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Supplementary Materials for

High-throughput 3D screening for differentiation of hPSC-derived cell therapy candidates

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Published 7 August 2020, *Sci. Adv.* **6**, eaaz1457 (2020)
DOI: 10.1126/sciadv.aaz1457

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/32/eaaz1457/DC1)

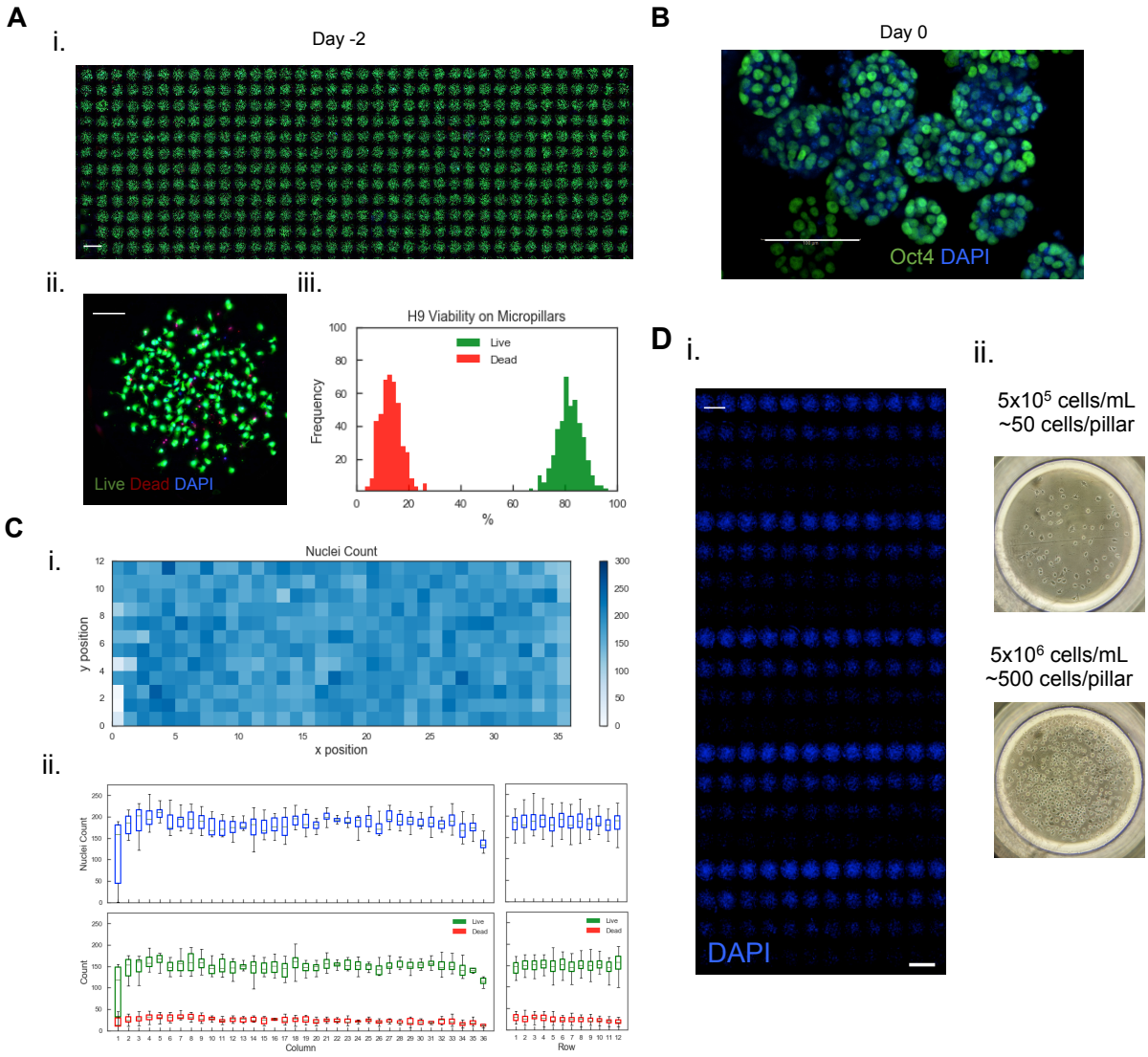

Figure S1. H9 seeding, viability, and pluripotency on the micropillar culture chip. A) i. Montage of 432 micropillar H9 culture environments stained with Hoechst (blue), calcein AM (green) and ethidium homodimer (red) to assess viability after the robotic dispensing process; scale bar represents 1 mm. ii. Image of a single microenvironment; scale bar represents 100 microns. iii. Histograms of percentage live and dead cells across the micropillar culture chip. B) Immunocytochemistry of 3D hPSC culture at Day 0 showing Oct4 (green) expression; scale bar represents 100 microns. C) i. Heatmap of total cell count across all positions on the micropillar culture chip. ii. Boxplots of i. total and ii. live/dead cell counts across every column and row of the micropillar chip. Scale bars represent 100 microns. D) i. Controlled modulation of seeding density of cells onto micropillars shown by fluorescence microscopy of Hoechst stained cells seeded at four different densities consistent across the micropillar chip; scale bar represents 100 microns. ii. Bright field images of micropillar cultures seeded at ii. $5x10^6$ cells/mL or roughly 500 cells per micropillar and $5x10^5$ cells/mL or roughly 50 cells per micropillar; pillar diameter is 750 microns.

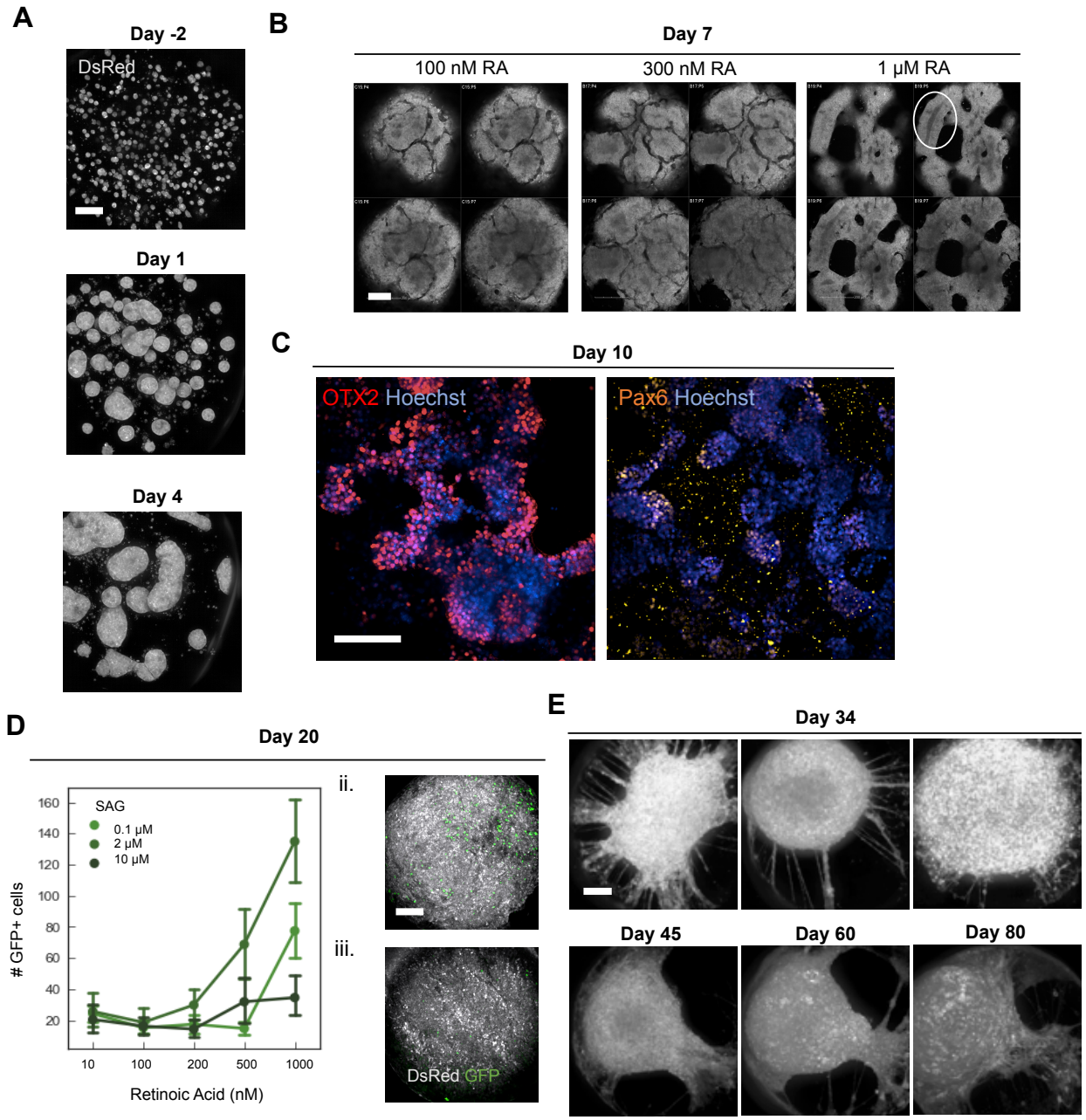

Figure S2. Longitudinal study of proliferation and differentiation on micropillar platform. A) Fluorescent confocal images of DsRed fluorescence from H9s cultured in 3D Matrigel from Day -2 to Day 4; scale bar represents 100 microns. B) Z-planes of 3D structures forming in the differentiating cultures at Day 7; scale bar represents 100 microns. C) OTX and Pax6 expression in differentiating cultures at Day 10; scale bar represents 100 microns. D) Quantification of GFP+ cells after 20 days of differentiation in 15 different combinations of RA and SAG concentrations with fluorescent confocal images of DsRed (gray) and GFP (green) at ii. 2 μM SAG + 1000 nM RA and iii. 10 μM SAG + 1000 nM RA conditions. Error bars represent 90% confidence interval from 4 technical replicates. E) Morphological changes into mature neural cells are visible by Day 34 and continue to develop beyond Day 45 to Day 80 of differentiation; scale bars represent 100 microns.

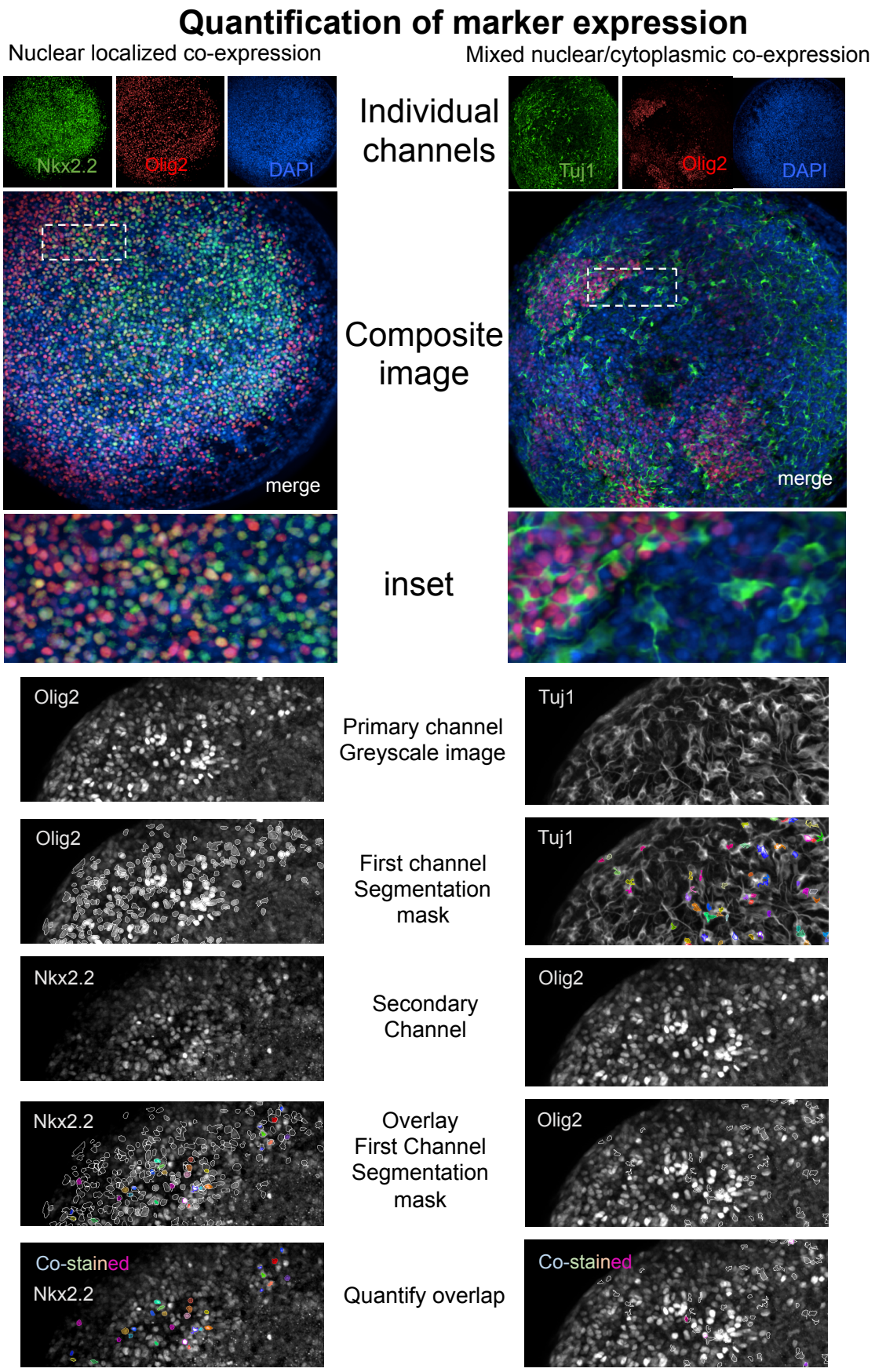

Figure S3. Image analysis pipeline for quantification of nuclear and cytoplasmic cellular markers and co-expression.

3-dimensional Image Scoring

The fluorescence microscopy readout used in this methodology is in the form of image data, which is commonly quantified by segmenting cells to score as positive or negative for marker expression. Dense cell populations can confound scoring methodology, which is further complicated if cellular markers are localized in the cytoplasm because of overlapping cell bodies. Therefore, if possible, it is advantageous to choose nuclear localized cell specific markers in the design of the screening experiment, such as Olig2 and Nkx2.2 expression for OPC differentiation. (Figure 3)

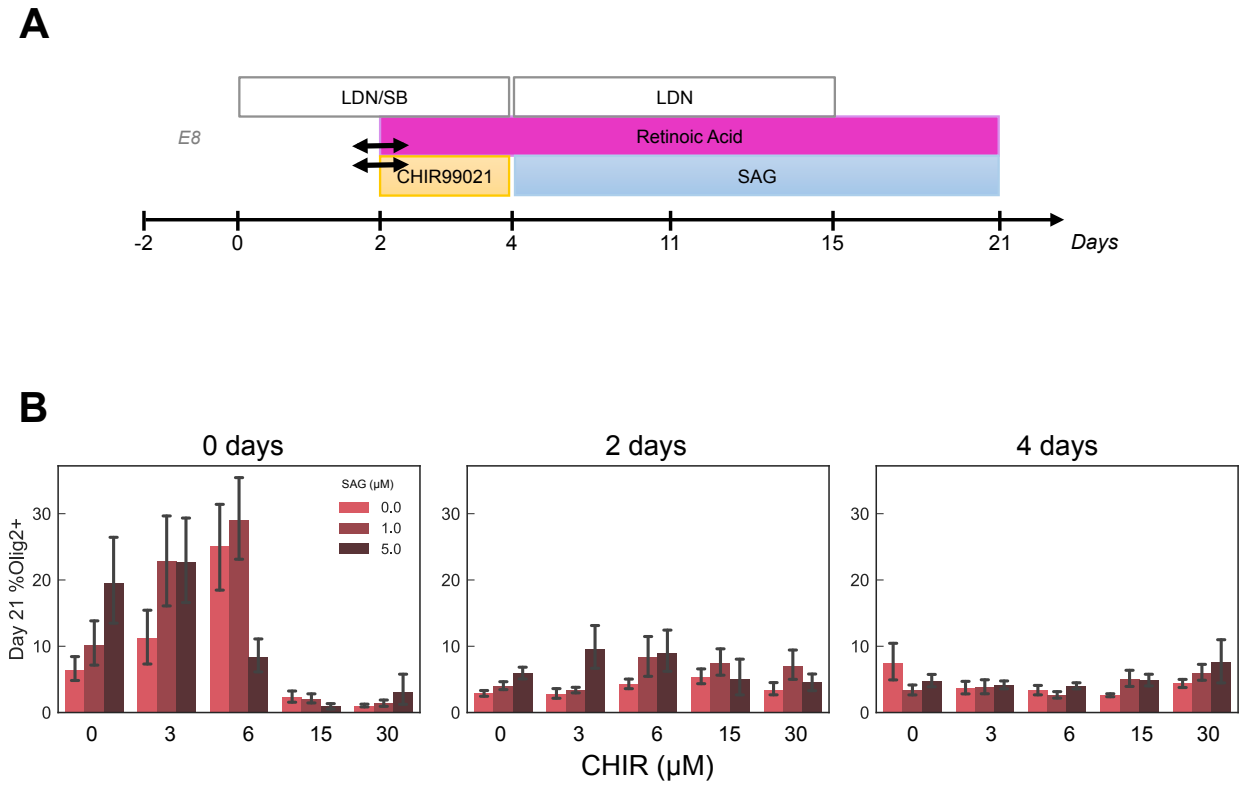

Figure S4. Timing of addition of signaling cues during neural induction affects OPC differentiation efficiency. A) Timeline of soluble signaling cues. B). Olig2+ cells at Day 21 in response to three different time points for addition of RA and CHIR, followed by SAG. Error bars represent 95% confidence intervals from 4 technical replicates.

Toxicity Artifacts

The bi-phasic relationship of Olig2 expression with respect to SAG and CHIR dosing is a unique trend and the cause may be difficult to attribute to biological signaling phenomena alone because of the DMSO solvent. Broadly, the use of DMSO as a solvent for numerous small molecules is a technical limitation that may constrain the upper limit of dosing assays, especially combinatorial assays, as previous reports have identified toxicity of hPSCs to DMSO above 0.5%.*(55)* and we have seen toxic effects at and above 2% (Figure S5C). To somewhat mitigate this confounding effect, it may be necessary to concentrate stock solutions of all small molecules as high as the solubility limit will allow. (Figure S5C)

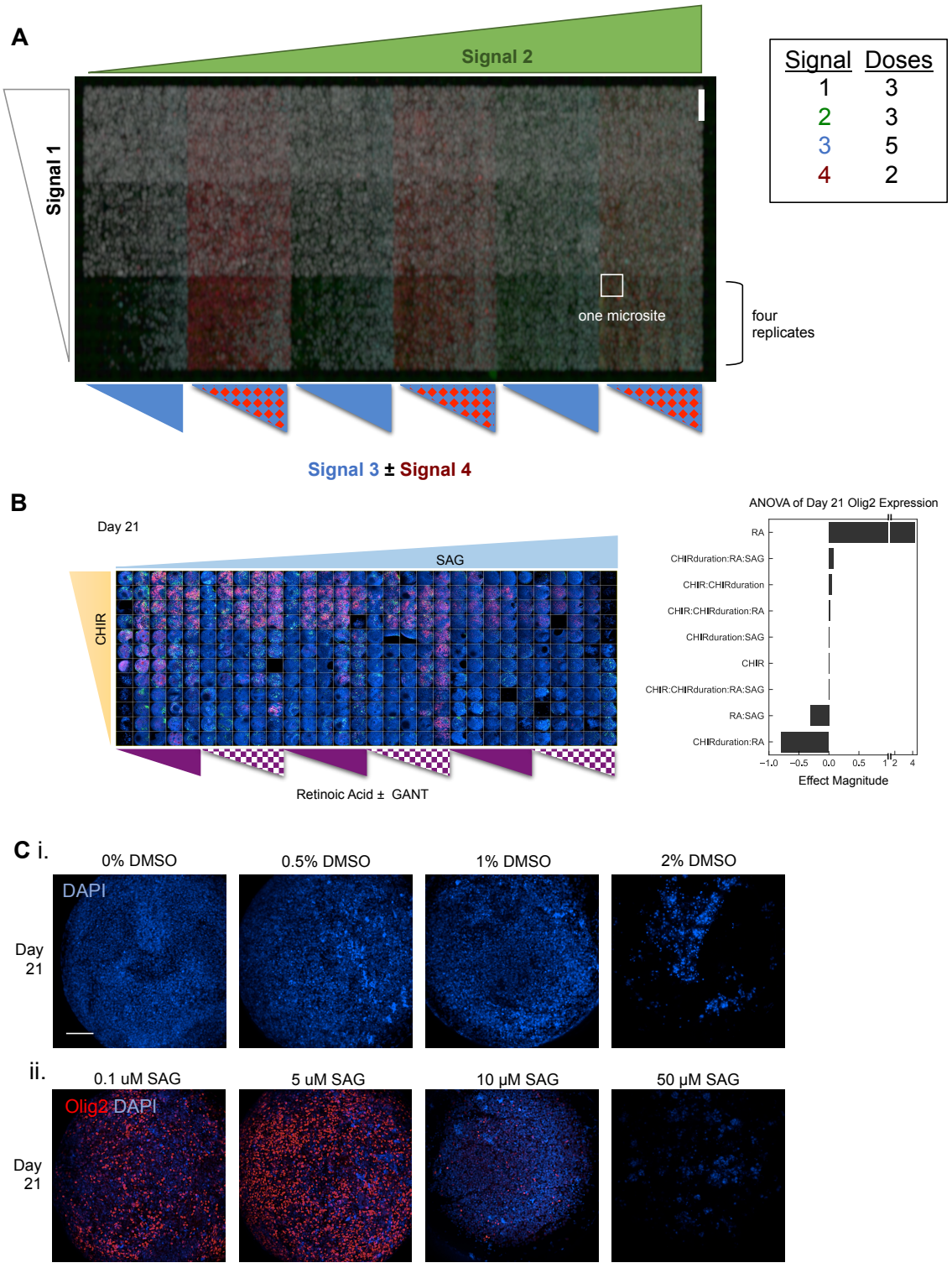

Figure S5. Combinatorial liquid dispensing and DMSO and SAG dose responses. A) Validation of liquid dispensing layout for full factorial combinatorial study. Full factorial design with n=4 factors at a range of doses dispensed into the microwell chip validated by using fluorescent beads representing the identity and concentration of each factor. One chip contains 90 unique culture combinations with four technical replicates each, totaling 360 independent microsites. Scale bar represents 1 mm. B) ANOVA model of effect magnitude for all single and combined variables in the factorial experiment with RA, CHIR, and SAG. C) i. Dose response of DMSO at Day 21 of culture and ii. dose response of SAG at Day 21 of differentiation with Olig2 expression (red); scale bar represents 100 microns.

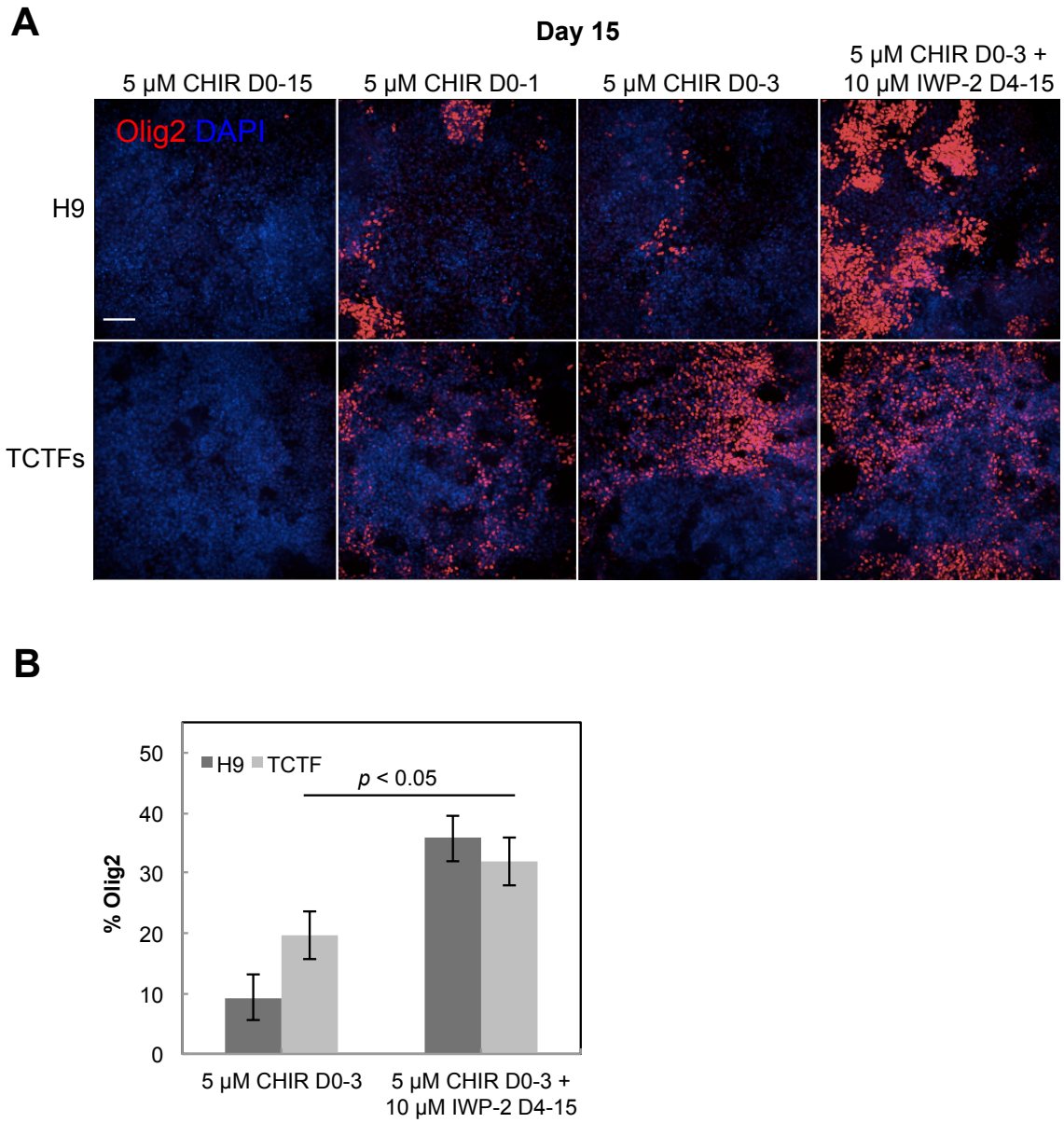

Figure S6. Replication of biphasic Wnt effect on Olig2 expression across hPSC lines. A) Olig2 expression at Day 15 across H9- and TCTF- derived OPCs in response to temporally changing Wnt signals (activated by small molecule agonist CHIR and inhibited by small molecule antagonist IWP-2) where D*x*-*y* represents period of exposure, in days, to CHIR or IWP-2; scale bar represents 100 microns. B) Quantification of Olig2 expression; error bars represent 95% confidence intervals.

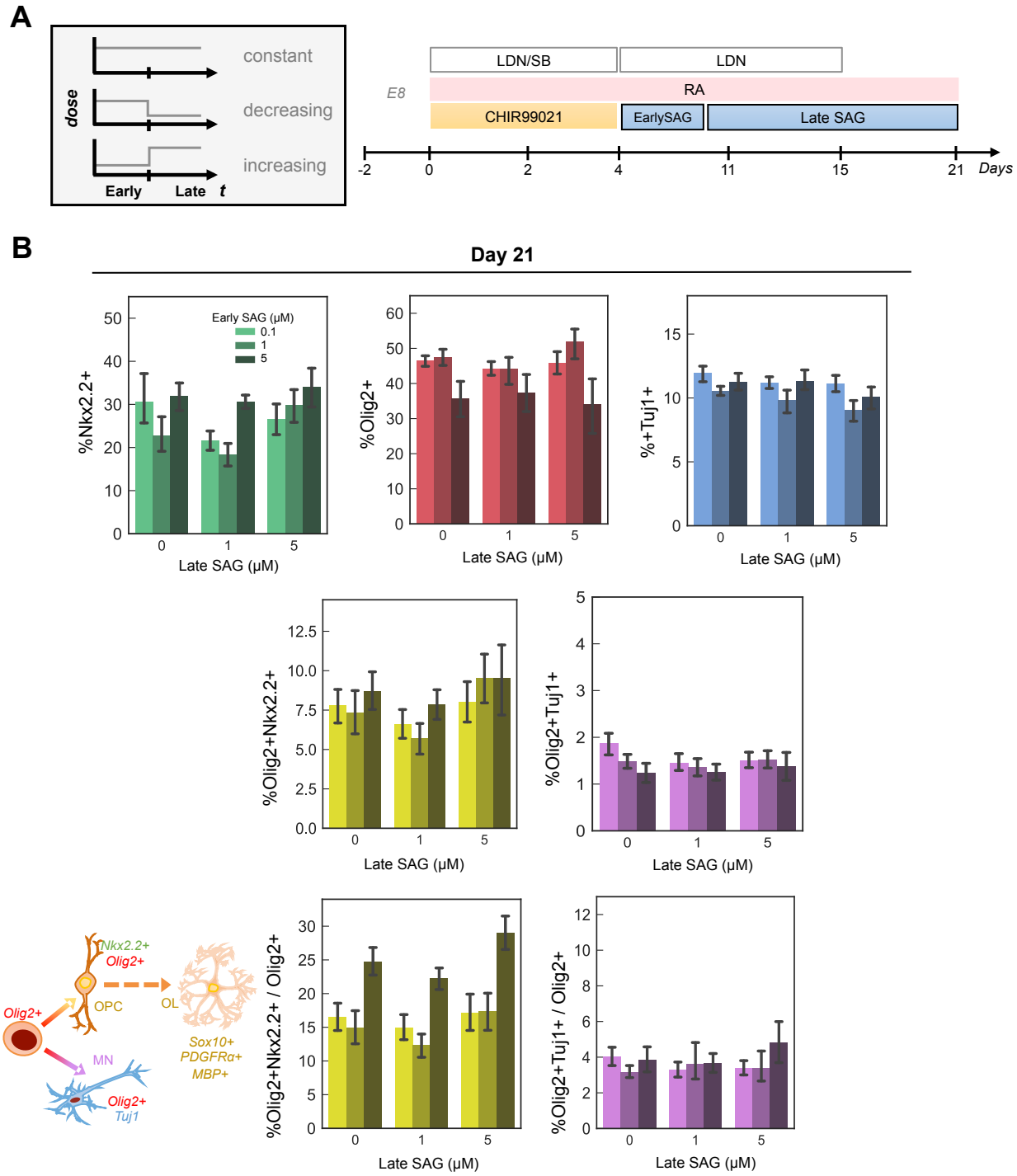

Figure S7. Temporal profiles of SAG influence OPC specification. A) Timeline of early and late windows for SAG exposure. B) Olig2+, Nkx2.2+, Tuj1+ and co-expression of markers in cells at Day 21 in response to time-varying doses of SAG. Error bars represent 95% confidence intervals from 4 technical replicates.

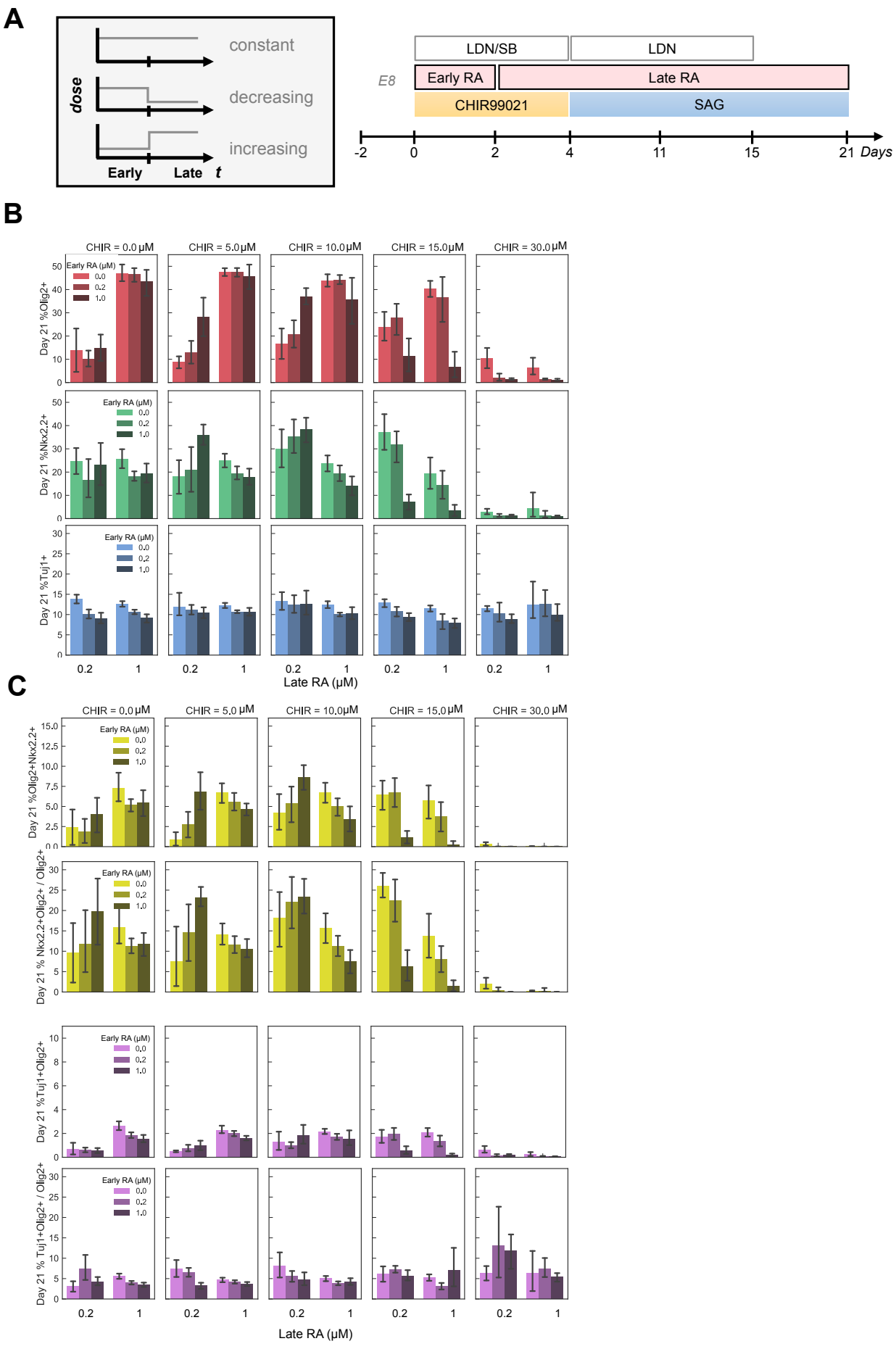
A
constant
decreasing
increasing
dose
Early
Late
t
E8
LDN/SB
LDN
Early RA
Late RA
CHIR99021
SAG
-2
0
2
4
11
15
21
Days
B
CHIR = 0.0 μM
CHIR = 5.0 μM
CHIR = 10.0 μM
CHIR = 15.0 μM
CHIR = 30.0 μM
Early RA (μM)
0.0
0.2
1.0
Day 21 %Olig2+
Day 21 %Nkx2.2+
Day 21 %Tuj1+
0.2
1
Late RA (μM)
C
Day 21 %Olig2+Nkx2.2+
Day 21 % Nkx2.2+Olig2+ / Olig2+
Day 21 %Tuj1+Olig2+
Day 21 % Tuj1+Olig2+ / Olig2+

Figure S8. Temporal profiles of RA influence OPC specification. A) Timeline of early and late windows for RA exposure. B) Olig2+, Nkx2.2+, and Tuj1+ cells at Day 21 in response to time-varying doses of RA at different CHIR doses. Error bars represent 95% confidence intervals from 4 technical replicates. C) Co-expression of Olig2, Nkx2.2 and Tuj1 cells, and proportions of co-expressing cells within Olig2+ population, at Day 21 in response to time-varying doses of RA at different CHIR doses. Error bars represent 95% confidence intervals from 4 technical replicates.

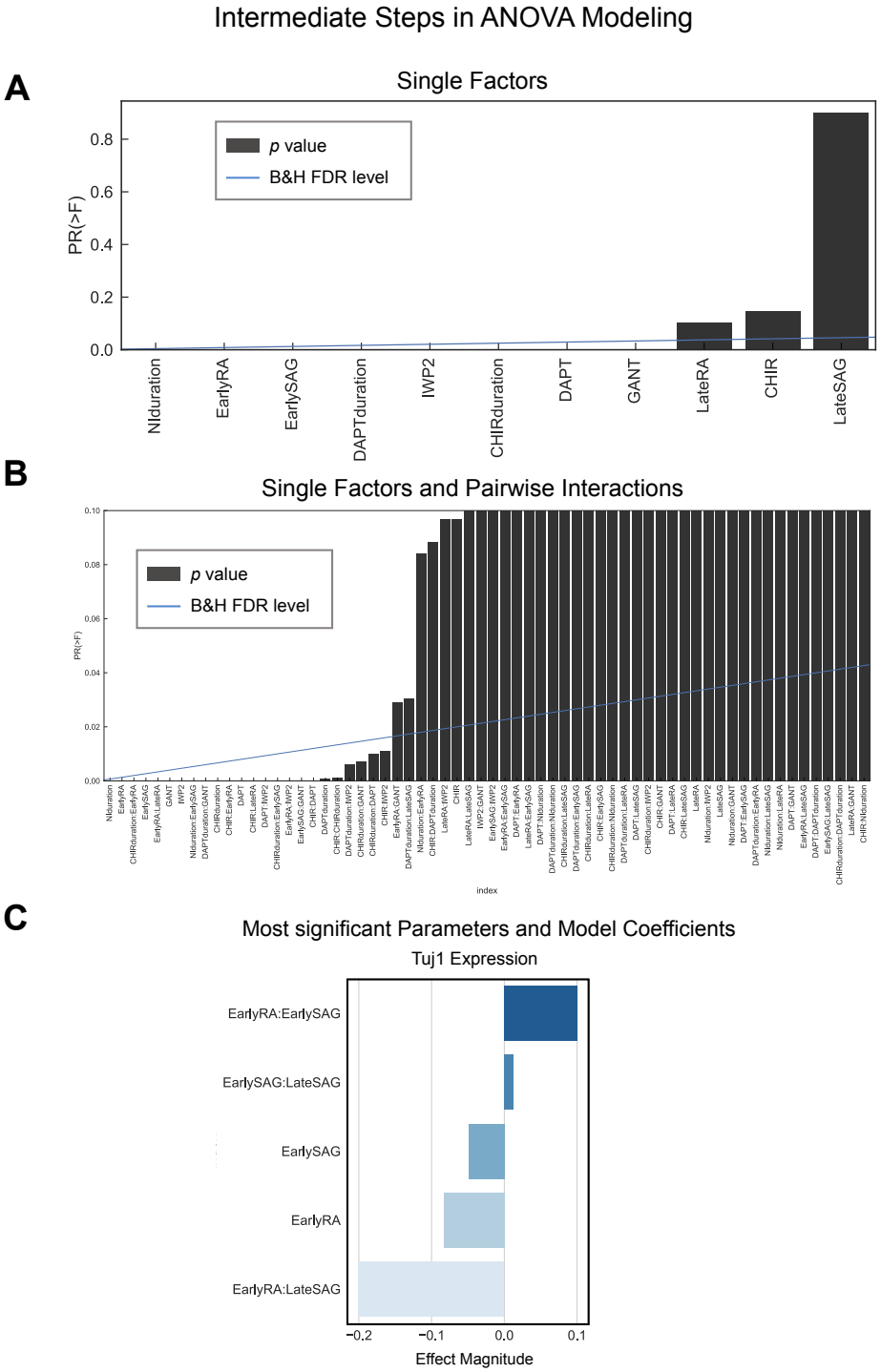

Figure S9. Iterative process to improve Factorial ANOVA model. A) Single factors ranked by their p-values with B&H False Discovery Rate correction. B) Single and pairwise interactions ranked by their p-values with B&H False Discovery Rate correction. C) Effect magnitude of statistically significant variables in the differentiation of Tuj1+ cells at Day 21.

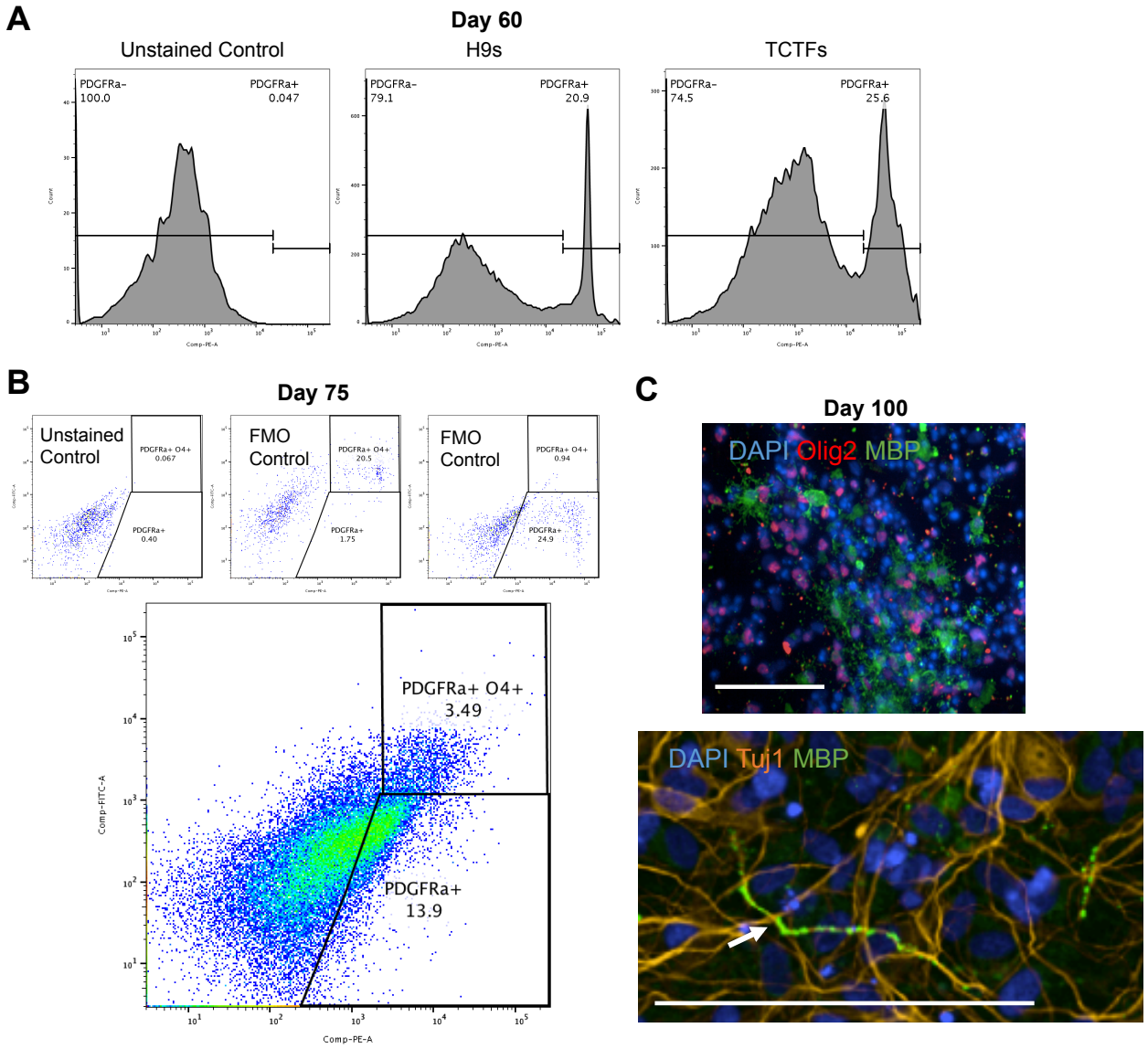

Figure S10. Later stage assessment of OPC differentiation protocol built from screening data. A) Flow cytometry indicating H9- and TCTF- derived OPCs express PDGFRα after 60 days of culture. B) Day 75 flow cytometry quantifying PDGFRα+ and O4+ expressing cells compared to controls: unstained (top left), PDGFRα-PE (top middle) and O4-Alexa 488 (top right) negative controls. C) MBP+ cells after 100 days of differentiation and Tuj1 and MBP co-localization (white arrow); scale bars represents 100 microns.

Movie S1. Z-scan of 3D hPSC neural differentiation on micro pillar at Day 15

Table S1. Reagent Details

Cell Culture Reagents	Concentration	Manufacturer, Cat. No
Y-27632 (Rock Inhibitor)	10 μM	SelleckChem, S1049
CHIR-99011	varied	Tocris, 4423
Retinoic acid, all trans	varied	Enzo, BML-GR100-0500
SAG dihydrochloride	varied	Xcessbio, M60081-2
DAPT	varied	SelleckChem, S2215
IWP-2	varied	Tocris, 3533
GANTT61	varied	Enzo, ALX-270-482-M001
IGF-1	10 ng/mL	Peprotech, 100-11
PDGF-AA	10 ng/mL	Peprotech, 100-13A
NT-3	10 ng/mL	Peprotech, 450-03
Insulin	25 μg/mL	Sigma, I2643
DMSO	100%	Sigma, D2650-5X5ML
Glutamax Supplement	1:100	ThermoFisher, 35050061
B27 supplement	1:50	ThermoFisher, 17504-044
N-2 Supplement	1:50	Life Technologies, 17502-048
Accutase Solution	100%	Life Technologies, A11105-01
Versene Solution	100%	ThermoFisher, 15040066
Essential-8 Media	100%	Life Technologies, A1517001
DMEM-F12 + HEPES + L-Glutamine	varied	Life Technologies, 11039-047
Neurobasal Media	varied	ThermoFisher, 10888022
hESC-qualified Matrigel	50% (3D), 1% (2D)	Corning, 354277
Penicillin/Streptomycin	0.5%	ThermoFisher, 15140122
Hoechst	1:2000	Life Technologies, H3570
Calcein AM	1:2000	Invitrogen, L-3224
Ethidium Homodimer	1:500	Invitrogen, L-3224
Mouse anti-Oct4	1:100	SCBT, sc-5279
Rabbit anti-Pax6	1:200	BioLegend, 901301
Goat anti-OTX2	1:300	R&D, AF1979-SP
Mouse anti-Nkx2.2	1:200	DSHB, 74.5A5
Goat anti-Olig2	1:40	R&D Systems, AF2418
Mouse anti-Tyrosine Hydroxylase	1:1000	Pel-Freez, P40101-150
Mouse anti-Tuj1	1:1000	Sigma, T8578-200UL
Rabbit anti-Tuj1	1:1000	Covance, MRB-435P
Mouse anti-MBP	1:1000	BioLegend, 808401
Anti PDGFRα-PE	1:5	BD Biosciences, 556002
Anti O4-Alexa488	1:100	Millipore, MAB345A4
Donkey anti-Rabbit Cy3	1:250	Jackson, 711-165-152
Donkey anti-Rabbit 647	1:250	Jackson, 711-605-152
Donkey anti-Mouse 488	1:250	Jackson, 711-545-152
Donkey Serum	5%	Sigma, D9663-10ML
Triton X-100	0.25%	Sigma, X100-100mL
Paraformaldehyde	4% in PBS	SCBT, sc-281692