

## High-Throughput Discovery of Synthetic Surfaces That Support Proliferation of Pluripotent Cells

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**Abstract:** Synthetic materials that promote the growth or differentiation of cells have advanced the fields of tissue engineering and regenerative medicine. Most functional biomaterials are based on a handful of peptide sequences derived from protein ligands for cell surface receptors. Because few proteins possess short peptide sequences that alone can engage cell surface receptors, the repertoire of receptors that can be targeted with this approach is limited. Materials that bind diverse classes of receptors, however, may be needed to guide cell growth and differentiation. To provide access to such new materials, we utilized phage display to identify novel peptides that bind to the surface of pluripotent cells. Using human embryonal carcinoma (EC) cells as bait, approximately  $3 \times 10^4$  potential cell-binding phage clones were isolated. The pool was narrowed using an enzyme-linked immunoassay: 370 clones were tested, and seven cell-binding peptides were identified. Of these, six sequences possess EC cell-binding ability. Specifically, when displayed by self-assembled monolayers (SAMs) of alkanethiols on gold, they mediate cell adhesion. The corresponding soluble peptides block this adhesion, indicating that the identified peptide sequences are specific. They also are functional. Synthetic surfaces displaying phage-derived peptides support growth of undifferentiated human embryonic stem (ES) cells. When these cells were cultured on SAMs presenting the sequence TVKHRPDALHPQ or LTTAPKLPKVTR in a chemically defined medium (mTeSR), they expressed markers of pluripotency at levels similar to those of cells cultured on Matrigel. Our results indicate that this screening strategy is a productive avenue for the generation of materials that control the growth and differentiation of cells.

### Introduction

The ability to grow isolated human cells outside of the body (ex vivo) has led to advances in fields ranging from fundamental cell biology to drug discovery. More recently, methods to expand human cells from progenitor and stem cells ex vivo have been developed, and this approach holds great therapeutic promise. Reaping this promise hinges on establishing conditions that reproducibly support the growth and differentiation of pluripotent cells. As in vivo, the ex vivo growth and differentiation of cells can be guided by the signals from soluble molecules and insoluble extracellular substrates, including those presented by the extracellular matrix and on the surface of other cells.<sup>1–3</sup> Historically, products of animal origin, such as the soluble components of the serum or the insoluble components of the basement membrane, supply the necessary signals. Still, the

heterogeneity of animal-derived components leads to variability in the responses of cultured cells. Moreover, the carryover of pathogens or immunogens from animal products can complicate the use of human cells in therapeutic applications.<sup>4</sup> The quest for “chemically defined” growth conditions was initiated early in the history of cell culture,<sup>5</sup> yet even now, the identification of such conditions requires trial-and-error screening of a plethora of formulations. Although the identification of soluble culture media can be performed rapidly via high-throughput screening, methods for the rapid identification of insoluble substrates have been lacking. To facilitate identification of synthetic substrates for cell adhesion and growth, we present a method that unites high-throughput screening for specific ligands with array-based screening of defined ligand-presenting surfaces.

We encountered a need for new approaches when we embarked on a project focused on identifying substrates that support undifferentiated proliferation of human embryonic stem (ES) cells. ES cells are pluripotent: they can give rise to any cell type.<sup>6</sup> The derivations of human ES cells and the related

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induced pluripotent stem (iPS) cells<sup>7,8</sup> have increased interest in cell therapies. Because human ES and iPS cells have the capacity to expand indefinitely *ex vivo*, they can provide unlimited access to any human cell types for research or therapeutic applications. Reaching this goal requires defined culture conditions for expansion of human ES cells in an undifferentiated state. Human ES cells were initially isolated and propagated in a coculture with murine embryonic fibroblasts.<sup>6</sup> Subsequently, growth media in which every soluble component is defined have been developed.<sup>9–11</sup> Nevertheless, even in chemically defined media, human ES cells are cultured on the insoluble substrate Matrigel, a mixture of murine sarcoma-derived extracellular matrix (ECM). Problems associated with the use of such a xenogeneic matrix can be alleviated by using human-isolated ECM<sup>9</sup> or recombinant ECM components.<sup>12–14</sup> As an alternative to top-down simplification of biological matrixes, we examined a bottom-up approach. Specifically, we sought to identify chemically defined, synthetic, functional substrates that support ES cell growth.

Our strategy is inspired by natural mechanisms that influence cellular decisions. Specific responses can be elicited by interactions between the ECM and cell-surface receptors. Cell adhesion to the ECM *in vivo* can be mediated by the binding of short peptide motifs within ECM proteins to integrin receptors on the cell surface.<sup>15–17</sup> This observation has been exploited by a number of research groups who have decorated materials with an integrin-binding Arg-Gly-Asp (RGD) sequence to yield peptide-modified substrates that support cell adhesion and growth.<sup>18</sup> The identification of peptide sequences such as RGD has been pivotal in advancing biomaterial research, because of the ease of synthesizing, manipulating, and tuning the properties of such materials.<sup>19</sup> Still, only a few adhesive peptide sequences have been found in natural proteins. We reasoned that the identification of cell growth substrates could be accelerated by the discovery of peptide ligands for cell-surface receptors.

To identify novel ligands for cells, we screened random peptide libraries using phage display. Billions of diverse sequences can be assessed by displaying random peptide

sequences on the coat proteins of bacteriophages,<sup>20</sup> rendering phage display a powerful means to identify peptides that function as cell-surface ligands.<sup>21–23</sup> To this end, intact cells *in vitro* and *in vivo* have been used as screening targets,<sup>22,24</sup> and peptides that recognize receptors on the surface of the target cell types have been identified.<sup>23,25</sup> We therefore envisioned that phage display could be used to find peptides that bind to the ES cell surface and that the resulting sequences could be used to fabricate novel ES cell-binding substrates. Although not every ES cell-binding material can support ES cell growth,<sup>26</sup> we postulated that the active peptide sequences from a phage display screen could be used in conjunction with cell array technology<sup>27,28</sup> to yield substrates that support long-term adhesion and proliferation of ES cells in an undifferentiated state.

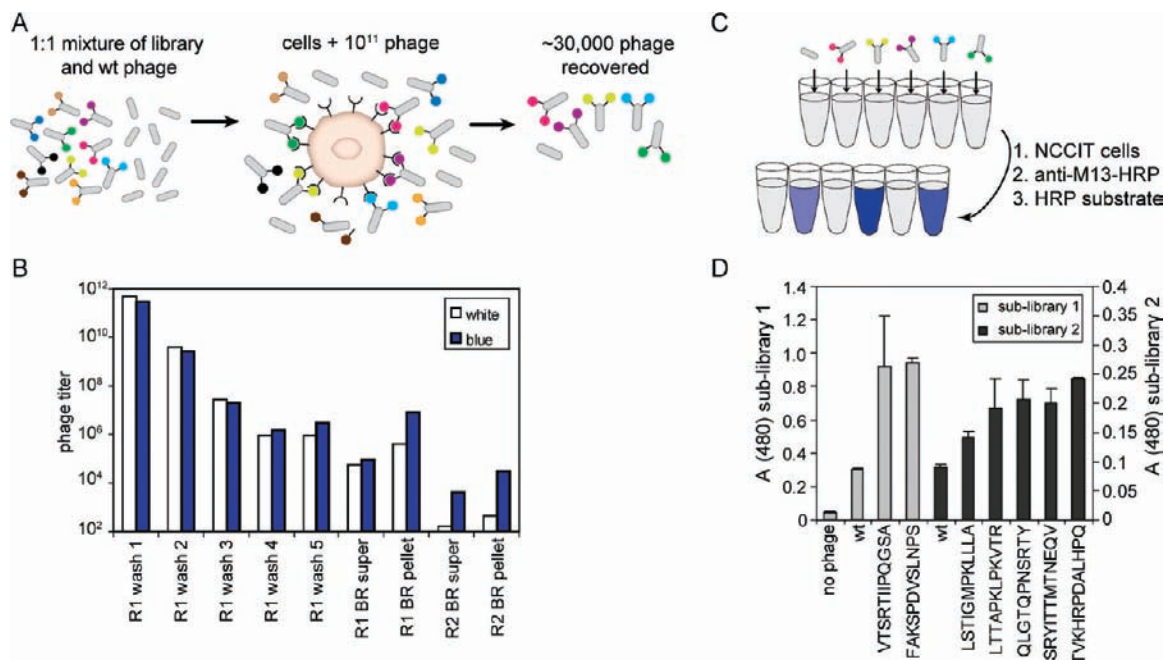
Although our long-term goal is focused on human ES cells, we postulated that ligands with the requisite properties could be identified using any cell line with a cell-surface receptor repertoire akin to that of human ES cells. Because DNA microarray analyses suggest that gene expression levels are similar for the human embryonal carcinoma (EC) cell line NCCIT and human ES cells,<sup>29</sup> we employed the former. Unlike human ES cells,<sup>30</sup> NCCIT EC cells rapidly grow in standard tissue culture conditions.<sup>31</sup> Thus, we used phage display panning with intact NCCIT cells to identify peptide ligands. The identified peptides were converted into insoluble substrates by their attachment to alkanethiols, and these were used to form self-assembled monolayers (SAMs) on gold.<sup>27,28</sup> From the materials that resulted, we identified substrates that not only bind NCCIT cells but also support the proliferation of human ES cells.

## Results and Discussion

**Cell-Binding Peptides from Phage Display.** Our phage display panning strategy is based on the “biopanning and rapid analysis for selective interactive ligands” (BRASIL) technique.<sup>24</sup> Suspensions of live ES or EC cells were used as targets. Previous investigations using phage display to identify cell-binding peptides have employed multiple rounds of panning and amplification.<sup>20,21,24</sup> To test this approach for our purposes, we conducted a screen employing three rounds of panning and amplification starting from a commercially available library of M13 phage displaying random 12-mer sequences on the N-terminus of pIII minor coat protein. Before or after each panning step, 48 clones were picked randomly for sequencing to monitor the diversity of the library. Because intact cells

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**Figure 1.** (A) A 1:1 mixture of peptide-presenting (library) and peptide-free (wt) phage was used to optimize the screen for NCCIT-binding phage clones. (B) The amount of peptide-bearing (blue) and wt (white) phage was monitored at each panning and washing step (see the Supporting Information for details). Through multiple panning steps, a steady increase in the blue/white ratio is observed. (C) The phage population that remained associated with cells after all washing steps (denoted as “R2 BR pellet” in (B)) was subcloned; the clones were amplified and tested using the cell suspension enzyme-linked immunosorbent assay (CS-ELISA) to identify those that bind to NCCIT cells. (D) Testing of 92 phage clones (sublibrary 1, left scale) and 276 phage clones (sublibrary 2, right scale) using the CS-ELISA yielded 2 and 5 clones, respectively, that bind to NCCIT cells significantly better than wt phage. The results are averages from two independent measurements. Error bars indicate 1 standard deviation.

present hundreds of different receptors, we expected to identify a large number of cell-binding peptides. In two independent screens, however, the library converged at the third round of screening, and 4–5 peptides dominated the sequence pool. A comparison of the number of unique sequences at each screening step revealed that convergence occurred not at the panning step but rather during amplification (Supporting Information Figure S1 and Table S1). The problem of amplification-induced convergence is exacerbated when cells are used as bait, because the number of potential cell-surface targets is high and therefore the pool of target-binding phage clones in the screen will be diverse. The pool can be narrowed by carrying out a negative selection step using “control cells”,<sup>24</sup> but for our experiments, there were no appropriate control cell lines. Thus, we developed a funnel screening protocol that provided the means to identify peptides with the requisite cell-binding properties.

In principle, amplification-induced convergence can be eliminated by deleting the amplification step from the screen. This change, however, complicates the isolation of cell-binding phage that act via peptide-specific interactions. Thus, we needed a strategy to optimize the screening conditions to maximize the identification of cell-binding phage. To this end, we used wild-type (wt) or “environmental” M13 phage, which contain no peptide insert, as reporters for “nonspecific” phage (Figure 1A). Phage that contain peptide inserts bear the *lacZ $\alpha$*  reporter gene and thereby give rise to blue plaques (upon infection of bacterial lawn on a solid medium containing the chromogenic substrate X-gal). “Environmental” phage, however, give rise to colorless plaques. Because only peptide-bearing phage should bind to cell-surface ligands, the ratio of blue to white plaques provides a measure of the specificity of the screen. If the screening conditions are nonselective, wt and library phage will bind similarly, and no change in the blue/white ratio will be observed.

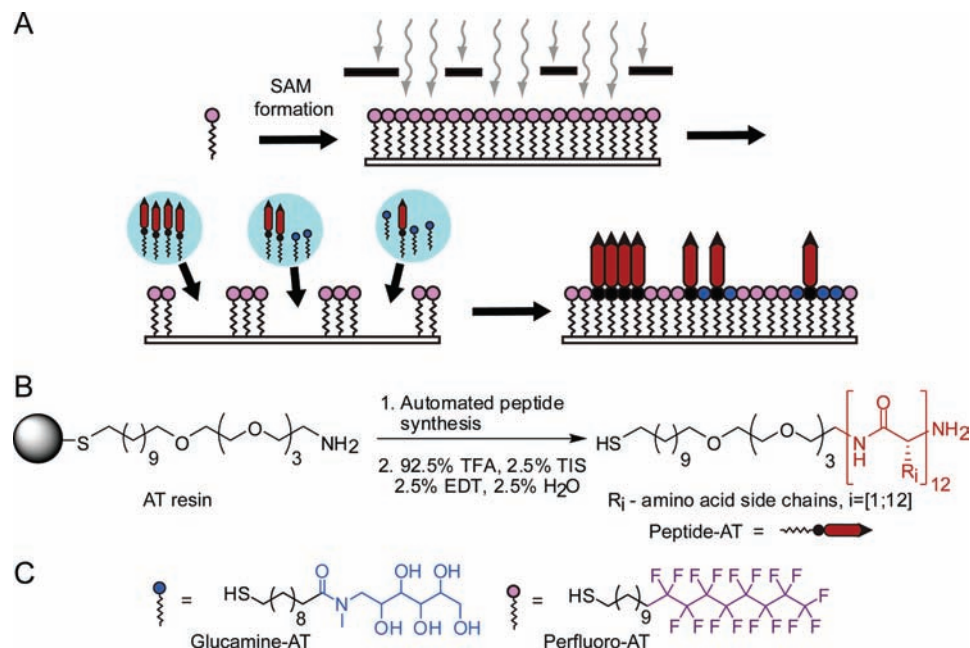
If peptide-specific interactions occur, an enrichment of the peptide-containing (“blue”) phage will be detected.

The aforementioned strategy was employed to develop an effective phage panning protocol against live NCCIT cells. A mixture of wt and library phage (1:1 ratio,  $10^{11}$  phage particles each) was incubated with EC cells, and the cells were pelleted by centrifugation to remove the unbound phage. After several washes, the cells were subjected to a more stringent protocol to remove unbound phage—centrifugation in a biphasic aqueous/organic system (a “BRASIL wash”).<sup>24</sup> The phage titer was monitored at each step of the screen (Figure 1B). After five rounds of centrifugation-based washes and one BRASIL wash, the phage associated with the cell pellet exhibited a 30-fold increase in the blue/white ratio (Figure 1B). The resulting phage-bearing pellet was mixed with EC cells and subjected to another round of panning and washing. Two consecutive rounds of panning resulted in a 50-fold enrichment in the amount of peptide-bearing phage and yielded  $3 \times 10^4$  phage particles. Although the panning procedure led to enrichment of a population of specific phage, many nonspecific phage were retained in the pool. To identify the EC-binding phage, we implemented a secondary screen.

**Narrowing the Pool: Identification of Cell-Binding Phage Clones with an Enzyme-Linked Immunosorbent Assay.** An enzyme-linked immunosorbent assay (ELISA) is a convenient technique for high-throughput detection of phage binding.<sup>32</sup> We developed an ELISA using an NCCIT cell suspension as a target, because this assay configuration minimizes false-positive signals that result from the binding of phage to the plate. Cells

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**Figure 2.** (A) Method for fabrication of surface arrays of peptides. Reprinted from ref 27. Copyright 2004 American Chemical Society. SAMs composed of perfluorinated alkanethiol on gold were photopatterned. Solutions that contain peptide-AT, glucamine-AT, or mixtures of the two were spotted onto the exposed gold regions. The ratio of peptide-AT and non-cell-adhesive glucamine-AT in solution was altered to vary the surface concentration of peptide. (B) Route for the synthesis of the peptide-AT starting from the AT resin. Reprinted from ref 37. Copyright 2007 American Chemical Society. (C) Structures of the glucamine-AT and perfluoro-AT. Reprinted from ref 27. Copyright 2004 American Chemical Society.

suspended in 96-well plates were incubated with subcloned phage, and a solution of anti-M13 antibody conjugated to horseradish peroxidase (HRP) was added. After the cells were washed via centrifugation, the cell pellet was probed with HRP substrate (Figure 1C, Supporting Information Figure S6). This cell suspension ELISA (CS-ELISA) provided the means to rapidly probe hundreds of phage clones. We tested a subpopulation of  $\sim 3 \times 10^4$  clones (1.3%) isolated by EC cell panning (Figure 1B), and from 370 clones tested, seven cell-binding phage clones were identified (Figure 1D). These data indicate that a combination of amplification-free panning and high-throughput CS-ELISA can readily yield hundreds of potential cell-binding peptide sequences.

**Evaluating Cell-Binding Properties of the Phage-Derived Peptides with Surface Arrays.** We sought to determine whether the identified peptides can be employed to generate materials that support cell adhesion and growth. To this end, we appended the identified cell-binding peptides to synthetic surfaces and evaluated their ability to support adhesion of NCCIT cells. The immobilization approach we employed has several key features. First, we avoided immobilization techniques that yield a random orientation of peptides on the surface (e.g., nonspecific adsorption), because they can afford nonproductive orientations of the peptide that decrease binding. Second, we noted that the attachment of peptides to the surface through their N-termini also might diminish binding, because phage-presented peptides possess N-termini that can contribute to target recognition.<sup>33</sup> Accordingly, we linked the identified peptides to defined synthetic surfaces through their C-termini. Finally, the surface distribution of the peptides can have dramatic effects on cell binding,<sup>34,35</sup> hence, we evaluated surfaces presenting different peptide densities.

The arrays we employed consist of SAMs of alkanethiols (AT) on gold. These surfaces are well-defined because the orientation and density of the surface-presented compounds can be easily controlled.<sup>27,36</sup> To create the desired display, we synthesized peptides with free N-termini and a C-terminal AT substituent.<sup>37</sup> When a solution of AT-peptide is spotted onto the gold surface (Figure 2B), an oriented C-terminal surface display of peptides is formed. Using a two-step fabrication strategy (Figure 2A), arrays presenting different peptides at different densities were generated and tested for their ability to support adhesion of NCCIT cells. We employed serum-free media for cell-binding studies to eliminate the nonspecific adsorption of cell-secreted proteins. To minimize the influence of cell-secreted proteins, we used short-term cell adhesion (1 h). Of the seven phage-derived peptides identified, six supported cell binding. The SAMs presenting LTTAPKLPKVTR were especially effective, as they supported cell adhesion over the broadest range of densities (Figure 3A,B).

**SAMs Displaying Phage-Derived Peptides Support Adhesion of EC Cells through Peptide-Specific Interactions.** We sought to determine whether cell binding is mediated by specific interactions with a peptide presented by the surface. Evidence for specificity can be obtained by assessing whether a soluble inhibitor blocks cell binding to the surface.<sup>38</sup> If the binding is due to nonspecific effects, a soluble competitor will have little effect. We therefore tested whether phage displaying a peptide sequence of interest inhibit cell adhesion to the peptide-presenting monolayer. The competition experiments were

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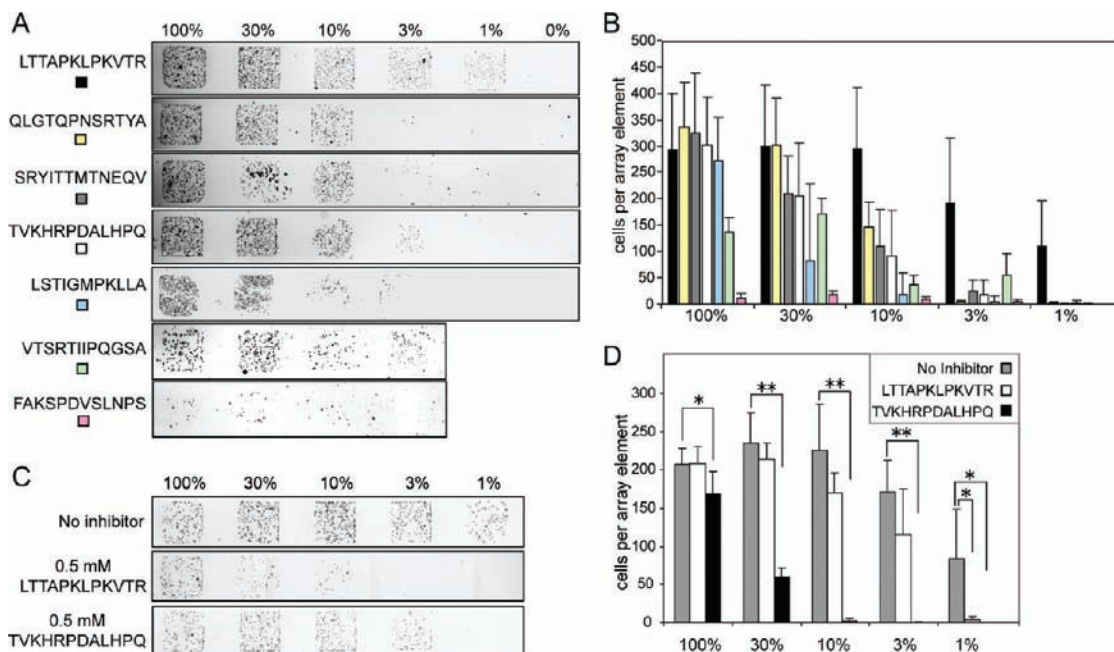
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**Figure 3.** (A) Arrays were prepared by spotting solutions containing mixtures of peptide-AT and glucamine-AT (1 mM total concentration) onto a patterned perfluoro-AT SAM (see Figure 2). The percentages are derived from the mole fraction of the peptide-AT in the solution from which the array element was generated. NCCIT cells were incubated with arrays for 1 h in serum-free media. The cells were then fixed with formaldehyde and stained with Hoechst 33342 to visualize cell nuclei. (B) The number of cells on each array element was determined by counting nuclei using ImageJ software. Data are averages of 3–6 measurements from three independent experiments; error bars indicate 1 standard deviation. (C) The short-term cell adhesion of NCCIT cells to monolayers presenting LTTAPKLPKVTR was inhibited with soluble LTTAPKLPKVTR peptide. Another phage-derived peptide that also supports cell adhesion, TVKHRPDALHPQ, had little effect. (D) The ability of the soluble peptides to inhibit binding was quantified using ImageJ software. Data presented are the averages of six measurements; error bars are 1 standard deviation. Key: (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$  as determined by the two-tailed unequal variance  $t$  test. (A) and (B) represent mosaic images obtained with a fluorescent microscope with a  $6.4\times$  objective. An inverted image of the blue-fluorescent channel is presented.

conducted using concentrations at which wt phage had no effect on cell binding (Supporting Information Figure S2). Under these conditions, the addition of VTSRTIIPQGSA-presenting phage diminished cell adhesion to the VTSRTIIPQGSA-substituted surface.

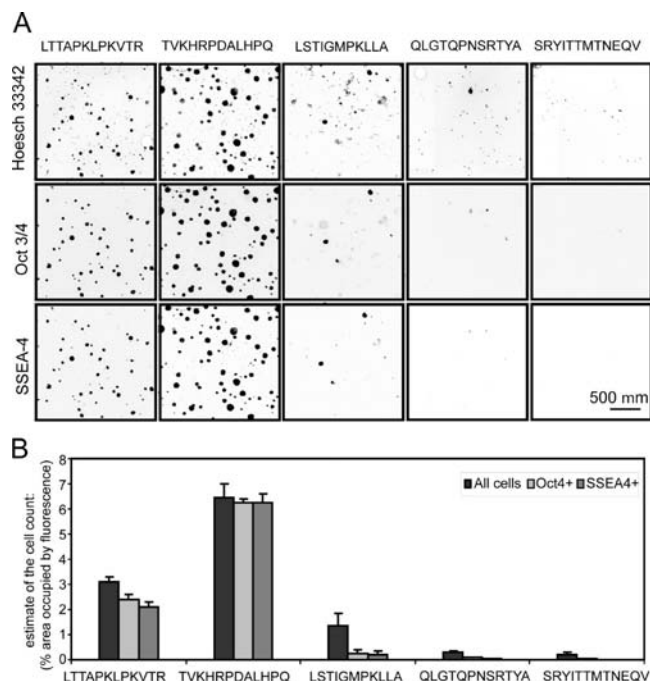
We also assessed the abilities of soluble peptides to block cell adhesion. These competition studies were carried out using soluble peptide concentrations that have no nonspecific effects (e.g., no general toxicity; see Supporting Information Figure S2). The data indicate that soluble LTTAPKLPKVTR can compete with cells for a surface presenting the cognate peptide sequence (Figure 3C,D). In contrast, another phage-derived peptide sequence (TVKHRPDALHPQ) had little effect. These findings indicate that adhesion to the LTTAPKLPKVTR-modified surface is specific. Moreover, the competition data suggest that LTTAPKLPKVTR and TVKHRPDALHPQ interact with different cell-surface targets. Thus, our phage display screen can yield surfaces that support EC cell adhesion via independent mechanisms.

**Human ES Cells Proliferate on LTTAPKLPKVTR- and TVKHRPDALHPQ-Substituted Monolayers.** Given the aforementioned similarity of the ES and EC cell gene expression profiles,<sup>29</sup> many receptors on the EC cell surface should be present on human ES cells. We therefore postulated that the sequences identified using EC cells could be used to generate synthetic substrates for human ES cell binding and proliferation. The ability of SAMs to support short-term cell adhesion (Figure 3), however, does not predict their performance in long-term growth assay. Many substrates that engage in short-term binding of human ES cells fail to support their proliferation over longer

time periods.<sup>9,12,13,39</sup> To determine whether any of the peptide-displaying SAMs can facilitate human ES cell proliferation, we cultured human ES cells on these surfaces for multiple days, using conditions that are stringent and chemically defined. Specifically, we used the medium mTeSR-1<sup>9,40</sup> supplemented with an inhibitor of Rho-associated kinase (ROCK inhibitor, Y-27632).<sup>41</sup> The latter was added because it can improve human ES cell culture by decreasing apoptosis of single hES cells.<sup>42</sup> To minimize cell adhesion resulting from adsorbed cell-secreted factors, we avoided using a high ES cell density (e.g.,  $10^6$  cells/mL) and employed the conditions ( $4 \times 10^4$  cells/mL) used in standard human ES cell culture.

Human ES cells (H9) were proliferated on the peptide-displaying SAMs, and the results were compared to those of cells cultured on Matrigel, the standard substrate employed in ES cell culture.<sup>30</sup> Of the six peptide-modified SAMs that support NCCIT cell adhesion, all were effective at mediating human ES cell adhesion. These results confirm our initial hypothesis that human EC cells could be used in screens to identify ligands for human ES cells. Three of the active surfaces, those presenting QLGTQPNRSRTYA, SRYITTMNEQV, or VTSRTIIPQGSA (data not shown), could promote short-term human ES cell adhesion (Supporting Information Figures S2B,C,E and

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**Figure 4.** (A) Monolayers that present phage-derived peptides were tested for their ability to support prolonged growth of human ES cells in a defined medium. Human ES cells (line H9) were plated in mTeSR1 medium in the presence of ROCK inhibitor (Y-27632) atop SAMs displaying the indicated peptide sequence. Cells were allowed to proliferate for 5 days; the medium was exchanged daily. At day 5, the cells were fixed and stained with anti-Oct4 goat IgG and anti-SSEA-4 mouse IgG, followed by Alexa 488 anti-goat-IgG and Alexa 555 anti-mouse-IgG, and Hoechst 33342. Inverted images of each fluorescent channel (Hoechst, Oct4, SSEA-4) are presented. (B) Quantification reveals that two of five peptides support the proliferation of undifferentiated human ES cells. SAMs presenting the sequence LSTIGMPKLLA support cell growth but induce differentiation. The cell count in each fluorescent channel was estimated by integrating the area of the fluorescent signal. The acquisition time and signal threshold used for the quantification of each fluorescent channel were set to identical levels for all images. For additional images see Supporting Information Figure S3.

S3A), but failed to sustain binding and proliferation over 5 days (Figure 4). In contrast, SAMs derived from the other three peptide sequences (LSTIGMPKLLA, LTTAPKLPKVTR, and TVKHRPDALHPQ) could promote human ES proliferation over a longer time period (Figure 4). Staining for two markers of pluripotency, Oct4 and SSEA-4,<sup>30</sup> revealed that most cells on the LSTIGMPKLLA surface had differentiated. In contrast, a majority of the cells proliferated on substrates presenting either LTTAPKLPKVTR or TVKHRPDALHPQ retained the features of pluripotent ES cells (Figures 4B and 5A and Supporting Information Figure S3B,C).

These successes led us to evaluate the utility of the surfaces over longer time periods. Specifically, human ES cells were proliferated on LTTAPKLPKVTR- and TVKHRPDALHPQ-substituted monolayers in defined (TeSR-ROCK) media for 20 days (three passages). The results were compared to those obtained using Matrigel either under the same conditions or with a differentiation-inducing medium.<sup>39</sup> Flow cytometry analysis after 20 days of culture indicated that cells grown on the LTTAPKLPKVTR or TVKHRPDALHPQ substrate maintained a high level of Oct4 expression. Indeed, it was similar to that of cells cultured on the Matrigel substrate in TeSR-ROCK medium (Figure 5B) and significantly higher than those cultured in conditions that induce differentiation. These results indicate

that the peptide-modified surfaces can maintain cells in an undifferentiated state even after several weeks of culture.

Because the culture of human ES cells in suboptimal conditions can lead to the accumulation of chromosomal abnormalities,<sup>43</sup> we examined the karyotype of the cells proliferated on peptide-modified surfaces (Supporting Information Figure S4). After 25 days of culture, nonclonal abnormalities were detected in a small number of cells from the TVKHRPDALHPQ-presenting monolayer (2 out of 40), but no clonal abnormalities were observed. No karyotypic abnormalities were detected in cells proliferated on the LTTAPKLPKVTR-displaying monolayer even after 49 days of culture. For both sequences, longer term growth is necessary to confirm the karyotypic stability of cells under these conditions. Nevertheless, the data suggest that these novel peptide-presenting surfaces can support the undifferentiated growth of human ES cells.

**Human ES Cells Bind to LTTAPKLPKVTR- and TVKHRPDALHPQ-Substituted SAMs and Matrigel-Coated Surfaces via Different Receptors.** As a step toward dissecting the mechanisms by which the active phage-derived peptides function, we tested the involvement of receptors known to mediate cell adhesion. The receptor for these peptides is not obvious, as a BLAST search indicated that they have no significant sequence similarities to known extracellular proteins. We therefore considered potential receptor candidates on the basis of proteins known to mediate cell adhesion. The interaction of human ES cells with surfaces coated with ECM proteins is mediated largely by cell-surface integrins.<sup>12,39</sup> Alternatively, cadherins are another protein family that can be critical for ES cell adhesion.<sup>44</sup> Both of these receptor classes require divalent metal cations ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ),<sup>45</sup> and their action is obliterated by the metal chelator EDTA. Other cell-surface entities known to be important for cell-ECM interactions are anionic glycosaminoglycans (e.g., heparan sulfate proteoglycan (HSPG)).<sup>46</sup> Many glycosaminoglycan-dependent, including HSPG-dependent, interactions can be blocked by soluble heparin.<sup>47</sup> We tested the effect of EDTA and heparin on human ES cell adhesion to different substrates. As a positive control for HSPG-dependent interactions, we utilized adhesion of human ES cells to poly-L-lysine-coated surfaces, as this binding interaction is mediated by Coulombic attraction: EDTA had no effect, but heparin inhibited human ES cell adhesion. Interestingly, cell adhesion to Matrigel also was reduced by 45% in the presence of soluble heparin. Moreover, as expected, the addition of EDTA reduced adhesion to Matrigel by >80%, an observation that indicates that human ES cell-Matrigel interactions are driven primarily by integrin interactions.<sup>39</sup> In contrast, the adhesion of human ES cells to LTTAPKLPKVTR-substituted monolayers was reduced modestly (~30%) in the presence of EDTA or heparin. Even more strikingly, neither inhibitor had an effect on the adhesion of human ES cells to TVKHRPDALHPQ-displaying monolayers (Figure 5C). These results suggest that phage-derived peptides, unlike Matrigel<sup>39</sup> or vitronectin,<sup>12</sup> bind neither integrin receptors nor proteoglycans. We therefore conclude that the interaction

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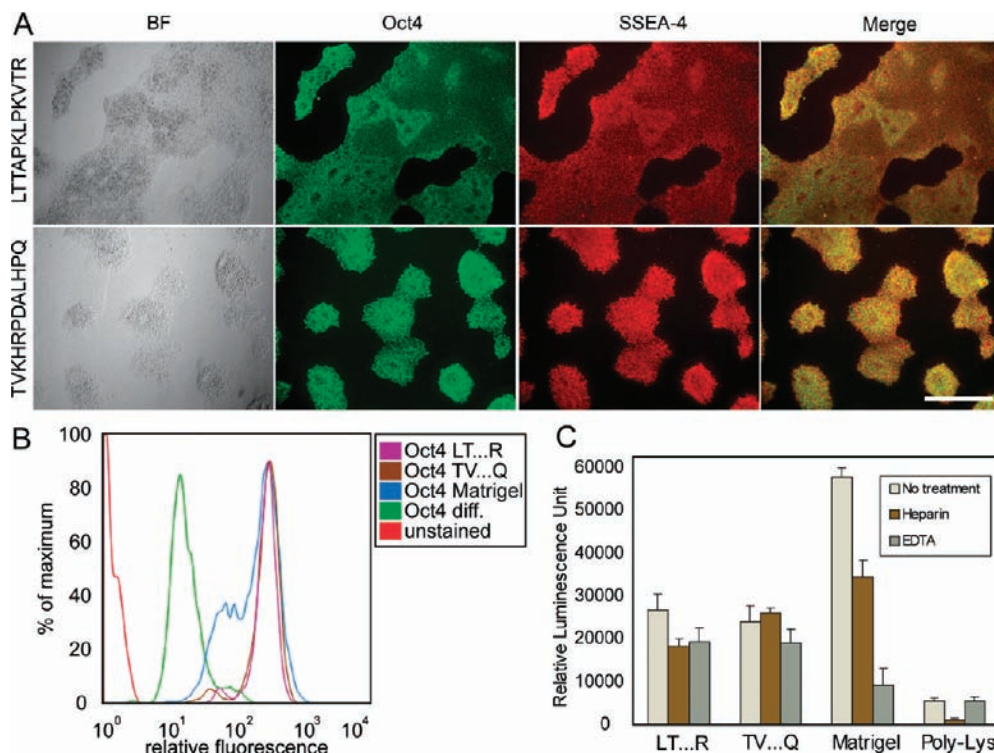
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**Figure 5.** Images depicting human ES cells proliferating on SAMs that present the peptide sequence LTTAPKLPKVTR or TVKHPDALHPQ (abbreviated as “LT...R” and “TV...Q”). (A) Human ES cells were plated onto the SAMs in mTeSR1 medium supplemented with 5  $\mu$ M Y27632 ROCK-1 inhibitor (mTeSR-ROCK). On day 5, the cells were fixed and stained with anti-Oct4 and anti-SSEA4 antibody to visualize markers associated with pluripotency. Scale bar 500  $\mu$ m. (B) Cells were cultured on peptide-substituted SAMs in mTeSR-ROCK medium for 20 days (three passages). The cells were then harvested and stained with anti-Oct4 PE-conjugate, and their staining was compared to that of cells proliferated on Matrigel (positive control) and those proliferated on Matrigel in a differentiation-inducing medium (negative control).<sup>39</sup> (C) Inhibition of human ES cell adhesion: A suspension of human ES cells containing 10 mM EDTA or 5 mg/mL heparin was added to wells coated with Matrigel or polylysine or wells with gold-coated peptide-SAM substrates. Cells were allowed to bind for 1 h, and the number of adherent cells was analyzed using CellTiter-Glo. Neither heparin nor EDTA treatment exhibits a significant effect on short-term human ES cell adhesion to peptide-presenting SAM substrates.

of peptides with human ES cells occurs through different types of receptors. As a consequence, the strategy that we have outlined affords entities that can illuminate new molecular mechanisms that can regulate the adhesion and self-renewal of pluripotent cells.

## Conclusions

Our results demonstrate that a bottom-up approach can be used for the *de novo* discovery of synthetic cell growth substrates. In this way, fully defined, synthetic substrates may replace complex biological matrixes of animal origin. Indeed, the ability of our peptide-displaying SAM to serve as a substrate that can support undifferentiated proliferation of human ES cells highlights the promise and the utility of our method. Although Matrigel and other matrixes that support stable long-term ES cell culture function through cell adhesion receptors, like the integrins, neither LTTAPKLPKVTR nor TVKHPDALHPQ acts through these receptors. These findings highlight that our screening strategy can yield new avenues for investigating the receptors and mechanisms that govern cell growth and differentiation.<sup>15</sup> The strategy we have described is applicable to

discovering new cell-surface receptor–ligand pairs for a wide variety of cell types, including hematopoietic stem cells, induced pluripotent stem cells, and cancer stem cells.

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**Supporting Information Available:** Experimental procedures, characterization of compounds, Figures S1–S6, Table S1, and complete ref 25. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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