Signals from the surface modulate differentiation of human pluripotent stem cells through glycosaminoglycans and integrins

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The fate decisions of human pluripotent stem (hPS) cells are governed by soluble and insoluble signals from the microenvironment. Many hPS cell differentiation protocols use Matrigel, a complex and undefined substrate that engages multiple adhesion and signaling receptors. Using defined surfaces programmed to engage specific cell-surface ligands (i.e., glycosaminoglycans and integrins), the contribution of specific matrix signals can be dissected. For ectoderm and motor neuron differentiation, peptide-modified surfaces that can engage both glycosaminoglycans and integrins are effective. In contrast, surfaces that interact selectively with glycosaminoglycans are superior to Matrigel in promoting hPS cell differentiation to definitive endoderm and mesoderm. The modular surfaces were used to elucidate the signaling pathways underlying these differences. Matrigel promotes integrin signaling, which in turn inhibits mesendoderm differentiation. The data indicate that integrin-activating surfaces stimulate Akt signaling via integrin-linked kinase (ILK), which is antagonistic to endoderm differentiation. The ability to attribute cellular responses to specific interactions between the cell and the substrate offers new opportunities for revealing and controlling the pathways governing cell fate.

biomaterials | cell signaling | human embryonic stem cells | proteoglycan | integrin-linked kinase

Human pluripotent stem (hPS) cells (embryonic stem cells and induced pluripotent stem cells) are promising sources of specialized cells because of two intrinsic properties: they can self-renew indefinitely and differentiate into diverse cell types (1). To selectively guide hPS cell differentiation, precise control cellular microenvironment is needed (2, 3). Considerable effort has been devoted to identify soluble factors that promote differentiation. Additionally, there is interest in developing matrices to support cells during differentiation (4), yet much less is known about how signals from the matrix influence cell fate.

Matrigel is the most widely used substratum for hPS cell propagation (5) and differentiation (6–12); it is derived from mouse sarcoma cells, and although its principal components are laminin, collagen, and entactin, Matrigel consists of up to 1,800 different proteins—including encapsulated growth factors—whose levels vary significantly from batch to batch (13). Accordingly, the contributions of Matrigel-delivered signals to specific phenotypic outcomes—self-renewal or differentiation—are difficult to characterize or control. Elucidating the effects of insoluble signals requires defined substrata. Such defined substrata have been developed for the long-term culture of undifferentiated hPS cells in defined media. A number of surfaces have been described including recombinant proteins (14–16), fully synthetic polymers (17–19), and peptide-modified surfaces (20–23). Surfaces that present bioactive peptides have the advantage that they can be programmed to interact with specific cell-surface macromolecules such as the glycosaminoglycans (GAGs) and integrins.

Defined surfaces can be exploited for differentiation via two distinct mechanisms. First, surfaces can be tailored to present ligands that promote adhesion of specific cell populations during differentiation (24). Second, surfaces can be devised to specifically activate (or mitigate) signal transduction pathways. Here, we use a modular approach to exploit the aforementioned modes to control hPS cell differentiation to each of the primary germ layers. Our results reveal the advantages of defined surfaces for decoding the influence of insoluble signals on cell fate.

Results

Surfaces Tailored for Ectoderm Cell Adhesion. We used a modular approach to program surfaces to engage specific cell-surface targets. Our method was to present biotinylated peptides on immobilized streptavidin (Fig. 1*A* and *SI Appendix*, Fig. S1). Surfaces generated in this way that display the GAG-binding peptide GKKQRFRHRNRKG (GBP) promote long-term hPS cell self-renewal in a defined medium (20). To evaluate the utility of these defined surfaces during ectoderm differentiation, we used a protocol that depends on inhibition of dual Smad signaling pathways. We employed a medium containing noggin, a bone morphogenic protein (BMP) family inhibitor, and a small molecule inhibitor of the Nodal/Activin signaling pathways

Significance

Human pluripotent stem (hPS) cells possess the extraordinary capacity to self-renew indefinitely and differentiate into specialized cell types. Elucidating the signaling pathways that underlie hPS cell differentiation is important for understanding human development and advancing regenerative medicine. Growth factors and small molecules can influence hPS cell differentiation. Many protocols rely on these soluble cues while using substrata composed of numerous biomolecules (e.g. Matrigel). Complex substrata engage multiple types of cellular receptors, thereby obscuring the influence of distinct signals on hPS cell fate. Modular peptide-modified surfaces that interact with specific targets can selectively activate signaling pathways, thereby facilitating hPS cell differentiation. This strategy can be used to dissect how cross-talk between soluble and insoluble signals influences cell fate.

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See Commentary on page 18104.

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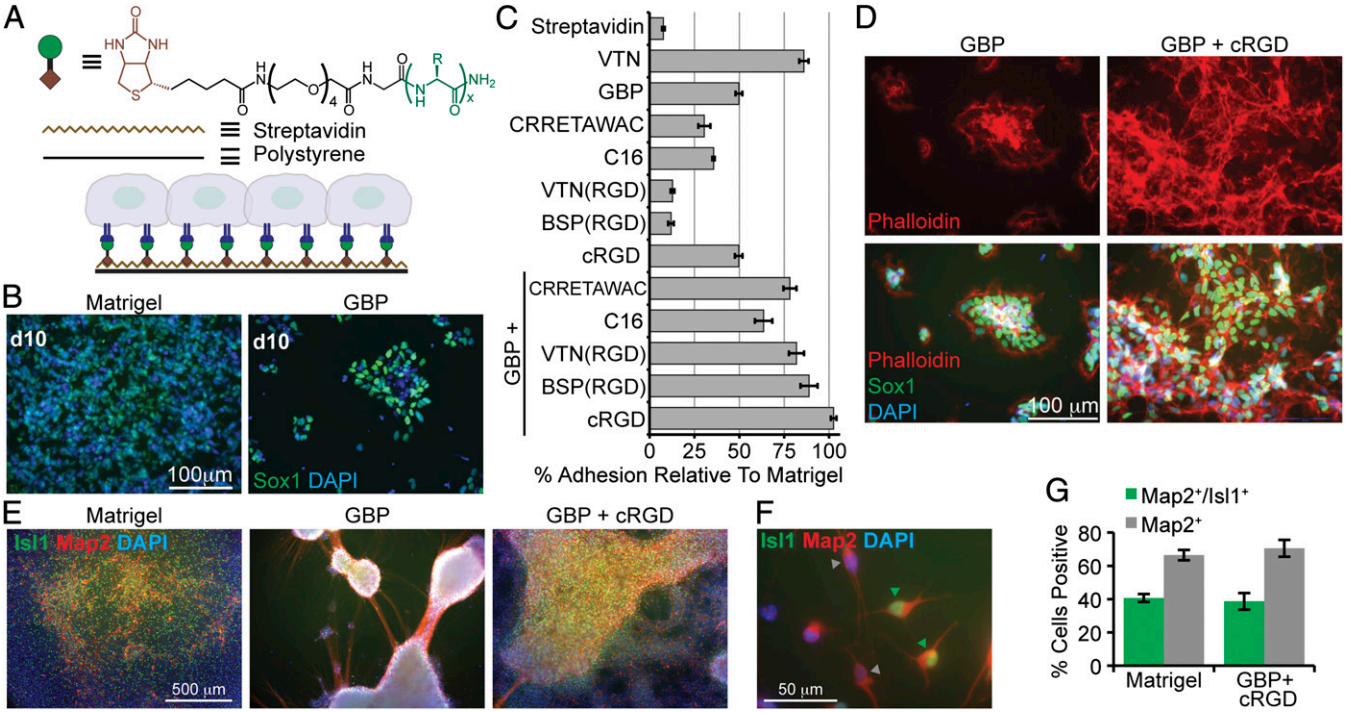

Fig. 1. Defined peptide-presenting surfaces for ectoderm differentiation of hPS cells. (*A*) Cell culture surface schematic consisting of streptavidin-coated polystyrene modified with a biotinylated peptide. (*B*) Human embryonic stem cells (H9) were cultured on Matrigel or on the surface displaying biotinylated GKKQRFRHRNRKG (glycosaminoglycan-binding peptide or GBP) for 10 d and then immunostained for the ectoderm marker Sox1 (green) and the DNA stain DAPI (blue). (*C*) Cells (H9) were exposed to ectoderm differentiation conditions (medium containing noggin and SB431542 on Matrigel) for 5 d, lifted, and allowed to adhere to the specified surfaces (bottom five bars indicate combination surfaces with GBP). Cell adhesion percentages were normalized to the values obtained upon plating on Matrigel. Error bars, SD ($n = 3$ individual surfaces). VTN, vitronectin. Peptide sequences are listed in *SI Appendix*, Table S1. (*D*) Immunostaining for Sox1 (green) with phalloidin (red) and DAPI counterstain (blue) of H9 cells cultured for 8 d in ectoderm-induction medium on the specified peptide surfaces. See *SI Appendix*, Fig. S4 for results using H1, H7, H14, and IMR90-4 cell lines. (*E*) The motor neuron differentiation protocol was applied to cells (H9) cultured on different surfaces. After for 13 d, cells were stained for markers Isl1 (green) and Map2 (red) and counterstained with DAPI (blue). (*F*) Higher-magnification image of individualized cells after 13 d of culture on the combination peptide surface. Gray arrows indicate $Map2^+$ neurons. Green arrows indicate $Map2^+/Isl1^+$ motor neurons. (*G*) Quantification of cellular fractions of $Map2^+$ neurons and $Map2^+/Isl1^+$ motor neurons after culture on the indicated surface for 13 d. Error bars, SD ($n = 3$ individual surfaces).

(SB431542) (6). Gene expression analysis of H9 hES cells (H9) cultured on either Matrigel or on the GBP surface revealed that *NANOG* expression decreased over time, whereas the expression of ectoderm markers *PAX6*, *OTX2*, and *OTX1* increased (*SI Appendix*, Fig. S2*A*). Immunocytochemistry was also consistent with ectoderm differentiation. After 6 d, Oct4, a pluripotency marker, was no longer detectable, but cells stained positive for Sox2, a marker of pluripotency and early ectoderm differentiation (*SI Appendix*, Fig. S2*B*). After 10 d, cells stained positive for Sox1, a hallmark of ectoderm differentiation (Fig. 1*B*). These results indicate defined surfaces displaying GBP can replace complex substrata during directed differentiation toward ectoderm.

Although surfaces displaying GBP can support ectoderm differentiation, cells cultured on such surfaces aggregated, and they expanded less robustly than cells cultured on Matrigel (Fig. 1*B*). The modular approach was used to optimize surfaces for differentiation to this lineage. As cells differentiate, the expression and utilization of cell adhesion receptors can change. To identify such changes, we cultured hES cells on Matrigel in ectoderm-inducing medium for 6 d and analyzed the expression levels of 84 genes related to cell adhesion or the extracellular matrix (ECM; *SI Appendix*, Fig. S3*A*). The genes encoding several integrin subunits and integrin-binding ECM proteins were expressed at higher levels than those in undifferentiated cells (*SI Appendix*, Table S1). These data suggest that a surface designed to engage integrins would be a more effective substratum.

Our screen suggested several integrin targets. The integrin α_5 subunit and fibronectin were up-regulated, implicating a role for integrin $\alpha_5\beta_1$. Expression levels of genes encoding vitronectin and tenascin-C also were increased implicating α_V-containing integrins, and expression of laminin- and collagen-encoding genes was augmented, implicating other β_1-containing integrins. Because integrins participate in cell adhesion and signaling, numerous peptide ligands possessing varying receptor specificities have been developed with the RGD peptide sequence being the most widely exploited (25). We therefore evaluated surfaces displaying a cyclic RGD-containing peptide (cRGD) known to interact with high affinity with the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (21). Additionally, we assessed RGD-containing peptides derived from bone sialoprotein [BSP(RGD)] (26) and vitronectin [VTN(RGD)] (22). We included a laminin-derived ligand for integrin $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (C-16) (27) and a cyclic peptide integrin $\alpha_5\beta_1$ ligand (CRRETAWAC) (28). We fabricated surfaces presenting these ligands alone or in combination with GBP and analyzed each surface for its capacity to support adhesion of hES cell-derived ectoderm (Fig. 1*C*). For comparison, we included a vitronectin-coated surface, which can engage both integrins and GAGs and is less complex than Matrigel (29). HES cells (H9) cultured on Matrigel surfaces were differentiated by treatment with noggin and SB431542 for 5 d and then lifted and allowed to adhere to each of the surfaces. Ectoderm differentiation was confirmed by immunostaining (*SI Appendix*, Fig. S3*B*). Compared with Matrigel, neither GBP-presenting surfaces nor any of the surfaces that only bind integrins could support greater than 50% cell adhesion. Intriguingly, the surfaces presenting both GBP and

an integrin-binding peptide supported adhesion, and these surfaces were as effective as Matrigel or vitronectin (Fig. 1*C*).

Given the ability of the defined surfaces to bind differentiated cells, we evaluated the capacity of combination surfaces presenting GBP and cRGD to support ectoderm differentiation. Human ES cells (H9) were cultured on the combination surfaces in ectoderm induction medium for 8 d. Sox1$^+$ cells were observed on both surfaces presenting GBP alone and surfaces presenting GBP and cRGD. Formation of distinct cytoskeletal stress fibers (filamentous actin) was observed only on surface presenting GBP and cRGD (Fig. 1*D*), indicating these substrata afford robust cell–matrix interactions (30). Differentiation was highly efficient on Matrigel, surfaces presenting both GBP and cRGD, and surfaces presenting GBP alone (*SI Appendix*, Fig. S2*C*). Thus, the advantage of integrin-binding surfaces lies with improved cell adhesion and expansion. These observations are broadly applicable, because the adhesion of hPS cell lines H1, H7, H14, and IMR90-4 during ectoderm differentiation also depended on the surface attributes (*SI Appendix*, Fig. S4).

We next tested whether the defined surfaces could support differentiation of hPS cells to spinal motor neurons, a more mature ectodermal cell type. Cells were subjected to dual SMAD inhibition for 6 d to produce neural progenitors, and then for 6 d using small molecule inhibitors of the Notch (DAPT) and FGF (SU 5402) pathways. Retinoic acid and a small molecule smoothened agonist (SAG) were included throughout for posterior and ventral patterning of the neurons (31). As expected, cells cultured on the GAG-binding peptide surface clustered and many detached, but surfaces displaying both GBP and cRGD were as effective for differentiation as Matrigel (Fig. 1*E*). By 13 d, we observed cells staining positive for Map2, a pan-neuronal marker, and Isl1, a marker indicative of motor neurons when expressed together with Map2 (Fig. 1 *E* and *F*). The percentage of cells that are Map2-positive (~70%) and Map2- and Isl1-positive (~40%) on day 15 was similar whether cells were cultured on Matrigel or a GBP/cRGD combination surface (Fig. 1*G*). This efficiency is consistent with previous protocols for motor neuron differentiation that use complex substrata (31). These results demonstrate that our modular approach can yield chemically defined substrates that are as effective for motor neuron differentiation as Matrigel.

Matrigel Is Inhibitory to Endoderm Differentiation. We next used defined surfaces for differentiation toward the other primary germ layers. Differentiation of hPS cells to definitive endoderm under completely defined conditions remains challenging. Typical procedures involve exposure of hPS cells to activin A, combinations of growth factors, and/or undefined components like FBS or Matrigel (7, 9, 10). To determine the effects of the insoluble substratum on definitive endoderm differentiation, we used serum-free medium supplemented with activin A. HES cells (H9) were cultured on Matrigel or GBP surfaces and exposed to differentiation medium for 3 d. Interestingly, the expression of pluripotency markers *POU5F1* (encodes Oct4) and *SOX2* was down-regulated earlier and more drastically in cells cultured on GBP surfaces vs. Matrigel. The primitive streak genes *T* and *MIXL1* were detected earlier in the cells cultured on GBP, and increases in the expression levels of definitive endoderm genes *CXCR4*, *CER1*, *SOX17*, and *FOXA2* all occurred earlier in the cells cultured on GBP (*SI Appendix*, Fig. S2*D*). This trend held when we used immunocytochemistry to analyze cells cultured for 4 d with activin A on GBP or Matrigel (Fig. 2*A*). GBP surfaces yielded a greater proportion of Sox17$^+$ cells compared with Matrigel (Fig. 2*A* and *SI Appendix*, Fig. S5). Further, many cells stained positive for both Sox17 and FoxA2 (*SI Appendix*, Fig. S2*E*), which is indicative of definitive rather than extraembryonic endoderm (3). These results illustrate that signals from the matrix influence hPS cell differentiation to endoderm.

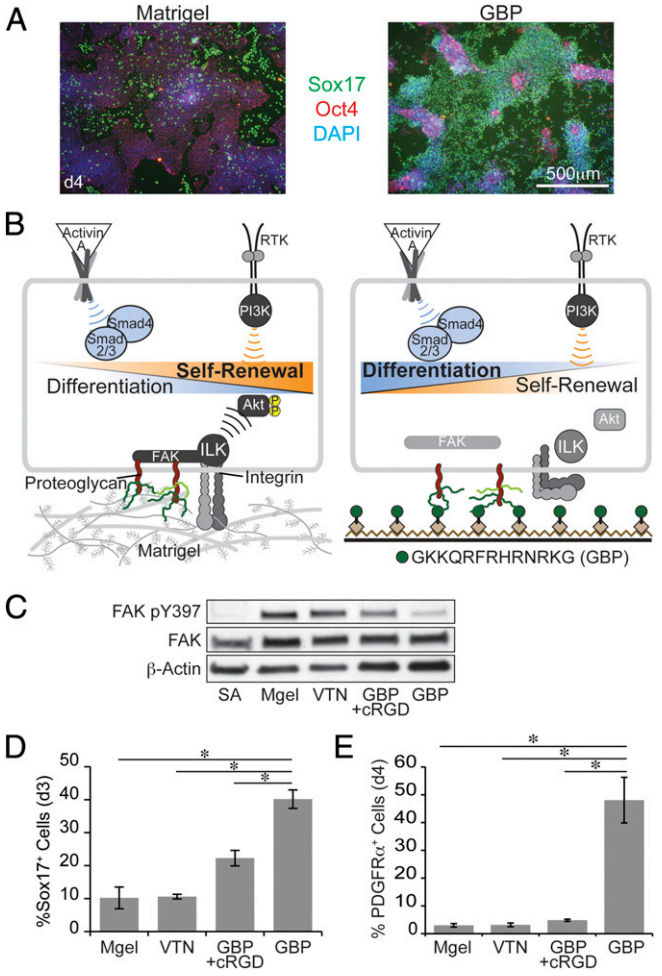

Fig. 2. Mesendoderm differentiation on GAG-binding and integrin-binding surfaces. (*A*) Immunocytochemistry analysis for Oct4 (red) and Sox17 (green) for cells (H9) cultured on the indicated surfaces treated with activin A for 4 d. Nuclei are counterstained with DAPI (blue). (*B*) Proposed model: Insoluble signals influence the balance between Smad2/3 and Akt signaling. Surfaces that engage integrins (Matrigel, vitronectin, and GBP + cRGD) activate ILK and Akt signaling. Surfaces engaging only GAGs (GBP alone) show reduced Akt activation. The influence of the substratum on Akt signaling tips the balance toward self-renewal (Matrigel) or differentiation (GBP). RTK, receptor tyrosine kinase. Yellow-circled P indicates phosphorylation. (*C*) Western blot analysis of phospho-Y397 FAK, total FAK, and β-actin in H9 cells adhering to the indicated surfaces. The streptavidin (SA) surface was used as a negative control because cells did not adhere and were gathered from suspension. Mgel, Matrigel. VTN, vitronectin. (*D*) Quantification of flow cytometry analyses for Sox17$^+$ cells after culture on the indicated surfaces with activin A for 3 d. Error bars, SD ($n = 3$ and $^*P < 0.01$). (*E*) Quantification of flow cytometry analyses for PDGFRα$^+$ cells after culture on the indicated surfaces with activin A for 1 d followed by BMP4 and bFGF for 3 d ($n = 3$ and $^*P < 0.01$).

Integrin-Binding Surfaces Inhibit Mesendoderm Differentiation. To understand why differentiation occurs efficiently on the synthetic surface, we probed the underlying molecular mechanism. Differentiation toward mesendoderm, the common progenitor for definitive endoderm and mesoderm, is regulated by the balance of two signaling pathways: PI3K/Akt and Smad2/3 (32). When PI3K/Akt signaling is high and Smad2/3 signaling low (but not absent), hPS cells favor self-renewal. When the balance shifts toward high Smad2/3 signaling and low (but not absent) Akt signaling, mesendoderm differentiation is favored. Soluble signals, such as insulin or bFGF, can promote PI3K/Akt signaling through receptor tyrosine kinases, whereas activin A and TGF-β ligands activate Smad2/3. We postulated that the substratum ligands could alter

the Akt/Smad signaling balance. Specifically, integrin engagement can activate Akt signaling (30). With its mixture of many ECM proteins, Matrigel engages many integrins (13), whereas surfaces displaying GBP bind cell-surface GAGs and not integrins (20).

The aforementioned analysis suggests that integrin-activating substrata will inhibit definitive endoderm differentiation (Fig. 2*B*). We monitored autophosphorylation at tyrosine 397 of focal adhesion kinase (FAK) to assess different substrata, including Matrigel, vitronectin-coated substrates, surfaces presenting both GBP and cRGD, or surfaces presenting GBP alone for their ability to activate signals downstream of integrins. As expected, Matrigel, vitronectin, and the surface displaying both GBP and cRGD induced greater FAK autophosphorylation than did GBP alone (Fig. 2*C*). We then tested the differentiation capacity of hPS cells cultured on surfaces that specifically activate integrin signaling. After 3 d of culture on each test surface in endoderm-induction medium, the cells were analyzed by flow cytometry for Sox17 expression (Fig. 2*D*). Differentiation was less efficient on each of the integrin-binding surfaces compared with that on GBP alone. To rule out the possibility that decreased GBP density on the combination surfaces caused the decreased differentiation, we generated surfaces with biotinylated GBP mixed 1:1 with unconjugated biotin, which allowed adhesion equal to that on full GBP surfaces and did not inhibit differentiation (*SI Appendix*, Fig. S6). On the GBP surfaces, 40% of the H9 cells and 66% of IMR90-4 cells expressed Sox17 after 3 d (Fig. 2*D* and *SI Appendix*, Fig. S5*E*), significantly higher proportions compared with integrin-binding surfaces.

Mesoderm and definitive endoderm share a common progenitor stage; therefore, we postulated that cells would also differentiate more efficiently toward mesoderm on defined GBP surfaces. To induce mesoderm differentiation, cells (H9) were cultured on GBP or Matrigel with activin A for 1 d and then with BMP4 and bFGF for 3 d (8, 11). Compared with cells cultured on Matrigel, cells cultured on GBP underwent a more pronounced decrease in expression of the pluripotency markers (*NANOG* and *POU5F1*) and expressed higher levels of primitive streak (*T* and *MIXL1*) and mesoderm marker genes (*PDGFRA* and *FOXF1*; *SI Appendix*, Fig. S2*F*). After 4 d, 48% of cells cultured on GBP stained positive for the mesoderm marker PDGFRα. In contrast, only 3% of cells cultured on Matrigel or vitronectin and only 5% of cells cultured on surfaces presenting GBP and cRGD were PDGFRα$^+$ (Fig. 2*E*). Every hPS cell line tested differentiated more efficiently on GBP surfaces than on Matrigel (*SI Appendix*, Fig. S7).

We then examined whether the advantage of GBP surfaces extends to differentiation to cardiomyocytes, a functional mesodermal cell type. To this end, we developed and characterized a cardiac reporter cell line that drives GFP expression under the cardiac troponin T (cTnT) promoter (H9-cTnT-GFP) (*SI Appendix*, Fig. S8). This reporter cell line enables the dynamic fluorescent tracking of cardiomyocyte generation. We cultured H9-cTnT-GFP cells on a GBP surface under the mesoderm differentiation conditions for 6 d and an additional 6 d without added growth factors (33). We observed spontaneous contracting of GFP$^+$ cells cultured on the synthetic surface (*SI Appendix*, Fig. S8 and Movie S1). We then compared cells cultured on Matrigel to cells on GBP and observed significantly higher proportions of cells expressed GFP after 10 and 15 d when cultured on GBP (*SI Appendix*, Fig. S8). Because cells can remodel their ECM, the long time periods of this experiment render it difficult to attribute the differentiation differences to specific matrix signals. Still, these results demonstrate that the substratum can influence mesoderm differentiation, and defined surfaces can present an advantage.

Integrin-Linked Kinase Links Matrix to Akt. To analyze whether culture on integrin- or GAG-binding surfaces influences the Smad/Akt balance, we compared activation of Smad2 and Akt during activin A treatment. When active, Smad2 is phosphorylated at Ser465 and Ser467 and Akt is phosphorylated at Ser473 and Thr308. We monitored levels of Akt and Smad2 phosphorylation over time. Levels of Smad2 phosphorylation increase quickly upon activin A treatment, but remain similar for cells cultured on all surfaces tested (*SI Appendix*, Fig. S9). After 12 h of exposure to activin A, however, significantly increased levels of Akt phosphorylation were detected in cells cultured on Matrigel versus those cultured on GBP (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S9). Because Matrigel is complex and could be acting via components besides integrins, we also compared vitronectin-coated surfaces to GBP surfaces. As expected for a surface that can bind integrins, Akt phosphorylation on vitronectin was higher than that on GBP at both 30 min and at 12 h (*SI Appendix*, Fig. S9*E*).

Akt signaling can control the cell cycle, proliferation, and survival. Thus, we hypothesized that cells cultured on integrin-binding surfaces, which activate Akt signaling, would self-renew and proliferate at the expense of differentiation. Despite equivalent initial cell binding (−24 h) and expansion (0 h), cells on integrin-binding surfaces proliferated upon exposure to activin A-medium, whereas cells on GBP ceased proliferation (Fig. 3*C*). In cells cultured on GBP surfaces, we also observed slightly increased levels of cleaved caspase-3 and cleaved poly ADP ribose polymerase-1 (PARP1). Thus, increased levels of apoptosis or anoikis on GBP surfaces may also contribute to the increase in differentiation efficiency (*SI Appendix*, Fig. S10). These differences reveal how the substratum composition can influence proliferation, survival, and differentiation.

Integrins transmit signals via intracellular signaling proteins, such as FAK or integrin-linked kinase (ILK), both of which are can activate Akt signaling (30). We used a chemical biology approach to dissect the contribution of these pathways. Cells (H9) were cultured on Matrigel with differentiation medium supplemented with the FAK inhibitor PF-573,228 (34) or ILK inhibitor Cpd 22 (35). At greater than 1 μM PF-537,228 or 0.7 μM Cpd 22, we observed cell toxicity. Cells were therefore cultured on Matrigel with activin A and the FAK inhibitor at concentrations that inhibit FAK but are not toxic. No change in Akt phosphorylation was detected, and no increase in differentiation was observed (Fig. 4 *A* and *B*). In contrast, cells treated with the ILK inhibitor displayed decreased levels of Akt phosphorylation (Fig. 4*A*). Further, ILK inhibition afforded more brachyury$^+$ cells after 24 h (*SI Appendix*, Fig. S11), and at 4 d, a significant increase in the percentage of Sox17$^+$ cells was observed (Fig. 4*B*). To account for the possibility of small molecule off-target effects, we tested the role of ILK using RNA interference. By treating H9 cells with two different shRNA sequences, we depleted ILK at the RNA (Fig. 4*C*) and protein (Fig. 4*D*) levels. When cultured on Matrigel (Fig. 4*E*) or vitronectin (Fig. 4*F*), cells with depleted ILK responded more efficiently to endoderm induction compared with cells transduced with a nontargeting shRNA. Together, these results indicate that signals from the insoluble substratum are transmitted via integrins and ILK to modulate the Akt signaling pathway and directly influence activin A-induced endoderm differentiation.

Discussion

Defined substrata have been designed to obviate the need for Matrigel for hPS cell culture; these include purified human ECM proteins coated on plastic or other polymers (5, 14, 16, 36, 37), fully synthetic polymers (17–19, 22, 38), or peptide-presenting surfaces (20–22, 39, 40). Several surfaces have been used for differentiation to specific cell types, such as cardiomyocytes (22, 33, 41), endothelial and bone cells (36), neurons (38, 42), or definitive endoderm (38, 43). Although polymers can be produced inexpensively, it can be difficult to characterize or control how these surfaces interact with cells. Recombinant ECM proteins, such as vitronectin or laminin, engage multiple classes of cell-surface receptors. Vitronectin, for example, binds cell-surface integrins, GAGs, and urokinase receptors, as well as extracellular proteins, including plasminogen, plasminogen activator

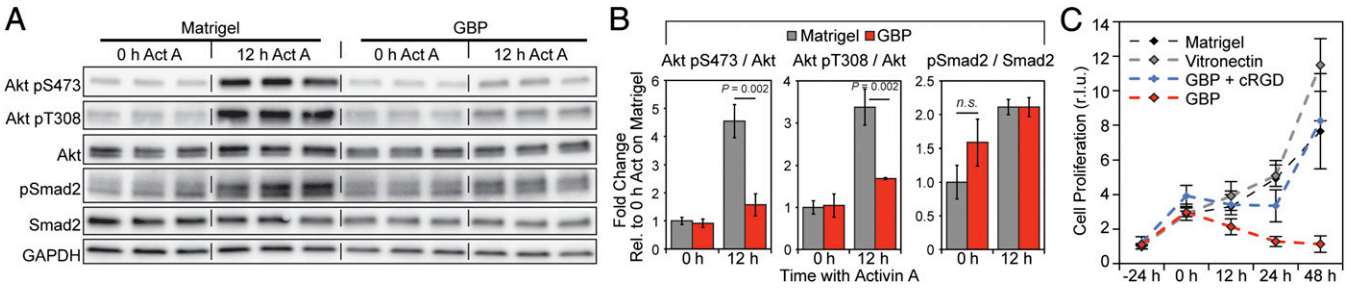

Fig. 3. Culture on Matrigel activates Akt signaling. (*A*) Western blot analyses for phospho-S473 Akt, phospho-T308 Akt, total Akt, phospho-S465/467 Smad2, and total Smad2. Cells were cultured on Matrigel or GBP and incubated for either 0 h or 12 h with activin A (Act A). (*B*) Quantification of Western blot analyses. Graphs show the mean and error bars indicate SD ($n = 3$ individual surfaces. n.s., $P > 0.05$). Rel., relative. (*C*) Analysis of cell proliferation on the indicated surfaces. Error bars, SD ($n = 5$ individual surfaces). r.l.u., relative light units. Samples normalized to those obtained when cells were allowed to adhere to Matrigel overnight (−24 h).

inhibitor-1, collagen, and thrombin-antithrombin III complex (29). As a result, separating the individual effects of specific interactions on cell fate is complicated. Moreover, many ECM proteins are difficult or costly to obtain in sufficient quantities for use as substrata (16). The modular, programmable approach we described can be tailored to yield surfaces that present peptides that bind to targeted receptors; in this way, it combines the simplicity of synthetic polymers with the bioactivity of recombinant proteins.

Peptide-presenting surfaces can be tailored to display ligands specific for desired cell populations by exploiting genomic, proteomic, or glycomic analysis of the desired cell types. The defined ectoderm differentiation conditions we devised illustrate this strategy. Although simple surfaces displaying the GBP support cells during ectoderm differentiation, cell adhesion to the surface was not robust. By analyzing the expression of genes encoding proteins involved in adhesion, we identified cell-surface integrins as potential targets. When surfaces presenting both the GBP and cRGD were fabricated, they supported hPS cell-derived ectoderm and motor neuron differentiation, and they were as effective as Matrigel. These investigations illustrate that a defined surface displaying two specific ligands can replace an undefined surface that presents over 1,800 proteins (13). Standardizing motor neuron differentiation protocols will facilitate understanding of degenerative diseases such as amyotrophic lateral sclerosis.

The surface strategy described herein is a powerful means of uncoupling the cross-talk between soluble signals and those from the matrix. The power is illustrated by our experiments focused on endoderm and mesoderm differentiation. Specifically, we found that GAG-binding surfaces favorably shift the relative balance of the Akt/Smad axis to favor differentiation. In contrast, substrata like Matrigel or vitronectin are not passively adhesive, but rather activate integrin signaling that is antagonistic for mesoderm and endoderm differentiation.

Further dissection of the role of integrin engagement in limiting differentiation revealed a role for ILK, a protein not previously implicated in pluripotency. ILK is largely studied for its role in cancer (35) and epithelial–mesenchymal transition (44). Although whether ILK contains a functional kinase domain is debatable (45), there is evidence that its role as an oncogene stems from its ability to activate its downstream effector Akt, a signaling hub in both cancer and pluripotency. The addition of Cpd 22, a ligand for ILK, reduced Akt phosphorylation. Cells cultured with Cpd 22 and cells with ILK depleted using shRNA exit the pluripotent state more efficiently in response to activin A. Other less-defined definitive endoderm differentiation protocols use more complex mixtures of growth factors (9, 10, 46), which may function to overcome the inhibitory signals from immobilized vitronectin or Matrigel. Our strategy of using completely defined soluble and insoluble components afforded new insight into mechanisms of differentiation, which enabled precise analysis and control of the relevant signaling pathways.

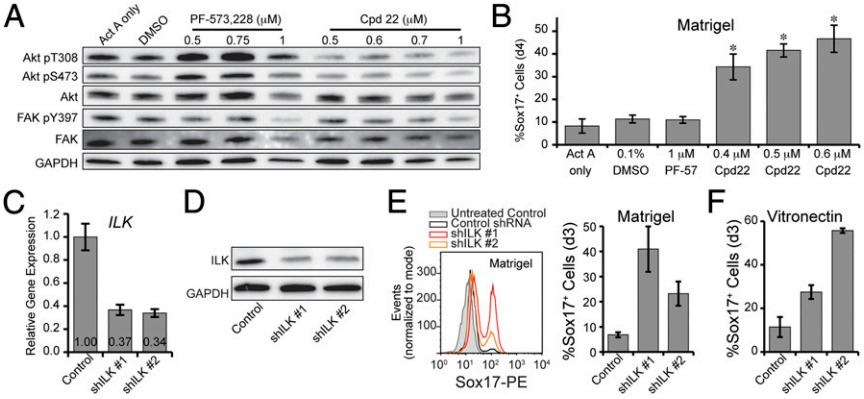

Fig. 4. ILK inhibition decreases Akt activity and increases endodermal differentiation. (*A*) Western blot analysis for phospho-Akt and phospho-FAK in cells (H9) treated for 12 h with activin A and the FAK inhibitor PF-573,228 or activin A and the ILK inhibitor Cpd 22. (*B*) Quantification of flow cytometry analyses of $Sox17^+$ cells after culture on Matrigel surfaces with activin A and the indicated small molecule inhibitors for 2 d and then activin A alone for 2 d. Graph depicts the mean, and error bars indicate SD ($n = 3$, $^*P < 0.005$ compared with DMSO control). (*C*) Quantitative PCR and (*D*) Western blot analyses of cells transduced with a nontargeting shRNA (control) or with shRNA sequences targeting *ILK*. Error bars indicate SD of quadruplicate quantitative PCR reactions. (*E*) Representative histograms and quantification of flow cytometry analyses for shILK cells cultured on Matrigel (*E*) or vitronectin (*F*). Error bars indicate SD ($n = 3$).

We anticipate that the simplicity of our approach using modular peptide-presenting surfaces will facilitate multifactorial screens to determine the combinatorial effects of insoluble and soluble signaling on stem cell fate. Defined surfaces that support cardiomyocyte differentiation (i.e., GBP-presenting surfaces), and the selectable H9 cTnT-GFP cell line we derived, will be useful tools study cardiogenesis (47). Many differentiation protocols rely on different stages in which the soluble factors are changed over time. We anticipate that altering the substrate during different stages could also improve differentiation outcomes. Because streptavidin-coated surfaces are compatible with typical multiwell-format plastic culture plates, this surface fabrication strategy can be used to immobilize new types of peptides (48) or other small molecules (24) to probe the cross-talk between soluble and insoluble signaling in diverse cell types and cells at different stages of differentiation.

Materials and Methods

HPS cell lines were cultured on GBP in mTeSR (Stem Cell Technologies) with 5 μM Y-27632 (Tocris) as described previously (20). All differentiation experiments began by plating hPS cells at 50,000 cells/cm^2, allowing them to adhere for 2 d before differentiation. For ectoderm, RPMI-1640 with B27 (RB27) (Invitrogen Gibco) was supplemented with 500 ng·mL^{-1} Noggin (R&D Systems) and 10 μM SB431542 (Tocris) for 5 d and RB27 without supplement afterward. Media for culture on peptide surfaces were supplemented with Y-27632. For endoderm, RB27 contained 100 ng·mL^{-1} activin A. For inhibitor experiments, PF573,228 (Tocris) and Cpd 22 (Millipore) were included for 2 d, then replaced with activin A medium without inhibitors for 2 d. For mesoderm, RB27 contained 100 ng·mL^{-1} activin A (R&D Systems) for 1 d, then 10 ng·mL^{-1} BMP4 (R&D Systems) and 10 ng·mL^{-1} bFGF (WiCell Research Institute, Inc.). For further experimental details, see *SI Appendix*.

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