**c**) Analysis of NPC regional identity via expression of typical fore- (OTX2), mid- (EN1, GBX2) and hindbrain (GBX2, KROX20) marker genes relative to NES expression. normalized to GAPDH; n = 3 experiments; mean ± s.e.m; no significant differences.

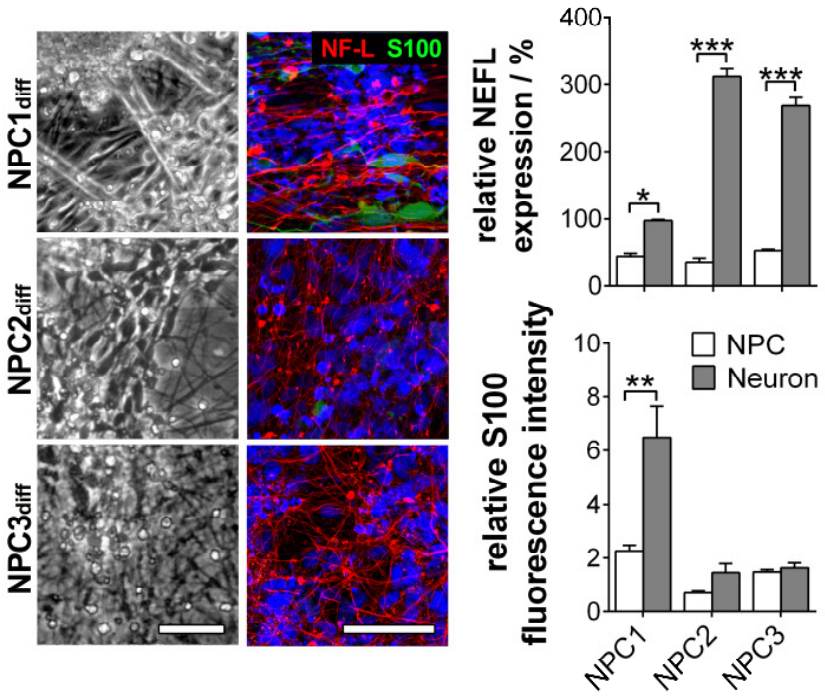


Fig. S5. NPC uniformly differentiate to a clear neural phenotype. Morphological and marker-specific characterization of the neural phenotype of 15-days differentiated NPC – NF-L (red) marks processes in neuronal cells, S100 staining (green) indicates astrocyte evolvement. Neural marker analysis via quantification of the gene expression and protein staining fluorescence intensity of NF-L and S100 before and after neuronal differentiation. scale 50 µm. n = 3 experiments; mean ± s.e.m; *p < 0.05, **p < 0.01, ***p < 0.001.

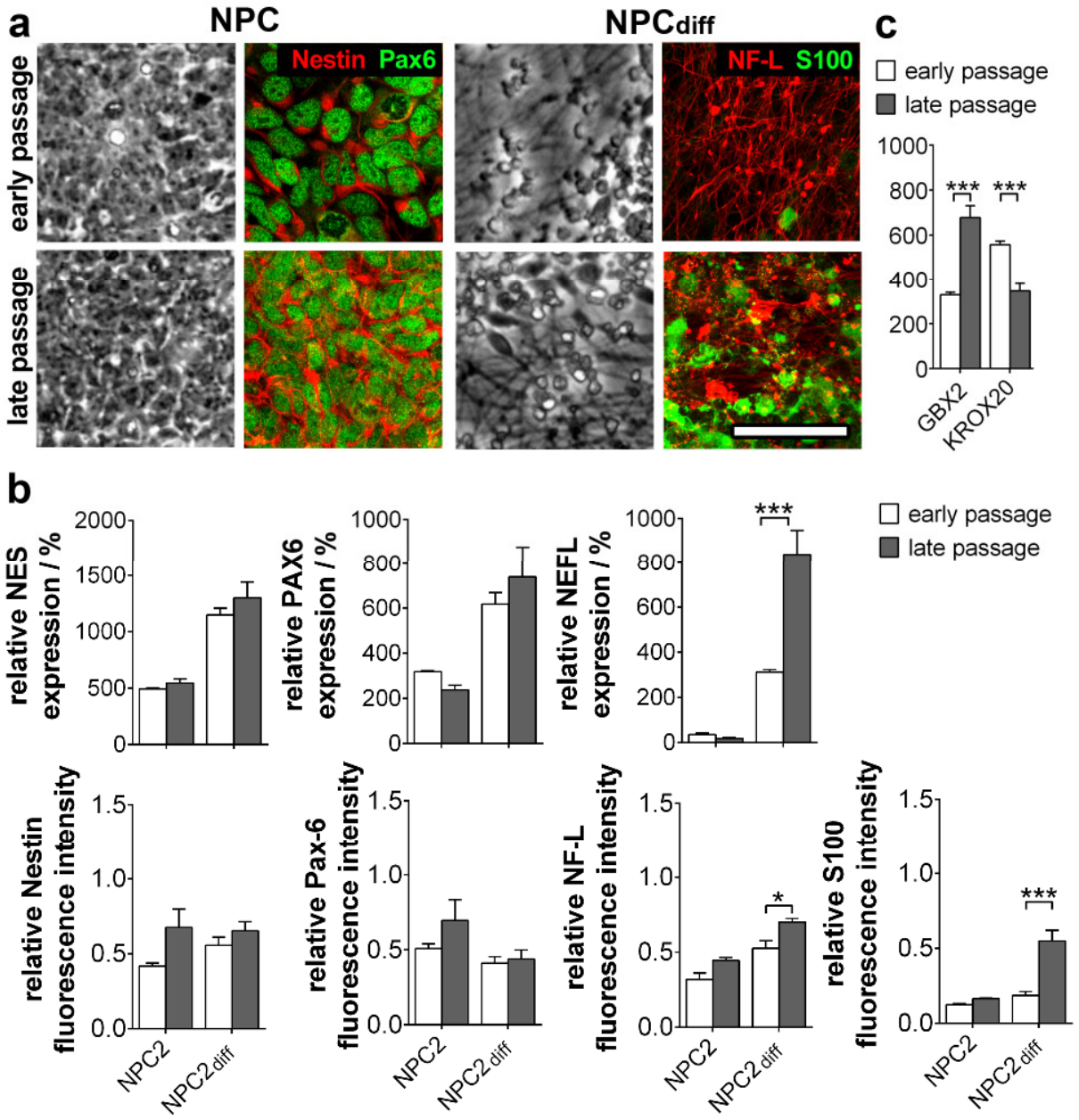


Fig. S6. Long-term cultured NPC showed no changes in neurogenic potential. (**a**) Cultured (young < 25 passages; old > 80 passages) and 15-day differentiated $NPC2_{diff}$ were stained for the NPC markers Nestin (red) and Pax6 (green), the neuronal marker NF-L (red) and the astrocyte marker S100 (green). Scale bar 50 µm. (**b**) Analysis of basal NPC and neuronal marker gene expression of NPC2 comparing an early passage (< 30) with a late passage (> 80) number (top). Fluorescence intensity of marker staining was as well quantified and compared between early and late passage NPC2 (bottom) and (**c**) Identification of regional patterning shifts during long-term cultivation via gene expression profile of young (p: 23 – 26) and old (p: 80 – 90) NPC2 before and after differentiation. n = 3 experiments; mean ± s.e.m; *p < 0.05, ***p < 0.001.

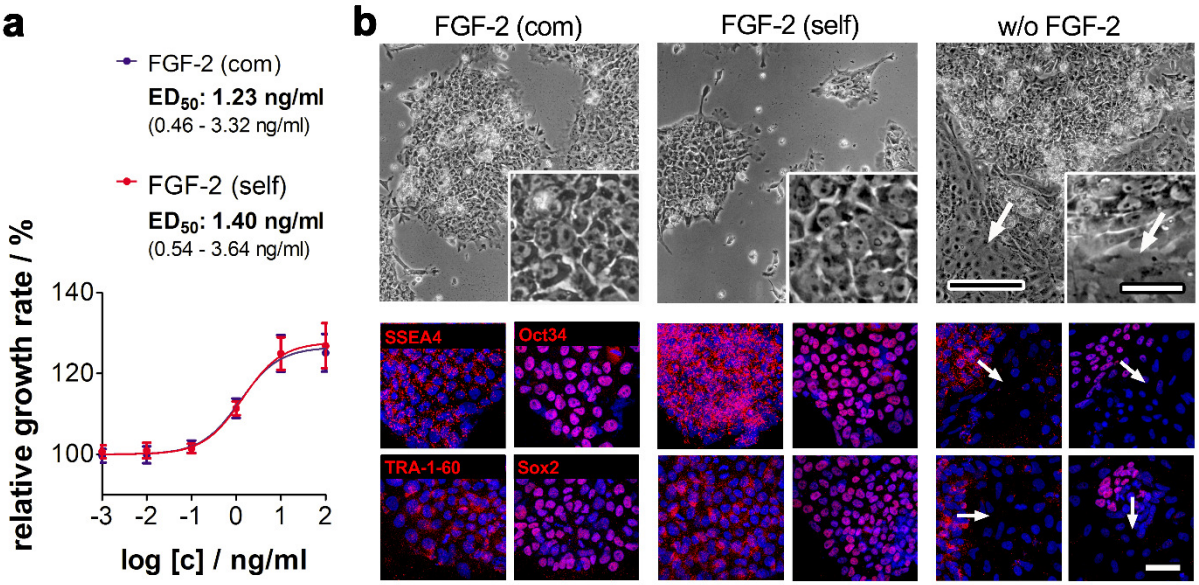


Fig. S7. Activity and functionality test of self-made hFGF-2 compared to commercial hFGF-2 in IMR90-4 cells. (**a**) Activity of FGF-2 was tested via a BHK-21 proliferation assay showing comparative $ED_{50}$ values for commercial and self-made FGF-2. N = 9, mean ± s.e.m. (**b**) Morphological and marker-specific analysis of stemness revealed high-quality stem cell culture for both tested FGF-2 products, whereas refusal of FGF-2 lead to diminished expression of Oct34, Sox2, SSEA4 and TRA-1-60 (arrows).

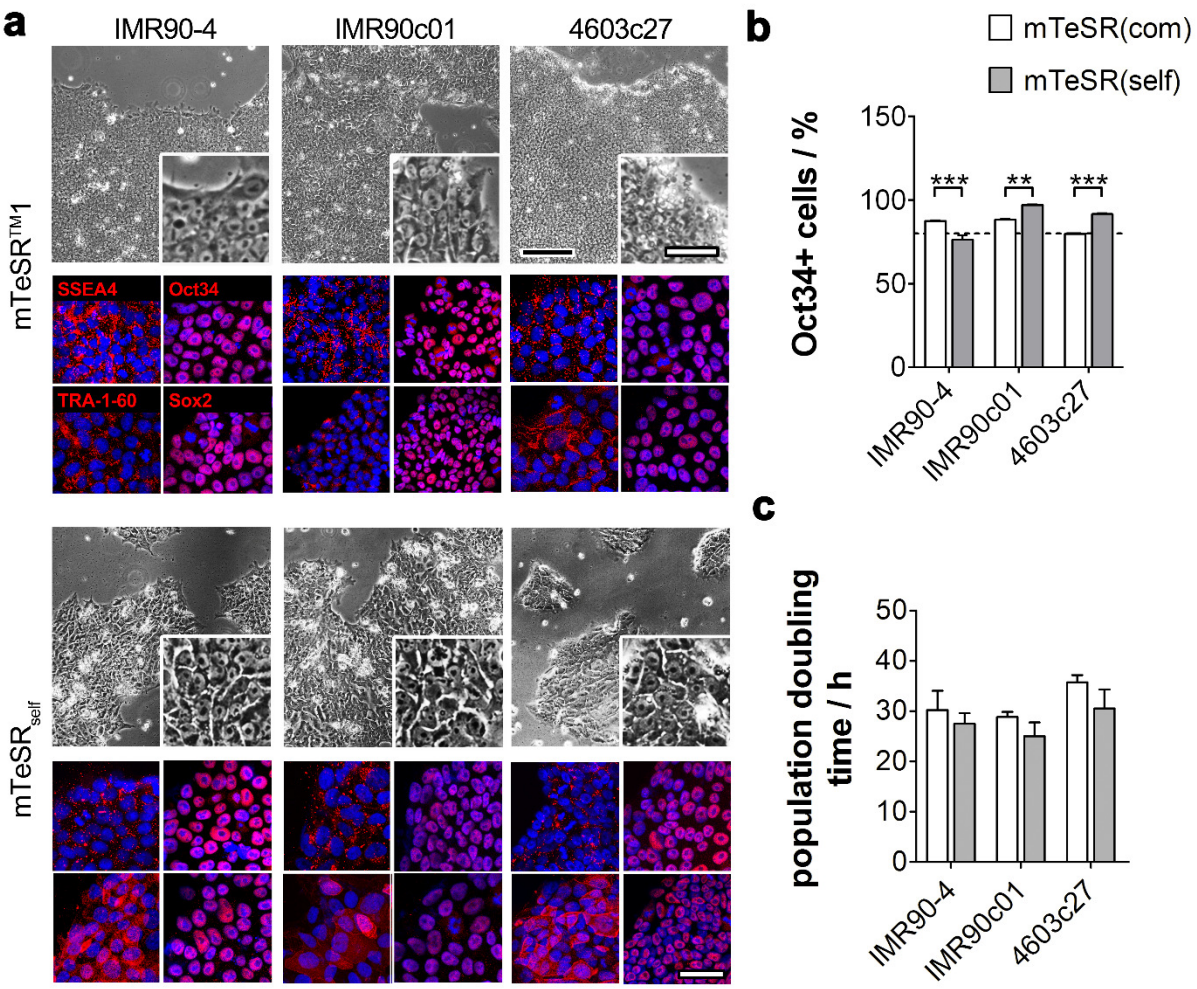


Fig. S8. Marker-based analysis of mTeSR$_{self}$-based culture quality in comparison to mTeSR$^{TM}$1. (**a**) Staining of Oct34, Sox2, SSEA4 and TRA-1-60 additionally supports high-quality stem cell culture using mTeSR$_{self}$ over 30 passages (black scale 200 µm; white scale 50 µm). (**b**) Flow cytometry revealed comparative or elevated Oct34-positive cell percentages for self-made vs. commercial mTeSR after 15 passages. n = 3 experiments; mean ± s.e.m; **p < 0.01, ***p < 0.001. The critical threshold for a good-quality stem cell culture (80 %) is indicated as dashed line. (**c**) Proliferation analysis showed no significant differences in population doubling time. n = 14; mean ± s.e.m.