Colloids and Surfaces B: Biointerfaces 171 (2018) 451–460

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Molecular basis for RGD-containing peptides supporting adhesion and self-renewal of human pluripotent stem cells on synthetic surface

Ping Zhou[a,b], Bo Yin[c], Rui Zhang[a], Zerong Xu[d], Yuqing Liu[e], Yubo Yan[e], Xiaohong Zhang[b], Siqi Zhang[b], Yongliang Li[f], Huanxiang Liu[d], Y. Adam Yuan[c,g,**], Shicheng Wei[b,f,*]

[a] School and Hospital of Stomatology, Lanzhou University, Lanzhou, 730000, PR China
[b] Center for Biomedical Materials and Tissue Engineering, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, 100871, PR China
[c] National University of Singapore (Suzhou) Research Institute, Suzhou Industrial Park, Suzhou, 215123, PR China
[d] School of Pharmacy, Lanzhou University, Lanzhou, 730000, PR China
[e] Shenzhen Cell Inspire Biotechnology CO., Ltd, Tao Huayuan Science and Technology Innovation Park, Shenzhen, 518102, PR China
[f] Central Laboratory, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, 100081, PR China
[g] Department of Biological Sciences and Centre for Bioimaging Sciences, National University of Singapore, Singapore, 117543, Singapore

ARTICLE INFO

Keywords:
Human pluripotent stem cells
Peptides
RGD sequences
Synthetic surface

ABSTRACT

The ability to obtain a large number of human pluripotent stem cells (hPSCs) under chemically defined conditions plays a key role in clinical application of hPSCs. Chemically defined, economical and effective synthetic peptide displaying surfaces should be the optimal choice for clinical applications involving hPSCs. However, synthetic peptide displaying surfaces are worse than Matrigel surface in supporting cell adhesion and self-renewal. Moreover, the correlations between peptide amino acid sequences and the ability of peptides to support cell survival has never been investigated in hPSCs. In this study, we focused on the Arg-Gly-Asp (RGD) sequence and integrin receptors, which constitute the major recognition system for cell adhesion. Several new RGD-containing peptides were designed by altering the amino acids surrounding the RGD sequence. We investigated the ability of these peptides to sustain hPSC survival, and identified the Ac-KGGPQVTRGDTYRAY sequence, which was capable of supporting cell reprogramming, long-term self-renewal and lineage differentiation. In addition, this report demonstrates that the introduction of mutations in the amino acids surrounding the RGD sequence is a good strategy to design peptides that display excellent adhesion properties and promote hPSC self-renewal. Our results will help improve the current understanding of the mechanisms by which RGD-containing peptides exhibit different abilities in sustaining hPSC culture, and will promote clinical application of both peptide displaying surfaces and hPSCs.

1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have remarkable abilities for both self-renewal and differentiation into any specialized human cell type and thus exhibit great potential for a wide range of applications in regenerative medicine [1,2]. Originally, hPSCs were cultured on feeder cell layers or on Matrigel in conditioned medium, which led to problems associated with immunogenicity, animal pathogens, lack of chemical definition and batch-to-batch variability [3–11]. Several chemically defined and xeno-free media have been designed to support cell self-renewal [12–16]. Moreover, many chemically defined surfaces, such as protein, protein fragment and synthetic surfaces, have been reported in recent years to support cell culture [11,13,17–34]. However, Matrigel is still the most commonly used culture substrate in most laboratories, indicating that much effort is still needed to develop economical, scalable and effective synthetic surfaces for both fundamental research and cell therapy.

Biological materials are expensive to manufacture and have batch-to-batch variability, which makes them unsuitable for large-scale cultivation of hPSCs. Thus, economic synthetic surfaces, especially most reported peptide displaying surfaces, should be the optimal choice for

* Corresponding author at: Center for Biomedical Materials and Tissue Engineering, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, 100871, PR China.
** Corresponding author at: National University of Singapore (Suzhou) Research Institute, Suzhou Industrial Park, Suzhou, 215123, PR China.
E-mail addresses: dbsyya@nus.edu.sg (Y.A. Yuan), sc-wei@pku.edu.cn, weishicheng99@163.com (S. Wei).

https://doi.org/10.1016/j.colsurfb.2018.07.050
Received 23 April 2018; Received in revised form 6 July 2018; Accepted 23 July 2018
Available online 26 July 2018

large-scale cell proliferation under defined conditions [27,28,32,35,36]. However, although several types of peptides are described in the literature for cell cultivation, drawbacks and limitations have been reported in both fundamental research and clinical applications [37]. First, most reported peptides display poor cell adhesion capacity. Second, only a few types of peptides permit long-term self-renewal of cells. Third, high seeding densities, dissociated as colony and supplementation with the Rho-associated protein_kinase (ROCK) inhibitor Y-27632 are needed to achieve good cell adhesion and self-renewal on peptide displaying surfaces. Finally, in general, the amino acid sequences of these adhesive peptides were derived from the active domains of extracellular matrix proteins, and the correlations between amino acid sequences and the ability of peptides to support cell survival have never been investigated in hPSCs. Therefore, much effort is still needed to develop novel peptide sequences for culturing of cells under defined conditions.

We speculated that the correlation between peptide amino acid sequences and support of hPSC survival could be very important in designing synthetic bioactive peptides for hPSC culture. In the literature, synthetic surface presented peptides, such as Ac-KGGPQVTRGDVFTMP, Ac-KGGNGEPRGDTYRAY and Ac-KGGAVTGRGDSPASS, have been reported to support hPSC adhesion [27]. Notably, although the compositions of these peptides were quite similar, their performance in supporting cell adhesion and self-renewal differed. All these sequences harbour an Arg-Gly-Asp (RGD) sequence, which directly binds with members of the integrin receptor family, constituting a major recognition system for cell adhesion and integrin-mediated cell migration, growth, differentiation, and apoptosis [38]. Moreover, it is well known that the sequences surrounding RGD have a large influence on the bioactivity of peptides. Hence, in this report, 12 novel RGD containing peptides derived from the amino acid sequences of these three adhesive peptides were designed and used for cell adhesion and self-renewal assays. Our study provides critical insights into the correlations between the amino acid sequence of RGD-containing peptides and their ability to support hPSC survival, which has great value for designing peptides with a good performance in large-scale cell proliferation.

2. Experimental section

2.1. Survival and short-term self-renewal of hPSCs on peptide displaying surfaces

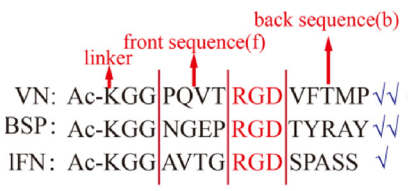
Twelve types of RGD-containing peptides (Fig. 1) derived from the amino acid sequence of vitronectin (VN), bone sialoprotein (BSP) and long fibronectin (IFN) peptides were, respectively, grafted to surface of polydopamine-carboxymethyl chitosan (PDA-CMC) modified culture plates. hNF-C1 hiPSCs colonies were seeded onto these peptide displaying surfaces at a splitting ratio of 1:3 in mTeSR1 medium without supplementation with the ROCK inhibitor Y-27632, and Matrigel surface was used as the control. After 24 h of culture, these surfaces were observed using an inverted microscope (CKX41, Olympus, Japan).

Then, H1 hESC and hNF-C1 hiPSC colonies were separately seeded onto peptide displaying surfaces at a splitting ratio of 1:3 in 12-well cell culture plates. Matrigel surface was used as the control. Y-27632 at 5 μM (Sigma-Aldrich, USA) was added only during passaging, and the medium was changed daily. The cell number at each 24 h interval for 96 h was determined using a cell counting kit-8 assay (CCK-8, Dojindo, Japan) as described previously [36]. Three parallel wells were prepared for each group, and every test was performed in triplicate. Moreover, using the same passaging method, H1 hESCs and hNF-C1 hiPSCs were consecutively cultured on peptide-displaying surfaces for 3 passages. Cells were photographed with a phase contrast microscope equipped with a CCD camera. The expression of pluripotency gene markers, such as *OCT-4*, *NANOG* and *SOX2*, in cells was measured *via* quantitative reverse-transcription PCR (RT-PCR) at the end of each passage.

2.2. Detailed cell adhesion assays on surface displaying Ac-KGGPQVTRGDTYRAY

The newly designed peptide with the sequence Ac-KGGPQVTRGDTYRAY that permitted short-term hPSC self-renewal was denoted as VB peptide. The cell adhesion of H1 hESC and hNF-C1 hiPSC on surfaces displaying VN or VB peptide was analyzed. First, to investigate the hPSC adhesion and migration on VN or VB peptide displaying surfaces, H1 hESCs were seeded as single cells and photographed after adhesion for various lengths of times (1 h, 4 h, 8 h, 12 h and 24 h). Second, PDA-CMC surfaces in 12-well plates were grafted with VN or VB peptide at different concentrations (0.125 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1 mM), and the number of H1 hESC and hNF-C1 hiPSC colonies after 24 h of culture on these peptide displaying surfaces was measured. Third, H1 hESC and hNF-C1 hiPSC colonies at densities of 6250 cell cm^{-2}, 12,500 cell cm^{-2}, 25,000 cell cm^{-2}, 50,000 cell cm^{-2} and 100,000 cell cm^{-2} were seeded onto surfaces displaying VN or VB peptide, and cell numbers were measured after culture for 24 h. Fourth, H1 hESCs and hNF-C1 hiPSCs dissociated as single cells or colonies were respectively seeded onto VN or VB peptide displaying surfaces, and the cell number after 4 days of culture was measured. Finally, colonies of H1 hESCs and hNF-C1 hiPSCs were seeded onto surfaces displaying VN or VB peptide, and the cell number was measured at each 24 h interval for 96 h.

In all studies, unless otherwise specified, peptide concentrations were 1 mM, hPSCs were seeded at a density of 25,000 cells cm^{-2}, cells were cultured in mTeSR1 medium and fed daily, the ROCK inhibitor Y-



Peptide sequence	Numbered	Cell adhesion
VNf: Ac-KGGPQVTRGD	1	×
VNb: Ac-KGGRGDVFTMP	2	√
BSPf: Ac-KGGNGEPRGD	3	×
BSPb: Ac-KGGRGDTYRAY	4	√
IFNf: Ac-KGGAVTGRGD	5	×
IFNb: Ac-KGGRGDSPASS	6	×
VNf+BSPb: Ac-KGG PQVT RGD TYRAY	7	√√
VNf+IFNb: Ac-KGG PQVT RGD SPASS	8	√
BSPf+VNb: Ac-KGG NGEP RGD VFTMP	9	√
BSPf+IFNb: Ac-KGG NGEP RGD SPASS	10	×
IFNf+VNb: Ac-KGG AVTG RGD VFTMP	11	√
IFNf+BSPb: Ac-KGG AVTG RGD TYRAY	12	√

Fig. 1. Schematic representations of the designed peptides. Twelve new RGD-containing peptides were designed based on the known peptides of vitronectin (VN), bone sialoprotein (BSP) and long fibronectin (IFN), which allow survival of hPSCs. The symbol "×" indicates that peptides do not support hPSCadhesion, "√" indicates that peptides allow hPSC survival, and "√√"indicates that peptides display an ability to support hPSC survival similar to that of the Matrigel control.

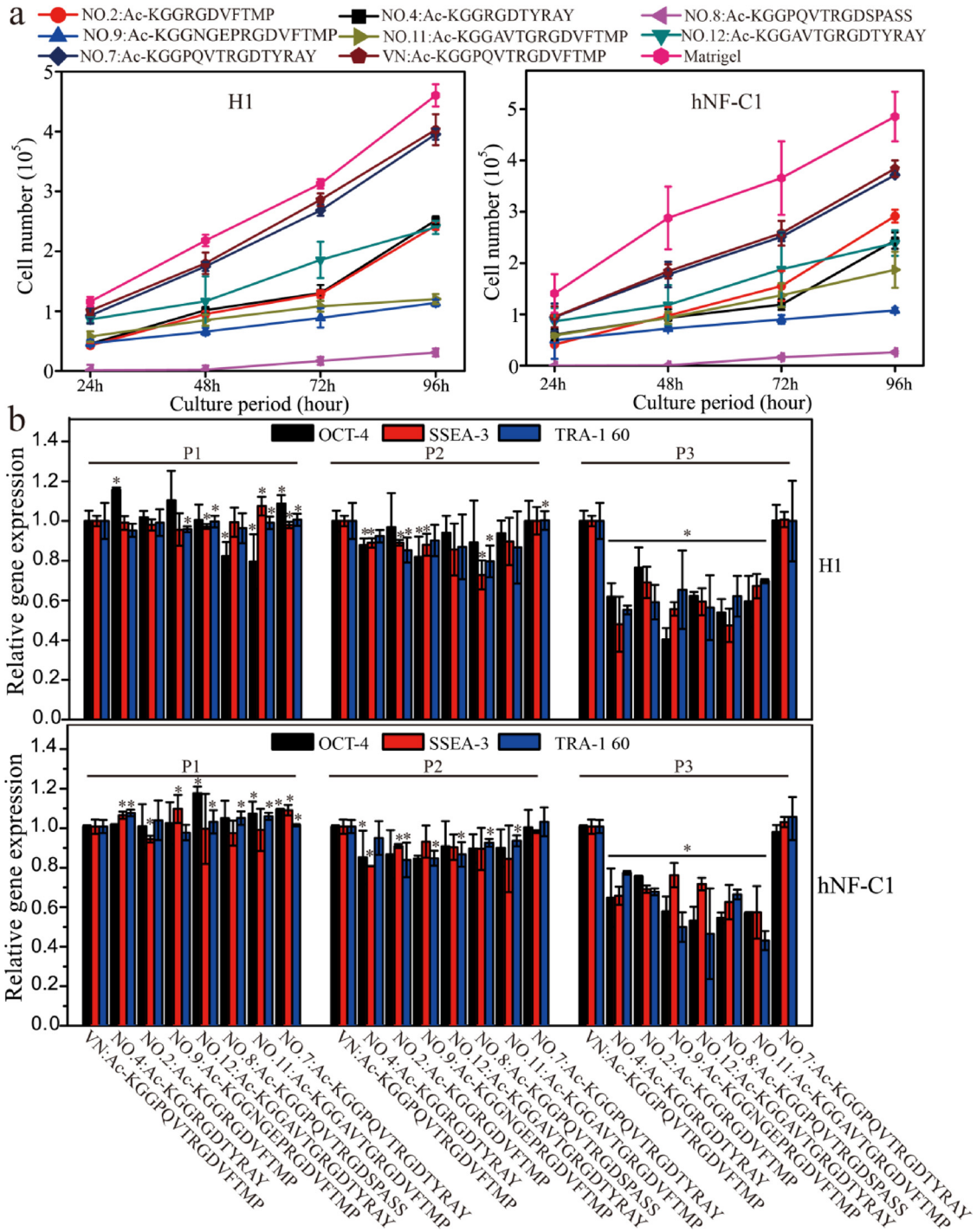


Fig. 2. Proliferation and short term self-renewal of hPSCs on peptide displaying surfaces. (a) H1 hESCs and hNF-C1 hiPSCs were seeded as colonies on surfaces displaying the indicated peptides in 12-well plates with Matrigel surface served as the control. The number of H1 hESC and hNF-C1 hiPSC was measured at each 24 h interval for 96 h. (b) Relative gene expression levels of *OCT-4*, *SOX2* and *NANOG* in H1 hESCs and hNF-C1 hiPSCs cultured on surfaces displaying the indicated peptides were measured by RT-PCR at the end of each passage for 3 passages. The expression levels of these genes in hPSCs cultured on Matrigel surface were standardized to 1; * represents $p < 0.05$ compared with cells on VN peptide displaying surface; n = 3.

27632 was added at a final concentration of 5 μM only during passaging, and the cell number of hPSCs was determined using a cell counting kit-8 assay.

2.3. Long-term self-renewal of multiple hPSCs on VB peptide displaying surface

For each passage of more than 20 passages, when cell colonies had grown to confluence, hESCs (H1 and H9) and hiPSCs (hNF-C1, UMC-C1 and ipsN004) were transferred onto new VB peptide displaying surface as colonies at a splitting ratio of 1:3 in mTeSR1 medium containing 5 μM Y-27632. Y-27632 was applied only during passaging, and the cells were fed every day. Microscopic examination of cell and colony morphology was performed daily. Differentiated cells were marked and removed mechanically with a negative pressure aspirator (YX932D, China). Besides, all hPSC lines at each of the passage for over 20

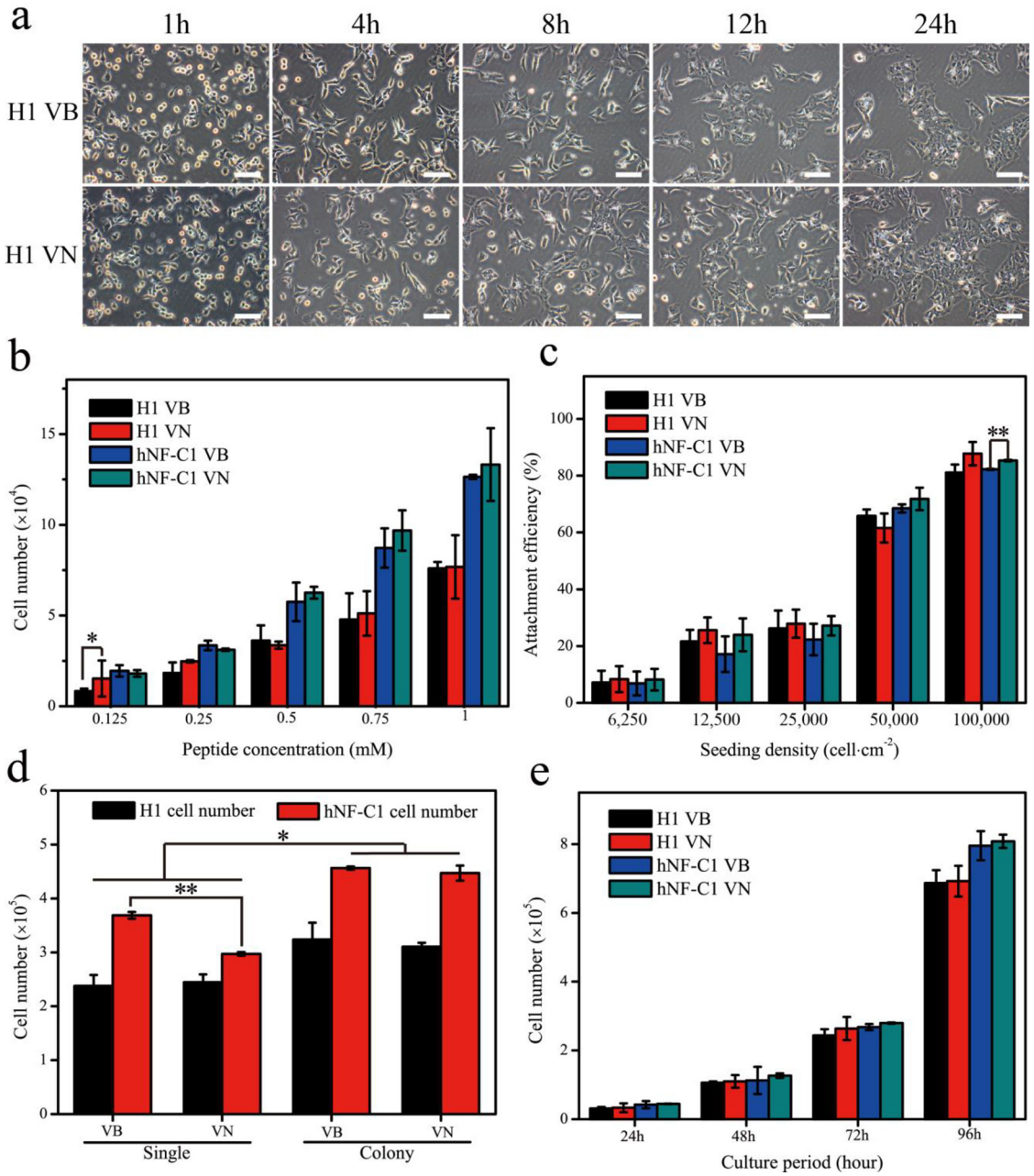


Fig. 3. Survival of hPSCs on Ac-KGGPQVTRGDTYRAY (VB) or VN peptide displaying surfaces. (a) Phase-contrast images of single H1 hESCs cultured on peptide displaying surfaces at different time points (1 h, 4 h, 8 h, 12 h and 24 h). Scale bars, 100 μm. (b) PDA-CMC modified 12-well plates were grafted with VN or VB peptide at the indicated concentrations. H1 hESCs and hNF-C1 hiPSCs were seeded on those plates as colonies at a density of 25,000 cell cm^{-2}. Cell numbers were measured after 24 h of culture. (c) Colonies of H1 hESCs and hNF-C1 hiPSCs were seeded onto surfaces displaying VB or VN peptide at the indicated densities. After culture for 24 h, the adhesion efficiency of hPSCs was measured. Values are shown as the average percentage of cells that attached. (d) H1 hESCs and hNF-C1 hiPSCs were dissociated as single cells or colonies and were seeded onto VB or VN peptide displaying surfaces at a density of 25,000 cell cm^{-2} for 4 days. (e) Colonies of H1 hESCs and hNF-C1 hiPSCs were seeded onto VB or VN peptide displaying surfaces, and the cell number was measured each 24 h for 96 h. (f) Cell doubling times of H1 hESCs and hNF-C1 hiPSCs over the course of 10 consecutive passages on VB peptide displaying surface. * represents $p < 0.05$, and ** represents $p < 0.01$ ($n = 3$).

passages were maintained in frozen stock solution containing 90% mTeSR1 medium and 10% dimethylsulfoxide (DMSO; AMRESCO, USA), and stored in liquid nitrogen for future use.

Cell doubling times of H1 hESCs and hNF-C1 hiPSCs on VB peptide displaying surface over ten passages were measured using the same method reported recently for VN peptide displaying surface and Matrigel surface [36]. The expression of the intracellular pluripotent marker OCT-4 in H1 hESCs and hNF-C1 hiPSCs cultured on VB peptide displaying surface was also detected at the end of each of ten passage using flow cytometry.

2.4. Identification of pluripotency

As provided in the Supporting information, for hPSCs cultured on peptide displaying surfaces, the expression of pluripotency genes, such as *OCT-4*, *NANOG* and *SOX-2*, was detected *via* reverse-transcription PCR (RT-PCR), and the expression of the pluripotency proteins OCT-4, SSEA-3 and TRA-1 60 was measured *via* fluorescence-activated cell sorting (FACS) and immunofluorescence. hPSC samples were transferred back onto the Matrigel surface for analysis of karyotype, embryoid body formation and teratoma formation.

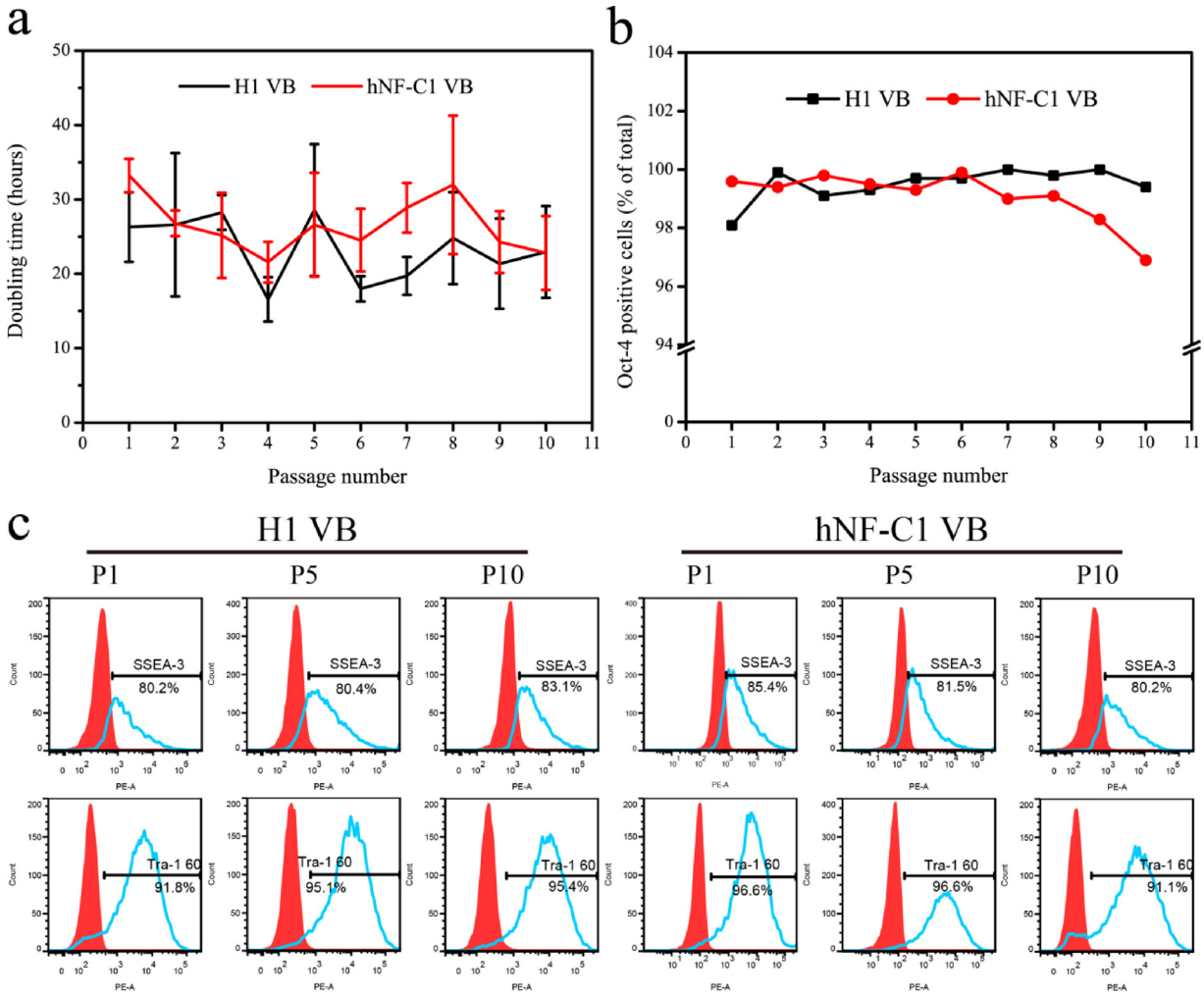


Fig. 4. Long-term self-renewal of hPSCs on Ac-KGGPQVTRGDTYRAY (VB) or VN peptide displaying surfaces. (a–b) OCT-4 expression in H1 hESCs (a) and hNF-C1 hiPSCs (b) cultured on VB peptide displaying surface for 10 sequential passages were measured by FACS. (c) FACS analysis of SSEA-3 and TRA-1 60 expressions in H1 hESCs and hNF-C1 hiPSCs on VB peptide displaying surface at passages (P) 1, 5 and 10. IgG and IgM controls: red peak; FITC-or PE-conjugated antibody counts: unfilled peak. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2.5. Statistical analysis

All data are expressed as the average of at least three replicate experiments and are presented as the mean ± standard deviation. Student's *t*-test was used to determine the significant differences among the groups, and p values < 0.05 were considered statistically significant. In this study, * represents $p < 0.05$, and ** represents $p < 0.01$.

3. Results and discussion

3.1. Testing the designed peptide-displaying surfaces for hPSC survival

We designed a total of 12 new RGD-containing peptides, derived from the amino acid sequence of vitronectin (VN) peptide, bone sialoprotein (BSP) peptide and long fibronectin (IFN) peptide (Fig. 1). These 12 new peptides were grafted onto polydopamine-carboxymethyl chitosan (PDA-CMC)-modified cell culture plates as we recently described [36]. To investigate whether these peptide displaying surfaces allowed for hPSC adhesion, we seeded hNF-C1 hiPSC colonies onto the peptide displaying surfaces for 24 h, and found that 7 of the peptides permitted cell adhesion (Fig. S1).

Then, cell proliferation and short-term self-renewal analyses were conducted to evaluate the abilities of those 7 adhesive peptides to support hPSC culture. The widely used VN peptide, which enables good cell survival [27,32,33,36,39], and Matrigel were used as positive controls. The growth curves of H1 hESCs and hNF-C1 hiPSCs cultured on the NO.7 peptide displaying surface were very similar to those from cells grown on the VN peptide displaying surface, and much better than those of cells cultured on surfaces displaying the other 6 peptides (Fig. 2a). Moreover, we serially passaged H1 hESCs and hNF-C1 hiPSCs on peptide displaying surfaces and the Matrigel surface for 3 passages, and the expression levels of pluripotent gene markers, such as *OCT-4*, *SOX2* and *NANOG*, in cell samples were measured using RT-PCR at the end of each passage. At passage 1, the gene expression level of *OCT-4* in H1 hESCs cultured on surfaces displaying the NO.8 or NO.11 peptide was slightly lower than that in cells cultured on the Matrigel control surface (Fig. 2b). Consistently, as shown in Figs. S1 and 2a, surfaces displaying the NO.8 or NO.11 peptide performed poorly in supporting cell adhesion and proliferation. Because a low density of attached colonies indicates disadvantages in pluripotency maintenance of hPSCs, the poor cell adhesion performance on surfaces displaying these two peptides were likely the reason for the down-regulated expression levels of pluripotent gene markers. Only on surface displaying the designed NO.7 peptide (Ac-KGGPQVTRGDTYRAY), the expression levels of pluripotent gene markers in both H1 hESCs and hNF-C1 hiPSCs was almost the same as that cultured on control surfaces throughout 3 passages, indicating a good ability to support hPSC proliferation and

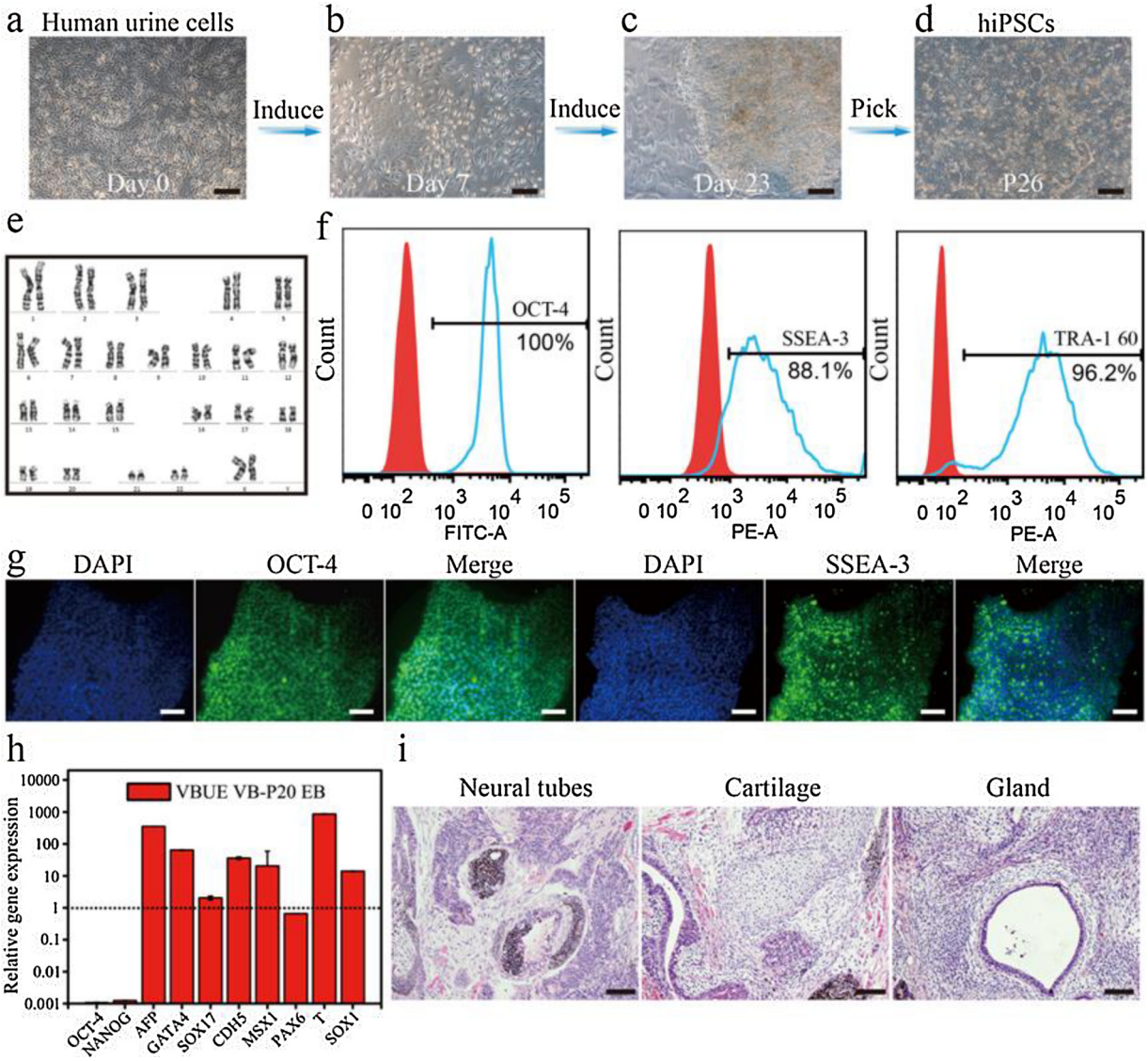


Fig. 5. Generation of hiPSCs from human urine-derived cells on Ac-KGGPQVTRGDTYRAY (VB) peptide displaying surface under defined conditions. (a) Cell morphology of human urine cells before reprogramming. (b–c) Morphology of ESC-like colonies that appeared after 7 days (b) and 23 days (c) under induction on the VB peptide displaying surface. (d) Colony morphology of picked VBUE hiPSCs on the synthetic surface at passage 26. (e) Karyotype analysis of VBUE hiPSCs at passage 20. (f) FACS analysis of OCT-4, SSEA-3 and Tra-1 60 expression in VBUE hiPSC samples. IgG and IgM controls: red peak; FITC- or PE-conjugated antibody counts: unfilled peak. (g) Immunostaining of OCT-4 (green) and SSEA-3 (green) in cells cultured on VB peptide displaying surface. Nuclei are visualized with DAPI staining (blue). (h) RT-PCR results for the indicated gene markers to identity the three-germ layers in spontaneously differentiated VBUE hiPSCs. The expression of these genes in hPSCs before differentiation was standardized to 1, which is marked by a single dotted threshold line. (i) Tissues from all three germ layers, representing secretory epithelium (endoderm), cartilage (mesoderm) and neuroepithelium (ectoderm), were identified in teratomas formed by VBUE hiPSCs (P20). White scale bar, 100 μm; black scale bar, 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

self-renewal.

To quantitatively calculate the surface densities of the 7 adhesive peptides grafted onto PDA-CMC surfaces, we synthesized 7 corresponding FITC-labelled versions of each peptide. Calibration curves showing fluorescence intensity and surface peptide density were generated for the NO.7 and NO.4 peptides as shown in Fig. S2. FITC-labelled peptides at a concentration of 1 mM were, respectively, grafted onto PDA-CMC surfaces. Four specimens were produced for each group, and each test was performed in triplicate using a microplate reader. Significant differences in surface density were detected among all 7 designed peptides and the VN peptide on PDA-CMC surfaces (Fig. S3). The NO.11 peptide (Ac-KGGAVTGRGDVFTMP) exhibited the highest surface density, while the NO.7 peptide (Ac-KGGPQVTRGDTYRAY) showed the best performance in hPSC adhesion and exhibiting a exhibited moderate surface density (Fig. S3). Given that the NO.11 peptide exhibited a poor ability to support cell adhesion and self-renewal compared with the NO.7 peptide, we concluded that the difference in the performance for these peptides was likely caused not by the surface density but by the amino acid sequences and composition surrounding the RGD sequence.

Based on the amino acid sequences of all 13 investigated peptides, our cell adhesion and self-renewal results showed the following. 1) C-terminal neighboring residues of the RGD sequence were indispensable for hPSC attachment to the surface. 2) N-terminal neighboring residues of the RGD sequence were crucial for hPSC survival. 3) The amino acids surrounding the RGD sequence apparently affect the bioactivities of the peptides in hPSC culture, and introducing mutations into the amino acids surrounding the RGD sequence is a good strategy for designing peptides for cell culture. In future studies, we will systematically screen a series of peptides by introducing mutations into the non-RGD

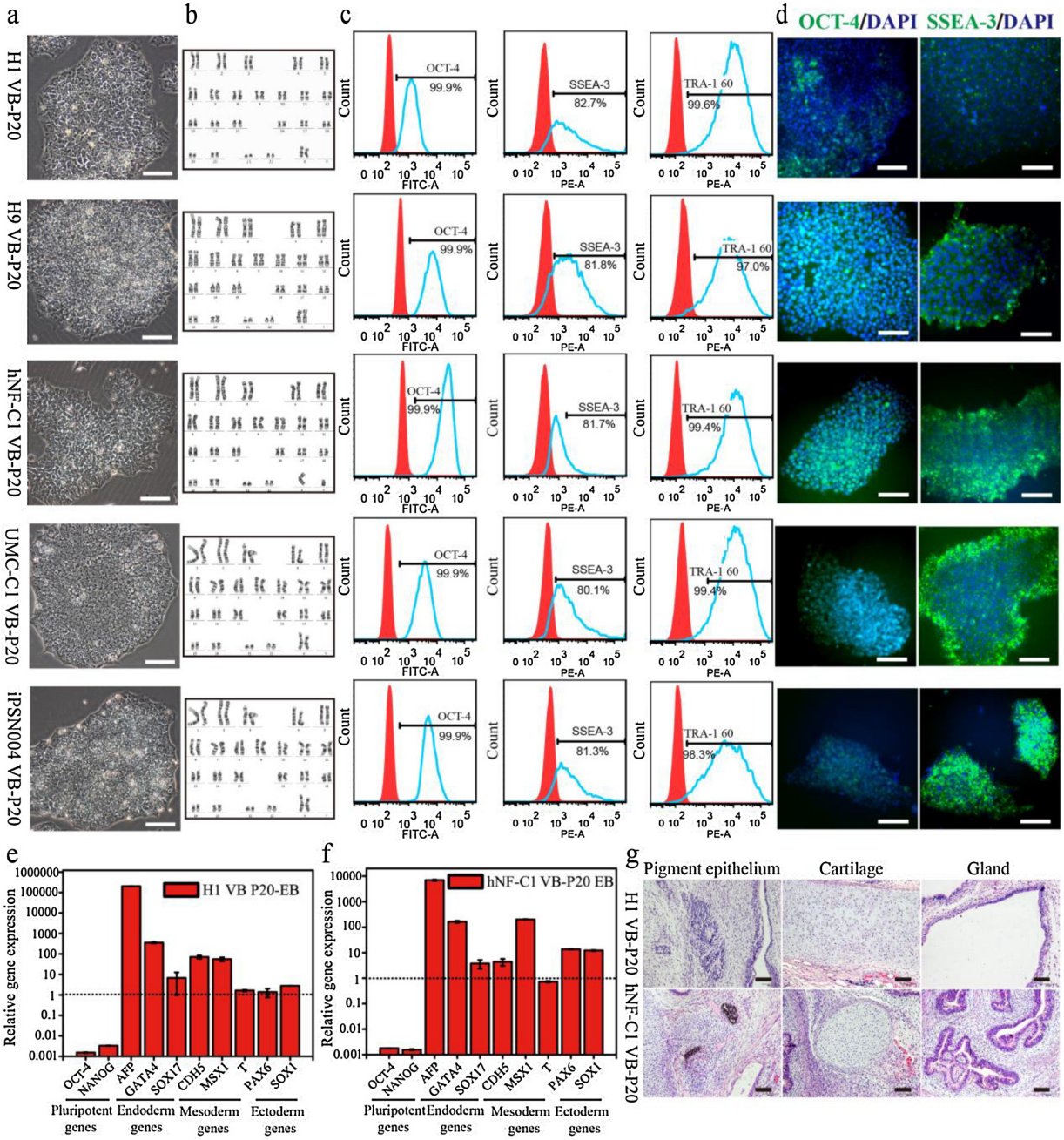


Fig. 6. Long-term self-renewal of hPSCs on surface displaying Ac-KGGPQVTRGDTYRAY (VB) peptide. (a–b) Colony morphology (a) and karyotype analysis (b) of all five hPSCs lines (H1 hESCs, H9 hESCs, hNF-C1 hiPSCs, UMC-C1 hiPSCs and ipsN004 hiPSCs) at P20 on VB peptide displaying surface. (c) FACS measurements of OCT-4, SSEA-3 and Tra-1 60 expression in hPSC samples. IgG and IgM controls: red peak; FITC- or PE-conjugated antibody counts: unfilled peak. (d) The expression of OCT-4 (green) and SSEA-3 (green) is shown using immunostaining. Nuclei are visualized with DAPI staining (blue). (e–f) Relative expression of the indicated gene markers to identity the three-germ layers in spontaneously differentiated H1 hESCs (e) or hNF-C1 hiPSCs (f) was investigated by RT-PCR. The expression of these genes in hPSCs before differentiation was standardized to 1, which is marked by a single dotted threshold line. (g) H1 hESC and hNF-C1 hiPSC samples were injected subcutaneously into SCID mice to form teratomas, and pluripotency was determined by the presence of tissues from all three germ layers, representing the secretory epithelium (endoderm), cartilage (mesoderm) and neuroepithelium (ectoderm). White scale bar, 100 μm; black scale bar, 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

sequences starting with the VN peptide sequence and examine hPSC survival on these peptide surfaces. Moreover, molecular dynamics simulation, biotin labelling, isothermal titration calorimetry and surface plasmon resonance sensing will be applied to reveal detailed insights into the mechanism by which amino acid sequences affect the bioactivity of RGD-containing peptides in hPSC culture.

3.2. NO.7 peptide exhibited comparable abilities in adhesion and self-renewal of hPSCs to reported VN peptide

The NO.7 peptide will be referred to as VB peptide in subsequent sections because its sequence consists of the front sequence of the VN peptide and the back sequence of the BSP peptide. VB and VN peptides

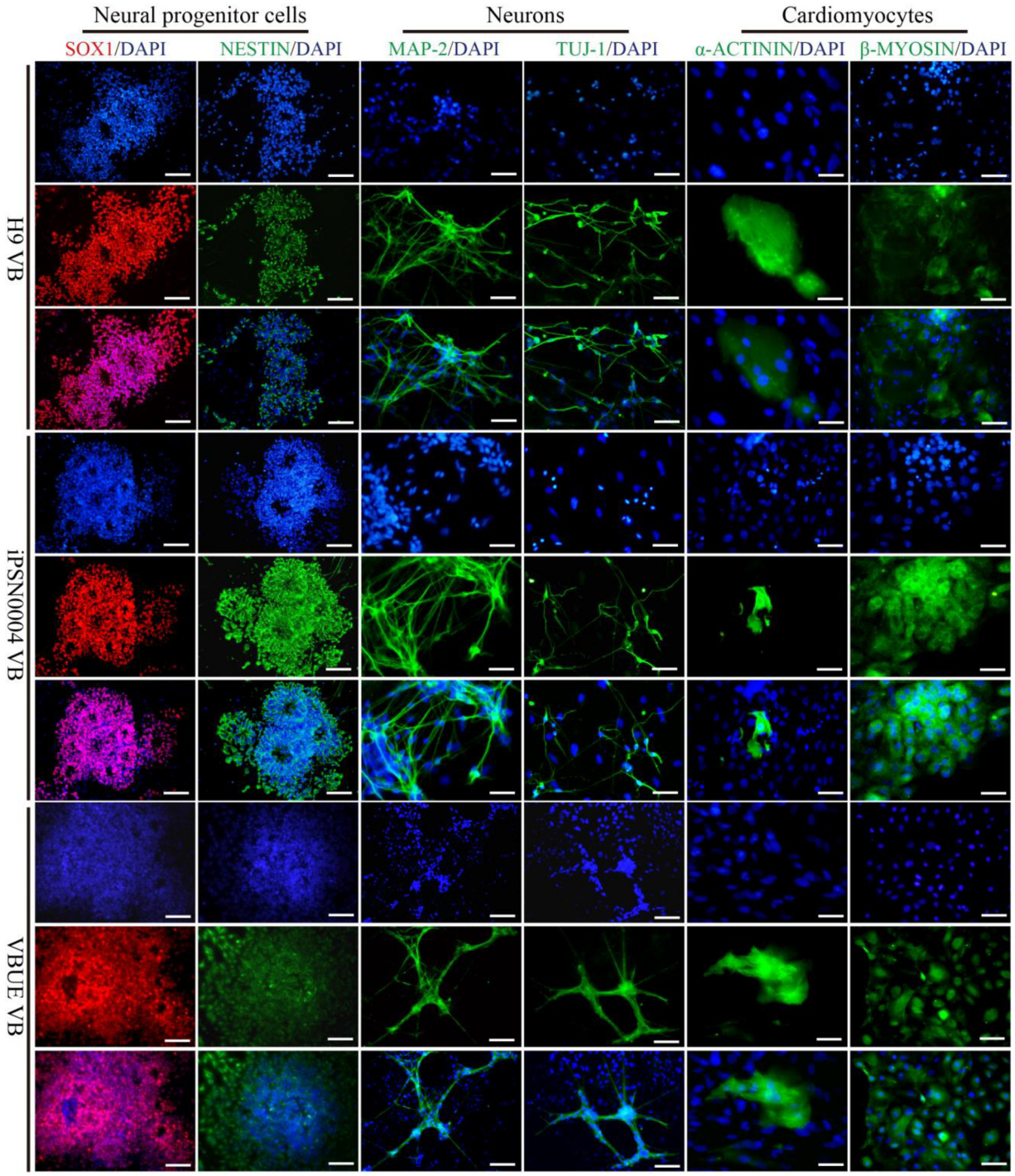


Fig. 7. *In vitro* differentiation of multiple hPSCs lines on surface displaying Ac-KGGPQVTRGDTYRAY (VB) peptide. H9 hESCs, iPSN004 hiPSCs and VBUE hiPSCs were induced towards neural progenitor-like cells, neural-like cells and cardiomyocytes, which were confirmed by immunostaining of SOX-1 (red) /NESTIN (green), MAP-2 (green)/TUJ-1 (green) and α-ACTININ (green)/β-MYOSIN (green). Nuclei are shown in blue (DAPI staining). Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

were grafted onto separate PDA-CMC surfaces. The cell morphology of H1 hESCs on the surfaces displaying VN or VB peptide were similar at time intervals of 1 h, 4 h, 8 h, 12 h and 24 h (Fig. 3a). As shown in Fig. 3b–e, the cell number of H1 hESC and hNF-C1 hiPSC on surfaces displaying VN or VB peptide was nearly the same when changing the following parameters: peptide concentrations (0.125–1 mM), seeding densities (6250–100,000 cell cm^{-2}), digestion as single cells or colonies, and incubation time (24–96 h). Moreover, RNA-seq was applied to compare the gene expression patterns in hiPSCs attached to VB peptide displaying surface or the Matrigel surface for 8 h and 24 h. The results confirmed that our VB peptide displaying surface presented cell adhesion and pluripotency maintenance abilities comparable with those of the VN peptide and Matrigel control surfaces (Figs. S5–6).

In addition, the cell doubling times of H1 hESC and hNF-C1 hiPSC on the VB peptide displaying surface were very close to those on the VN peptide displaying surface throughout a ten-passage study (Fig. 4a), and the expression of the pluripotency markers OCT-4, SSEA-3 and Tra-1 60 in cells cultured on these surfaces were similar based on FACS analysis

(Fig. 4b–c). Our data indicated that the newly developed VB peptide, compared with VN peptide, exhibits nearly the same hPSC adhesion and self-renewal abilities when grafted onto PDA-CMC surfaces, thus displaying great promise for sustaining long-term hPSCs cultivation under defined conditions.

3.3. VB peptide displaying surfaces supporting derivation, long-term self-renewal and differentiation of hPSCs

Under chemically defined conditions, we obtained one hiPSC line derived from human urine-derived cells (denoted as VBUE hiPSCs) (Fig. 5). Then, two hESC lines (H1 and H9) and four hiPSC lines (VBUE hiPSCs, hNF-C1, UMC-C1 and ipsN004) were sequentially passaged on VB peptide displaying surface in defined mTeSR1 medium for over 20 passages. After being passaged on VB peptide displaying surface for 20 passages, all six lines showed an unaltered morphology (Figs. 5d, 6 a) and retained the normal karyotypes (Figs. 5e, 6b). For each hPSC line, most cells co-expressed pluripotency markers, such as OCT-4, SSEA-3 and Tra-1 60, as demonstrated by FACS (Figs. 5f, 6c). In addition, immunofluorescence results showed that OCT-4 and SSEA-3 were expressed at high levels throughout the colonies (Figs. 5g, 6d). To investigate the maintenance of hPSC multipotent differentiation ability on the VB peptide displaying surface, *in vitro* embryoid body (EB) and *in vivo* teratoma formation for H1 hESCs and hNF-C1 hiPSCs at passage 20 were assessed. Notably, spontaneously differentiated cells from all three germ layers were observed for both H1 hESCs and hNF-C1 hiPSCs, as revealed by RT-PCR (Figs. 5h, 6e–f). H1 hESCs and hNF-C1 hiPSCs formed teratomas and generated cells from all three germ layers approximately 2 months after injection of the cells into the flanks of nude mice (Figs. 5i, 6g). These results confirmed that the multilineage potential of hPSCs was maintained after culture on synthetic VB peptide displaying surface for 20 passages. H9 hESCs, iPSN004 hiPSCs and VBUE hiPSCs cultured on the VB peptide displaying surface were further differentiated towards neural cells and cardiomyocytes under defined conditions (Fig. 7). Notably, we achieved reprogramming, long-term self-renewal and lineage differentiation of VBUE hiPSCs on the VB peptide displaying surface under defined conditions, which will be of great value for clinical application of hiPSCs.

4. Conclusions

In summary, we designed twelve novel RGD-containing peptide sequences based on three previously reported peptide sequences and investigated their ability to support adhesion and self-renewal of hPSCs as a function of the amino acid sequences surrounding RGD. Seven of the peptides allowed cell survival, and only one of these peptides supported cell self-renewal in three passages. Fortunately, this Ac-KGGPQVTRGDTYRAY peptide exhibited excellent properties in human somatic cell reprogramming, long-term self-renewal and lineage differentiation of hPSCs and thus could be applied for clinical use. Notably, based on these results, we preliminary explored correlations between the amino acid sequence surrounding the RGD motif and the ability of peptides to support hPSC culture, and our findings can be used to design new peptide sequences with better performance than the currently reported peptides. Our research will surely promote the application of synthetic peptides for surface modification in both fundamental research and clinical application of hPSCs.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (nos. 81371697 and 81571824), Teaching Reform Research Project of Lanzhou University (2018001), and Fundamental Research Funds for the Central Universities (lzujbky-2015-295 and lzujbky-2018-27). We thank Miss. Junhua Zou from the Department of Medical Genetics, Peking University for generous support in the karyotyping analysis, and Dr. Andrew Hutchins from Guangzhou Institutes of Biomedicine and Health for help with the bioinformatic analysis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2018.07.050.

References

[1] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[2] B.E. Reubinoff, M.F. Pera, C.Y. Fong, A. Trounson, A. Bongso, Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, Nat. Biotechnol. 18 (2000) 399–404.
[3] O. Genbacev, A. Krtolica, T. Zdravkovic, E. Brunette, S. Powell, A. Nath, E. Caceres, M. McMaster, S. McDonagh, Y. Li, R. Mandalam, J. Lebkowski, S.J. Fisher, Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders, Fertil. Steril. 83 (2005) 1517–1529.
[4] I. Rodriguez-Piza, Y. Richaud-Patin, R. Vassena, F. Gonzalez, M.J. Barrero, A. Veiga, A. Raya, J.C. Izpisua Belmonte, Reprogramming of human fibroblasts to induced pluripotent stem cells under xeno-free conditions, Stem Cells 28 (2010) 36–44.
[5] J.B. Lee, J.E. Lee, J.H. Park, S.J. Kim, M.K. Kim, S. Il Roh, H.S. Yoon, Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition, Biol. Reprod. 72 (2005) 42–49.
[6] J. Inzunza, K. Gertow, M.A. Stromberg, E. Matilainen, E. Blennow, H. Skottman, S. Wolbank, L. Arlund-Richter, O. Hovatta, Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells, Stem Cells 23 (2005) 544–549.
[7] J.A. Thomson, Embryonic stem cell lines derived from human blastocysts, Science 282 (1998) 1145–1147.
[8] C. Xu, M.S. Inokuma, J. Denham, K. Golds, P. Kundu, J.D. Gold, M.K. Carpenter, Feeder-free growth of undifferentiated human embryonic stem cells, Nat. Biotechnol. 19 (2001) 971–974.
[9] M.J. Martin, A. Muotri, F. Gage, A. Varki, Human embryonic stem cells express an immunogenic nonhuman sialic acid, Nat. Med. (2005) 228–232.
[10] J. Lu, R. Hou, C.J. Booth, S.-H. Yang, M. Snyder, Defined culture conditions of human embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 5688–5693.
[11] S. Rodin, A. Domogatskaya, S. Strom, E.M. Hansson, K.R. Chien, J. Inzunza, O. Hovatta, K. Tryggvason, Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511, Nat. Biotechnol. 28 (2010) 611–615.
[12] H. Tsutsui, B. Valamehr, A. Hindoyan, R. Qiao, X. Ding, S. Guo, O.N. Witte, X. Liu, C.-M. Ho, H. Wu, An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells, Nat. Commun. 2 (2011) 167.
[13] G. Chen, D.R. Gulbranson, Z. Hou, J.M. Bolin, V. Ruotti, M.D. Probasco, K. Smuga-Otto, S.E. Howden, N.R. Diol, N.E. Propson, R. Wagner, G.O. Lee, J. Antosiewicz-Bourget, J.M.C. Teng, J.A. Thomson, Chemically defined conditions for human iPSC derivation and culture, Nat. Methods 8 (2011) 424–429.
[14] T.E. Ludwig, M.E. Levenstein, J.M. Jones, W.T. Berggren, E.R. Mitchen, J.L. Frane, L.J. Crandall, C.A. Daigh, K.R. Conard, M.S. Piekarczyk, R.A. Llanas, J.A. Thomson, Derivation of human embryonic stem cells in defined conditions, Nat. Biotechnol. 24 (2006) 185–187.
[15] V. Akopian, P.W. Andrews, S. Beil, N. Benvenisty, J. Brehm, M. Christie, A. Ford, V. Fox, P.J. Gokhale, L. Healy, F. Holm, O. Hovatta, B.B. Knowles, T.E. Ludwig, R.D.G. McKay, T. Miyazaki, N. Nakatsuji, S.K.W. Oh, M.F. Pera, J. Rossant, G.N. Stacey, H. Suemori, Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells, In Vitro Cell. Dev. Biol. Anim. 46 (2010) 247–258.
[16] Y. Liu, Z. Song, Y. Zhao, H. Qin, J. Cai, H. Zhang, T. Yu, S. Jiang, G. Wang, M. Ding, H. Deng, A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells, Biochem. Biophys. Res. Commun. 346 (2006) 131–139.
[17] N. Montserrat, E.G. Bahima, L. Batlle, S. Hafner, A.M. Rodrigues, F. Gonzalez, J.C. Izpisua Belmonte, Generation of pig iPS cells: a model for cell therapy, J. Cardiovasc. Transl. Res. 4 (2011) 121–130.
[18] S. Wu, J. Johansson, P. Damdimopoulou, M. Shahsavani, A. Falk, O. Hovatta, A. Rising, Spider silk for xeno-free long-term self-renewal and differentiation of human pluripotent stem cells, Biomaterials 35 (2014) 8496–8502.
[19] M. Baker, Stem cells in culture: defining the substrate, Nat. Methods 8 (2011) 294–297.
[20] S.R. Braam, L. Zeinstra, S. Litjens, Dorien Ward-van Oostwaard, Stieneke van den Brink, Linda van Laake, Franck Lebrin, Peter Kats, Ron Hochstenbach, Robert Passier, Arnoud Sonnenberg, C.L. Mummery, Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alpha V beta 5 integrin, Stem Cells 26 (2008) 2257–2265.
[21] A.B.J. Prowse, M.R. Doran, J.J. Cooper-White, F. Chong, T.P. Munro, J. Fitzpatrick, T.-L. Chung, D.N. Haylock, P.P. Gray, E.J. Wolvetang, Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media, Biomaterials 31 (2010) 8281–8288.
[22] M. Widhe, H. Bysell, S. Nystedt, I. Schenning, M. Malmsten, J. Johansson, A. Rising, M. Hedhammar, Recombinant spider silk as matrices for cell culture, Biomaterials 31 (2010) 9575–9585.

[23] T. Miyazaki, S. Futaki, H. Suemori, Y. Taniguchi, M. Yamada, M. Kawasaki, M. Hayashi, H. Kumagai, N. Nakatsuji, K. Sekiguchi, E. Kawase, Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells, Nat. Commun. 3 (2012) 1236.
[24] S. Rodin, L. Antonsson, C. Niaudet, O.E. Simonson, E. Salmela, E.M. Hansson, A. Domogatskaya, Z. Xiao, P. Damdimopoulou, M. Sheikhi, J. Inzunza, A.-S. Nilsson, D. Baker, R. Kuiper, Y. Sun, E. Blennow, M. Nordenskjöld, K.-H. Grinnemo, J. Kere, C. Betsholtz, O. Hovatta, K. Tryggvason, Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment, Nat. Commun. 5 (2014).
[25] H.F. Lu, C. Chai, T.C. Lim, M.F. Leong, J.K. Lim, S. Gao, K.L. Lim, A.C.A. Wan, A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells, Biomaterials 35 (2014) 2816–2826.
[26] L.G. Villa-Diaz, H. Nandivada, J. Ding, N.C. Nogueira-de-Souza, P.H. Krebsbach, K.S. O'Shea, J. Lahann, G.D. Smith, Synthetic polymer coatings for long-term growth of human embryonic stem cells, Nat. Biotechnol. 28 (2010) 581–583.
[27] Z. Melkoumian, J.L. Weber, D.M. Weber, A.G. Fadeev, Y. Zhou, P. Dolley-Sonneville, J. Yang, L. Qiu, C.A. Priest, C. Shogbon, A.W. Martin, J. Nelson, P. West, J.P. Beltzer, S. Pal, R. Brandenberger, Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells, Nat. Biotechnol. 28 (2010) 606–610.
[28] J.R. Klim, L. Li, P.J. Wrighton, M.S. Piekarczyk, L.L. Kiessling, A defined glycosaminoglycan-binding substratum for human pluripotent stem cells, Nat. Methods 7 (2010) 989–994.
[29] E.F. Irwin, R. Gupta, D.C. Dashti, K.E. Healy, Engineered polymer-media interfaces for the long-term self-renewal of human embryonic stem cells, Biomaterials 32 (2011) 6912–6919.
[30] D.A. Brafman, C.W. Chang, A. Fernandez, K. Willert, S. Varghese, S. Chien, Long-term human pluripotent stem cell self-renewal on synthetic polymer surfaces, Biomaterials 31 (2010) 9135–9144.
[31] L. Liu, M. Yoshioka, M. Nakajima, A. Ogasawara, J. Liu, K. Hasegawa, S. Li, J. Zou, N. Nakatsuji, K.-i. Kamei, Y. Chen, Nanofibrous gelatin substrates for long-term expansion of human pluripotent stem cells, Biomaterials 35 (2014) 6259–6267.
[32] Y. Deng, X. Zhang, X. Zhao, Q. Li, Z. Ye, Z. Li, Y. Liu, Y. Zhou, H. Ma, G. Pan, D. Pei, J. Fang, S. Wei, Long-term self-renewal of human pluripotent stem cells on peptide-decorated poly(OEGMA-co-HEMA) brushes under fully defined conditions, Acta Biomater. 9 (2013) 8840–8850.
[33] H.J. Park, K. Yang, M.J. Kim, J. Jang, M. Lee, D.W. Kim, H. Lee, S.W. Cho, Bio-inspired oligovitronectin-grafted surface for enhanced self-renewal and long-term maintenance of human pluripotent stem cells under feeder-free conditions, Biomaterials 50 (2015) 127–139.
[34] K. Kandasamy, K. Narayanan, M. Ni, C. Du, A.C. Wan, D. Zink, Polysulfone membranes coated with polymerized 3,4-dihydroxy-l-phenylalanine are a versatile and cost-effective synthetic substrate for defined long-term cultures of human pluripotent stem cells, Biomacromolecules 15 (2014) 2067–2078.
[35] Kevin G. Chen, Barbara S. Mallon, Ronald D.G. McKay, Pamela G. Robey, Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics, Cell Stem Cell 14 (2014) 13–26.
[36] P. Zhou, F. Wu, T. Zhou, X. Cai, S. Zhang, X. Zhang, Q. Li, Y. Li, Y. Zheng, M. Wang, F. Lan, G. Pan, D. Pei, S. Wei, Simple and versatile synthetic polydopamine-based surface supports reprogramming of human somatic cells and long-term self-renewal of human pluripotent stem cells under defined conditions, Biomaterials 87 (2016) 1–17.
[37] A. Higuchi, Q.-D. Ling, S.S. Kumar, M. Munusamy, A.A. Alarfajj, A. Umezawa, G.-J. Wu, Design of polymeric materials for culturing human pluripotent stem cells: progress toward feeder-free and xeno-free culturing, Prog. Polym. Sci. 39 (2014) 1348–1374.
[38] E. Ruoslahti, RGD and other recongnition sequences for intergrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.
[39] X. Zhang, P. Zhou, Y. Zhao, M. Wang, S. Wei, Peptide-conjugated hyaluronic acid surface for the culture of human induced pluripotent stem cells under defined conditions, Carbohydr. Polym. 136 (2016) 1061–1064.