Nanoscale regulates motor neuron differentiation of human pluripotent stem cells†

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The regulation of human pluripotent stem cell (hPSC) behaviors has been mainly studied through exploration of biochemical factors. However, the current directed differentiation protocols for hPSCs that completely rely on biochemical factors remain suboptimal. It has recently become evident that coexisting biophysical signals in the stem cell microenvironment, including nanotopographic cues, can provide potent regulatory signals to mediate adult stem cell behaviors, including self-renewal and differentiation. Herein, we utilized a recently developed, large-scale nanofabrication technique based on reactive-ion etching (RIE) to generate random nanoscale structures on glass surfaces with high precision and reproducibility. We report here that hPSCs are sensitive to nanotopographic cues and such nanotopographic sensitivity can be leveraged for improving directed neuronal differentiation of hPSCs. We demonstrate early neuroepithelial conversion and motor neuron (MN) progenitor differentiation of hPSCs can be promoted using nanoengineered topographic substrates. We further explore how hPSCs sense the substrate nanotopography and relay this biophysical signal through a regulatory signaling network involving cell adhesion, the actomyosin cytoskeleton, and Hippo/YAP signaling to mediate the neuroepithelial induction of hPSCs. Our study provides an efficient method for large-scale production of MNs from hPSCs, useful for regenerative medicine and cell-based therapies.

Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs)1 and induced pluripotent stem cells (hiPSCs),2 can be induced to become functional motor neurons (MNs), thus providing reliable and direct access to human MNs for fundamental studies and cell-based therapies for the treatment of MN-related diseases.3–6 However, the current hPSC MN differentiation protocols, which rely completely on biochemical factors, remain suboptimal due to poorly defined in vitro culture conditions, a prolonged differentiation process, and low differentiation yield and purity.7,8

The extracellular matrix (ECM) in vivo regulates the fate and function of a myriad of stem cells by dynamically modulating nanoscale topographic cues embedded in the stem cell niche through biological processes such as embryogenesis and tissue maintenance and repair.9–11 Such an in vivo ECM contains abundant hierarchical filamentous proteins, which present adhesive ligands on a structured landscape with spatial organizations and characteristic dimensions of a few to hundreds of nanometers.12 The cell membrane, being in direct contact with the ECM, is also enriched with adhesion molecules including integrins and protrusive structures (i.e., nanopodia) with characteristic nanometer length scales. These cell surface molecules and structures have been shown to be critically involved in cellular sensing of extracellular nanotopographic features.9–11 Indeed, substrates with nanoscale topography, which mimic the nanoscale topographic cues of the stem cell niche, have recently been shown to regulate self-renewal and differentiation of adult stem cells including mesenchymal,13,14 neural,15–17 and hematopoietic18 stem cells in vitro. More recent studies have further shown the functional modulation of mouse pluripotent stem cells (mPSCs)9,20 and hPSC-derived progenitors21,22 by substrate nanotopography.23–25 However, it remains to be determined whether nanoscale topo-
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graphic cues can provide potent regulatory signals to mediate the differentiation of hPSCCs towards specific neurological lineages such as MNs.

Herein, using nanoengineered nanotopographic glass substrates, we explicitly demonstrate and leverage the intrinsic nanotopographic sensitivity of hPSCCs for improving early neuroepithelial (NE) conversion and MN progenitor production. We explore how nanotopographic signals in the extracellular environment are transduced through cell–ECM interactions into intracellular biochemical and transcriptional responses through a regulatory network involving cell adhesion, the actomyosin cytoskeleton (CSK), and Hippo/YAP signalling that ultimately control the differentiation of hPSCCs towards the MN fate.

Results and discussion

Nanotopographic substrates promote hPSC neural conversion

Various nanotechnology tools and synthesis methods have been successfully developed and utilized for generating nanotopographic surfaces and scaffolds for in vitro stem cell research. However, previous techniques including electron beam and nanoimprint lithography for generating the nanotopography are complex and costly. Furthermore, the intrinsic random features of the nanotopography in the in vivo cell microenvironment may not be fully recapitulated by patterning regular nanoscale structures. Herein, we utilized a recently developed, large-scale nanofabrication technique based on reactive-ion etching (RIE) to generate random nanoscale structures on glass surfaces with high precision and reproducibility. AFM assays further confirmed that the nanoroughness of unprocessed rough surfaces with \( R_q = 1 \) nm did not significantly change after vitronectin coating. Our X-ray Photoelectron Spectroscopy (XPS; Kratos Axis Ultra DLD, Kratos Analytical Ltd, Manchester, UK) analysis confirmed that there is no material property change or undesired chemical residue left on glass surfaces after RIE and the cleaning process. It is known that the absorption of the ECM or serum proteins may also affect cell–substrate interactions and thus cell behaviours. To exclude this possible effect, detailed surface characterization was performed and confirmed that the density of protein absorbed on glass surfaces was independent of the nanoroughness \( R_q \). hPSCCs were first seeded as single cells at a density of 20,000 cells per cm\(^2\) in growth medium onto vitronectin-coated glass surfaces of varying surface roughness (\( R_q = 1 \) and 100 nm). Expression of pluripotency (DNMT3B, TERT, GABRB3, GABRB7, and UTF1) and neural (PAX6 and NEUROD1) genes was analyzed using qRT-PCR after 7 days of culture in growth medium (Fig. 1a). qRT-PCR results showed that for unprocessed smooth glass surfaces with \( R_q = 1 \) nm, mRNA expression of pluripotency related genes remained unchanged, whereas it was significantly reduced for nanorough glasses with \( R_q = 100 \) nm. Expression of neural genes, on the other hand, increased significantly for nanorough glasses with \( R_q = 100 \) nm compared with smooth glass surfaces with \( R_q = 1 \) nm. These results suggest that unprocessed smooth glass surfaces were conducive to hPSC self-renewal and pluripotency maintenance under growth medium conditions, whereas nanorough glasses promoted spontaneous differentiation of hPSCCs towards a neuronal fate, even without using a neural induction medium.

To specifically examine the effect of nanotopographic cues on hPSC NE conversion, a critical step for generating neural progenitor cells, singly dissociated hPSCCs were seeded at a density of 20,000 cells per cm\(^2\) onto vitronectin-coated glass surfaces with different nanoroughness and were treated with a neural induction medium containing dual Smad inhibitors, SB 431542 (SB, a TGF-β inhibitor) and LDN 193189 (LDN, a BMP4 inhibitor) for 8 days (Fig. 1b). Neural induction was monitored by expression of PAX6, a marker of early neuroectodermal differentiation. On nanorough substrates with \( R_q = 200 \) nm, PAX6+ neuroepithelial cells (NECs) were detected as early as day 2, and reached 88.6% for hPSCs and 95.5% for hESCs by day 8 (Fig. 1b–e & Fig. S3†). In distinct contrast, on smooth glasses (\( R_q = 1 \) nm) PAX6+ NECs appeared at day 4 and constituted only 32.2% of total cells at day 8. qRT-PCR was also performed to measure the temporal expression of pluripotency (OCT4 and NANOG) and neuroectodermal genes (PAX6 and SOX1) during neural induction. Nanorough surfaces with \( R_q = 200 \) nm accelerated the disruption of the transcriptional circuitry that maintains the pluripotency of hPSCCs, while simultaneously promoting neuroectodermal gene expression (Fig. S4†). Altogether, our data support that nanorough substrates with \( R_q = 200 \) nm led to significantly improved production of NECs, followed by nanorough substrates with \( R_q = 100 \) nm (Fig. 1f). Unprocessed smooth glass surfaces with \( R_q = 1 \) nm, even though effective for hPSC pluripotency maintenance, were the least conducive to NE differentiation from hPSCCs.

Consistent with the results reported previously, treatment of hPSCCs with dual Smad inhibitors led to not only PAX6+ NECs but also PAX6– cells expressing neural crest (NC) markers AP2, p75 and HNK-1 (Fig. 1c, g & Fig. S5†). At day 8, 18.0% and 14.4% cells were AP2+ and p75+ on smooth glass controls (\( R_q = 1 \) nm), respectively. Strikingly, only 1.6% and 3.4% AP2+ and p75+ NCs were evident on nanorough surfaces with \( R_q = 200 \) nm. Furthermore, immunoblot analysis confirmed higher expression of PAX6 and SOX1 (neuroectodermal transcription factors) but lower expression of AP2 and HNK-1 for hPSCCs at day 8 on nanorough surfaces with \( R_q = 200 \) nm, compared with...
smooth glass controls ($R_q = 1$ nm) (Fig. 1h). PAX6+ NECs derived from nanorough substrates were responsive to bFGF treatment and readily formed polarized neural tube-like rosettes (a functionally distinct early neural stem cell stage32–34 (Fig. 1i)). Altogether, our data show that the intrinsic nanotopographic sensitivity of hPSCs could be leveraged to achieve significant improvement in the NE conversion of hPSCs.

**Nanotopographic substrates promote hPSC motor neuron progenitor cell differentiation**

We next examined whether NECs derived from nanorough glass surfaces could be specified into spinal MN progenitors using oligodendrocyte transcription factor 2 (Olig2) as an early MN marker (Fig. 2). When hPSC-derived NECs were cultured...
continuously for another 8 days in the presence of the ventraliza-
tion factor purmorphamine (Pur) and the caudalization factor
retinoic acid (RA), 58.5% cells on nanorough surfaces with
$R_q = 200 \text{ nm}$ emerged as Olig2+ MN progenitors, whereas only
11.2% cells became Olig2+ on smooth glasses ($R_q = 1 \text{ nm}$;
Fig. 2b & c).

To further investigate whether MN production could be
expedited on nanorough surfaces, hPSCs were first cultured on
vitronectin-coated glass substrates for 16 days to allow neural
induction and caudalization prior to passage onto poly-L-
ornithine/laminin-coated coverslips and treatment with a MN
maturation medium containing brain-derived neurotrophic factor
(BDNF), ascorbic acid, cyclic adenosine monophosphate (cAMP)
and insulin-like growth factor 1 (IGF-1) 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 8 days. At day 24, 16.3% and 35.3% of cells
derived from nanorough substrates with $R_q = 200 \text{ nm}$ became HB9+ (MN-specific
transcription factor) and Tuj1+ ($\beta$-III tubulin, general neuron marker), respectively
(Fig. 2d–f). The purity of MNs, defined as the percentage of
HB9+ cells in Tuj1+ neurons, from nanorough substrates was
45%, a more than four-fold increase compared with smooth
controls (10%; Fig. 2f). Furthermore, culture on nanorough
substrates with $R_q = 200 \text{ nm}$ led to 3.9- and 15.6-fold increases
in the numbers (and therefore yields) of Tuj1+ and HB9+ cells,
respectively, compared with smooth controls (Fig. S6†). The
purity and yield of the derived MNs were further improved on
nanorough substrates with a 32-day differentiation protocol, as
confirmed by immunostaining for HB9 and Tuj1 (Fig. S7†).
Altogether, the yield and purity of MN progenitors were signifi-
cantly improved using nanorough glass substrates for directed
differentiation of hPSCs. Electrophysiological properties are a
defining property of neuronal maturation. In the course of
neurodevelopment, neuronal electrophysiological properties
exhibit significant alterations. Therefore, electrophysiological
characterization is desirable to further confirm the functional
status of MNs derived from nanorough glass surfaces.

Nevertheless, our detailed temporal analysis using multiple
neural lineage markers (PAX6, AP2, HNK-1, Tuj1, Olig2, and

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Fig. 2 Nanotopographic substrates promote hPSC motor neuron progenitor cell differentiation. (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 ($R_q = 200 \text{ nm}$) 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 8
days. (b) Representative immunofluorescence images showing Tuj1+, Olig2+, and HB9+ cells at day 16 and day 24 as indicated. (c–f) Bar plots showing
percentages of Olig2+ cells at day 16 (c), and percentages of HB9+ (d) and Tuj1+ (e) cells and percentages of HB9+ cells in Tuj1+ cells (f) at day 24.
Data represent the mean ± s.e.m. with $n = 3$. $P$-Values were calculated using the Student’s paired sample $t$-test. **, $P < 0.01$. 

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HB9) for different differentiation stages strongly supports the notion that nanorough glass substrates can promote hPSC NE conversion and MN lineage.

**Functional roles of cell adhesion, actomyosin CSK, and Hippo/YAP signaling in nanotopography-mediated neural induction of hPSCs**

Bidirectional integrin-mediated adhesion signaling has been implicated in nanotopography-mediated adherent stem cell behaviors by regulating integrin activation and clustering, which can in turn mediate dynamic organization and activation of adaptor and signaling proteins in focal adhesions (FAs) including focal adhesion kinase (FAK). To investigate the involvement of adhesion signaling in the nanotopographic sensitivity of hPSCs, we examined integrin activation in hPSCs by immunostaining for activated β1 integrin. The activated β1 integrin level on nanorough glass surfaces with \( R_q = 200 \) nm was significantly higher compared with smooth controls (Fig. 3a & c), whereas total β1 integrin levels were comparable (Fig. S8†). We further investigated the effect of the nanotopography on the integrin-mediated FA formation by immunostaining for vinculin and FAK. On smooth glass surfaces with \( R_q = 1 \) nm, vinculin-containing mature FAs localized primarily on the cell periphery (Fig. 3a). However, hPSCs on nanorough glass surfaces with \( R_q = 200 \) nm exhibited randomly distributed, punctate FAs throughout the entire cell areas (Fig. 3b–f & Fig. S9†). An important signaling axis downstream of adhesion signaling is the FAK-Src pathway. Nanotopographic glass surfaces enhanced FAK phosphorylation (pFAK; Fig. 3a & g), supporting FAK activation in response to nanotopographic sensing by hPSCs.

It has been shown that the inhibition of BMP/Smad signaling is required for neural induction of hPSCs. Indeed, the immunoblot results in Fig. 4a confirmed an enhanced inhibitory effect on the phosphorylation of Smad1/5/8, a downstream target of BMP/Smad signaling, by nanotopography. This observation confirms a functional link between hPSC neural conversion and BMP signaling inhibition by nanotopography. How the nanotopographic sensitivity of hPSCs is relayed to BMP/Smad signaling remains to be determined. It is likely that integrin activation by nanotopography may lead to an inhibition of BMP/Smad signaling through co-internalization of integrin and BMP type-I receptor (i.e., BMPRIA), which has been shown to co-localize and form a complex with \( \beta_1 \) integrin.

Another layer of regulation of BMP/Smad signaling in hPSCs is through the canonical Hippo pathway. YAP, the transcriptional co-activator in the Hippo pathway, has recently been shown to bind phosphorylated Smads (phosphoSmads) and control their nucleocytoplasmic shuttling in hPSCs. In addition, nuclear accumulation of YAP (and phosphoSmads) is required for hPSC pluripotency maintenance, and cytoplasmic retention of YAP prevents the nuclear translocation of YAP.
phosphoSmads and results in hPSC neuroectoderm differentiation.40,41 Indeed, our results showed that the phosphorylation and nucleocytoplasmic shuttling of YAP were responsive to the nanotopography in hPSCs (Fig. 4a, b and Fig. S10†). Specifically, immunoblots demonstrated that nanorough glass surfaces (with $R_q = 200 \text{ nm}$) significantly promoted YAP phosphorylation on serine 127 (p-YAP S127), a key target of Lats1/2 kinase downstream of the Hippo pathway (Fig. 4a).42,43 While YAP was predominantly localized in the nucleus of undifferentiated hPSCs on smooth glass substrates, more than 60% hPSCs on nanorough surfaces (with $R_q = 200 \text{ nm}$) showed cytoplasmic YAP (Fig. 4b & c). This nanotopography-dependent nucleocytoplasmic localization of YAP was also observed after 2–4 days of neural induction. For the drug treatment experiments, hPSCs were cultured for 2–8 days in neural induction medium supplemented with DMSO (vehicle control), ROCK inhibitor Y27632, actin polymerization inhibitor cytochalasin D (CytoD), and RhoA activator lysophosphatidic acid (LPA), as indicated. For c–e, 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. 4** Nanotopography regulates YAP phosphorylation and nuclear shuttling in hPSCs by actomyosin contractility and actin CSK integrity. (a) Western blotting showing total and phosphorylated Smad 1/5/8 (p-Smad 1/5/8), phosphorylated YAP on serine 127 (p-YAP S127) and YAP in hPSCs differentiated for 4 days on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass surfaces. (b) Representative immunofluorescence images showing the nanoroughness-dependent subcellular localization of YAP in hPSCs at day 0 and 4 on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass surfaces as indicated. (c) Bar plot showing nanoroughness-dependent subcellular localization of YAP at day 0, 2 and 4 on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass surfaces as indicated. (d & e) Bar plots showing percentages of hPSCs with nuclear YAP after 2 days (d), and percentages of PAX6+ NECs derived from hPSCs after 8 days of culture (e) on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass surfaces under different drug treatments as indicated. (f) Immunofluorescence images showing PAX6+ NECs derived from hPSCs on smooth ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 200 \text{ nm}$) glass surfaces after 8 days of differentiation. For the drug treatment experiments, hPSCs were cultured for 2–8 days in neural induction medium supplemented with DMSO (vehicle control), ROCK inhibitor Y27632, actin polymerization inhibitor cytochalasin D (CytoD), and RhoA activator lysophosphatidic acid (LPA), as indicated. For c–e, data represent the mean ± s.e.m. with $n = 3$. $P$-Values were calculated using the Student’s paired sample $t$-test. **, $P < 0.01$."

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both smooth and nanorough glass surfaces (Fig. 4e). Notably, CytoD treatment significantly promoted the cytoplasmic localization of YAP and inhibited neural induction on both smooth and nanorough glass surfaces (Fig. 4d & e and Fig. S1†).

### Experimental

#### Fabrication and surface characterization of nanorough glass samples

Glass wafers (Borofloat 33; Plan Optik) were processed with RIE (LAM 9400, Lam Research) for different periods of time to generate the nanoscale surface roughness (ranging from 1 nm to 200 nm). The corresponding RIE process conditions were selected as SF$_6$ (8 sccm), C$_4$F$_8$ (50 sccm), He (50 sccm), Ar (50 sccm), chamber pressure (1.33 Pa), bias voltage (100 V), and radio frequency power (500 W). All the processed glass wafers were cut into small pieces (1 cm × 1 cm or 1.5 cm × 1.5 cm) using the ADT7100 dicing saw (Advanced Dicing Technologies) before placing into standard 24-well or 12-well tissue culture plates. To promote cell attachment, glass substrates were functionalized with human vitronectin (Trevigen) by immersing the substrates in a vitronectin solution (20 μg mL$^{-1}$) in distilled water overnight. It has been reported that vitronectin can support the self-renewal of hPSCs. 47 Glass substrates were rinsed twice with PBS before they were used for cell seeding.

Nanoroughness of the glass surfaces was measured at room temperature with a Veeco NanoMan Atomic Force Microscope (AFM, Digital Instruments) using a non-contact, tapping mode and standard Si tapping mode AFP tips. The AFM scan image size was 10 μm × 10 μm with a scan rate of 1 Hz. The resulting map of the local surface height was represented using the AFM topographs. Unprocessed bare glass wafers had an intrinsic surface roughness $R_q$ of 1 nm.

#### Cell culture

NIH approved hESC lines H1 (WiCell Research Institute, Madison, WI) and CHB10 (Children’s Hospital Corporation, Boston, MA) and hiPSCs derived from human foreskin fibroblasts (hFF) in Dr Paul H. Krebsbach’s laboratory at University of Michigan were used in this study. hESCs (H1; WiCell) were cultured on mitotically inactive mouse embryonic fibroblasts (MEFs; GlobalStem) in growth medium at 37 °C in 5% CO$_2$ and 100% humidity with a humidified atmosphere. Single hPSCs were then seeded at a density of 20,000 cells per cm$^2$ onto glass substrates, and were then allowed to spread out overnight before other assays.

### Culture and differentiation medium conditions

#### Growth medium.

The growth medium contains DMEM/F12 (GIBCO), 20% KnockOut serum replacement (GIBCO), 0.1 mM β-mercaptoethanol (GIBCO), 2 mM glutamax (GIBCO), 1% non-essential amino acids (GIBCO), and 4 ng mL$^{-1}$ human recombinant basic fibroblast growth factor (bFGF; GlobalStem).

#### Neural induction medium.

The growth medium was used as neural induction medium from day 1 to day 3. N2 medium containing DMEM/F12, 1% N2 supplement (GIBCO), 2 mM glutamax, and 1% non-essential amino acid was used to gradually replace hPSC growth medium from day 4 as follows: 25% N2 media and 75% growth medium at day 4, 50% N2 media and 50% growth medium at day 5 and day 6, 75% N2 medium and 25% growth medium at day 7, 100% N2 medium at day 8. To promote neural induction, TGF-β inhibitor SB 431542 (10 μM; Cayman Chemical) and BMP4 inhibitor LDN 193189 (0.1 μM, unless stated otherwise; Selleckchem) were added into the growth medium from day 1 till the end of neural induction.

#### MN differentiation medium.

MN differentiation medium contained N2 medium supplemented with 1 μM retinoic acid (RA; Cayman Chemical), 1 μM purmorphamine (Pur; Cayman Chemical), and 20 ng mL$^{-1}$ bFGF.

#### MN maturation medium.

MN maturation medium contained basal medium that was a 1:1 mixture of N2 and B-27 media. The B-27 medium contained neurobasal media (GIBCO), 2% B-27 supplement (GIBCO), and 2 mM Glutamax. The following chemicals were added to basal medium freshly before each medium change: 10 ng mL$^{-1}$ brain-derived neurotrophic factor (BDNF; R&D systems), 10 ng mL$^{-1}$ insulin-like growth factor 1 (IGF-1; Peprotech), 1 μM cyclic adenosine 3'5' monophosphate (cAMP).
monophosphate (cAMP; Sigma), 0.2 μg mL\(^{-1}\) ascorbic acid (Sigma), 0.1 μM RA, and 1 μM Pur.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min and then permeabilized with 0.1% Triton X-100 (Roche Applied Science) for 20 min at room temperature. Fixed cells were then incubated with 10% goat serum (Invitrogen) for 1 h and then primary antibodies for 1 h. Alexa Fluor 488 and 555 conjugated goat anti-mouse (or anti-rabbit) IgG secondary antibodies (Invitrogen) were used as secondary antibodies. Alexa Fluor 555 conjugated phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) were used for visualization of actin microfilaments and the nucleus, respectively. The percentage of marker-positive cells was quantified with a custom-developed MATLAB program (MathWorks) based on a watershed segmentation algorithm.

**Western blotting**

Whole cell lysates were prepared from cells, separated on SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with 5% milk in PBS for 1 h and then incubated with primary antibodies overnight at 4 °C. Blots were incubated with peroxidase-coupled secondary antibodies (Promega) for 1 h, and protein expression was detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**RNA isolation and RT-qPCR analysis**

Total RNA was isolated from hPSCs grown on glass substrates using the RNeasy kit (Qiagen). Real-time PCR (RT-PCR) was performed and monitored using an ABI 7300 system (Applied Biosystems). RT-qPCR was also performed with either Taqman-probes or SYBR Green PCR mastermix. Human GAPDH or 18S primers were used as an endogenous control for relative quantifications. All analyses were performed with three replicates. Relative expression levels were determined by calculating \(2^{-\Delta\Delta Ct}\) with the corresponding s.e.m.

**SEM specimen preparation**

Cell samples were washed three times with 50 mM Na-cacodylate buffer (pH 7.3; Sigma-Aldrich), fixed for 1 h with 2% glutaraldehyde (Electron Microscopy Sciences) in 50 mM Na-cacodylate buffer, and dehydrated in a graded series of ethanol concentrations through 100% over a period of 1.5 h. Dehydration in 100% ethanol was performed three times. Afterwards, dehydrated substrates were dried with liquid CO\(_2\) using a super critical point dryer (Samdri®-PVT-3D, Tousimis). Samples were mounted on stubs, sputtered with gold palladium, observed and photographed under a Hitachi SU8000 Ultra-High Resolution SEM machine (Hitachi High Technologies America).

**Statistical analysis**

The \(P\) value was calculated using the Student \(t\)-test function in Excel (Microsoft). All data presented in the manuscript represent the mean ± standard error of the mean (s.e.m.) with \(n \geq 3\).

**Conclusions**

The culture environment of hPSCs comprises two main elements, soluble culture medium and culture substrates. By leveraging the knowledge of developmental pathways that allow neural induction and lineage specification, stem cell biologists have achieved considerable progress in developing a culture medium containing small molecules and growth factors for MN production.\(^7,8,30\) Despite the understanding of hPSC fate regulation by soluble factors,\(^7,30\) little is known about the role of insoluble, “solid-state” signals of the cell microenvironment in regulating the hPSC fate.\(^51–53\) It has recently become evident that the regulation of the stem cell fate by soluble factors is strongly influenced by the coexisting insoluble adhesive, topological, and mechanical cues inherently contained within the cell microenvironment. Thus, to achieve optimal conditions for hPSC self-renewal and lineage-specific differentiation and to unlock the full potential of these cells for cell-based therapies, the influence of nanoscale topographic signals in the culture environment on hPSC behaviors should be elucidated.

This work introduces a large-scale nanofabrication technique to generate nanoscale structures on glass surfaces with high precision and reproducibility. Our results show that hPSCs are sensitive to nanotopographic cues and such nanotopographic sensitivity can be leveraged for improving the directed neuronal differentiation of hPSCs. We demonstrate that both early stage NE conversion and MN progenitor cell differentiation of hPSCs can be promoted by using nanoengineered topographic substrates. Although careful electrophysiological characterization of MNs is still desirable for determining the functional state of neuronal maturation, our detailed investigation using different neuronal lineage markers has confirmed that the nanotopographic sensitivity of hPSCs can be leveraged for shortened MN differentiation with concurrent high yield and purity. Such a nanotopographic effect could be incorporated in the future development of new biomaterial systems for large-scale production of clinical grade MNs from hPSCs for treating MN-related degenerative diseases.

We further demonstrate how hPSCs could sense the substrate nanotopography and relay such biophysical signals through a regulatory network involving cell adhesion, the actin cytoplasmic signal transducers, such as FAK, RhoA/ROCK signaling, and CSK contractility in regulating the nanotopographic sensitivity of adherent mammalian cells, a vital link between such diverse “cytoplasmic signal transducers” and downstream “transcriptional regulators” is still missing. Particularly, it remains unclear how nanotopography-mediated adhesion signaling, RhoA/ROCK signaling, and CSK contractility could contribute to hPSC transcriptional activity and lineage specification. Our results revealed that nanotopography-mediated integrin signaling contributed to hPSC neural lineage specification through the Hippo/YAP pathway.
Phosphorylation and nucleocytoplasmic shuttling of YAP were sensitive to the nanotopography, which in turn regulated Smad signaling to control the neural induction of hPSCs. How the nanotopographic sensitivity of hPSCs is relayed from integrins to Hippo/YAP remains to be determined. Integrons transmit topographic signals via intracellular signaling proteins including integrin-linked kinase (ILK), which has recently been shown to suppress the Hippo pathway. By inhibiting MYPT1 through direct phosphorylation, ILK prevents Merlin dephosphorylation and activation, leading to the inhibition of the Hippo kinase cascade and the nuclear accumulation of YAP/TAZ. Thus, it is likely that nanotopographic signals are transmitted from the cell–ECM interface toward the nucleus through a regulatory pathway involving integrin/ILK adhesion signaling, MYPT1/Merlin signaling, and finally the Hippo/YAP pathway to control the hPSC fate decision.

Collectively, our study revealed how the nanotopography-sensitive cellular machinery including integrin-mediated adhesion signaling, Rho GTPase and actomyosin CSK, and the Hippo/YAP signaling functions or collaborates synergistically to control the hPSC fate decision. Recent studies showed that functional MNs can be derived from both hESCs and hiPSCs and functionally integrated into animal models. Thus, hPSC-derived MNs provide an exciting cell source for neurodegenerative disease modelling and development of therapeutic strategies. Altogether, our work using nanotopographic surfaces to improve functional MN production from hPSCs will have significant implications for future cell-based therapies for a number of debilitating MN-related diseases and pathological conditions such as spinal cord injury, amyotrophic lateral sclerosis, and spinal muscular atrophy.

Conflicts of interest
There are no conflicts to declare.

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