Substratum-induced differentiation of human pluripotent stem cells reveals the coactivator YAP is a potent regulator of neuronal specification

Samira Musah[a,1], Paul J. Wrighton[b], Yefim Zaltsman[b], Xiaofen Zhong[c], Stefan Zorn[d], Matthew B. Parlato[d], Cheston Hsiao[e], Sean P. Palecek[e], Qiang Chang[c,f,g], William L. Murphy[d,h], and Laura L. Kiessling[a,b,2]

Departments of [a]Chemistry, [b]Biochemistry, [d]Biomedical Engineering, [e]Chemical and Biological Engineering, and [g]Medical Genetics and Neurology, [c]Waisman Center, and [f]Genetics Training Program, University of Wisconsin–Madison, Madison, WI 53706; and [h]Department of Orthopedics and Rehabilitation, University of Wisconsin, Wisconsin Institute for Medical Research, Madison, WI 53706

Contributed by Laura L. Kiessling, August 13, 2014 (sent for review April 25, 2014); reviewed by Kristi S. Anseth, Ali Khademhosseini, and Jason A. Burdick

Physical stimuli can act in either a synergistic or antagonistic manner to regulate cell fate decisions, but it is less clear whether insoluble signals alone can direct human pluripotent stem (hPS) cell differentiation into specialized cell types. We previously reported that stiff materials promote nuclear localization of the Yes-associated protein (YAP) transcriptional coactivator and support long-term self-renewal of hPS cells. Here, we show that even in the presence of soluble pluripotency factors, compliant substrata inhibit the nuclear localization of YAP and promote highly efficient differentiation of hPS cells into postmitotic neurons. In the absence of neurogenic factors, the effective substrata produce neurons rapidly (2 wk) and more efficiently (>75%) than conventional differentiation methods. The neurons derived from substrate induction express mature markers and possess action potentials. The hPS differentiation observed on compliant surfaces could be recapitulated on stiff surfaces by adding small-molecule inhibitors of F-actin polymerization or by depleting YAP. These studies reveal that the matrix alone can mediate differentiation of hPS cells into a mature cell type, independent of soluble inductive factors. That mechanical cues can override soluble signals suggests that their contributions to early tissue development and lineage commitment are profound.

mechanotransduction | neuronal differentiation | YAP/TAZ | biomaterials | glycosaminoglycans

Human pluripotent stem (hPS) cells, which include human embryonic (hES) and human induced pluripotent stem cells, possess the remarkable capacity to self-renew indefinitely and differentiate into almost any specialized cell type (1, 2). They represent a potentially unlimited supply of cells for regenerative medicine, drug screening, and studies of human development. These applications require efficient and reproducible conditions to direct hPS cell differentiation into specialized cell types, including neuronal cells. To date, the focus has been on identifying soluble factors, such as growth factors and small molecules, that can influence hPS cell differentiation. The ability of insoluble signals to promote hPS cell-lineage specification remains less clear.

Studies in murine ES cells (3, 4) and tissue-specific stem cells (5–10) indicate that the adhesive and mechanical properties of the substratum used can influence cell fate decisions (11). For example, human mesenchymal stem (hMS) cells are sensitive to changes in substrate elasticity and respond by differentiating toward distinct cell lineages depending on the stiffness of the matrix (5). These hMS cells, however, tend to exist in heterogeneous cell populations and lack a specific and unique cell characterization marker (12). Their differentiation capacity is restricted to a few tissues that arise from the mesoderm lineage, such as bone, fat, and cartilage. Indeed, there are questions about whether these cells undergo transdifferentiation to cell types, such as neurons (12–14). With the unique ability to differentiate into almost any cell type, hPS cells serve as an excellent model for understanding the roles of extracellular signals on lineage specification and tissue morphogenesis.

In examining the influence of substrate mechanics on hPS cell propagation, we found that stiff surfaces facilitate hPS cell expansion (15). Key to this activity is their ability to promote the nuclear localization of the coactivator Yes-associated protein (YAP) (15, 16), which is critical for pluripotency (15, 17). Alternatively, compliant matrices inhibit nuclear localization of YAP and are unable to support hPS cell self-renewal (15). YAP acts with TEAD transcription factors to drive cell cycle progression (18, 19), and YAP depletion or inhibition of YAP–TEAD interactions can promote neuronal differentiation (19, 20). Fu and coworkers (21) reported that polydimethylsiloxane micropost arrays that inhibit Hippo/YAP signaling can improve neuronal differentiation of hPS cells induced by soluble neurogenic factors. We postulated that the mechanical properties of the substrate alone would be powerful enough to poise cells for neuronal differentiation. Using synthetic hydrogels as a tunable platform (22), we tested this hypothesis by evaluating the differentiation of hPS cells on surfaces of different stiffness. These investigations revealed that compliant hydrogels induce rapid and efficient differentiation of hPS cells into neurons that express mature neuronal markers and possess action potentials.

Significance

Human pluripotent stem (hPS) cells can self-renew indefinitely and differentiate into almost any cell type. Thus, hPS cells represent a potentially unlimited supply of cells for regenerative medicine, drug screening, and developmental studies. Realizing the full potential of hPS cells requires efficient protocols to direct their differentiation into desired cell types. Most efforts to control hPS cell differentiation have focused on soluble signaling factors, while the roles of insoluble signals, such as the mechanical properties of the ECM, have been less explored. We show that matrix mechanics alone can robustly induce neuronal differentiation of hPS cells, independent of soluble neurogenic factors. These results can guide the design of materials to influence stem cell fate.

Author contributions: S.M., S.P.P., Q.C., W.L.M., and L.L.K. designed research; S.M., P.J.W., Y.Z., X.Z., S.Z., and C.H. performed research; S.M., S.Z., and M.B.P. contributed new reagents/analytic tools; S.M., P.J.W., Y.Z., and L.L.K. analyzed data; and S.M. and L.L.K. wrote the paper.

Reviewers: K.S.A., Howard Hughes Medical Institute, University of Colorado Boulder; A.K., Brigham and Women's Hospital, Harvard Medical School; J.A.B., University of Pennsylvania.

The authors declare no conflict of interest.

[1]Present address: Harvard Medical School, Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA 02115.

[2]To whom correspondence should be addressed. Email: kiessling@chem.wisc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1415330111/-/DCSupplemental.

The molecular mechanism underlying the substratum-induced differentiation is through modulation of the subcellular localization of YAP. Our results indicate that mechanical properties of the ECM can be a principal factor in directing the lineage-specific differentiation of hPS cells.

Results and Discussion

Substratum-Induced Neuronal Differentiation of hPS Cells. Exploiting the chemoselective synthesis of peptide-bearing hydrogels, we previously found that stiff hydrogels (elastic modulus of ~10 kPa) displaying a glycosaminoglycan (GAG)-binding peptide, CGKKQRFRHRNRKG, can support long-term self-renewal of hPS cells but that the corresponding compliant hydrogels (~0.7 kPa) do not (15). The inability of compliant hydrogels to support hPS expansion suggests they might facilitate hPS cell differentiation. To test this possibility, we cultured hES cells on compliant polyacrylamide (PA) hydrogels (~0.7 kPa) functionalized with the aforementioned GAG-binding peptide (15, 23). For initial experiments, we used a defined hPS cell culture medium utilized for hPS cell self-renewal, mTeSR (24), supplemented with Y27632, a small-molecule inhibitor of Rho-associated protein kinase (ROCK) (25). The ROCK inhibitor was added to facilitate hPS cell survival. After ~14 d of culture on the compliant hydrogels, hES cells differentiated selectively. The cells' loss of expression of key pluripotency marker Oct4 was accompanied by their adoption of a cell morphology consistent with neurons and expression of neuronal marker Tuj1 (neuron-specific class III β-tubulin) (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1). Given that the mTeSR culture medium is designed to promote pluripotency and lacks the soluble signaling factors used to induce neuronal differentiation, these observations were notable.

The effect of the compliant hydrogels prompted us to evaluate the efficiency of this differentiation protocol. We primed hES cells for differentiation to facilitate their adhesion to the compliant hydrogels (*SI Appendix*, Figs. S2 and S3 and *Supplementary Notes*) and then cultured them with a defined medium. The tested medium consisted of mTeSR or mTeSR lacking the key soluble signaling component basic FGF (*b*FGF), TGF-β, or GABA. We found that the cells exhibited neuronal morphology and stained positive for Tuj1 neuronal marker, irrespective of the composition of the medium (Fig. 1*C* and *SI Appendix*, Fig. S4 *A* and *B*). The differentiation time frame was conspicuous. The cells rapidly (within 5–10 d) adopted a neuronal morphology and expressed Tuj1, and they did so more efficiently in defined medium lacking the self-renewal factor *b*FGF. None of the medium components was required to induce differentiation. The primed cells developed neuronal characteristics in defined medium depleted of all three soluble factors (*b*FGF, TGF-β, and GABA; referred to as "depleted medium") or even in basal (DMEM/F12) medium (*SI Appendix*, Fig. S4*C*). Although it improves cell survival (23, 25), the small molecule Y27632 was also dispensable for substratum-induced neuronal differentiation (23, 25) (*SI Appendix*, Fig. S4*D*). Together, these results indicate that the appearance of neuronal traits by the hPS cells was not triggered by soluble inductive factors.

To explore whether the inductive influence of the compliant hydrogel depended on the GAG-binding peptides, we tested different classes of cell-binding ligands (26). We had shown that GAGs can transmit mechanical signals to cells (15), but integrins are well known as mechanoreceptors (26). We therefore synthesized hydrogels functionalized with either linear or cyclic Arg–Gly–Asp (RGD) peptides, both of which can support cell adhesion through integrins. The compliant hydrogels functionalized with the integrin-binding peptides also promote neuronal differentiation of hPS cells without exogenous neurogenic factors (*SI Appendix*, Fig. S5). Similarly, compliant hydrogels functionalized with hPS cell-binding peptides identified from phage display (whose binding partners are unknown) (27) also enable neuronal differentiation of hPS cells (*SI Appendix*, Fig. S5). These results indicate that the observed hPS cell-lineage restriction that results from mechanotransduction is not confined to a specific cell-surface receptor. Under all conditions, it was the compliant hydrogels that consistently resulted in rapid and efficient neuronal differentiation.

Characterization of Substratum-Derived Neurons. To evaluate the developmental status of the substratum-differentiated cells, we first examined their proliferative capacity, because neurons are postmitotic. After 2 wk of differentiation on compliant hydrogels, the majority of the resulting cells ceased proliferation (Fig. 2 *A* and *B*). Immunocytochemistry analysis revealed that the hydrogel-derived neurons were positive for the neuronal marker Tuj1. For prolonged culture on the compliant hydrogels, we allowed the cells to differentiate for 12 d on the compliant hydrogels in depleted medium and then switched to N2B27 neuronal maintenance medium. After 4 wk, the cells stained positive for the mature neuronal marker microtubule-associated protein 2 (*SI Appendix*, Fig. S7*A*). Using a transgenic hES cell line engineered with a GFP reporter for synapsin (28), we found that the differentiated cells express synapsin, a marker of synaptic vesicles (*SI Appendix*, Fig. S7*B*). Together, these results indicate that the substratum-differentiated cells are postmitotic and possess molecular markers indicative of mature neuronal cells.

We analyzed the gene expression profile of the hydrogel-differentiated cells by assessing lineage identification gene markers specific for pluripotent stem cells; progenitor cells from all three embryonic germ layers (ectoderm, mesoderm, and endoderm); and terminally differentiated cells, such as astrocytes, neurons, cardiomyocytes, and β-cells (functional gene grouping is provided in *SI Appendix*, Table S1). After 2 wk of differentiation on the compliant hydrogels, pluripotency genes (*POU5F1*, *ZFP42*, *PODXL*, and *LEFTY1*) were significantly down-regulated (Fig. 2*C* and *SI Appendix*, Table S2). Additionally, expression levels were decreased for the genes encoding endoderm markers *KRT19* and *ITGB4*, as well as the mesoderm marker *T* (brachyury). Ectoderm and neuroectoderm markers *ZIC1* and *NEUROG2* were highly up-regulated, as were neuronal genes, including *NEUROD1*, *DCX*, *HES5*, and *FABP7* (Fig. 2*C* and *SI Appendix*, Fig. S7 and Table S2). These results are consistent with the morphological and immunocytochemical findings that the compliant substratum facilitates neuronal differentiation of hPS cells.

Most of the hydrogel-derived neurons exhibit bipolar projections (Fig. 2*D*), a morphological feature of interneurons (29). To

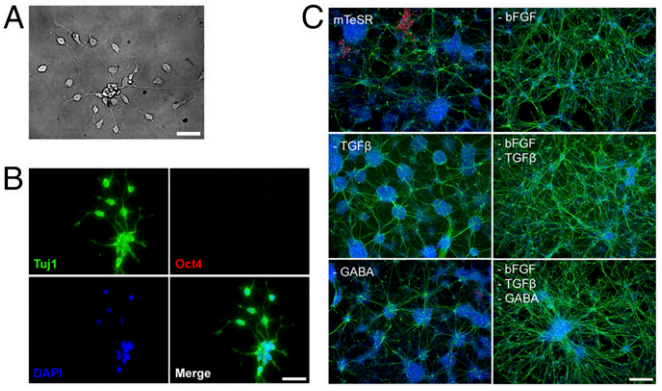

Fig. 1. Neuronal differentiation of hES cells on compliant (0.7 kPa) PA hydrogels. Bright-field (*A*) and immunofluorescence (*B*) images of naive hES cells (SA02 line) cultured for 14 d on the hydrogels with defined mTeSR medium. (*C*) Primed hES cells (SA02 line) differentiated for 5 d on compliant hydrogels with defined mTeSR medium or mTeSR lacking the indicated soluble signaling factors. Cells were immunostained for Tuj1 (green) and Oct4 (red), and counterstained with DAPI (blue). (Scale bars: *A* and *B*, 50 μm; *C*, 250 μm.)

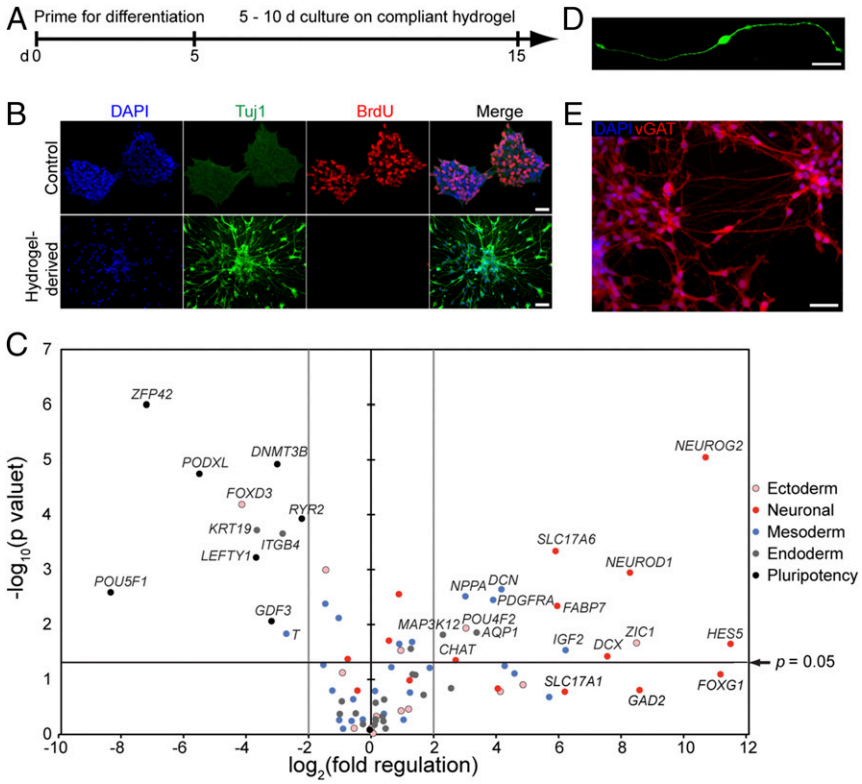

Fig. 2. Characterization of substratum-derived neurons. (*A*) Schematic representation of the procedure for neuronal induction of hES cells on a compliant hydrogel. (*B*) BrdU labeling analysis of hES cells (H9 line) that were undifferentiated or differentiated for 2 wk on a compliant hydrogel. Cells were immunostained for Tuj1 (green) and BrdU (red), and counterstained with DAPI (blue). (*C*) Gene expression analysis of substratum-derived neurons. The hES cells (H9 line) were cultured for 2 wk on compliant PA hydrogels, and their levels of neuronal, pluripotency, and germ layer markers were examined relative to undifferentiated cells. Values shown are $\log_2$ of mean fold regulation (*x* axis) and the statistical significance [*y* axis: $-\log_{10}$ (*P* value)] for $n = 3$ biological replicates. (*D*) Representative image of a Tuj1-positive (green) cell showing dual axon morphology of the substratum-derived neurons. (*E*) Microscopy image of substratum-derived neurons expressing vesicular GABA transporter (vGAT). Cells were counterstained with DAPI (blue). (Scale bars: 50 μm.)

determine whether the differentiated cells express markers of glutamatergic (excitatory) or GABAergic (inhibitory) interneurons, we evaluated their gene expression profile and immunoreactivity. Immunocytochemistry analyses confirmed expression of the proteins vesicular GABA transporter (encoded by *SLC32A1*) and glutamate decarboxylase 67 (GAD67, encoded by *GAD1*) (Fig. 2*E* and *SI Appendix*, Fig. S8). These results indicate that the compliant hydrogel can function without soluble inductive factors to induce hPS cell differentiation rapidly and efficiently into a specific neuronal subtype. Standard protocols for hES cell differentiation into GABAergic neurons involve multiple phases of treatment with soluble signaling factors and require a timeline of 45 d or longer (30). Using our defined substratum, hES cells differentiated into cells characteristic of GABAergic neurons within 2 wk.

We next assessed the functional attributes of the substratum-derived neurons using electrophysiology. After 2 wk of differentiation on the compliant hydrogel, whole-cell patch-clamp recordings indicated that the differentiated cells express large inward and outward currents indicative of neurons (Fig. 3*A*). The substratum-derived cells also display spontaneous postsynaptic currents (Fig. 3*B*), and a subset (3 of 21 cells) of the tested cells had spontaneous action potentials (Fig. 3*C*). Thus, the substratum-derived neurons are electrophysiologically functional and electrochemically similar to neurons derived by standard hES cell differentiation protocols that require a differentiation timeline of 4 wk (31).

We examined whether the induction of neuronal differentiation by compliant hydrogels was applicable to other hPS cell lines. We tested the hES cell lines H1, H7, H9, and SA02 using depleted medium (Fig. 1*C*). Within 2 wk of culture on the compliant hydrogels, all of the hES cell lines undergo neuronal differentiation (*SI Appendix*, Fig. S9*A*). Additionally, the substratum-induced neuronal differentiation is highly efficient; up to 85% of the cells express the neuronal marker Tuj1 (*SI Appendix*,

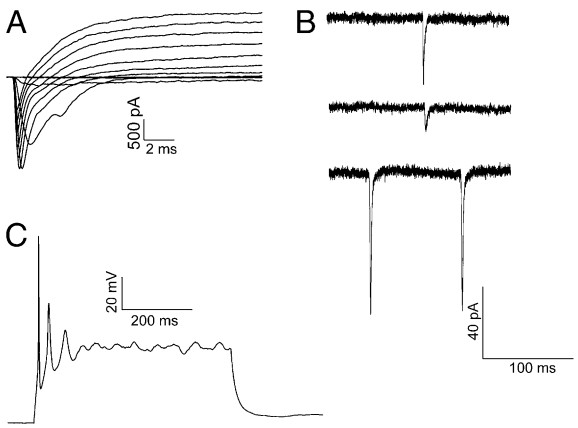

Fig. 3. Functional characterization of substratum-derived neurons. The hES cells (H9 line) were differentiated for 2 wk on compliant PA hydrogels and analyzed by electrophysiology. (*A*) Step-induced currents revealed large, rapidly inactivating inward currents followed by sustained outward currents. Traces of spontaneous postsynaptic currents (*B*) and action potentials (*C*) detected in substratum-derived neurons.

Fig. S9*B*). Neuronal differentiation on the compliant hydrogel was rapid (within 2 wk) for all cell lines examined.

Compliant Hydrogel Does Not Selectively Bind Neuronal Cells. One explanation for the efficient neuronal derivation on the substratum is that compliant hydrogels selectively bind neuronal cells. If the substratum selectively binds neurons, its exposure to a mixed population of cells would lead to selective neuronal cell adhesion. To test for this possibility, we spontaneously differentiated hES cells by treatment with embryoid body medium for at least 25 d to afford a mixed population of cells, including neuronal cells. These spontaneously differentiated cells were transferred onto complaint hydrogels, and the bound cells were characterized by morphology and immunostaining (*SI Appendix*, Fig. S10). Cells adhering to the compliant hydrogel exhibited a variety of morphologies. After an additional 15 d of culture on these surfaces, only a minor subset of the cells was positive for the neuronal marker Tuj1. These results indicate that the observed effects of the compliant hydrogels on neuronal differentiation are not merely due to selective neuronal cell binding.

Molecular Mechanism of Substratum-Induced Neuronal Differentiation of hPS Cells. To determine whether the primed hES cells undergo selective differentiation on stiffer hydrogels, we tested PA hydrogels of varying elasticity (0.7, 3, and 10 kPa) and functionalized them with the GAG-binding peptide CGKKQRFRHRNRKG (15, 23). We selected this range of elasticity based on previously observed differences in the nuclear localization of YAP in hPS cells cultured on such matrices (15). Specifically, YAP is localized in the nucleus on the stiffest hydrogels (10 kPa), whereas it is mainly excluded from the nucleus on the compliant (0.7 kPa) hydrogels. Primed hES cells were cultured on the hydrogels in depleted medium. After 10 d, only the compliant hydrogel (0.7 kPa) promoted efficient and reproducible neuronal differentiation. Although a majority of cells on the stiff hydrogels differentiated (indicated by loss of Oct4 expression), only a small population (~5%) expressed the neuronal marker Tuj1 (Fig. 4 *A* and *B*). Similarly, other stiff substrates, such as polystyrene (~10^6 kPa) displaying the GAG-binding peptide or polystyrene functionalized with Matrigel (BD Biosciences) (a standard ECM for hPS cell culture), also failed to promote neuronal differentiation (Fig. 4*B* and *SI Appendix*, Fig. S11). These results indicate that compliant hydrogels are robust substrata for inducing neuronal differentiation.

Cells bound to compliant surfaces can exhibit cytoskeletal changes, including a decrease in F-actin polymerization (15, 16, 32). If these cytoskeletal changes are important for eliciting neuronal differentiation of hES cells, a small-molecule inhibitor of F-actin polymerization should promote neuronal differentiation even on stiff surfaces. Accordingly, we cultured hES cells on a stiff substratum (polystyrene functionalized with Matrigel) in depleted medium supplemented with an inhibitor of F-actin polymerization, latrunculin A (33) (Fig. 4*C*). The addition of this small molecule promoted neuronal differentiation of hES cells on the stiff substratum (Fig. 4*D* and *SI Appendix*, Fig. S12 *A* and *B*). The similarity between the cells propagated on the stiff surface with latrunculin A (Fig. 4*D*) and the compliant hydrogel (Fig. 4*A*) is striking.

A decrease in F-actin polymerization and stress fiber formation has been linked to a decrease in transcriptional regulatory activity of the paralogous coactivators YAP and transcription

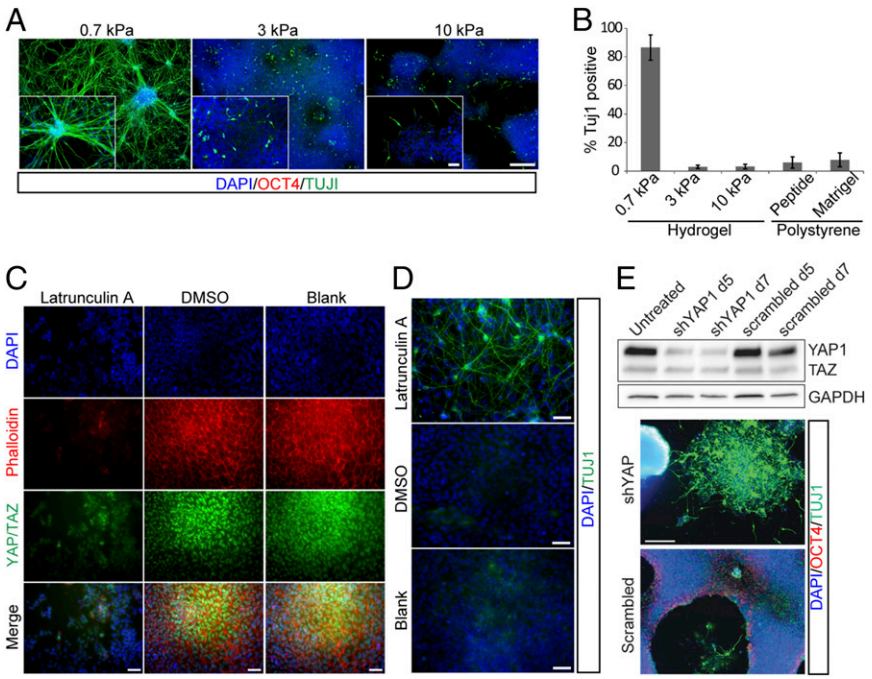

Fig. 4. Compliant hydrogel induces neuronal differentiation by exclusion of YAP from the nucleus. (*A*) H9 hES cells differentiated for 10 d on PA hydrogels that vary in elasticity. Cells were immunostained for the pluripotency marker Oct4 (red) and neuronal marker Tuj1 (green), and counterstained with DAPI (blue). (*B*) Quantification of neuronal induction efficiency (H9 cell line) on hydrogels of varying elasticity, or on polystyrene functionalized with either the peptide displayed on the hydrogels (CGKKQRFRHRNRKG) or Matrigel. Error bars represent SD of the mean ($n = 4$). (*C*) Small-molecule (0.5 μM latrunculin A) inhibition of F-actin polymerization and YAP/TAZ expression in hES cells (H9 line) cultured on a stiff (polystyrene coated with Matrigel) surface. Control samples were treated with depleted medium (blank) or depleted medium with 0.01% DMSO. (*D*) Microscopy analysis of neuronal differentiation resulting from latrunculin A treatment on a stiff polystyrene surface. Cells were immunostained for Tuj1 (green) and counterstained with DAPI (blue). (*E*) Western blot (*Upper*) and immunocytochemistry (*Lower*) analyses of YAP knockdown in H9 hES cells cultured on polystyrene coated with Matrigel. Cells were immunostained for pluripotency (Oct4, red) and neuronal (Tuj1, green) markers, and counterstained with DAPI (blue). shYAP, short hairpin YAP. [Scale bars: *A*, 250 μM; *E*, 200 μM; *A* (*Insets*), *C*, and *D*, 50 μm.]

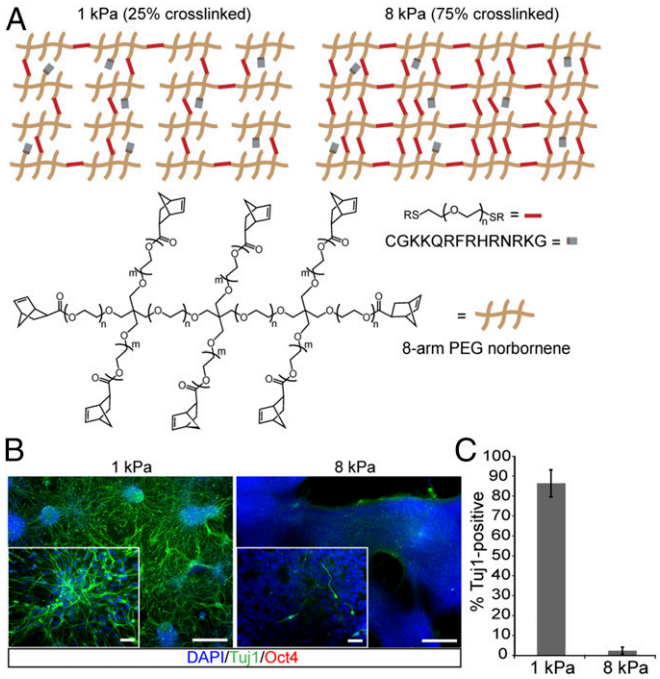

Fig. 5. hES cell differentiation on PEG hydrogels of varying elasticity. (*A*) Schematic of compliant (1 kPa, 25% cross-linked) and stiff (8 kPa, 75% cross-linked) PEG hydrogels. (*B*) Microscopy images of hES cells (H9 line) differentiated for 2 wk on compliant or stiff PEG hydrogels. Cells were immunostained for pluripotency marker Oct4 (red) and neuronal marker Tuj1 (green), and counterstained for DAPI (blue). (*C*) Quantification of neuronal differentiation efficiency on compliant and stiff PEG hydrogels. Error bars denote SD of the mean ($n = 4$). [Scale bars: *B*, 250 μm; *B* (*Insets*), 50 μm.]

coactivator with PDZ-binding domain (TAZ, also known as WWTR1) in hMS cells (16) and hES cells (15). YAP and TAZ have been implicated in signaling pathways elicited by either chemical or mechanical stimuli (34). When in the nucleus, the paralogs YAP and TAZ modulate gene expression, but upon phosphorylation, they are sequestered in the cytoplasm (15). Treatment with latrunculin A resulted in a decrease in YAP/TAZ localization in the nucleus (Fig. 4*C*). This change in subcellular localization was accompanied by an increase in phosphorylated YAP (*SI Appendix*, Fig. S13*A*).

If the absence of YAP or TAZ in the nucleus is important for substratum-induced differentiation, depleting the transcriptional coactivator on a stiff substrate could result in neuronal differentiation. We knocked down YAP using lentiviral-mediated RNAi and observed a decrease in the expression of *CTGF* (an indicator of YAP activity) but not *WWTR1* (encodes TAZ) (*SI Appendix*, Fig. S13*B*). Moreover, YAP depletion resulted in neuronal differentiation of hES cells cultured on a rigid polystyrene surface (Fig. 4*E*). This lineage restriction occurred even in the presence of mTeSR medium. Thus, by preventing nuclear localization of YAP, compliant substrata override soluble signals and robustly induce neuronal differentiation of hPS cells. The activity of the compliant surfaces reveals that the substrata can be as powerful as soluble factors in influencing hPS cell-lineage specification.

Neuronal Differentiation Across Hydrogel Platforms. The compliant (0.7 kPa) PA hydrogel was the most effective at inducing hES differentiation to neurons (Fig. 4 *A* and *B*); consequently, we postulated that other cell culture substrates with similar elastic properties would also direct neuronal specification. We therefore synthesized a different class of hydrogels and varied the elasticities. PEG hydrogels were generated by step-growth polymerization of eight-arm PEG norbornene (PEGNB) monomers and PEG dithiol cross-linker (35). The cross-linking density of the PEGNB hydrogel was varied so that a compliant (~1 kPa) or stiffer (~8 kPa) substratum was obtained (Fig. 5*A* and *SI Appendix*, Fig. S14). These hydrogels were functionalized with the cell-adhesive GAG-binding peptide CGKKQRFRHRNRKG used previously. Primed hES cells were cultured on the PEGNB hydrogels with depleted medium for up to 2 wk. As with the PA hydrogels, only the compliant PEGNB hydrogels induced efficient hES cell neuronal differentiation. (Fig. 5*B* and *SI Appendix*, Fig. S15). Induction of neuronal differentiation on the complaint PEGNB hydrogel was rapid (within 2 wk) and highly efficient (Fig. 5*C*). These results highlight the generality of the substratum-induced differentiation of hPS cells, because the strategy described herein is applicable to different biomaterials.

In summary, our data indicate that insoluble signals from cell culture substrata can have a significant impact on hPS cell-lineage specification. Compliant substrates (~1 kPa) across different hydrogel platforms can rapidly and efficiently direct terminal differentiation of hPS cells into neurons. Thus, neuronal specification of hPS cells need not depend only on soluble inductive factors but also on substratum features. Our data highlight the profound influence of the substratum and underscore the benefits of exploiting substratum features to design protocols to guide hPS cell differentiation.

It is intriguing that the hydrogels with elasticity similar to brain tissue are the most effective at inducing neuronal differentiation. We propose that substratum elasticity controls neuronal differentiation of hPS cells by regulating the activity of YAP (Fig. 6). YAP activity is decreased during mammalian neurogenesis (19, 36, 37) in conjunction with Sonic Hedgehog (36, 37) and Smad (38) signaling pathways, which are ubiquitous targets of soluble factors used to promote neuronal differentiation of hES cells (39–46).

Efforts to differentiate hPS cells to neurons have focused on genes that encode two proneural basic helix–loop–helix (bHLH) transcription factors: *NEUROD1* and *NEUROG2*. Lentiviral-mediated overexpression of either of these genes in hPS cells promotes neuronal differentiation. Intriguingly, *NEUROG2*, a master regulator of neuronal development (20, 47), is up-regulated in cells cultured on the compliant hydrogels. We also observe elevated expression of *NEUROD1* (Fig. 2*C* and *SI Appendix*, Table S2).

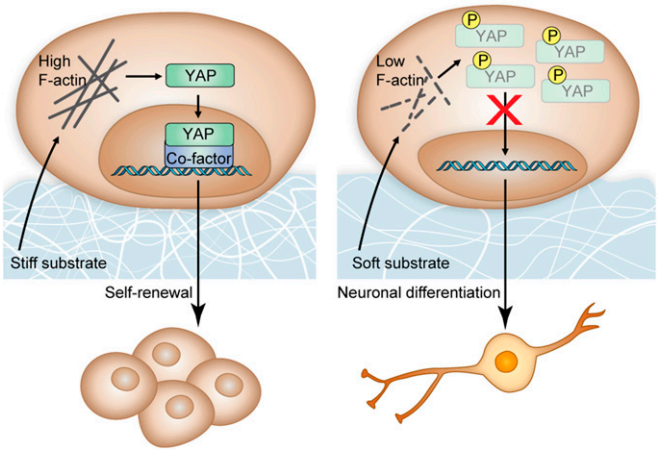

Fig. 6. Proposed model for substratum-induced neuronal differentiation of hPS cells. Stiff substrates promote F-actin polymerization and stress fiber formation, which results in the translocation of YAP to the nucleus, where it regulates gene expression to support self-renewal of hPS cells. Compliant substrates decrease F-actin polymerization, afford increased phosphorylation of YAP, and result in YAP localization in the cytoplasm. Inhibition of YAP coactivator function induces neurogenesis in hPS cells. P, phosphorylation.

Because YAP is excluded from the nucleus in cells cultured on the compliant hydrogels and knockdown of YAP is sufficient to induce neurogenesis, YAP may directly or indirectly repress the production of these master regulators. Our findings suggest that modulating YAP localization via substrate elasticity can circumvent the need for overexpression of exogenous transcription factors to drive neuronal differentiation. Another gene encoding a bHLH transcription factor that is likely regulated by YAP in hPS cells is *NEUROD4*, also known as *NEUROM*, which is repressed by YAP overexpression in the chick neural tube (19). *NEUROD4* is transiently expressed in cells approaching the postmitotic phase and persists in bipolar neurons until terminal differentiation (18). The substratum-induced neurons possess bipolar projections (Fig. 2*D*), a characteristic morphology of interneurons (29). It is therefore possible that YAP inhibition by the compliant hydrogels promotes *NEUROG2* and *NEUROD1* expression along with that of *NEUROD4*, which subsequently guides hPS cell differentiation into interneurons (18, 29). Given the importance of YAP signaling in organ development and function (48, 49), our results suggest that synthetic materials could mimic the inductive power of the embryo and drive hPS cells to specialize.

ACKNOWLEDGMENTS. We thank J. R. Jones for helpful discussions. We acknowledge the W. M. Keck Foundation for support of the Center for Chemical Genomics, and the WiCell Research Institute for providing hES cell lines. P.J.W. and Y.Z. thank the University of Wisconsin–Madison Stem Cell and Regenerative Medicine Center for fellowships. This work was supported by National Institutes of Health (NIH) Grants R01 GM49975 (to L.L.K.), R01 EB007534 (to S.P.P.), R01 HL093282 (to W.L.M.), and R21 NS081484 (to Q.C.) and by National Science Foundation (NSF) Grant CBET 0745563 (to W.L.M.). S.M. was supported by an NSF Graduate Research Fellowship (ID 2007058921), an NIH Chemical-Biology Training Grant (T32 GM008505), and a University of Wisconsin–Madison Graduate Research Fellowship. P.J.W. was supported by an NIH Molecular Biosciences Training Grant (5T32 GM00721535).

1. Thomson JA, et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391):1145–1147.
2. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131(5):861–872.
3. Chowdhury F, et al. (2010) Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. *Nat Mater* 9(1):82–88.
4. Chowdhury F, et al. (2010) Soft substrates promote homogeneous self-renewal of embryonic stem cells via downregulating cell-matrix tractions. *PLoS ONE* 5(12):e15655.
5. Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. *Cell* 126(4):677–689.
6. Holst J, et al. (2010) Substrate elasticity provides mechanical signals for the expansion of hemopoietic stem and progenitor cells. *Nat Biotechnol* 28(10):1123–1128.
7. Mammoto T, Ingber DE (2010) Mechanical control of tissue and organ development. *Development* 137(9):1407–1420.
8. Moore SW, Sheetz MP (2011) Biophysics of substrate interaction: Influence on neural motility, differentiation, and repair. *Dev Neurobiol* 71(11):1090–1101.
9. Yang C, Tibbitt MW, Basta L, Anseth KS (2014) Mechanical memory and dosing influence stem cell fate. *Nat Mater* 13(6):645–652.
10. Li J, Han D, Zhao Y-P (2014) Kinetic behaviour of the cells touching substrate: The interfacial stiffness guides cell spreading. *Sci Rep* 4:3910.
11. Murphy WL, McDevitt TC, Engler AJ (2014) Materials as stem cell regulators. *Nat Mater* 13(6):547–557.
12. Neirinckx V, Coste C, Rogister B, Wislet-Gendebien S (2013) Concise review: Adult mesenchymal stem cells, adult neural crest stem cells, and therapy of neurological pathologies: a state of play. *Stem Cells Transl Med* 2(4):284–296.
13. Krabbe C, Zimmer J, Meyer M (2005) Neural transdifferentiation of mesenchymal stem cells—A critical review. *APMIS* 113(11-12):831–844.
14. Lu P, Blesch A, Tuszynski MH (2004) Induction of bone marrow stromal cells to neurons: Differentiation, transdifferentiation, or artifact? *J Neurosci Res* 77(2):174–191.
15. Musah S, et al. (2012) Glycosaminoglycan-binding hydrogels enable mechanical control of human pluripotent stem cell self-renewal. *ACS Nano* 6(11):10168–10177.
16. Dupont S, et al. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* 474(7350): 179–183.
17. Lian I, et al. (2010) The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes Dev* 24(11):1106–1118.
18. Roztocil T, Matter-Sadzinski L, Alliod C, Ballivet M, Matter JM (1997) NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. *Development* 124(17):3263–3272.
19. Cao X, Pfaff SL, Gage FH (2008) YAP regulates neural progenitor cell number via the TEA domain transcription factor. *Genes Dev* 22(23):3320–3334.
20. Zhang H, Deo M, Thompson RC, Uhler MD, Turner DL (2012) Negative regulation of Yap during neuronal differentiation. *Dev Biol* 361(1):103–115.
21. Sun Y, et al. (2014) Hippo/YAP-mediated rigidity-dependent motor neuron differentiation of human pluripotent stem cells. *Nat Mater* 13(6):599–604.
22. Gauvin R, Parenteau-Bareil R, Dokmeci MR, Merryman WD, Khademhosseini A (2012) Hydrogels and microtechnologies for engineering the cellular microenvironment. *Nanobiotechnol* 4(3):235–246.
23. Klim JR, Li L, Wrighton PJ, Piekarczyk MS, Kiessling LL (2010) A defined glycosaminoglycan-binding substratum for human pluripotent stem cells. *Nat Methods* 7(12): 989–994.
24. Ludwig TE, et al. (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24(2):185–187.
25. Watanabe K, et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 25(6):681–686.
26. Wang N, Butler JP, Ingber DE (1993) Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260(5111):1124–1127.
27. Derda R, et al. (2010) High-throughput discovery of synthetic surfaces that support proliferation of pluripotent cells. *J Am Chem Soc* 132(4):1289–1295.
28. Du ZW, Hu BY, Ayala M, Sauer B, Zhang SC (2009) Cre recombination-mediated cassette exchange for building versatile transgenic human embryonic stem cells lines. *Stem Cells* 27(5):1032–1041.
29. Maxwell DJ, Belle MD, Cheunsuang O, Stewart A, Morris R (2007) Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *J Physiol* 584(Pt 2):521–533.
30. Liu H, Zhang SC (2011) Specification of neuronal and glial subtypes from human pluripotent stem cells. *Cell Mol Life Sci* 68(24):3995–4008.
31. Johnson MA, Weick JP, Pearce RA, Zhang S-C (2007) Functional neural development from human embryonic stem cells: Accelerated synaptic activity via astrocyte coculture. *J Neurosci* 27(12):3069–3077.
32. Discher DE, Mooney DJ, Zandstra PW (2009) Growth factors, matrices, and forces combine and control stem cells. *Science* 324(5935):1673–1677.
33. Spector I, Shochet NR, Kashman Y, Groweiss A (1983) Latrunculins: Novel marine toxins that disrupt microfilament organization in cultured cells. *Science* 219(4584):493–495.
34. Guo X, Zhao B (2013) Integration of mechanical and chemical signals by YAP and TAZ transcription coactivators. *Cell Biosci* 3(1):33.
35. Fairbanks BD, et al. (2009) A versatile synthetic extracellular matrix mimic via thiol-norbornene photopolymerization. *Adv Mater* 21(48):5005–5010.
36. Lin YT, et al. (2012) YAP regulates neuronal differentiation through Sonic hedgehog signaling pathway. *Exp Cell Res* 318(15):1877–1888.
37. Fernandez-L A, et al. (2009) YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. *Genes Dev* 23(23):2729–2741.
38. Alarcón C, et al. (2009) Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell* 139(4):757–769.
39. Neely MD, et al. (2012) DMH1, a highly selective small molecule BMP inhibitor promotes neurogenesis of hiPSCs: Comparison of PAX6 and SOX1 expression during neural induction. *ACS Chem Neurosci* 3(6):482–491.
40. Surmacz B, et al. (2012) Directing differentiation of human embryonic stem cells toward anterior neural ectoderm using small molecules. *Stem Cells* 30(9):1875–1884.
41. Chambers SM, et al. (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27(3):275–280.
42. LaVaute TM, et al. (2009) Regulation of neural specification from human embryonic stem cells by BMP and FGF. *Stem Cells* 27(8):1741–1749.
43. Shimada T, et al. (2012) A simplified method to generate serotonergic neurons from mouse embryonic stem and induced pluripotent stem cells. *J Neurochem* 122(1):81–93.
44. Chambers SM, et al. (2012) Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. *Nat Biotechnol* 30(7):715–720.
45. Ma L, et al. (2012) Human embryonic stem cell-derived GABA neurons correct locomotion deficits in quinolinic acid-lesioned mice. *Cell Stem Cell* 10(4):455–464.
46. Liu H, Zhang SC (2011) Specification of neuronal and glial subtypes from human pluripotent stem cells. *Cell Mol Life Sci* 68(24):3995–4008.
47. Zhang Y, et al. (2013) Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron* 78(5):785–798.
48. Zhao B, Tumaneng K, Guan KL (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nat Cell Biol* 13(8):877–883.
49. Boggiano JC, Fehon RG (2012) Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. *Dev Cell* 22(4):695–702.