A defined glycosaminoglycan-binding substratum for human pluripotent stem cells

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To exploit the full potential of human pluripotent stem cells for regenerative medicine, developmental biology and drug discovery, defined culture conditions are needed. Media of known composition that maintain human embryonic stem (hES) cells have been developed, but finding chemically defined, robust substrata has proven difficult. We used an array of self-assembled monolayers to identify peptide surfaces that sustain pluripotent stem cell self-renewal. The effective substrates displayed heparin-binding peptides, which can interact with cell-surface glycosaminoglycans and could be used with a defined medium to culture hES cells for more than 3 months. The resulting cells maintained a normal karyotype and had high levels of pluripotency markers. The peptides supported growth of eight pluripotent cell lines on a variety of scaffolds. Our results indicate that synthetic substrates that recognize cell-surface glycans can facilitate the long-term culture of pluripotent stem cells.

Human pluripotent stem cells have the remarkable capacity to both self-renew indefinitely and differentiate into many different cell types[1–3]. Progress in developing defined conditions for human embryonic stem (hES) cell propagation has resulted from elucidating the roles of soluble factors[4–6]. In contrast, it has proven challenging to identify defined substrata for this purpose. Typical mixtures used to date comprise extracellular matrix proteins from animal or human sources[4,7]. The batch-to-batch inconsistency that accompanies the use of complex mixtures complicates hES cell propagation. Moreover, the use of animal-derived extracellular matrix proteins exposes cells to potentially hazardous pathogens and allows for the transfer of immunogenic epitopes[8]. Individual extracellular matrix proteins[9–11] have been used as substrates for hES cell self-renewal, but even single extracellular matrix proteins have multiple domains that can engage a variety of cell-surface receptors. As a result, it is difficult to identify the key interactions that result in reproducible culture conditions.

We sought to replace the substrata used for hES cell culture with a fully defined, synthetic alternative. A synthetic surface could minimize the exposure of pluripotent cells to hazardous contaminants and yield a more homogenous cell culture. Moreover, it could illuminate the minimum requirements for adhesion and self-renewal. Although synthetic substrata have been described[12–20], none have been shown to be effective for the long-term culture of both hES cells and induced pluripotent stem (iPS) cells. Most of these substrata either have been used in combination with a conditioned medium[12–14] or have been tested with only a limited number of cell lines[15–20]. Additionally, the mechanisms by which these substrates function are largely unknown. Here we describe a synthetic surface that engages cell-surface glycosaminoglycans and supports the long-term propagation of multiple hES and iPS cell lines in fully defined conditions.

RESULTS

Peptide substrates for hES cell adhesion

To seek a substrate with the desired activity, we used a defined-surface array[21] composed of peptide-substituted alkanethiol conjugates, which form self-assembled monolayers on gold[22]. Self-assembled monolayers can present various ligands with control over surface density, making them valuable probes for testing the consequences of engaging cell-surface ligands[23]. Previous screens using surface arrays that present peptides derived from laminin at high densities[12] or cell-binding peptides identified by phage display[18] yielded synthetic surfaces capable of supporting the short-term propagation of hES cells. To identify a synthetic surface for long-term propagation, we expanded our array screen[12,21] to assess surfaces with bioactive peptide sequences reported to bind to diverse cell-surface receptors (**Supplementary Table 1**). We did not limit our screen to known protein-binding sequences (for example, the integrin ligand arginine–glycine–aspartic acid (RGD) tripeptide[24]) and included peptides that can interact with glycosaminoglycans. Because cell adhesion is influenced not just by the identity of the adhesive epitopes but by their density on the substrate and whether they are present alone or in combination with other ligands[21,25], we used the array to present bioactive peptides alone, in different combinations and at varying surface densities. We screened over 500 unique surfaces based on 18 bioactive peptides.

First we evaluated the surfaces for their abilities to support hES cell adhesion. We dissociated H1 or H9 cells that

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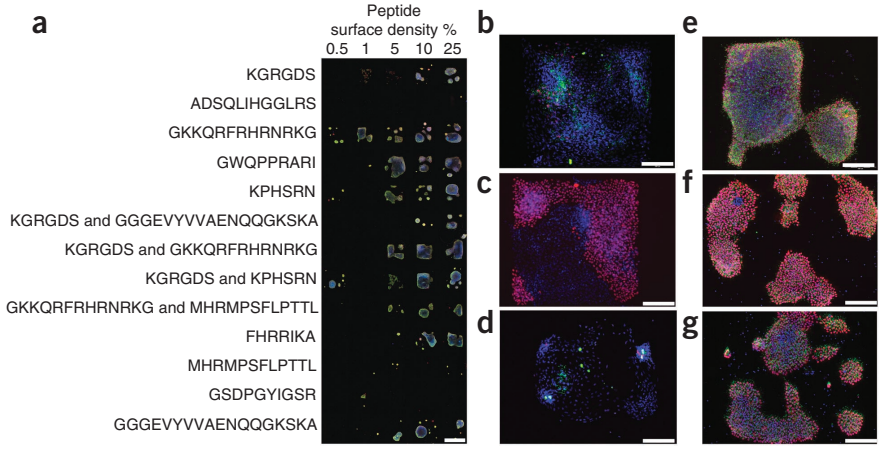

Figure 1 | Strategy for the identification of peptide-substituted surfaces for hES cell adhesion and survival. (**a**) H9 cells bound to a representative array presenting the shown bioactive peptides at indicated surface densities (indicated as percentage of peptide-substituted alkanethiol in the mixed self-assembled monolayer). Cells were fixed, immunostained for Oct-4 (red) or SSEA-4 (green) and counterstained with DAPI (blue). (**b**–**g**) Representative higher magnification images of H9 cells (**b**–**e**) and H1 cells (**f**,**g**) on surfaces presenting the fibroblast growth factor receptor–binding peptide GGGEVYVVAENQQGKSKA and the integrin-binding peptide KGRGDS (**b**), KGRGDS and another bioactive peptide derived from fibronectin, KPHSRN (**c**), the laminin-derived peptide GSDPGYIGSR (**d**) or on surfaces presenting heparin-binding peptides GKKQRFRHRNRKG from vitronectin (**e**), GWQPPRARI from fibronectin (**f**) or FHRRIKA from bone sialoprotein (**g**). Scale bars, 1 mm (**a**) and 200 μm (**b**–**g**).

had been grown in culture (cultured) on Matrigel and applied them to the array in a protein-free basal medium, to minimize the adsorption of proteins from the medium. After 1 h, we replaced the basal medium with defined mTeSR medium[4] supplemented with Y-27632, an inhibitor of rho-associated kinase (ROCK). This small molecule aids the survival of dissociated hES cells[26]. Using these conditions, we propagated hES cells on the array for 6 d, and then fixed and stained them for pluripotency markers Oct-4 and SSEA-4 (**Fig. 1a**). In this way we identified several surfaces that could sustain cell adhesion during the experiment. Adhesion to the self-assembled monolayers required the ROCK inhibitor, even after colonies were established (**Supplementary Fig. 1**).

Analysis of the surfaces capable of supporting adhesion revealed that those substituted with the integrin ligand KGRGDS routinely bound to cells, but their ability to maintain hES cell markers was inconsistent (**Fig. 1b**,**c**). Another integrin-binding peptide, GSDPGYIGSR, also mediated hES cell adhesion, but the resulting cells had markedly less Oct-4 and SSEA-4 (**Fig. 1d**). In contrast, surfaces presenting heparin-binding peptides (GKKQRFRHRNRKG, FHRRIKA and GWQPPARARI) consistently mediated hES cell adhesion and allowed for hES cell propagation: cells cultured on these surfaces for 6 d had high levels of Oct-4 and SSEA-4 (**Fig. 1e**–**g**). Surfaces displaying the heparin-binding peptide GKKQRFRHRNRKG[27] derived from vitronectin not only supported adhesion but did so at the lowest peptide substitution levels (**Fig. 1a**,**e**). Other peptide-substituted surfaces either did not mediate hES cell adhesion or did so inconsistently (**Supplementary Table 1**). Thus, surfaces presenting heparin-binding peptides effectively supported short-term hES cell survival.

Adhesion through cell-surface glycosaminoglycans

We anticipated that surfaces displaying heparin-binding peptides would promote cell adhesion and spreading by interacting with cell-surface glycosaminoglycans. Glycosaminoglycans have a variety of roles: they participate in receptor-signaling complexes, cell-cell recognition, cell-cell adhesion and cell-matrix interactions[28]. To test whether glycosaminoglycans participate in hES cell binding to the heparin-binding peptide–substituted surfaces, we exposed cells to the enzyme chondroitinase ABC. Although this enzyme catalyzes the hydrolysis of only a subset of glycosaminoglycans, cells exposed to it exhibited decreased binding to the synthetic surfaces presenting GKKQRFRHRNRKG (**Supplementary Fig. 2**). Soluble heparin, which should compete with cell-surface glycosaminoglycans, also inhibited binding (**Fig. 2a**). In addition to completely blocking cell interactions to the synthetic GKKQRFRHRNRKG-presenting surfaces, heparin mitigated cell adhesion to Matrigel and recombinant vitronectin protein, suggesting that these substrates also bind to hES cells through glycosaminoglycan interactions (**Fig. 2a**). These data suggest that glycosaminoglycans found on hES cell surfaces can be important in mediating attachment to the substratum. Additionally, a substratum consisting of even a single extracellular matrix protein, such as recombinant vitronectin, supported cell adhesion through multiple interactions, and synthetic surfaces presenting a heparin-binding peptide enabled cell adhesion through a single binding epitope.

Growth rates of hES cells

We compared hES cell growth rates on surfaces presenting synthetic peptides and on more complex surfaces. First, we used a colorimetric assay to assess cell numbers and generate growth curves for H9 and H13 cells cultured on Matrigel, recombinant vitronectin–coated surfaces and streptavidin-modified surfaces presenting biotinylated GKKQRFRHRNRKG. The results for Matrigel culture and for surfaces presenting GKKQRFRHRNRKG were similar (**Supplementary Fig. 3a**,**b**). Second, we monitored cell division using a fluorescence assay: cells cultured on synthetic surfaces presenting the heparin-binding peptide GKKQRFRHRNRKG or a combination of GKKQRFRHRNRKG and KGRGDS propagated at a rate similar to those cultured on Matrigel (**Supplementary Fig. 3c**). In contrast, fewer cell divisions occurred on recombinant vitronectin–coated or KGRGDS-presenting surfaces (**Supplementary Fig. 3c**). We also compared our surface to one coated with polylysine because polylysine had been reported to support hES cell propagation in a defined medium for multiple passages[15]. Our results indicated that cells cultured on polylysine-coated surfaces did not readily divide (**Supplementary Fig. 3c**). We conclude that, for hES cell proliferation, surfaces presenting GKKQRFRHRNRKG are comparable to Matrigel and superior to polysine-coated surfaces.

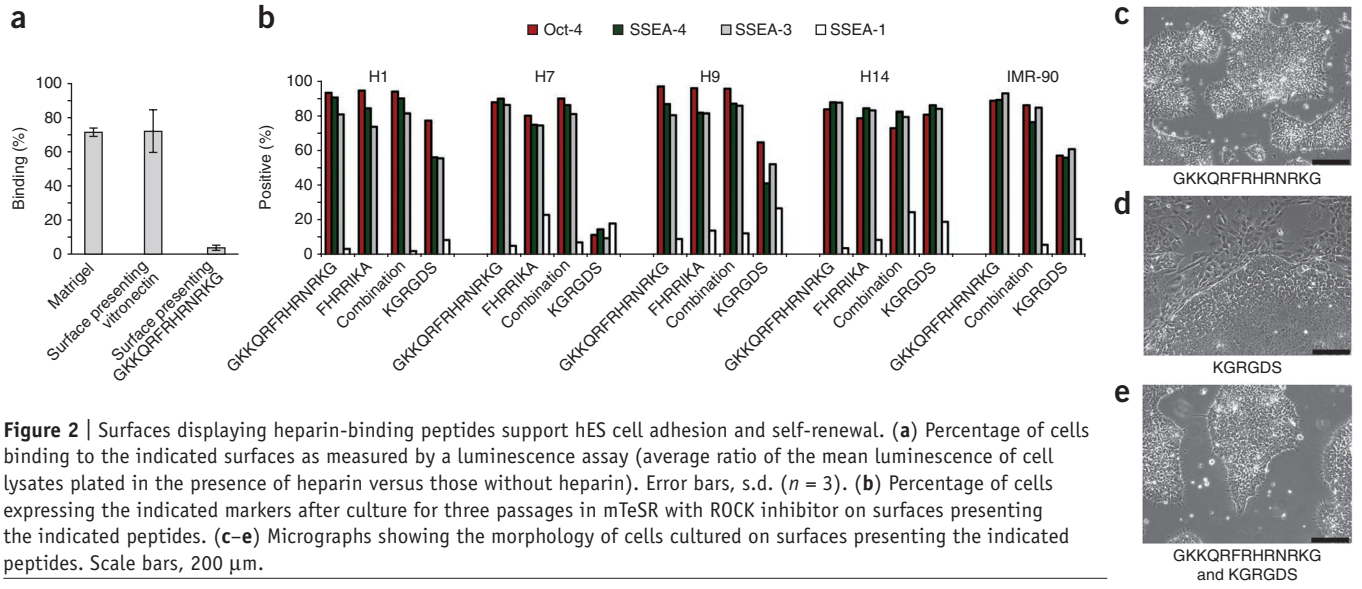

Figure 2 | Surfaces displaying heparin-binding peptides support hES cell adhesion and self-renewal. (**a**) Percentage of cells binding to the indicated surfaces as measured by a luminescence assay (average ratio of the mean luminescence of cell lysates plated in the presence of heparin versus those without heparin). Error bars, s.d. (n = 3). (**b**) Percentage of cells expressing the indicated markers after culture for three passages in mTeSR with ROCK inhibitor on surfaces presenting the indicated peptides. (**c**–**e**) Micrographs showing the morphology of cells cultured on surfaces presenting the indicated peptides. Scale bars, 200 μm.

Analysis of stem cell markers

We examined whether surfaces with heparin-binding peptides could support H1, H7, H9 and H14 hES cell self-renewal over multiple passages. We transferred cells from Matrigel culture to surfaces presenting the heparin-binding peptides GKKQRFRHRNRKG or FHRRIKA. After three passages or 21 d, the cells maintained high levels of pluripotency markers (**Fig. 2b**) and grew in the compact colonies characteristic of undifferentiated pluripotent stem cells (**Fig. 2c**). We obtained similar results for IMR-90-1 iPS cells cultured on GKKQRFRHRNRKG peptide–presenting surfaces (**Fig. 2b**). For comparison, we analyzed hES cells grown on a surface displaying the KGRGDS sequence. When we used self-assembled monolayers presenting the KGRGDS peptide at densities comparable to those used for the heparin-binding peptides (3 pmol mm^{-2}), the integrin-binding surfaces yielded a heterogeneous cell population (**Fig. 2d** and **Supplementary Fig. 4a**–**c**). Comparison of this population to cells grown on Matrigel or the heparin-binding peptides revealed that the KGRGDS peptide–substituted surfaces afforded fewer cells that displayed pluripotency markers (**Fig. 2b**). Overall, cells cultured on KGRGDS peptide–substituted self-assembled monolayers had various morphologies and differentiated into all three embryonic germ layers, as determined by immunostaining (**Supplementary Fig. 4**). These data are consistent with those from our array screen in which KGRGDS peptide–presenting surfaces were inferior to other substrata for pluripotent stem cell propagation.

To determine whether KGRGDS peptide–displaying self-assembled monolayers induce differentiation, we cultured cells on synthetic surfaces presenting a combination of KGRGDS and GKKQRFRHRNRKG peptides. The resulting cells cultured on these surfaces maintained high levels of pluripotency markers and grew in the compact colonies characteristic of undifferentiated pluripotent stem cells (**Fig. 2b**,**e**). Thus, our KGRGDS–substituted surfaces did not induce differentiation but were inadequate substrates for maintaining a homogenous population of undifferentiated pluripotent cells.

Long-term culture of human pluripotent cells

A major challenge for defined culture conditions is long-term pluripotent stem cell propagation. To test whether our surface can meet this challenge, we cultured human pluripotent stem cells (H9, H13, H14 and DF19-9 7T) on self-assembled monolayers presenting the heparin-binding peptide GKKQRFRHRNRKG for 2–3 months. We used surfaces displaying this peptide because this peptide enabled strong, consistent attachment and promoted colony spreading at low surface densities. Human pluripotent stem cells in long-term culture continued to grow as compact colonies and maintained markers of pluripotency (**Fig. 3a**–**c**).

We compared cells cultured on our surfaces to those propagated on Matrigel by examining the expression of genes associated with pluripotency and differentiation. Flow-cytometry analysis of H9 and H14 cells cultured for three months (17 passages) indicated that the cells maintained high levels of markers both integral to and associated with pluripotent stem cells. They also had low levels of the differentiation marker SSEA-1 (**Fig. 3d** and **Supplementary Fig. 5**). Consistent with these results, real-time quantitative PCR (qPCR) for H13 cells cultured for 2 months on a synthetic surface indicated that the relative expression of key transcription factors required for pluripotency was similar to that of cells cultured on Matrigel (**Fig. 3e**). For H9 cells that we propagated for 3 months on the synthetic surface or on Matrigel, we profiled the expression of 84 genes associated with pluripotency or early differentiation on Matrigel (**Supplementary Fig. 6a**). The cells maintained similar expression levels of genes associated with pluripotency in both culture conditions. Most genes with expression differing by fourfold or greater were associated with differentiation, and their levels were lower for the cells cultured on our synthetic surface (**Supplementary Fig. 6b**). hES cell lines H1, H7, H9, H13, H14 and iPS cell line IMR-90 cultured for 1–3 months on our synthetic surface maintained a normal karyotype (**Supplementary Fig. 7**). These data demonstrate that our chemically defined synthetic surface can be used to propagate homogenous populations of pluripotent stem cells.

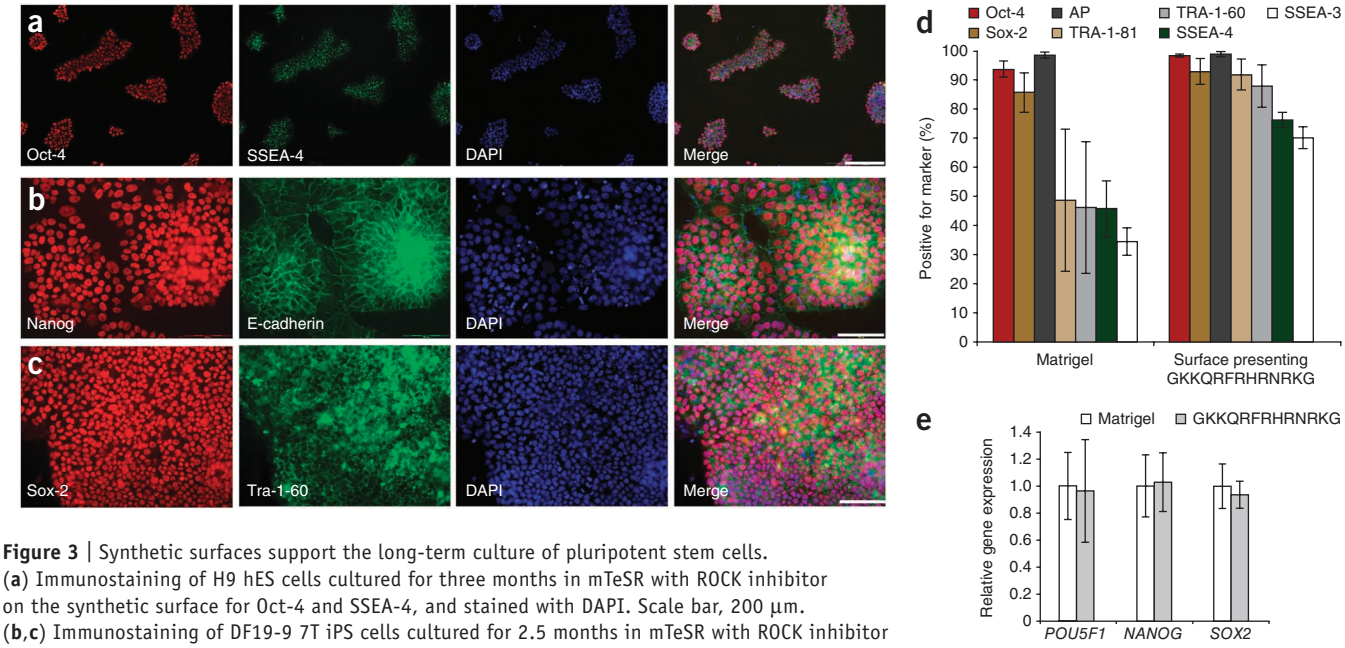

Figure 3 | Synthetic surfaces support the long-term culture of pluripotent stem cells. (**a**) Immunostaining of H9 hES cells cultured for three months in mTeSR with ROCK inhibitor on the synthetic surface for Oct-4 and SSEA-4, and stained with DAPI. Scale bar, 200 μm. (**b**,**c**) Immunostaining of DF19-9 7T iPS cells cultured for 2.5 months in mTeSR with ROCK inhibitor on the synthetic surface for nanog and E-cadherin (**b**) or for Sox2 and Tra-1-60 (**c**). Scale bars, 100 μm. (**d**) Percentage of H9 cells staining positive for the indicated markers as measured by flow cytometry after three months (17 passages). Cells were cultured in mTeSR with ROCK inhibitor on the synthetic surface or in mTeSR on Matrigel. Data represent the average (± s.d.) of three consecutive passages. (**e**) Relative expression of the indicated genes as measured by real-time qPCR analysis of H13 hES cells maintained in long-term culture (14 passages) in mTeSR with ROCK inhibitor on a surface displaying the peptide GKKQRFRHRNRKG and cells cultured concurrently in mTeSR on Matrigel. *POU5F1* encodes Oct-4. Error bars, s.d. (n = 3).

Differentiation of human pluripotent cells

To evaluate the pluripotency of the cells that had been cultured on the synthetic surfaces, we assessed their ability to differentiate. When placed in suspension culture, pluripotent stem cells form embryoid bodies that differentiate into derivatives of all three embryonic germ layers. We subjected H1, H7, H9, H13 and H14 hES cell lines and IMR-90 and DF19-9 7T iPS cell lines cultured on the synthetic substrate for 1–3 months to this differentiation protocol. All lines differentiated into a heterogenous population containing cells that stained for markers of ectoderm, endoderm and mesoderm (**Fig. 4a** and **Supplementary Fig. 8a**–**f**). Additionally, H1 and H9 cell lines cultured on a synthetic surface for 6 and 19 passages, respectively, formed teratomas (**Fig. 4b**–**d** and **Supplementary Fig. 8g**).

The aforementioned investigations revealed that cells cultured on our synthetic surfaces can differentiate, but they did not address whether these culture conditions render the cells less prone to do so. To explore this issue, we used procedures developed to differentiate human pluripotent cells to specific lineages in culture. We applied three differentiation protocols to H14 cells that we had propagated for 3 months on either surfaces displaying GKKQRFRHRNRKG or Matrigel. To monitor the differentiation rate, we determined the relative expression of lineage-specific genes using real-time qPCR. For each germ layer, after long-term culture on GKKQRFRHRNRKG peptide–substituted surfaces, cells expressed lineage-specific genes at a level and timescale similar to those obtained for cells cultured on Matrigel (**Fig. 4e**–**g** and **Supplementary Fig. 9**). Together the data indicate that pluripotent cells propagated on the synthetic surface had the differentiation potential of those cultured on Matrigel.

Portability of heparin-binding peptides

One advantage of synthetic peptides is that they can be incorporated into a wide variety of materials. To determine the portability of the heparin-binding peptides, we tested whether displaying the GKKQRFRHRNRKG peptide on alternative scaffolds could yield materials that support cell adhesion and growth. To this end, we chemically conjugated the peptide CGKKQRFRHRNRKG to glass coverslips functionalized with bromoacetamide groups. The resulting material supported excellent cell attachment and allowed for colony spreading (**Supplementary Fig. 10a**,**b**). We also tested streptavidin-coated surfaces because of their availability and ease of modification with biotinylated peptides. Surfaces presenting biotinylated heparin-binding peptides supported iPS and hES cell adhesion (**Supplementary Fig. 10c**,**d**) and propagation (data not shown). As with the self-assembled monolayers, these surfaces required that the culture medium be supplemented with the ROCK inhibitor.

We also examined a combination surface. A streptavidin-coated surface modified with the glycosaminoglycan-binding peptide GKKQRFRHRNRKG and cyclic RGD peptide[20] was a robust substrate for hES cell adhesion (**Fig. 5a**). Notably, after colonies had formed on this surface, we could remove the ROCK inhibitor from the culture medium. hES cell lines H9, H14 and SA02 propagated on streptavidin-coated surfaces modified with GKKQRFRHRNRKG and the cyclic RGD peptides for 1–2 months maintained high levels of pluripotency markers and a stable karyotype in the absence of the ROCK inhibitor (**Fig. 5b**,**c** and **Supplementary Figs. 6c**,**d** and **7g**,**h**). In contrast, streptavidin-coated surfaces displaying biotinylated cyclic RGD peptide alone did not support hES cell adhesion in the absence of the ROCK inhibitor (**Supplementary Fig. 11b**–**h**), and in these

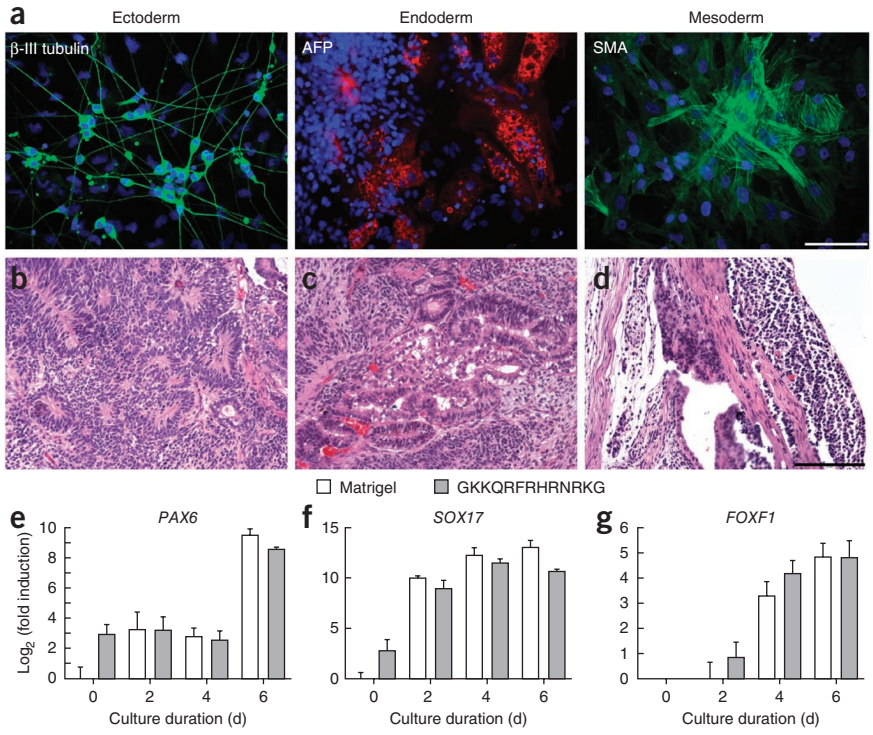

Figure 4 | Pluripotent stem cells grown on synthetic surfaces maintain their ability to differentiate. (**a**) Micrographs illustrating *in vitro* differentiation of DF19-9 7T human iPS cells maintained in mTeSR with ROCK inhibitor on surfaces presenting the heparin-binding peptide GKKQRFRHRNRKG for 3 months. Differentiated cells were stained for markers of ectoderm (β-III tubulin), endoderm (α-fetoprotein (AFP)) and mesoderm (α-smooth muscle actin (SMA)) and counterstained with DAPI. Scale bar, 100 μm. (**b**–**d**) Micrographs showing teratoma formation by H9 cells maintained on self-assembled monolayers presenting the heparin-binding peptide GKKQRFRHRNRKG for 3 months in mTeSR with ROCK inhibitor. Teratomas contained a mixture of tissues resembling the neural tube (**b**), the gut (**c**) and the mesenchyme (**d**). Scale bars, 200 μm. (**e**–**g**) Fold induction (relative gene expression) of lineage-specific genes representing (**e**) ectoderm, (**f**) endoderm and (**g**) mesoderm after directed differentiation of H14 hES cells maintained in long-term cultures (17 passages) on surfaces presenting GKKQRFRHRNRKG in mTeSR with ROCK inhibitor, compared to Matrigel culture. Expression was analyzed via real-time qPCR. Error bars, s.d. ($n = 3$). On day 0, *FOXF1* was not detected after 40 amplification cycles.

cells Oct-4 levels were inconsistent over multiple passages even in the presence of the ROCK inhibitor (**Supplementary Fig. 11i**). Additional investigations are needed to understand how simultaneous glycosaminoglycan and integrin engagement influences pluripotent stem cell propagation.

DISCUSSION

The most effective peptide substrates we found for pluripotent stem cell adhesion and propagation were those that can bind anionic polysaccharides such as heparin, supporting a model in which the synthetic surfaces act by engaging the glycosaminoglycans on pluripotent cells. That these simple synthetic substrata can replace Matrigel for pluripotent cell culture is consistent with the proposition that embryonic stem cells have an innate capacity for self-renewal and do not require a rich milieu of external signals[29].

Still, our data indicate that adhesion is not the sole prerequisite for self-renewal. We and others[14,16,20] have found that materials decorated with RGD-containing peptides vary in their ability to support hES cell self-renewal despite their ability to support adhesion. Many previous investigations to develop substrata for hES cell culture have focused on the importance of integrins binding to extracellular matrix proteins[10,11,19]. The RGD-containing peptide–substituted material that is most successful at maintaining hES proliferation requires high densities of the integrin epitope KGGNGEPRGDTYRAY or KGGPQVTRGDVFTMP[16]. Such high extent of substitution could yield surfaces that manifest their activities through bulk properties rather than their ability to engage a particular type of cell-surface receptor. Alternatively, the more complex sequence may bind specific integrins or other crucial cell-surface receptors that the shorter RGD sequences cannot. The origins of the varying outcomes obtained with different surfaces that present RGD-containing peptides are still unknown.

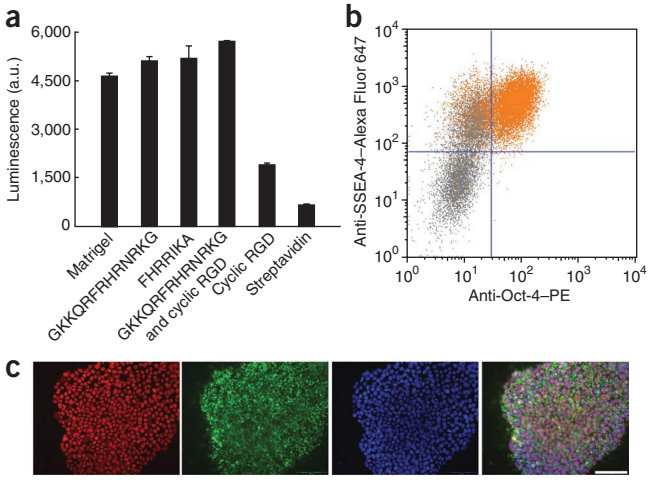

Figure 5 | Streptavidin-coated surfaces presenting heparin-binding peptides support robust adhesion and self-renewal. (**a**) H9 hES cell adhesion to the indicated surfaces as measured by a luminescence assay. Error bars, s.d. ($n = 3$). (**b**) Flow-cytometric analysis with phycoerythrin (PE)-conjugated antibodies to Oct-4 (anti-Oct-4–PE) and Alexa Fluor 647–conjugated antibodies to SSEA-4 (anti-SSEA-4–Alexa Fluor 647) of H14 hES cells (orange) cultured for one month (ten passages) on surfaces presenting a combination of GKKQRFRHRNRKG and cyclic RGD peptide in mTeSR alone. Data from partially differentiated cells also are shown (gray) and were used to set gates between positive and negative staining. (**c**) Micrographs of H9 hES cells cultured in mTeSR for two months (17 passages) on a combination of GKKQRFRHRNRKG and cyclic RGD peptide and immunostained for Oct-4 (red), SSEA-4 (green) and counterstained with DAPI (blue). Scale bar, 100 μm.

Though integrin ligands alone were not effective in maintaining long-term pluripotent stem cell culture on our surfaces, they augmented the activity of the glycosaminoglycan-binding substrates. Adhesion of pluripotent cells to surfaces presenting a heparin-binding peptide alone required ROCK inhibition even after the formation of colonies. In contrast, streptavidin-coated surfaces presenting a combination of the high-affinity cyclic RGD and GKKQRFRHRNRKG did not require ROCK inhibition after colonies had formed.

The heparin-binding peptides described here can be incorporated into a wide variety of materials[25,30] for culturing pluripotent human cells. Because GKKQRFRHRNRKG only needs to be present at a low surface density to serve as a substrate, additional signals or cell-binding epitopes can be incorporated easily to study their effects on self-renewal and differentiation. Moreover, the active surfaces in our array screen are portable. For instance, peptides presented on strepavidin-coated surfaces provide a defined, modular and facile method for propagating pluripotent stem cells that is inexpensive and readily accessible. Surfaces presenting the heparin-binding peptide derived from vitronectin are the most robust of all the synthetic surfaces we have tested to date[12,18,21].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

J.R.K., L.L., P.J.W. and L.L.K. conceived the experiments and interpreted the results. J.R.K. performed the *in vitro* experiments. L.L. synthesized and purified the molecules used to fabricate the surfaces. J.R.K. and M.S.P. conducted the teratoma assay, and P.J.W. conducted the directed differentiation assays. J.R.K. and L.L.K. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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ONLINE METHODS

Cell culture. hES cell lines (H1, H7, H9, H13, H14 and SA02) and iPS cell lines (IMR90-1 and the vector-free DF19-9 7T) were maintained on Matrigel (BD)–coated plates using the mTeSR1 medium (StemCell Technologies). Cells were maintained at 37 °C in 5% CO_2. They were passaged manually every 5–6 d after treatment with the protease dispase (Gibco; 2 mg ml^{-1}) for 5–6 min[31]. hES cells grown on synthetic surfaces were maintained in mTeSR1 medium supplemented with ROCK inhibitor Y-27632 (Calbiochem; 5 μM) or H-1152 (Alexis; 0.5 μM). Cells were passaged manually every 5–7 d after treatment with an enzyme-free, Hanks' solution–based cell dissociation buffer (Sigma) for 10–15 min. Cells were seeded onto new surfaces at 25,000 cells cm^{-2}.

Differentiation assays. Embryoid bodies were formed in flasks (Greiner Bio-One) coated with poly(2-hydroxyethyl methacrylate) (Sigma) and cultured in medium consisting of Iscove's modified Dulbecco's medium (Gibco), 15% FBS (Gibco), 1% nonessential amino acids (Gibco) and 0.1 mM β-mercaptoethanol (Gibco). Teratomas were generated as described previously[32]. A detailed protocol can be obtained from the WiCell research institute (http://www.wicell.org/). This protocol was approved by the University of Wisconsin–Madison Research Animal Resources Center and was performed by personnel listed on the Stem Cell Research Oversight Committee protocol after completing the Research Animal Resources Center animal handling course and the appropriate animal facilities orientation. For the directed differentiation assays, cells were dissociated with enzyme-free, Hanks' solution–based cell dissociation buffer (Sigma) and plated onto new Matrigel surfaces at 17,500–30,000 cells cm^{-2} in mTeSR1 supplemented with 5 μM Y-27632. The basal differentiation medium (RDM) consisted of Advanced RPMI 1640 (Gibco) supplemented with B27 (Gibco), penicillin-streptomycin (Gibco) and Glutamax (Invitrogen). For mesoderm differentiation, cells were treated with RDM supplemented with 100 ng ml^{-1} activin A (R&D Systems) for 1 d followed by RDM supplemented with 10 ng ml^{-1} BMP-4 (R&D Systems) for 5 d[33]. For endoderm differentiation, cells were treated with RDM supplemented with 100 ng ml^{-1} activin A for 6 d[34]. For ectoderm differentiation, cells were treated with RDM supplemented with 10 μM SB421542 (Tocris) and 500 ng ml^{-1} Noggin (R&D Systems) for 6 d[35].

Fabrication of peptide-presenting surfaces. Peptide–alkane thiols were synthesized as described previously[12,21]. Briefly, bioactive peptides were synthesized on PAL-polystyrene resin (Applied Biosystems) via standard Fmoc chemistry using an automated peptide synthesizer. Trityl-protected alkanethiol with a carboxyl group was coupled to the N terminus of the peptides on the solid support. Peptide–alkane thiols were cleaved off the resin using 92.5% trifluoroacetic acid (TFA), 2.5% triisopropylsilane, 2.5% ethalenedithiol and 2.5% H_2O. The resulting material was precipitated in ice-cold ether and purified by high-performance liquid chromatography. The glucamine–alkane thiol and the perfluorinated alkane thiol were synthesized as reported prevously[21,36]. Peptide–alkane thiols and glucamine–alkane thiol were dissolved at a 1 mM concentration in 30% DMSO in H_2O. Perfluorinated alkane thiol was dissolved at a 1 mM concentration in ethanol.

Gold-coated glass slides (250 Å gold-, 10 Å chromium-coated slides, 22 × 22 mm square, 0.16-mm thick) were purchased from EMF Corporation. Arrays were prepared as described previously[12]. When larger areas of peptide–alkane thiol self-assembled monolayers were needed, whole chips presenting the same self-assembled monolayer were fabricated by sandwiching a solution of peptide–alkane thiol and glucamine–alkane thiol between two gold-coated slides. Self-assembled monolayers were allowed to form in a moist, glass Petri dish for 24 h before use.

To fabricate peptide-displaying glass slides, glass surfaces were functionalized with amino groups using an established protocol[37]. The resulting dried surfaces were treated with a mixture of 50 mM bromoacetic anhydride and 50 mM triethylamine in anhydrous dimethyl formamide for 4 h. The slides were washed with water and exposed overnight to the cell-adhesive peptide CGKKQRFRHRNRKG, which can undergo reaction via its N-terminal cysteine residue with the bromoacetamide-substituted surface. Any remaining bromoacetamide groups were capped by treatment with 50 mM cysteine in PBS (pH 8.5) for 2 h.

To display the peptide on polystyrene, non-tissue-culture–treated plates (Falcon) were coated with 10 μg ml^{-1} streptavidin in Hanks' balanced salt solution (Gibco). Wells were washed with Hanks' balanced salt solution and then coated with 5 μM biotin-6-aminohexanoate-GKKQRFRHRNRKG (Biomatik), biotin-6-aminohexanoate-GRGDS (Anaspec), or cyclo RGD–D-Phe–Lys–diethylene glycol-biotin (Peptides International) in Hanks' balanced salt solution. Surfaces presenting a 7:3 ratio of biotin-6-aminohexanoate-GKKQRFRHRNRKG to RGD–D-Phe–Lys–diethylene glycol-biotin were used to propagate hES cells without ROCK inhibitor after the formation of colonies.

Microscopy and immunostaining. Images were collected with a Hamamatsu digital camera mounted onto an Olympus IX81 microscope. Primary antibodies used in this study were antibodies to Oct-4 (R&D Systems, 1:400), nanog (R&D Systems, 1:100), Sox-2 (R&D Systems, 1:250), SSEA-4 (Santa Cruz Biotechnology, 1:400), TRA-1-60 (Santa Cruz Biotechnology, 1:500), β-III tubulin (R&D Systems, 1:3,000), nestin (1:3,000), α-fetoprotein (Sigma, 1:250), FoxA2 (R&D Systems, 1:100), α-smooth muscle actin (Sigma, 1:1,000) and fatty acid binding protein 4 (R&D Systems, 1:250). Cells were fixed with PBS containing 4% formaldehyde and 0.15% picric acid for 20 min at 37 °C, and then permeabilized and blocked with PBS containing 0.1% Triton X-100 and 2% BSA. All antibodies were incubated in blocking buffer overnight at 4 °C, except for the antibodies to β-III tubulin, nestin and α-smooth muscle actin, which were incubated for 1 h at room temperature (25 °C). Secondary staining was performed with Alexa Fluor 488–, rhodamine- or Alexa Fluor 594–conjugated antibodies (Invitrogen, 1:1,000), which were diluted in blocking buffer and exposed to cells for 1 h at room temperature. Cells were counterstained with 4′,6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen). Peptide array mosaics were generated using the AnalySIS acquisition software. Image overlays were generated using ImageJ.

Flow cytometry. hES cells were dissociated with 0.05% trypsin-EDTA with 2% chicken serum (Gibco). Surface-marker staining was performed in PBS containing 2% BSA (w/v) at 4 °C for 30 min with antibodies to: alkaline phosphatase, allophycocyanin–conjugated (R&D Systems); Tra 1–60, Alexa Fluor 488–conjugated

(BD); Tra 1–81, Alexa Fluor 647–conjugated (BD); SSEA-4 Alexa Fluor 647–conjugated (BD); SSEA-3 phycoerythrin (PE)–conjugated (BD); and SSEA-1, PE–conjugated (R&D Systems). Cells were stained in PBS containing 2% BSA for 30 min. Antibody exposure was followed by one 5 min wash in PBS containing 2% BSA and then a 30-min fixation with 2% formaldehyde in PBS at room temperature. For intracellular staining, hES cells were fixed with 2% formaldehyde in PBS at room temperature for 30 min. For Oct-4 and nanog staining, cells were permeabilized with saponin permeabilization buffer (SPB) (0.1% saponin and 0.1% BSA in PBS) for 30 min at room temperature and then stained with a PE–conjugated Oct-4 antibody or PE-conjugated nanog antibody (BD) overnight. Cells were washed twice with SPB before analysis. For Sox-2 staining, cells were permeabilized with 90% ice-cold methanol, washed with SPB, incubated with Alexa Fluor 488– or 647–conjugated Sox-2 antibody (BD) for 1 h at 4 °C and then washed twice with SPB. Data were obtained using a FACSCalibur or a BD LSR II and analyzed using FlowJo software. The percentage of positive cells was established by comparing experimental cells to differentiated hES cells. Gating for 'positive' and 'negative' populations was established by analyzing the bimodal peaks of partially differentiated hES cells.

Real-time quantitative PCR analysis. Total RNA was isolated from samples using the RNeasy Plus Kit (Qiagen). We reverse-transcribed 200–600 ng of RNA via AffinityScript QPCR cDNA Synthesis Kit (Stratagene). qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosciences) using SYBR Green JumpStart Taq ReadyMix (Sigma) and lineage-specific gene primers. The cycling parameters were 95 °C for 2 min for initial denaturation followed by 40 cycles of 95 °C for 15 s for denaturation, then 56 °C for 1 min for primer annealing and extension. Melting curve analysis ensured primer specificity, and no-reverse-transcripase samples controlled for genomic DNA contamination. Relative gene expression levels (fold induction) were determined using the ΔΔCt method, and the error bars represent the propagated error determined from the s.d. of triplicate reactions. Briefly, gene-of-interest threshold cycle (Ct) values were normalized to Ct values for the housekeeping gene *SRP72* and compared to the Ct value determined for undifferentiated cells cultured long-term on Matrigel. In the event that the Ct value for the gene of interest was not detected in the undifferentiated sample, the earliest time point with detectable expression was substituted. The primer sets are listed in **Supplementary Table 2**.

The hES cell RT^2 Profiler PCR Array (SABiosciences) was performed according the manufacturer's protocol.

Collection and G-banding of hES cells for cytogenetic analysis. The collection procedure for hES cells was adapted from standard cytogenetics protocols. Detailed protocols can be obtained from the WiCell research institute.

Adhesion assay for glycosaminoglycan involvement. H9 cells cultured on Matrigel were dissociated using an enzyme-free, Hanks' solution–based cell dissociation buffer for 10–15 min. Cells were resuspended in DMEM/F12 (Gibco), DMEM/F12 supplemented with 2 units ml^{-1} chondroitinase ABC (Sigma) or 500 μg ml^{-1} heparin (Sigma). Cells treated with the glycosaminoglycan-degrading enzymes were incubated for 1 h in suspension at 37 °C. Cell suspensions were seeded on to Matrigel-coated surfaces, recombinant vitronectin–coated surfaces (10 μg ml^{-1}, R&D Systems), or self-assembled monolayers presenting the peptide GKKQRFRHRNRKG at a 5% surface density. After 1 h, surfaces were washed three times with PBS and the cells were lysed with M-PER buffer (Pierce). The cell lysate was mixed with CellTiter-Glo (Promega), which is a homogenous and sensitive method to determine the number of viable cells in culture based on the presence of ATP. The luminescence was measured on 20/20^n luminometer (Turner Biosystems).

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