SUPPLEMENTARY INFORMATION

Nanotopography Regulates Motor Neuron Differentiation of Human Pluripotent Stem Cells

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**Supplementary Figures 1 to 12**

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**Fig. S1.** Characterization of nanoengineered nanotopographic surfaces. (a) Schematic of nanotopography generated by RIE on glass surfaces. (b) Nanoroughness as a function of RIE process time. (c) AFM topographs of glass substrates before (left; $R_q = 1.3$ nm) and after (middle & right; $R_q = 101$ nm & 203 nm) RIE processing. (d) XPS survey spectra measured for glass substrates before (blue curve) and after RIE nanoetching (red curve). (e) Merged phase-contrast and anti-vitronectin immunofluorescence image of micropatterned glass substrate with vitronectin coating. (f) Normalized fluorescence intensity at the dash lines (I-III) in e. No significant difference in fluorescence intensity of the adsorbed vitronectin proteins was observed in the unprocessed smooth and RIE-processed nanorough regions.
Fig. S2. (a&b) Photograph showing a 4-inch glass wafer with nanotopographic features (a) before cut and placed into tissue culture dishes (b). (c) Representative SEM images showing hPSCs plated on smooth ($R_g = 1$ nm) and nanorough ($R_g = 150$ nm) glass surfaces.
**Fig. S3.** Representative immunofluorescence images showing temporal expression of pluripotency (Oct3/4; red) and neuroectodermal (PAX6; green) markers during neural induction of hPSCs in neural induction medium on smooth (top; $R_q = 1$ nm) and nanorough (bottom; $R_q = 200$ nm) glass substrates as indicated.
Fig. S4. qRT-PCR analysis for temporal expression of pluripotency (OCT4 and NANOG; a&b) and neuroectodermal (PAX6 and SOX1; c&d) markers during neural induction of hPSCs. hPSCs were cultured in neural induction medium on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) surfaces. Expression level of OCT4, a pluripotency marker, was reduced on both nanorough and smooth glass substrates at day 2. NANOG, another gene associated with pluripotency, decreased more significantly at both day 4 and 6 on nanorough ($R_q = 200 \text{ nm}$) glass substrates compared with smooth controls ($R_q = 1 \text{ nm}$). Genes associated with neural lineages, including PAX6 and SOX1, showed greater levels of expression on nanorough surfaces after day 2 when compared to smooth controls. Expression level of each gene was normalized to data from undifferentiated hPSCs. Data represent the mean ± s.e.m with $n = 3$. $P$-values were calculated using the Student’s paired sample $t$-test. *, $P < 0.05$; **, $P < 0.01$. 
Fig. S5. (a) Representative immunofluorescence images showing PAX6+ NEs and p75+ NCs after 8 days of culture on smooth ($R_q = 1$ nm) and nanorough ($R_q = 100$ nm & 200 nm) glass surfaces. (b) Percentages of p75+ NC cells derived from hPSCs at day 8 as a function of nanoroughness. Data represent the mean ± s.e.m. with $n = 3$. $P$-values were calculated using the Student’s paired sample $t$-test. **, $P < 0.01$. 
Fig. S6. (a) Representative immunofluorescence images showing Tuj1+ cells after 24 days of differentiation in MN differentiation medium on smooth ($R_q = 1$ nm) and nanorough ($R_q = 200$ nm) glass substrates. (b-c) Bar plots showing normalized Tuj1+ (b) and HB9+ (c) cell numbers at day 24 as a function of surface nanoroughness as indicated.
Fig. S7. Purity and yield of motor neurons (MNs) derived from hPSCs are improved on nanorough substrates with a 32-day differentiation protocol. (a) Schematic diagram showing experimental design for sequential neural induction, patterning, and maturation of MNs from hPSCs. hPSCs were cultured on vitronectin-coated smooth \((R_q = 1 \text{ nm})\) and nanorough \((R_q = 200 \text{ nm})\) glass substrates in neural induction medium containing the dual Smad inhibitors SB and LDN for 8 days and then in MN differentiation medium containing purmorphamine (Pur), basic fibroblast growth factor (bFGF) and retinoic acid (RA) for an additional 8 days. Putative MN progenitor cells collected at day 16 were transferred onto coverslips and cultured in MN maturation medium containing brain-derived neurotrophic factor (BDNF), ascorbic acid, cyclic adenosine monophosphate (cAMP) and insulin-like growth factor 1 (IGF-1) for another 16 days. (b) Representative immunofluorescence images showing Tuj1+ and HB9+ cells at day 32. (c-e) Bar plots showing percentages of HB9+ (c) and Tuj1+ (d) cells and percentages of HB9+ cells in Tuj1+ cells (e) at day 32. Data represent the mean ± s.e.m. with \(n = 3\). \(P\)-values were calculated using the Student’s paired sample \(t\)-test. **, \(P < 0.01\).
Fig. S8. (a) Immunofluorescence images showing total $\beta_1$ integrin in undifferentiated hPSCs on smooth ($R_q = 1$ nm) and nanorough ($R_q = 200$ nm) glass substrates after 48 hr of culture. (b) Bar graphs showing quantitative results of normalized total $\beta_1$ integrin for Oct3/4+ hPSCs cultured on substrates with different nanoroughness as indicated. Error bars represent ± s.e.m. with $n = 10$. $P$-values were calculated using the Student’s paired sample $t$-test. ns, $P > 0.05$. 
Fig. S9. Subcellular analysis of focal adhesion (FA) in hPSCs cultured on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass substrates.
**Fig. S10.** Representative immunofluorescence images showing nanoroughness-dependent subcellular localization of YAP in hPSCs at day 2 and PAX6+ NEs derived from hPSCs at day 8 on smooth ($R_q = 1$ nm) and nanorough ($R_q = 200$ nm) glass surfaces under different drug treatments as indicated.
Fig. S11. Immunofluorescence images showing actin CSK (green), including cap actin (left panels) and basal actin (right panels) filament organization, in Oct3/4+ hPSCs cultured on smooth ($R_q = 1$ nm) and nanorough ($R_q = 200$ nm) glass substrates as indicated. The confocal microscopy sections show the actin filament network at the apical surface (cap actin) and basal surface (basal actin) of Oct3/4+ hPSCs cultured on smooth ($R_q = 1$ nm) and nanorough ($R_q = 200$ nm) glass substrates. There are thick, parallel, and highly contractile perinuclear cap actin filament bundles observed in the hPSCs on the nanorough substrates, while well-developed basal stress fibers were found and apical perinuclear actin cap is absence in hPSCs on the smooth substrates.
Fig. S12. Nanotopography-triggered signaling controls hPSC behaviors.