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# Xeno-free and feeder-free culture and differentiation of human embryonic stem cells on recombinant vitronectin-grafted hydrogels



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ARTICLE

# Xeno-free and feeder-free culture and differentiation of human embryonic stem cells on recombinant vitronectin-grafted hydrogels

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Recombinant vitronectin-grafted hydrogels were developed by adjusting surface charge of the hydrogels with grafting of poly-L-lysine for optimal culture of human embryonic stem cells (hESCs) in xeno- and feeder-free culture conditions, with elasticity regulated by crosslinking time (10-30 kPa), in contrast to conventional recombinant vitronectin coating dishes, which have a fixed stiff surface (3 GPa). hESCs proliferated on the hydrogels for over 10 passages and differentiated into the cells derived from three germ layers indicating the maintenance of the pluripotency. hESCs on the hydrogels differentiated into cardiomyocytes in xeno-free culture conditions with much higher efficiency (80% of cTnT$^+$ cells) than those on conventional recombinant vitronectin or Matrigel-coating dishes just only after 12 days of induction. It is important to have the optimal design of cell culture biomaterials where biological cues (recombinant vitronectin) and physical cues (optimal elasticity) are combined for high differentiation of hESCs into specific cell lineages, such as cardiomyocytes, in xeno-free and feeder-free culture conditions.

## Introduction

Human pluripotent stem cells (hPSCs), including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), are attractive cell sources for cell therapy and drug discoveries.[1-19] hPSCs can be induced to differentiate into any cell type in our bodies and could be an unlimited cell source for cell therapy.[20-23] hPSCs have already been used in stem cell therapy for four major diseases[24,25]: diabetes,[20,21] acute myocardial infarction,[24,26] spinal cord injury,[27,28] and age-related macular degeneration and Stargardt's macular dystrophy.[25,29-32] The safe production and differentiation of hPSCs requires the development of hPSC culture biomaterials in feeder-free and xeno-free culture conditions while maintaining cell pluripotency[33,34] and permitting the induction of differentiation. One of the difficulties of hPSC culture is that hPSCs cannot be cultivated on tissue culture polystyrene (TCP) dishes that are used for the cultivation of adult stem cells and most cell lines and primary cells in general,[1,34] although there are some exceptions that hPSCs can culture on conventional TCP dishes with some supplement.[35,36] Several studies have tried to develop cell culture biomaterials that sustain hPSC pluripotency.[37-48] Matrigel-coated dishes are typically used instead of culturing hPSCs on mouse embryonic fibroblasts (MEFs). Both methods are currently the gold standards of hPSC culture. However, both processes involve xeno-containing culture conditions. Xeno-free culture of hPSCs is necessary for the clinical usage of hPSCs. In our previous studies, hydrogels grafted with oligopeptide derived from extracellular matrix (ECM) were developed for expansion of hPSCs that remained

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pluripotent.[49,50] It was suggested that adjustment of the elasticity of the hydrogels and scaffolds is important to maintain hPSC pluripotency with high proliferation speed.[51-53]

Dishes coated with recombinant vitronectin (rVN),[49,50,54-57] laminin 521, or laminin 511[47,58,-62] have become a popular choice for the cultivations of hPSCs in xeno-free culture media. Furthermore, synthetic dishes that have been developed for the cultivation of hPSCs in chemically-defined and xeno-free media include poly(methyl vinylether-alt-maleic anhydride) (PMVE-alt-MA),[39] poly[2-(methacryloyloxy)ethyldimethyl-(3-sulfopropyl) ammoniumhydroxide] (PMEDSAH),[40,63] aminopropylmethacryl amide (APMAAm),[41] and copoly[2-(acryloyloxyethyl)]trimethylammonium-co-2-(diethylamino) ethylacrylate [copoly(AEtMA-co-DEAEA)],[38,64] and copoly(acrylamide-co-sodium 4-vinylbenzenesulfonate) [$PAm_6$-co-$PSS_2$].[65] However, replacing the use of ECM-coated dishes by these synthetic cell culture materials has been hampered by the lack of information on the reproducibility of hPSC culture using the synthetic cell culture materials.[34] Other than MEFs and Matrigel-coated dishes, the most reliable cell culture materials are dishes coated with rVN,[49,50,54-57] laminin 511, or laminin 521,[47,58-62] and biomaterials that are immobilized with ECM-derived oligopeptides.[37,44,49,50,66-68]

An oligopeptide-grafted polymeric surface in which over 1000 μg/mL of vitronectin-derived oligopeptide was grafted on the polymer via N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (NHS/EDC) has been described.[37] A similar polymer is commercially marketed as Synthemax. The differentiation rate of hPSCs was typically 10-20% when cultured on dishes coated with 25 μg/mL of Synthemax I or Synthemax II in xeno-free cell culture media.[49,50] There are two studies that utilized novel polyvinylalcohol-co-itaconic (PVI) hydrogels grafted with several molecular designs of ECM-derived oligopeptides having different elasticities for hPSC proliferation.[49,50] A drawback of oligopeptide-immobilized dishes in previous studies, including ours[37,49,50,67] is that the concentration of oligopeptide reaction solution (25-2000 μg/mL, typically 1000-2000 μg/mL) should be higher than the coating concentration of ECM on the ECM-coated dishes (typically 5-20 μg/mL). ECM-coated dishes for the culture of hPSCs can be prepared using much less oligopeptide reaction solution than is used for oligopeptide-immobilized dishes. Currently, however, only stiff TCP dishes (3-10 GPa) are used for ECM-coated dishes. There is only few information concerning the effect of elasticity of cell culture biomaterials on the culture and differentiation of hPSCs on an ECM-immobilized surface having different elasticity. Several recent studies indicated that physical cues, especially elasticity, of biomaterials can regulate the proliferation and differentiation of stem cells.[1,69-76] Soft biomaterials with an elasticity of 0.6 kPa are superior to ECM-coated TCP dishes for the culture of mouse ESCs to maintain cell pluripotency.[77] In our previous study,[50] PVI hydrogels grafted with ECM-derived oligopeptide with an elasticity of 25 kPa were optimal among hydrogels having an elasticity ranging from 10.3-30.4 kPa for expansion of hESCs as well as hiPSCs in xeno-free cell culture medium. The elasticity of the PVI hydrogels was controlled by the crosslinking time, although a high concentration of ECM-derived oligopeptides, such as 1000 μg/mL, should be used for the grafting of ECM-derived oligopeptides on PVI hydrogels.

In this study, we developed ECM-grafted hydrogels with different elasticity for the optimal proliferation and differentiation of hPSCs. PVI hydrogels were used as the base cell culture biomaterial and rVN was selected as the ECM for hPSC binding sites via $\alpha_V\beta_5$ integrin with a low concentration of rVM reaction solution (5-20 μg/mL). In contrast, a high concentration of ECM-derived oligopeptides was required to prepare PVI hydrogels grafted with ECM-derived oligopeptides in our previous studies.[49,50] Since hESCs could not be cultured on PVI hydrogels grafted with rVN (P-rVN) using a direct and simple reaction, we investigated the effect of the surface density of rVN and hydrophilicity, as well as the zeta potential of P-rVN hydrogels on the proliferation of hESCs. We describe the design of optimal PVI hydrogels grafted with rVN for the proliferation of hPSCs and their differentiation to cardiomyocytes. Although several cell culture biomaterials are proposed for hPSC differentiation into cardiomyocytes,[22,78] we further evaluated the efficiency of hPSC differentiation into cardiomyocytes on the optimal PVI hydrogels grafted with rVN after long culture of hPSCs on the PVI hydrogels (ten passages) in this study.

## Materials and Methods

### Materials

The materials used in this investigation are summarized in Table 1. The other chemicals were purchased from Sigma-Aldrich.

### Preparation of cross-linked PVI hydrogels grafted with or without rVN and PLL

Three types of PVI hydrogels were prepared: PVI hydrogel grafted with rVN, PVI hydrogel grafted with poly-L-lysine (PLL) and rVN to produce different elasticity by controlling the crosslinking time of PVI hydrogels, and PVI hydrogels grafted with vitronectin-derived oligopeptide (OVN). These hydrogels were prepared as described below.

PVI films were prepared as previously described.[49,50] PVI hydrogels were prepared by crosslinking PVI films with an aqueous crosslinking solution composed of 20.0% (w/v) sodium sulfate, 1.0% (w/v) sulfuric acid, and 1.0% (w/v) glutaraldehyde (Fig. 1a). The degree of PVI hydrogel crosslinking was adjusted by the reaction time (2, 6, 12, 24, and 48 h). P-*X*h hydrogels indicate PVI hydrogels cross-linked for *X* h. After washing, the hydrogels were activated with 0.01 g/mL N-hydroxysuccinimide (NHS) and 0.01 g/mL 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) for 6.0-6.1 h at 24-25 °C, followed by immersion of the PVI hydrogels in aqueous solutions containing 1, 5, 10, 20, or 50 μg/mL rVN to graft rVN to the hydrogels. The design of rVN-grafted PVI hydrogels is shown in Fig. 1a. P-*X*h-rVN indicates hydrogels grafted with rVN, which were crosslinked for *X*= 2, 6, 12, 24, or 48 h in the crosslinking solution.

PVI hydrogels grafted with PLL and rVN (P-PLL-*X*h-rVN) were prepared as follows. The cross-linked PVI (P-*X*h) hydrogels prepared as just described were activated with 0.01 g/mL NHS and 0.01 g/mL EDC for 6.0-6.1 h at 24-25 °C, followed by immersion of the P-*X*h hydrogels in aqueous solutions containing 1 mg/mL PLL to graft PLL on the PVI hydrogels (P-*X*h-PLL hydrogels). P-*X*h-PLL hydrogels were further activated with 0.01 g/mL NHS and 0.01 g/mL EDC for 6.0-6.1 h at 24-25 °C, followed by immersion of the P-*X*h-PLL hydrogels in aqueous solutions containing 10 μg/mL rVN to graft rVN on the PVI hydrogels (P-*X*h-PLL-rVN). The design of PVI hydrogels grafted with PLL and rVN is also described in Fig. 1a.

PVI hydrogels grafted with vitronectin-derived oligopeptide (P-*X*h-OVN and P-*X*h-PLL-OVN) was also prepared as described for P-*X*h-rVN and P-*X*h-PLL-rVN, respectively, except for the

Table 1 Materials used in this study

| Materials | Abbreviation | Catalog No. | Company |
|---|---|---|---|
| **Polymer** | | | |
| Poly(vinyl alcohol-co-vinyl acetate-co-itaconic acid) | PVI | AF-17 | Japan VAM & Poval Co., Ltd. (Sakai, Osaka, Japan) |
| Poly-L-lysine | PLL | P4707 | Sigma-Aldrich (St. Louis, MO, USA) |
| **Oligopeptides** | | | |
| GCGGKGGPQVTRGDVFTMP | OVN | (derived from vitronectin) | PH Japan Co., Ltd. (Hiroshima, Japan) |
| **ECM** | | | |
| Matrigel | Matrigel | #356230 | Corning (Corning, NY, USA) |
| Reconvinant vitronectin | rVN | A14700 | Thermo Fisher Scientific Inc. (Waltham, MA, USA) |
| **Cell culture dishes** | | | |
| 6-well polystyrene plate | TCP | #353046 | Corning (Corning, NY, USA) |
| Ultra-low attachment plate | Ultra-low attachment plate | #3471 | Corning (Corning, NY, USA) |
| **Chemicals** | | | |
| Dispase II | Dispase | D4693-1G | Sigma-Aldrich (St. Louis, MO, USA) |
| N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride | EDC | 3450 | Sigma-Aldrich (St. Louis, MO, USA) |
| N-hydroxysuccinimide | NHS | 13062 | Sigma-Aldrich (St. Louis, MO, USA) |
| **Cell culture medium and component** | | | |
| Essential 8 medium | Essential 8 | A1517001 | Thermo Fisher Scientific Inc. (Waltham, MA, USA) |
| Essential 6 medium | Essential 6 | A1516401 | Thermo Fisher Scientific Inc. |
| DMEM/F12 medium | DMEM/F12 medium | 11330-057 | Thermo Fisher Scientific Inc. |
| Cardiac differentiation meidum | | A29212-01 | Life Technologies (Carlsbad, CA, USA) |
| Alkaline Phosphatase Assay Kit | SensoLyte® pNPP | AS-72146 | AnaSpec, Inc. (Fremont, CA, USA) |
| Hoechst 33342 | Hoechst | PA-3014 | Lonza (Basel, Switzerland) |
| **Antibodies** | | | |
| Anti-Oct3/4 antibody | Anti-Oct3/4 antibody | sc-5279 | Santa Cruz Biotechnology (Dallas, TX, USA) |
| Anti-Sox2 antibody | Anti-Sox2 antibody | AB5603 | Merck KGaA (Darmstadt, Germany) |
| Anti-SSEA-4 antibody | Anti-SSEA-4 antibody | ab16287 | Abcam (Cambridge, MA, USA) |
| Anti-Nanog antibody | Anti-Nanog antibody | MA1-017 | Thermo Fisher Scientific |
| Anti-AFP antibody | Anti-AFP antibody | PA5-21004 | Thermo Fisher Scientific |
| Anti-SMA antibody | Anti-SMA antibody | PA5-19465 | Thermo Fisher Scientific |
| Anti-GFAP antibody | Anti-GFAP antibody | MA5-15086 | Thermo Fisher Scientific |
| Alexa Fluor 488 goat anti-mouse IgG | Alexa Fluor 488 goat anti-mouse IgG | A11001 | Thermo Fisher Scientific |
| Alexa Fluor 488 goat anti-rabbit IgG | Alexa Fluor 488 goat anti-rabbit IgG | A11008 | Thermo Fisher Scientific |
| Alexa Fluor 594 donkey anti-rabbit IgG | Alexa Fluor 594 donkey anti-rabbit IgG | A21207 | Thermo Fisher Scientific |
| **Mice** | | | |
| NOD.CB17-Prkdcscid/Jnarl | NOD-SCID mice | NOD.CB17-Prkdcscid/Jnarl | National Laboratory Animal Center (Taipei, Taiwan) |

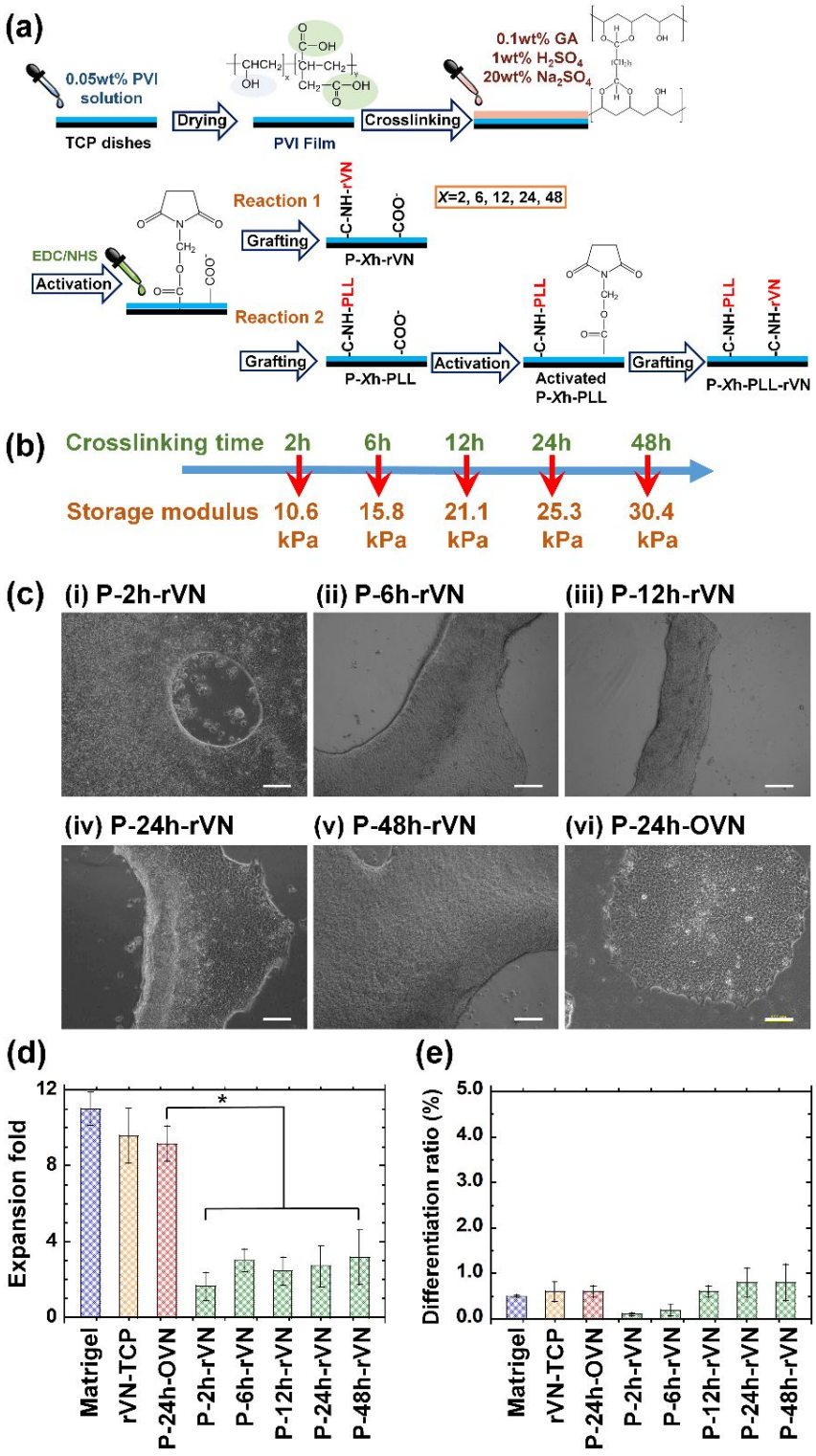


**Fig. 1** Preparation and design of PVI hydrogels grafted with recombinant vitronectin (rVN) and poly-L-lysine (PLL), and H9 human pluripotent stem cells (hPSCs) cells cultivated on PVI hydrogels grafted with rVN under feeder-free and xeno-free culture conditions, with a different storage modulus. (a) Preparation of PVI hydrogels grafted with rVN (P-*X*h-rVN) and with rVN and PLL (P-*X*h-PLL-rVN). (b) The storage modulus of P-*X*h-rVN hydrogels prepared at several crosslinking time (*X*=h). (c) Morphology of H9 hESCs cultured on PVI hydrogels grafted with rVN prepared at several crosslinking times: (i) P-2h-rVN, (ii) P-6h-rVN, (iii) P-12h-rVN, (iv) P-24h-rVN, (v) P-48h-rVN, and (vi) PVI hydrogels grafted with oligovitronectin (OVN) at passage 3. Scale bar indicates 100 µm. (d, e) Average fold-change of expansion (d) and differentiation rate (e) of H9 hESCs cultured on Matrigel-coated dishes, rVN-coated TCP (rVN-TCP) dishes, PVI hydrogels grafted with rVN (P-2h-rVN, P-6h-rVN, P-12h-rVN, P-24h-rVN, P-48h-rVN) during passages 1-3. *$p < 0.05$.

usage of 1000 μg/mL of oligopeptide (GCGGKGGPQVTRGDVFTMP) instead of 1-50 μg/mL of rVN.

PVI hydrogels grafted with or without PLL and/or rVN were sterilized by immersion in a 75% (v/v) ethanol solution overnight, followed by washing in ultrapure water. The hydrogels were kept in ultrapure water until use.

**Characterization of PVA hydrogels grafted with or without oligopeptides**

The chemical composition (N1s, O1s, and C1s) of the PVI hydrogels grafted with and without rVN and/or PLL was analyzed using X-ray photoelectron spectroscopy (XPS) with a K-Alpha spectrometer equipped with a monochromatic Al-K X-ray source (Thermal Scientific, Inc., Amarillo, TX, USA). Data were collected at a photoelectron take-off angle of 45° with respect to the sample surface. The binding energy scale was adjusted by setting the peak maximum in the C1s spectrum to 284.6 eV. The obtained high-resolution C1s spectra were fitted using Shirley background subtraction and a series of Gaussian peaks.

The storage modulus of PVI hydrogels prepared from a 5.0% (w/v) PVI solution and crosslinking for 2-48 h (P-*X*h, P-*X*h-rVN, and P-*X*h-PLL-rVN) were evaluated using a rheometer (Physica MCR 101; Anton Paar GmbH, Ostfildern, Germany) with a 5.0% strain at 1.0 Hz.

The zeta potential of PVI hydrogels grafted with and without rVN and/or PLL (1 cm × 2 cm) were determined using streaming potential and streaming current measurement with phosphate buffered saline (PBS, pH 7.4) using SurPASS™ 3 (Anton Paar GmbH).

Hydrophilicity of PVI hydrogels grafted with and without rVN and/or PLL was analyzed using the sessile drop method (contact angle measurement) using a contact angle goniometer instrument (OCA 15EC; DataPhysics Instruments GmbH, Filderstadt, Germany).

**hPSC culture**

The experiments were approved by the ethics committees of the Cathay Medical Research Institute (CT099012), National Central University (NCU-106-007), and the Taiwan Landseed Hospital (IRB-13-05). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of National Central University and approved by the Animal Ethics Committee of National Central University.

H9 hESCs (WA09; WiCell Research Institute, Madison, WI, USA) or HPS0077 hiPSCs (Riken BRC, Tsukuba, Japan) were maintained on Matrigel-coated dishes in Essential 8 medium using standard protocols. Near-confluent cell clusters were treated with dispase for 1-2 min at 37 °C. The hPSCs were extensively dispersed into the medium by repeated pipetting. After centrifugation at 160.0 x g for 4-5 min at 4.0 °C, the hPSCs were inoculated at an appropriate density of 5 x $10^4$ cells/$cm^2$ into new culture dishes (e.g., PVI hydrogels grafted with and without rVN and/or PLL). The medium was exchanged daily for all the experiments.

**hPSC characterization**

Alkaline phosphatase (AP) activity of hPSCs was evaluated using an AP detection kit according to the manufacturer's instructions.[49,50] Differentiation ratios were evaluated for the hPSC colonies after culture on the various biomaterials using the following equation:

Differentiation ratio (%) = Percentage of partially differentiated colonies x 0.5 (%) + percentage of differentiated colonies (%) (1)

where "differentiated colonies" are defined as the colonies expressing no AP activity and "partially differentiated colonies" refer the colonies expressing AP activity in the center of the colonies but not at the edge of the colonies.[49,50]

The hPSCs and differentiated cells were immunostained for Sox2, SSEA-4, Oct3/4, Nanog, α-SMA, AFP, and GFAP to analyze pluripotency and differentiation ability by following a standard protocol.[49,50] The stained cells were measured using fluorescence microscopy (Eclipse Ti-U fluorescence inverted microscope; Nikon Instruments, Inc., Tokyo, Japan).

The expression levels of the pluripotent genes *Oct4*, *Sox2*, and *Nanog* were analyzed by qRT-PCR. Probes for *Oct4* (Hs01895061_u1), *Sox2* (Hs00602736_s1), Nanog (Hs02387400_g1), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs03929097_g1) were obtained from Life Technologies (Carlsbad, CA, USA). Each sample ($n$ = 4) was evaluated in duplicate, and the expression level of the *GAPDH* housekeeping gene was utilized as a control to normalize the results. Data were processed using the ΔΔCt method.

**EB formation**

The potential of hPSCs to induce differentiation into cells originated from three germ layers was evaluated by determining EB generation at passage 10 using a standard protocol.[49,50] The cells were immunostained with antibodies against differentiation markers (α-SMA, AFP, and GFAP) of all three germ layers using a standard protocol,[49,50] and the staining was evaluated using fluorescence microscopy.

**Teratoma formation**

hPSCs were collected by treatment with a non-enzymatic cell-dissociation solution. After centrifugation, the cell pellets were suspended in DMEM/F12 medium with Matrigel. In total, 5.0 x $10^6$ cells were injected subcutaneously into NOD.CB17-*Prkdc*$^{scid}$/JNarl mice. After 5-8 weeks, the teratomas, which had developed, were dissected and fixed in formaldehyde solution. Paraffin-embedded teratomas were sectioned and stained with hematoxylin and eosin using a standard protocol.[49,50]

**hPSC differentiation into cardiomyocytes**

Cardiomyocyte differentiation of hPSCs was conducted using a commercially available xeno-free cardiomyocyte differentiation kit following the manufacturer's instructions.

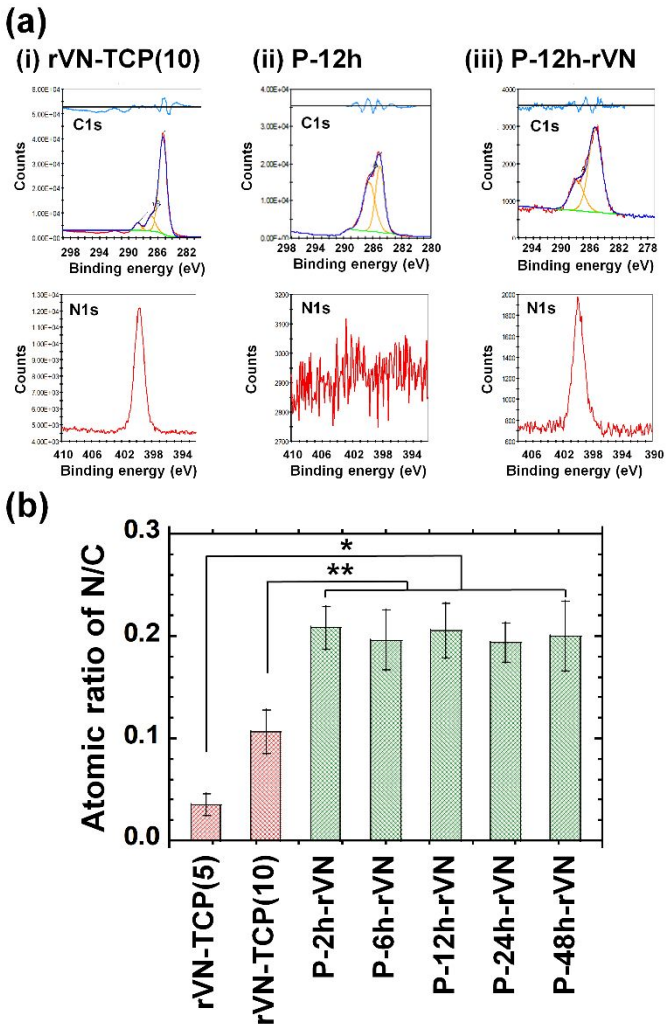


**Fig. 2** Characterization of PVI hydrogels grafted with rVN (P-*X*h-rVN) to produce a different elasticity. (a) High-resolution XPS spectra of the C1s and N1s peaks on the surface of (i) rVN-TCP dishes prepared with 10 μg/mL rVN coating solution, (ii) unmodified PVI hydrogels (P-12h), and (iii) P-12h-rVN hydrogels. (b) The nitrogen to carbon (N/C) atomic ratios in rVN-TCP dishes prepared with 5 and 10 μg/mL rVN coating solution (rVN-TCP(5) and rVN-TCP(10), respectively) and PVI hydrogels grafted with rVN (P-2h-rVN, P-6h-rVN, P-12h-rVN, P-24h-rVN, P-48h-rVN) where 10 μg/mL rVN solution was used for the preparation of the hydrogels. *$p < 0.05$; **$p < 0.05$.

### Statistical analysis

All quantitative results were evaluated from four independent samples with duplicated experiments on each condition. The data are expressed as the mean ± SD (standard deviation). Statistical analyses were done using Student's t-test in Excel (Microsoft Corporation). Probability values ($p$) < 0.05 were considered statistically significant.

## Results

### Preparation and characterization of PVI hydrogels grafted with rVN

H9 hESCs were cultured on PVI hydrogels cross-linked for 2, 6, 12, 24, and 48 h and grafted with rVN (P-*X*h-rVN hydrogels). This approach was used because the gold standard of hPSC culture using rVN-coated TCP (rVN-TCP) dishes is based on the data of interaction between rVN and hPSCs only on the extremely stiff surface of TCP dishes (3 GPa). Our aim was to investigate the effect of elasticity on the proliferation and differentiation of hPSCs cultured on rVN immobilized surfaces with different elasticity. PVI hydrogels were selected as the base biomaterials for hPSC culture. PVI hydrogels were grafted with rVN using solutions containing 1-50 μg/mL rVN, which had a different storage modulus (elasticity) depending on the crosslinking time (Fig. 1a). The thickness of PVI hydrogels was fixed to be 1.5±0.1 μm for hPSC culture and differentiation, whereas self-standing PVA hydrogels having thickness of 20-30 μm were used for a rheometer measurement. The estimated storage modulus of self-standing PVI hydrogels (P-*X*h and P-*X*h-rVN, where *X* indicates the crosslinking time in h) was 10.6, 15.8, 21.1, 25.3, and 30.4 kPa for a crosslinking time of 2, 6, 12, 24, and 48 h, respectively (Fig. 1b). The water content of PVI hydrogels, P-2h-rVN, P-6h-rVN, P-12h-rVN, P-24h-rVN, and P-48h-rVN was evaluated to be 70±3%, 59±2%, 49±2%, 35±1%, and 30±1%, respectively. These values were similar to those previously reported[49,50] for P-*X*h-OVN hydrogels (PVI hydrogels cross-linked for *X* h and grafted with oligovitronectin, OVN). These results indicated that surface grafting of ECM-derived-oligopeptide and rVN does not influence the bulk elasticity of PVI hydrogels grafted with rVN or OVN.

### Culture of hESCs on P-*X*h-rVN hydrogels under xeno-free culture conditions

H9 hESCs were cultured on P-*X*h-rVN hydrogels having a different elasticity under xeno-free culture conditions. hESCs cultured on Matrigel-coated dishes served as the control where Matrigel is derived from Engelbreth-Holm-Swarm mouse sarcoma cells, which includes unknown xeno-containing components. The hESCs were cultured in Essential 8 xeno-free culture medium (Fig. 1c). Fig. 1d depicts the average expansion (fold-change) of hESCs during passages 1-3 during culture on P-*X*h-rVN hydrogels and Matrigel-coated TCP dishes, rVN-TCP dishes coated using 5 μg/mL coating solution, and P-24h-OVN hydrogels as the control where the expansion fold was calculated from the averaged expansion fold evaluated during (a) passage 1, (b) passage 2, and (c) passage 3. Matrigel- and rVN-coated dishes are the current gold standard methods of hPSC culture. P-24h-OVN dishes can also support the proliferation of hESCs.[49,50] Presently, hESCs only weakly adhered to the novel P-*X*h-rVN hydrogels (Fig. 1c) and hESC proliferation was significantly less compared to proliferation on Matrigel- and rVN-coated dishes, and on P-24h-OVN dishes (P-24h-OVN vs. P-*X*h-rVN, $p < 0.05$; Fig. 1d). However, hESCs retained their pluripotency on all the culture biomaterials, as

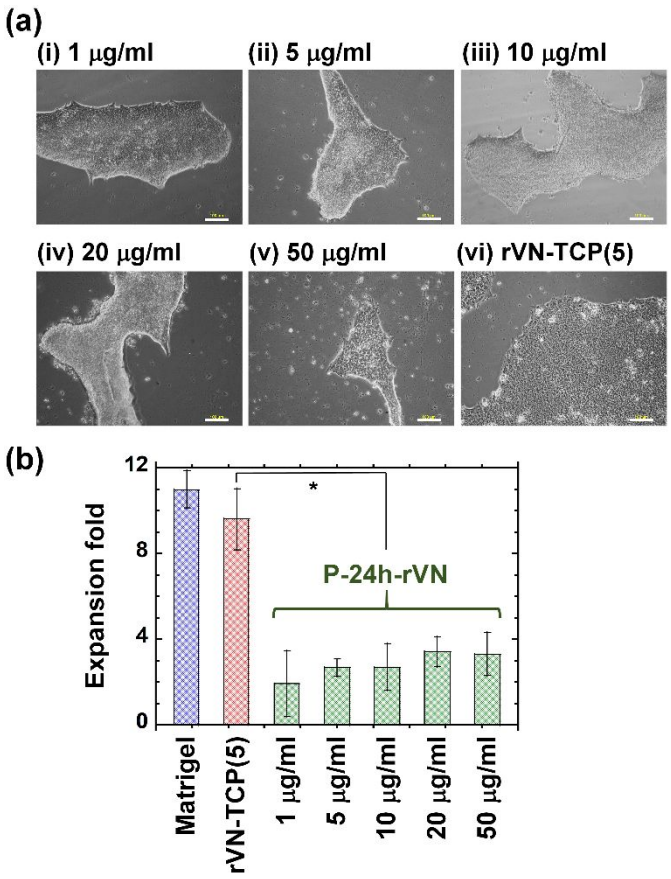


**Fig. 3** H9 human embryonic stem cells (hESCs) cultivated on PVI hydrogels grafted with rVN under feeder-free and xeno-free culture conditions, which were prepared with different concentrations of rVN. (a) Morphology of H9 hESCs cultured on PVI hydrogels grafted with rVN prepared after 24 h of crosslinking (P-24h-rVN). The hydrogels were prepared as follows: (i) 1 μg/ml rVN, (ii) 5 μg/ml rVN, (iii) 10 μg/ml rVN, (iv) 20 μg/ml rVN, and (v) 50 μg/ml rVN, as well as (vi) rVN-TCP dishes prepared with 5 μg/mL of rVN coating solution (rVN-TCP(5)) at passage 3. Scale bar indicates 100 μm. (b) Average expansion fold of H9 hESCs cultured on PVI hydrogels grafted with rVN (P-24h-rVN) prepared with 1, 5, 10, 20 and 50 μg/ml rVN solution, Matrigel-coated dishes, and rVN-coated TCP dishes (rVN-TCP(5)) during passages 1-3. *$p < 0.05$.

apparent by the extremely low differentiation ratio (<1%; Fig. 1e) where the differentiation ratio of Fig. 1e was calculated from the averaged differentiation ratio evaluated at (a) passage 1, (b) passage 2, and (c) passage 3 just before each passaging process.

One reason why the P-*X*h-rVN hydrogels did not support hESC proliferation might be the low quantity of grafted rVN on PVI hydrogels. To explore this, X-ray photoelectron spectroscopy (XPS) was used to determine the surface density of rVN, based on the presence of a nitrogen peak originating from the amino acids of rVN. In the high-resolution XPS spectra of the C1s and N1s peaks of rVN-TCP dishes, and P-12h (no rVN grafting) and P-12h-rVN hydrogels, an N1s peak of approximately 400 eV was clearly detected on the rVN-TCP dishes and P-12h-rVN hydrogels, but not the P-12h hydrogels (Fig. 2a).

The nitrogen-to-carbon (N/C) atomic ratios of rVN-TCP, where the rVN coating concentration was 5 μg/mL [rVN-TCP(5)] and 10 μg/mL [rVN-TCP(10)], and P-*X*h-rVN with a different elasticity (*X*=2, 6, 12, 24, and 48 h) were determined from the XPS spectra. The results are shown in Fig. 2b. The surface density of rVN on P-*X*h-rVN hydrogels with any elasticity prepared using 10 μg/mL of rVN solution was significantly higher than that on rVN-TCP dishes prepared with 5 and 10 μg/mL of rVN solution (rVN-TCP(5) vs. P-*X*h-rVN, $p < 0.05$; rVN-TCP(10) vs. P-*X*h-rVN, $p < 0.05$; Fig. 2b), because the N/C atomic ratio on the P-*X*h-rVN hydrogels was much higher than that on rVN-TCP dishes. These results indicate that low adhesion and low expansion of hESCs cultured on P-*X*h-rVN hydrogels (Fig. 1c and d) were not due to the low surface density of rVN on P-*X*h-rVN hydrogels.

To conclusively assess this idea, hESCs were cultured on P-24h-rVN hydrogels prepared using 1-50 μg/mL of rVN solution (Fig. 3a). Even P-24h-rVN hydrogels prepared with a high concentration of rVN solution (e.g., 50 μg/mL) could not satisfactorily support hESC adhesion and the cells proliferated more slowly than hESCs cultured on Matrigel-coated or rVN-TCP dishes prepared using 5 μg/mL of rVN solution (Fig. 3a and b) (rVN-TCP(5) vs. P-24h-rVN; $p < 0.05$; Fig. 3b).

The surface density of rVN on P-24h-rVN hydrogels prepared using 1-50 μg/mL of rVN solution was also evaluated by the presence of the nitrogen peak originating from amino acids of rVN using XPS. Fig. 4a presents the high-resolution XPS spectra of the C1s and N1s peaks of rVN-TCP dishes prepared with 5 μg/mL of rVN solution [rVN-TCP(5)] and P-24h-rVN prepared with 1 μg/mL of rVN solution [P-24h-rVN(1)] and 20 μg/mL of rVN solution [P-24h-rVN(20)]. The N1s peak was clearly observed in the rVN-TCP(5), P-24h-rVN(1), and P-24h-rVN(20) samples. The N/C atomic ratios of rVN-TCP(5), rVN-TCP(10), P-24h-rVN(1), P-24h-rVN(5), P-24h-rVN(10), P-24h-rVN(20), and P-24h-rVN(50) were determined from the XPS spectra (Fig. 4b). The surface density of rVN on P-*X*h-rVN hydrogels prepared with 5-50 μg/mL of rVN solution was significantly higher than that on rVN-TCP dishes prepared with rVN solution of 5 and 10 μg/mL (rVN-TCP(5) vs. P-24h-rVN(*Y*), $p < 0.05$; rVN-TCP(10) vs. P-24h-rVN(*Y*) [*Y*=5, 10, 20, or 50], $p < 0.05$; Fig. 4b) because the N/C atomic ratio of the P-24h-rVN hydrogels, except for P-24h-rVN(1), was much higher than that of the rVN-TCP dishes prepared. These results further suggested that the low adhesion and expansion of hESCs cultured on P-*X*h-rVN hydrogels (Fig. 1c, 1d, and 3) did not reflect the low surface density of rVN on P-*X*h-rVN hydrogels.

Another possibility for the weak adhesion and poor expansion of hESCs cultured on P-*X*h-rVN hydrogels is that the surface that was too hydrophilic or hydrophobic, and thus was unsuitable for hESC culture. This is consistent with several reports that biomaterials having optimal contact angles of 50-70˚ are preferable for cell culture.[79,80] To explore this suggestion, the water contact angle on rVN-TCP and TCP dishes, as well as P-24h-OVN, P-24h-rVN, and PVI hydrogels (P-24h) were measured using the sessile drop method with rVN-TCP and

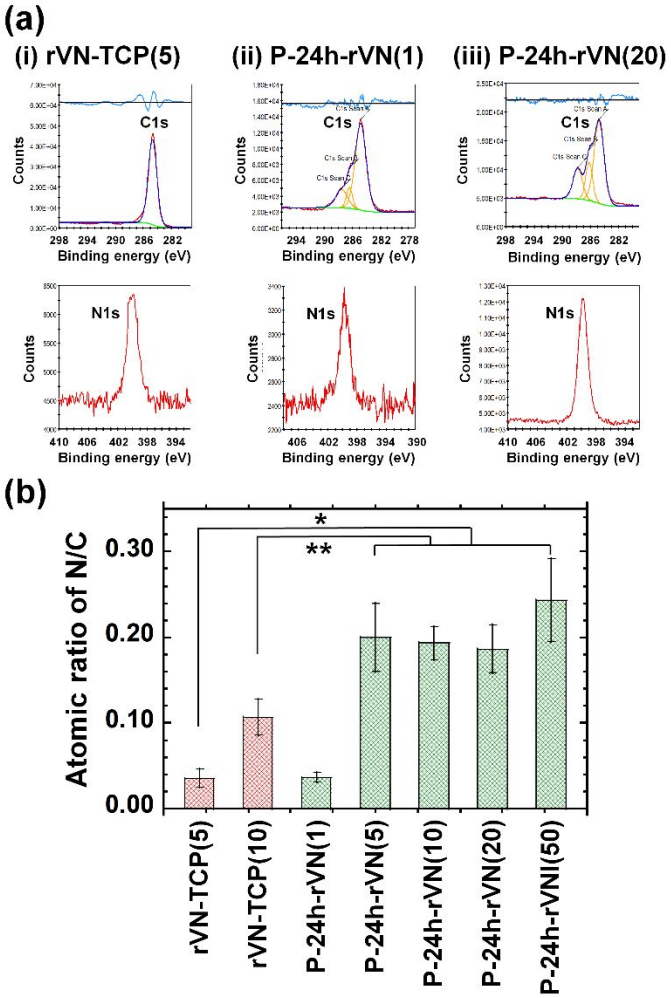


**Fig. 4** Characterization of PVI hydrogels grafted with rVN (P-*X*h-rVN) prepared with rVN solution with different concentrations. (a) High-resolution XPS spectra of the C1s and N1s peaks analyzed on the surface of (i) rVN-TCP dishes prepared with 5 μg/mL rVN coating solution, (ii) P-24h-rVN hydrogels prepared with 1 μg/mL rVN solution, and (iii) P-24h-rVN hydrogels prepared with 20 μg/mL rVN solution. (b) The nitrogen to carbon (N/C) atomic ratios in rVN-TCP dishes prepared with 5 and 10 μg/mL rVN coating solution (rVN-TCP(5) and rVN-TCP(10), respectively) and PVI hydrogels grafted with rVN (P-24h-rVN) in which 1, 5, 10, 20, and 50 μg/mL rVN solution was used for the preparation of the hydrogels [P-24h-rVN(1), P-24h-rVN(5), P-24h-rVN(10), P-24h-rVN(20), and P-24h-rVN(50), respectively]. *$p < 0.05$; **$p < 0.05$.

P-*X*h-rVN hydrogels prepared using 10 μg/mL of rVN and P-24h-OVN hydrogels prepared with a high concentration of OVN (1000 μg/mL). The water contact angle on P-24h-rVN hydrogels was the same as that on rVN-TCP dishes and P-24h-OVN hydrogels within experimental error (rVN-TCP vs. P-24h- rVN, $p >> 0.05$; P-24h-OVN vs. P-24h-rVN, $p >> 0.05$; Fig. 5a), which indicated that the P-24h-rVN hydrogel surface had a similar hydrophilicity to rVN-TCP dishes. Therefore, the hydrophilicity of the surface on P-*X*h-rVN hydrogels cannot explain the low

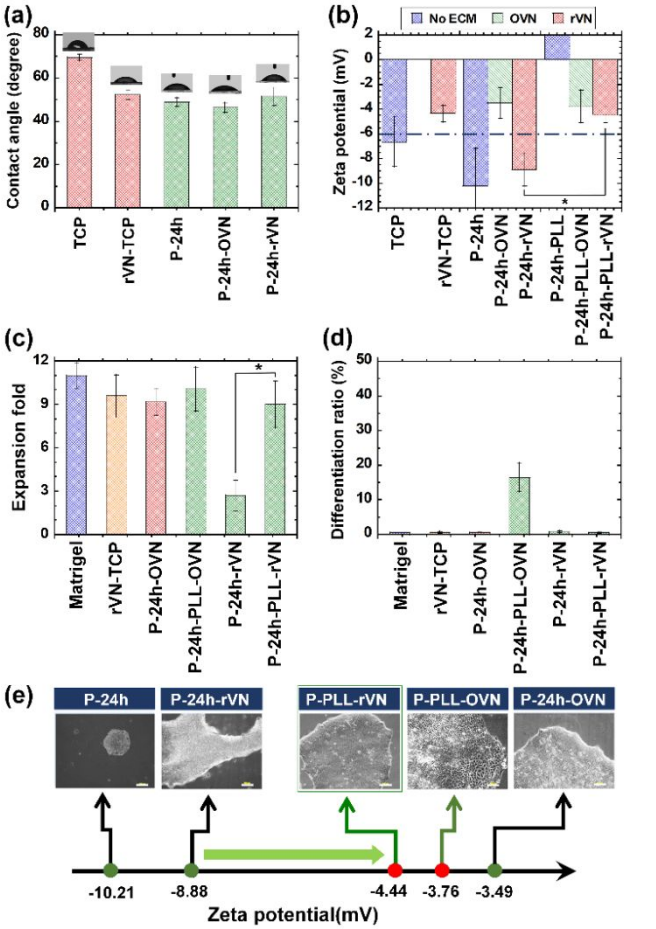


**Fig. 5** Characterization of PVI hydrogels grafted with rVN (P-*X*h-rVN) or rVN and PLL (P-*X*h-PLL-rVN), and H9 hESCs cultivated on PVI hydrogels grafted with rVN and/or PLL under feeder-free and xeno-free culture conditions. (a) Water contact angle on TCP dishes and rVN-TCP dishes prepared with 5 μg/mL of rVN coating solution, P-24h hydrogels, P-24h-OVN hydrogels, and P-24h-rVN hydrogels. The angle was measured using the sessile drop method. (b) Zeta potential of TCP dishes, rVN-TCP dishes, P-24h hydrogels, P-24h-OVN hydrogels, P-24h-rVN hydrogels, P-24h-PLL hydrogels, P-24h-PLL-OVN hydrogels, and P-24h-PLL-rVN hydrogels. The zeta potential was measured using a streaming potential and streaming current measurement. (c) Average fold-change in expansion of H9 hESCs cultured on Matrigel-coated dishes, rVN-coated dishes prepared with 5 μg/mL rVN coating solution (rVN-TCP), P-24h-OVN hydrogels, P-24h-rVN hydrogels, P-24h-PLL-OVN hydrogels, and P-24h-PLL-rVN hydrogels during passages 1-3. (d) Differentiation rate (%) of H9 hESCs cultured on Matrigel-coated dishes, rVN-coated dishes prepared with 5 μg/mL rVN coating solution (rVN-TCP), P-24h-OVN hydrogels, P-24h-rVN hydrogels, P-24h-PLL-OVN hydrogels, and P-24h-PLL-rVN hydrogels during passages 1-3. (e) Scale lines of the zeta potential of several PVI hydrogels (P-24h, P-24h-rVN, P-24h-PLL-rVN (P-PLL-rVN), P-24h-PLL-OVN (P-PLL-OVN), and P-24h-OVN) and morphologies of H9 hESCs cultivated on the PVI hydrogels. Scale bar indicates 100 μm. *$p < 0.05$.

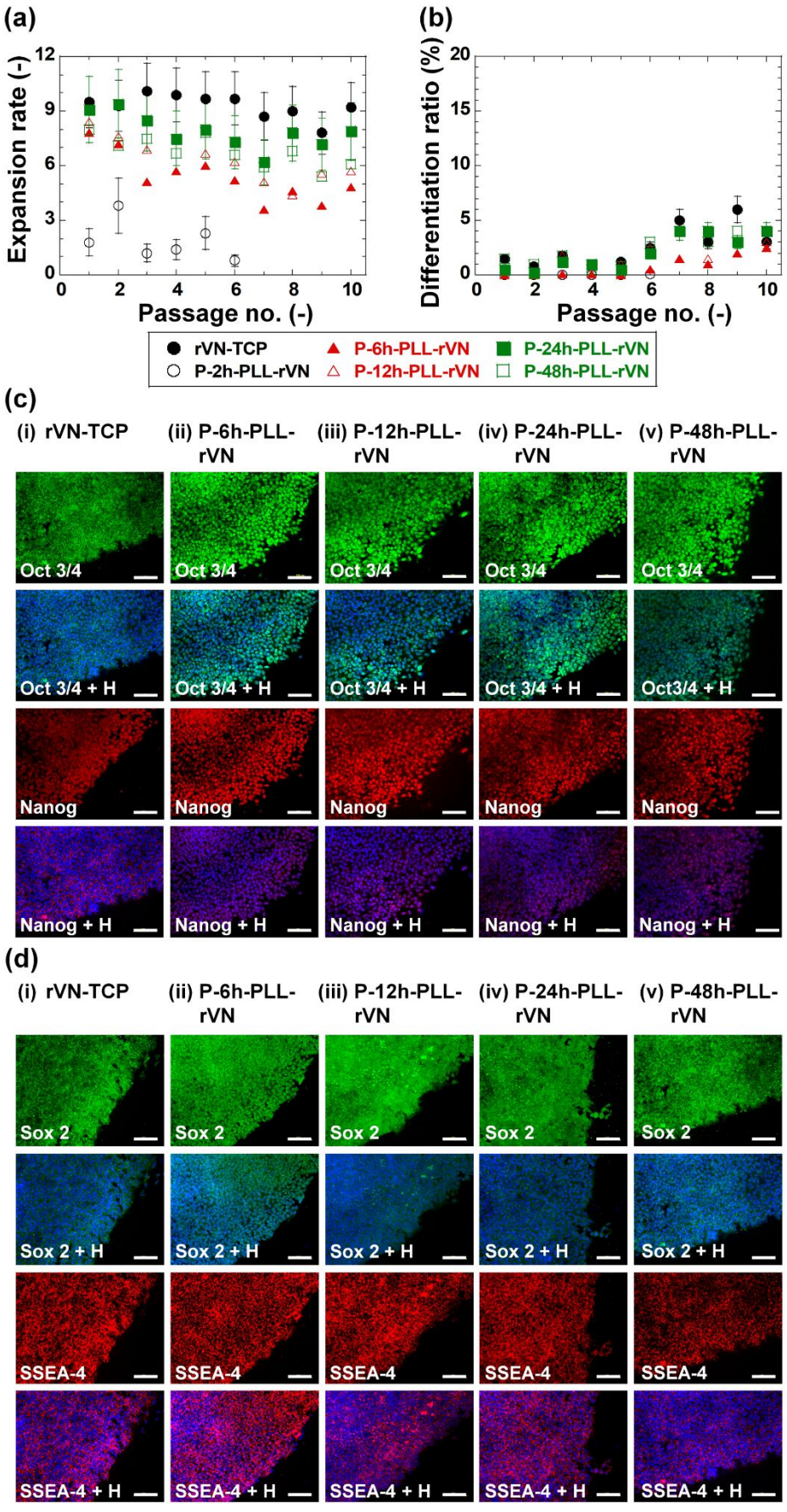
(a)
Expansion rate (-)
Passage no. (-)
(b)
Differentiation ratio (%)
Passage no. (-)
rVN-TCP
P-2h-PLL-rVN
P-6h-PLL-rVN
P-12h-PLL-rVN
P-24h-PLL-rVN
P-48h-PLL-rVN
(c)
(i) rVN-TCP
(ii) P-6h-PLL-rVN
(iii) P-12h-PLL-rVN
(iv) P-24h-PLL-rVN
(v) P-48h-PLL-rVN
Oct 3/4
Oct 3/4 + H
Nanog
Nanog + H
(d)
Sox 2
Sox 2 + H
SSEA-4
SSEA-4 + H

**Fig. 6** Long-term culture and characterization of H9 human embryonic stem cells (hESCs) on PVI hydrogels grafted with PLL and rVN under feeder-free and xeno-free culture conditions. (a, b) Expansion rate (a) and differentiation ratio (b) of H9 hESCs on rVN-TCP dishes prepared with 5 μg/mL rVN coating solution (closed circles), P-2h-PLL-rVN hydrogels (open circles), P-6h-PLL-rVN hydrogels (closed triangles), P-12h-PLL-rVN hydrogels (open triangles), P-24h-PLL-rVN hydrogels (closed squares), and P-48h-PLL-rVN hydrogels (open squares) for 10 passages. P-*X*h-PLL-rVN hydrogels were prepared with 1 mg/mL PLL solution and 10 μg/mL rVN solution. (c,d) Expression of pluripotency proteins SSEA-4 (red), Sox2 (green), Nanog (red), and Oct3/4 (green) on H9 hESCs evaluated by immunostaining with dual staining with Hoechest33342 (H) for nuclear (blue) after culturing on (i) rVN-TCP dishes prepared with 5 μg/mL rVN coating solution, (ii) P-6h-PLL-rVN hydrogels, (iii) P-12h-PLL-rVN hydrogels, (iv) P-24h-PLL-rVN hydrogels, and (v) P-48h-PLL-rVN hydrogels for 10 passages. The merged fluorescence images of (1) Hoechest 33342 and (2) Oct3/4 (Oct3/4 + H), Nanog (Nanog + H), Sox2 (Sox2 + H), or SSEA-4 (SSEA-4 + H) were also shown. P-*X*h-PLL-rVN hydrogels were prepared with 1 mg/mL PLL solution and 10 μg/mL rVN solution. Scale bar indicates 100 μm.

adhesion and expansion of hESCs cultured on P-*X*h-rVNhydrogels (Fig. 1 c, 1d, and 3).

We finally considered that the surface charge of P-*X*h-rVN hydrogels might affect hESC attachment and proliferation. To evaluate the surface charge of P-*X*h-rVN hydrogels, the zeta potential of P-24h-rVN hydrogels, as well as that of P-24h-OVN and P-24h hydrogels and rVN-TCP and TCP dishes, were measured using a streaming potential and streaming current measurement at pH 7.4 (Fig. 5b). P-24h-rVN and P-24h hydrogels displayed an extremely low zeta potential (< -8 mV), whereas the zeta potential of rVN-TCP and P-24h-OVN hydrogels were -4.4 mV and -3.5 mV, respectively. The use of a high concentration of OVN (1000 μg/mL) on PVI hydrogels seem to compensate for the negative surface charge, which led to a charge that was relatively less (> -6 mV). However, rVN grafted on PVI hydrogels did not seemingly contribute to a lower negative charge on P-*X*h-rVN hydrogels. P-24h-rVN hydrogels displayed a zeta potential of -8.9 mV. It appears that the surface zeta potential exceeding -6 mV was necessary for the culture of hESCs in the present study.

**hPSC culture on P-*X*h-PLL-rVN hydrogels under xeno-free culture conditions**

Poly-L-lysine (PLL) is positively charged and is used as a coating material to attach different kind of cells.[33] However, the PLL-immobilized surface cannot support hPSC proliferation because the interaction between PLL and cells is solely an electrostatic interaction. Therefore, we designed PVI hydrogels grafted with PLL and rVN (P-*X*h-PLL-rVN), where rVN contributes to the maintenance of the pluripotency of hPSCs and PLL supports hPSC adhesion. The nitrogen to carbon atomic ratio of P-24h-PLL-rVN was evaluated to be 0.241±0.035 as well as that of P-24h-PLL-OVN was 0.227±0.038. The nitrogen to carbon atomic ratio of P-24h-PLL-rVN and P-24h-PLL-OVN was found to be similar to that of P-24h-rVN(10) (P-24h-PLL-rVN vs. P-24h-rVN(10), $p >> 0.05$; P-24h-PLL-OVN vs. P-24h-rVN(10), $p >> 0.05$). The zeta potential of P-24h-PLL and P-24h-PLL-rVN was also evaluated. P-24h-PLL showed a positive zeta potential value of 10.2 mV as expected, whereas the zeta potential of P-24h-PLL-rVN was -4.4 mV (P-24h-rVN vs. P-24h-PLL-rVN, $p < 0.05$), which was identical to rVN-TCP (-4.4mV) and similar to P-24h-OVN (-3.5 mV), where 1 mg/mL of PLL and 10 μg/mL of rVN were used to prepare P-24h-PLL-rVN hydrogels (Fig. 5b). The zeta potential of P-24h-PLL-OVN and P-24h-OVN were -3.5 and -3.7 mV, respectively. The values were within experimental error. This is probably because the 1000 μg/mL concentration of the OVN oligopeptide used to prepare both hydrogels was too high to discern the contribution of PLL on the P-24h-PLL-OVN and P-24h-OVN hydrogels.

hESCs were cultured on P-24h-PLL-rVN hydrogels as well as Matrigel-coated and rVN-TCP dishes. P-24h-OVN, P-24h-rVN, P-24h-PLL-OVN hydrogels were used for three passages to evaluate whether the P-24h-PLL-rVN hydrogels could support the adhesion and pluripotency of hESCs under xeno-free culture conditions, with the exception of hESCs cultured on Matrigel-coated dishes. The average expansion of hESCs during passages 1-3 on P-24h-PLL-rVN hydrogels and the other cell culture biomaterials are presented in Fig. 5c. The hESCs were successively cultured on P-24h-PLL-rVN hydrogels with similar expansion of 9.0 to rVN-TCP dishes, whereas P-24h-rVN hydrogels lacking PLL grafted on the PVI surface displayed a low expansion value (2.7) of hESCs (P-24h-rVN vs. P-24h-PLL-rVN, $p < 0.05$). Furthermore, the differentiation ratio of hESCs on P-24h-PLL-rVN was <1%, which suggests that hESCs maintained their pluripotency on P-24h-PLL-rVN (Fig. 5d). Excellent attachment and retention of morphology were observed when hESCs were cultured on PVI hydrogels having a zeta potential exceeding -6 mV (Fig. 5e).

**Long-term culture of hPSCs on P-*X*h-PLL-rVN hydrogels of different elasticity under xeno-free culture conditions**

We developed PVI hydrogels grafted with rVN and PLL having different elasticity (P-Xh-PLL-rVN hydrogels) to evaluate the effect of elasticity of the hydrogels grafted with rVN on hPSC proliferation and differentiation. We evaluated the hPSCs during long-term culture (10 passages) on P-*X*h-PLL-rVN hydrogels with different elasticity in xeno-free culture conditions. Fig. 6a and b and Fig. 1S(a) and (b) (ESI†) depict the expansion rate and differentiation ratio of H9 hESCs cells and HPS0077 hiPSCs, respectively cultivated on P-2h-PLL-rVN, P-6h-PLL-rVN, P-12h-PLL-rVN, P-24h-PLL-rVN, and P-48h-PLL-rVN hydrogels, and rVN-TCP dishes. The storage modulus of P-*X*h-PLL-rVN hydrogels was the same to P-*X*h-rVN hydrogels and P-*X*h hydrogels at the same crosslinking time within experimental error ($p > 0.05$) indicating that the amount of rVN and PLL was insufficient to contribute to an increase or decrease of the storage modulus of PVI hydrogels. P-2h-PLL-rVN could not support the proliferation of hPSCs and no cells remained on P-2h-PLL-rVN hydrogels after seven passages. The hPSCs could be cultured on P-6h-PLL-rVN, P-12h-PLL-rVN, P-24h-PLL-

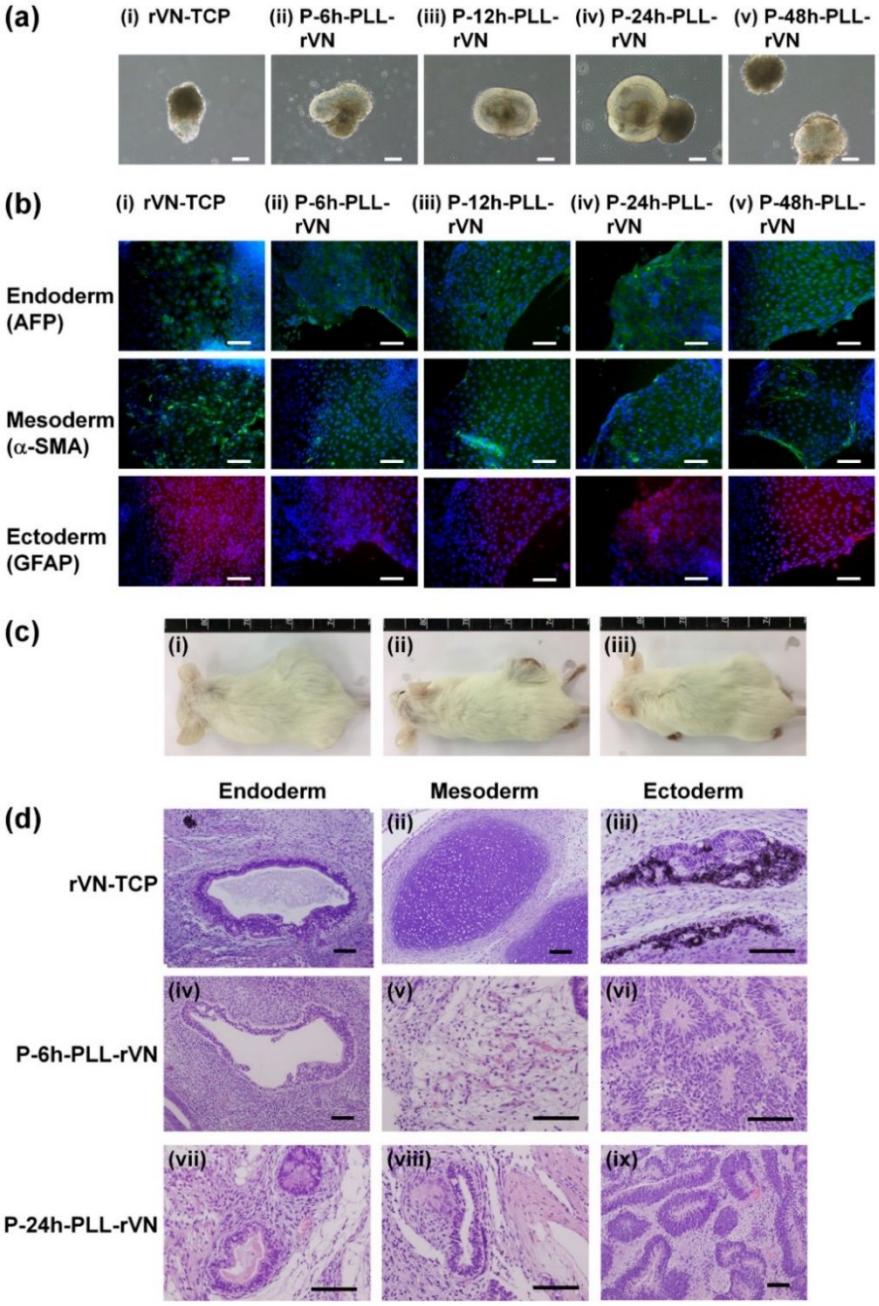


**Fig. 7** Characterization of the differentiation ability of H9 hESCs in vitro and in vivo after culturing on PVI hydrogels. (a) Morphology of EBs differentiated from H9 hESCs in suspension after culturing on (i) rVN-TCP(5) dishes and (ii) P-6h-PLL-rVN, (iii) P-12h-PLL-rVN, (iv) P-24h-PLL-rVN, and (v) P-48h-PLL-rVN hydrogels for 10 passages. Scale bar indicates 100 μm. (b) Expression of an ectoderm protein (GFAP, red), mesoderm protein (α-SMA, green), and endoderm protein (AFP, green) in H9 hESCs analyzed by immunostaining with dual staining with Hoechest33342 for nuclear (blue) after culturing on (i) rVN-TCP(5) dishes and (ii) P-6h-PLL-rVN, (iii) P-12h-PLL-rVN, (iv) P-24h-PLL-rVN, and (v) P-48h-PLL-rVN hydrogels for 10 passages. Scale bar indicates 100 μm. (c) Photograph of teratomas generated in NOD-SCID mice by injection with hESCs after culturing on (i) rVN-TCP(5) dishes, (ii) P-6h-PLL-rVN hydrogels, and (iii) P-24h-PLL-rVN hydrogels for 10 passages. (d) Photograph of teratoma resulting from the injection of hESCs after culturing on rVN-TCP(5) dishes, P-6h-PLL-rVN hydrogels, and P-24h-PLL-rVN hydrogels for 10 passages. Tissues including intestinal duct (endoderm) (i), duct (endoderm) (iv and vii), cartilage (mesoderm) (ii), vessels, adipocytes, mesenchymal spindle cells (mesoderm) (v), bone-like tissue (mesoderm) (viii) melanocytes (ectoderm) (iii), and immature neuroepithelium (ectoderm) (vi and xi) can be observed. Scale bar indicates 100 μm.

rVN, and P-48h-PLL-rVN hydrogels for more than 10 passages. The expansion rate of hPSCs on P-6h-PLL-rVN and P-12h-PLL-rVN hydrogels was less than that on rVN-TCP dishes, whereas the

expansion rate on P-24h-PLL-rVN and P-48h-PLL-rVN was similar to that on rVN-TCP.

No statistical difference of the expansion rate of hPSCs cultured on P-24h-PLL-rVN and rVN-TCP was found ($p$>0.05), although the expansion rate on P-24h-PLL-rVN was relatively less than that on rVN-TCP. Relatively stiff hydrogels, such as P-24h-PLL-rVN and P-48h-PLL-rVN hydrogels, seem to be preferable for fast expansion of hPSCs, although the low expansion rate of hPSCs on P-6h-PLL-rVN may be advantageous for the maintenance of hPSCs, with less medium being consumed (up to half the amount) depending on the purpose of the hPSC culture.

The differentiation ratio of hPSCs cultivated on P-2h-PLL-rVN, P-6h-PLL-rVN, P-12h-PLL-rVN, P-24h-PLL-rVN, and P-48h-PLL-rVN hydrogels were <5%, whereas those on rVN-TCP dishes were < 7-8%, indicating that hPSCs growing on P-*X*h-PLL-rVN hydrogels retained their pluripotency more than cells grown in conventional rVN-TCP dishes. Relatively soft P-*X*h-PLL-rVN hydrogels having a storage modulus < 31 kPa seemed to be preferable to maintain the pluripotency of hPSCs compared to rVN-TCP having an elasticity of 3 GPa.

hPSC pluripotency was also investigated based on the expression of pluripotent marker proteins (Sox2, SSEA-4, Nanog, and Oct3/4) on the cells by immunostaining after 10 passages on P-6h-PLL-rVN, P-12h-PLL-rVN, P-24h-PLL-rVN, and P-48h-PLL-rVN hydrogels, and on rVN-TCP dishes (Fig. 6c and Fig. 1S(c) (ESI†)). The merged fluorescence images of (1) Hoechest 33342 and (2) Oct3/4, Nanog, Sox2, or SSEA-4 were shown. The hPSCs abundantly expressed these pluripotency proteins during the 10 passages on P-*X*h-PLL-rVN hydrogels and rVN-TCP dishes in xeno-free culture conditions (Fig. 6c and Fig. 1S(c) (ESI†)).

Pluripotent gene expression of *Oct4*, *Sox2*, and *Nanog* was also evaluated after 10 passages of hESCs on P-24h-PLL-rVN hydrogels and rVN-TCP dishes and the results are shown in Fig. 2S (ESI†). hESCs cultured on P-24h-PLL-rVN hydrogels and rVN-TCP dishes were found to express pluripotent genes extensively and no difference of pluripotent gene expression on hESCs, which were cultured on P-24h-PLL-rVN hydrogels and rVN-TCP dishes, was statistically observed ($p$ >> 0.05).

Karyotyping analysis of hiPSCs after culture on P-24h-PLL-rVN for ten passages was performed and the results are shown in Fig. 3S (ESI†). hiPSCs after culture on P-24h-PLL-rVN for ten passages were found to show a normal euploid karyotype.

**Differentiation of hPSCs *in vitro* and *in vivo***

As described above, hPSCs retained their pluripotency during culture on PVI hydrogels grafted with rVN and PLL (P-*X*h-PLL-rVN hydrogels) for >10 passages. It was also necessary to investigate whether hPSCs maintained their ability of induced differentiation into the cells of all three germ layers *in vitro* in the embryoid body (EB) formation assay and *in vivo* in the teratoma formation assay. hPSCs were cultivated on P-6h-PLL-rVN, P-12h-PLL-rVN, P-24h-PLL-rVN, and P-48h-PLL-rVN hydrogels, and rVN-TCP dishes in xeno-free culture conditions for 10 passages, followed by cultivation in suspension to form EBs (Fig. 7a). EBs were cultivated on Matrigel-coated surfaces for another 3-5 weeks to produce spreading morphologies on the dishes.

The spread cells were evaluated to be identified whether these cells were differentiated into alpha-smooth muscle actin (α-SMA) expressing mesoderm cells, alpha-fetoprotein (AFP) expressing endoderm cells, and glial fibrillary acidic protein (GFAP) expressing ectoderm cells. The hPSCs differentiated into the three cell types (Fig. 7b for hESCs and Fig. 4S (ESI†) for hiPSCs), demonstrating the retention of differentiation capability and indicating that hPSCs can maintain their pluripotency *in vitro* after cultivation on P-*X*h-PLL-rVN hydrogels for 10 passages under feeder-free and xeno-free culture conditions.

We also evaluated the potential of hESCs to differentiate into cells derived from all three of the aforementioned germ layers *in vivo* by a teratoma formation analysis. hESCs cultured on P-6h-PLL-rVN and P-24h-PLL-rVN hydrogels, as well as rVN-TCP dishes for 10 passages were subcutaneously xenotransplanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice to generate teratomas *in vivo* (Fig. 7c). The teratomas were separated from the NOD.CB17-*Prkdc*$^{scid}$/JNarl mice and teratoma tissue sections were fixed, sliced, and stained with hematoxylin and eosin. The teratomas harbored cells derived from all three germ layers, specifically ectoderm cells (melanocytes and neuroepithelium), mesodermal cells (cartilage, mesenchymal spindle cells, and bone-like cells), and endoderm cells for the duct (Fig. 7d). The findings suggested that hESCs cultured on P-6h-PLL-rVN and P-24h-PLL-rVN hydrogels for 10 passages remained capable of induced differentiation to cells of all three germ layers, which was evidence of retained pluripotency *in vivo*.

**Differentiation of hPSCs to cardiomyocytes during culture on P-24h-PLL-rVN hydrogels**

We also evaluated whether hPSCs cultivated for 10 passages on P-*X*h-PLL-rVN hydrogels in xeno-free culture conditions could differentiate into a specific cell lineage. The lineage we selected was cardiomyocytes.[81-84] Cardiomyocyte differentiation was evaluated using a commercially available, xeno-free cardiomyocyte differentiation kit (A29212-01, Life Technologies (Carlsbad, CA, USA)) by following the manufacturer's instruction. The kit contains three different mediums: Medium A, Medium B, and maintenance medium. Although the exact components in the medium is unknown because of the commercial kit, Medium A is expected to contain the GSK-3β inhibitor and/or Wnt activator for hPSC induction into the mesoderm lineage of the cells because cardiomyocytes are classified as the cells of mesoderm lineage. Medium B probably contains Wnt inhibitor for the cardiomyocyte differentiation of the cells. The differentiation protocol is shown in Fig. 8a. Data of the morphological change of differentiated cells into cardiomyocytes during culture on P-6h-PLL-rVN, P-24h-PLL-rVN, P-48h-PLL-rVN hydrogels, and Matrigel-coated dishes and rVN-

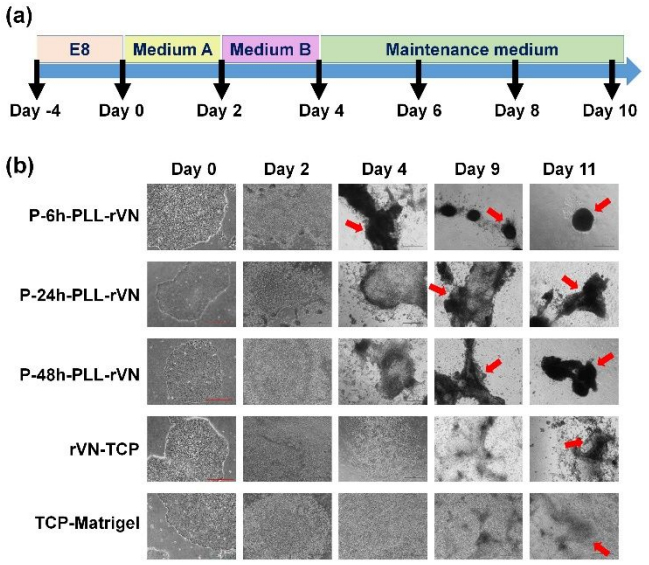


**Fig. 8** Differentiation of H9 human embryonic stem cells (hESCs) into cardiomyocytes cultured on PVI hydrogels grafted with PLL and rVN under feeder-free and xeno-free culture conditions. (a) Timeline of protocol for differentiating H9 hESCs into cardiomyocytes. (b) Morphology of hESCs after induction of differentiation into cardiomyocytes at day 0, 2, 4, 9, and 11, which were cultured on P-6h-PLL-rVN hydrogels, P-24h-PLL-rVN hydrogels, P-48h-PLL-rVN hydrogels, rVN-TCP dishes prepared with 5 μg/mL rVN coating solution, and Matrigel-coated dishes. P-*X*h-PLL-rVN hydrogels were prepared with 1 mg/mL PLL solution and 10 μg/mL rVN solution. The red arrow indicates beating cells. Scale bar indicates 500 μm.

TCP dishes are presented in Fig. 8b. Beating cells were more frequently observed on soft P-*X*h-PLL-rVN hydrogels than on Matrigel-coated dishes and rVN-TCP dishes at an early stage of differentiation. Beating cells were evident as early as 4 days following induction when cultured on the softest P-6h-PLL-rVN, whereas beating cells were not apparent until 10 days following induction for the cells cultured on conventional Matrigel-coated dishes and rVN-TCP dishes. The expression of the cardiomyocyte marker, cTnT, was investigated for the cells cultured on P-*X*h-PLL-rVN hydrogels, Matrigel-coated dishes, and rVN-TCP dishes after 12 days of induction. The results using hESCs are shown in Fig. 9a. The rate of differentiation into cardiomyocytes was evaluated from cTnT expression (Fig. 9b for hESCs and Fig. 5S(a) (ESI†) for hiPSCs). Differentiation into cardiomyocytes was not as evident for the cells cultured on Matrigel-coated dishes and rVN-TCP dishes, whereas hPSCs cultured on P-24h-PLL-rVN (Supporting Video S1) and P-48h-PLL-rVN successively differentiated into cardiomyocytes. It should be noted that the differentiation rate of hESC-derived cardiomyocytes shown in Fig. 9(b) was obtained from the evaluation of the cells without selection of the beating cells nor enrichment process (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Enrichment_and_Replating_of_PSC_ Derived_Cardiomyocytes _UB.pdf). The differentiation rate of hESC-derived cardiomyocytes even cultured on rVN-TCP dishes and Matrigel coated dishes should enhance more than 60-80% after the selection of the beating cells or the enrichment process. Especially hPSCs cultured on P-24h-PLL-rVN hydrogels differentiated into cardiomyocytes with an efficiency rate of approximately 80%, which was higher than that of hPSC-differentiated cardiomyocytes on P-48h-PLL-rVN hydrogels (P-24h-PLL-rVN vs. P-48h-PLL-rVN, $p < 0.05$). hPSCs less efficiently differentiated into cardiomyocytes on P-6h-PLL-rVN hydrogels because there was greater detachment of the cells during induction. The absolute number of cardiomyocytes on each condition after 12 days of induction can be calculated from the following equation;

Seeding number of hPSCs x Differentiation rate (shown in Fig. 9b) x Survival rate (shown in Fig. 9c) (2)

where the seeding number of hPSCs on each condition is $10^5$ cells in this study. The absolute number of cardiomyocytes on P-6h-PLL-rVN hydrogels, P-24h-PLL-rVN hydrogels, P-48h-PLL-rVN hydrogels, rVN-TCP dishes, and Matrigel coated dishes are 80, 14,000, 6,900, 450, and 4,200, respectively. Therefore, P-24h-PLL-rVN hydrogels were the most suitable for differentiation into cardiomyocytes in this study, whereas conventional Matrigel-coated dishes and rVN-TCP dishes were less promising for the differentiation of hPSCs into cardiomyocytes.

Since cell detachment occurred during the induction of cardiomyocyte differentiation, we evaluated the survival ratio. The ratio was calculated as the number of cells remaining at day 12 divided by the number of cells inoculated on day 0, with the resulting number multiplied by 100. The survival rate of the cells cultured on several biomaterials is shown in Fig. 9c for hESCs and Fig. 5S(b) (ESI†) for hiPSCs where survival rate of hPSCs, which is over 100% because of expansion of pluripotent hPSCs, is also plotted as 100%. The survival rate of cells cultured on Matrigel-coated dishes and rVN-TCP dishes was higher than the survival rate on P-*X*h-PLL-rVN hydrogels. hPSCs cultured on softer P-*X*h-PLL-rVN hydrogels displayed a lower survival ratio (P-6h-PLL-rVN vs. P-24h-PLL-rVN, $p < 0.05$), but higher differentiation rate (higher cTnT expression), except for P-6h-PLL-rVN hydrogels (Fig. 9c and Fig. 5S(b) (ESI†)). The findings suggested that P-24h-PLL-rVN hydrogels can sort cardiomyocytes from undifferentiated hPSCs during induction of differentiation into cardiomyocytes. Thus, P-*X*h-PLL-rVN hydrogels are preferred for hPSC proliferation and the differentiation of hPSCs into cardiomyocytes. We analyzed the expression of specific cardiomyocyte proteins, α-actinin, MLV2c (myosin light chain), and cTnT on hPSC-derived cardiomyocytes via an immunostaining method, which were differentiated on P-24h-PLL-rVN hydrogels after culture on P-24h-PLL-rVN hydrogels for ten passages (Fig. 10). The hPSC-derived cardiomyocytes differentiated on P-24h-PLL-rVN hydrogels were found to express α-actinin, MLV2c, and cTnT extensively. Because the sarcomere structure was clearly observed from α-actinin expression analyzed by a confocal laser microscopy on hPSC-derived cardiomyocytes (Fig. 10(f) and 10(g)), the average

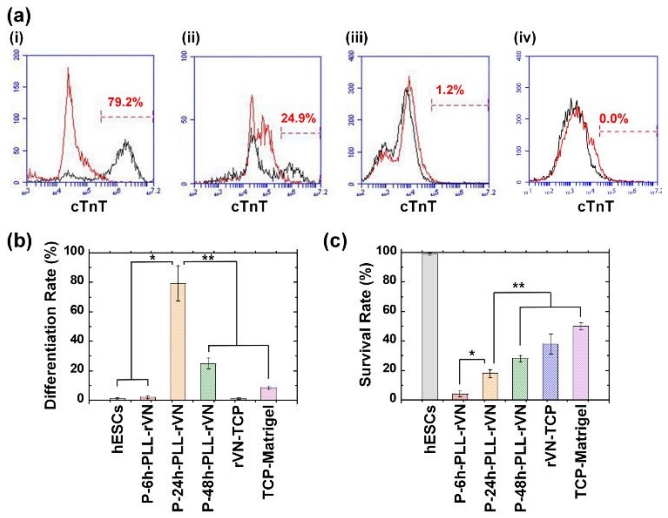


**Fig. 9** Characterization of differentiation of H9 human embryonic stem cells (hESCs) into cardiomyocytes during culture on PVI hydrogels grafted with PLL and rVN under feeder-free and xeno-free culture conditions. (a) Flow cytometry analysis of the expression of cTnT of the differentiated hESCs cultivated on (i) P-24h-PLL-rVN hydrogels, (ii) P-48h-PLL-rVN hydrogels, and (iii) rVN-TCP dishes prepared with 5 μg/mL rVN coating solution after 12 days of induction as well as (iv) undifferentiated hESCs. P-Xh-PLL-rVN hydrogels were prepared with 1 mg/mL PLL solution and 10 μg/mL rVN solution. The red lines indicate the plots of differentiated hESCs stained with an isotype antibody as the negative control. (b) Differentiation rate of cells differentiated from hESCs cultivated on cultured on P-6h-PLL-rVN hydrogels, P-24h-PLL-rVN hydrogels, P-48h-PLL-rVN hydrogels, rVN-TCP dishes prepared with 5 μg/mL rVN coating solution, and Matrigel-coated dishes after 12 days of induction. Differentiation rate of hESCs is also plotted. (c) Survival rate of differentiated hESCs cultivated on P-6h-PLL-rVN hydrogels, P-24h-PLL-rVN hydrogels, P-48h-PLL-rVN hydrogels, rVN-TCP dishes prepared with 5 μg/mL rVN coating solution, and Matrigel-coated dishes after 12 days of induction. Survival rate of hESCs, which is over 100% because of expansion of pluripotent hESCs, is also plotted as 100%. *$p < 0.05$.

sarcomere length of hPSC-derived cardiomyocytes was evaluated and was estimated to be 1.4-1.7 μm, which is approximately the length of sarcomeres in healthy human heart tissue (1.5 μm[85]).

## Discussion

Several cell culture biomaterials have been developed for cultivation of hPSCs in feeder-free and xeno-free culture conditions. Synthetic polymers do not bind specifically to hPSCs. These polymers have been studied as potential hPSC culture biomaterials. Copoly(AEtMA-co-DEAEA),[38] APMAAm,[41] PMVE-alt-MA,[39] and PMEDSAH[40,63] were reported to maintain hPSC cultivation in xeno-free culture media. However, it is unknown whether hPSCs can be extensively cultivated on these synthetic materials without any problems for a long time, given the lack of knowledge of the attachment mechanism of these stem cells to synthetic polymer substrates. Currently, the most reliable and gold-standard xeno-free cell culture materials for hPSCs are ECM-coated dishes and ECM-derived oligopeptide-immobilized dishes. ECMs used for this purpose are rVN,[49,50,54-57] laminin-521, and laminin-511.[47,58-62] Especially, rVN-TCP dishes have been widely used for hPSC culture.[49,50,54-57] Oligopeptide-immobilized dishes have been recently developed to support the proliferation of hPSCs.[37,44,49,50,66-68,86] However, one of the demerits of oligopeptide-immobilized dishes is the requirement for a high concentration of oligopeptide (typically over 1000 μg/mL) and/or the relatively high differentiation ratio of hPSCs cultured on oligopeptide-immobilized dishes, such as Synthemax II-coated dishes.[37] The P-*X*h-PLL-rVN hydrogels developed in this study have rVN-immobilized biomaterials with a different elasticity (10-30 kPa) that are regulated by the crosslinking time. These biomaterials were prepared with a relatively low concentration of rVN (10 μg/mL), whereas conventional rVN-TCP dishes have a fixed and extremely stiff surface (3 GPa).

The elasticity of cell culture biomaterials of P-*X*h-PLL-rVN can tune the proliferation speed of hPSCs, which is preferable for hPSC culture depending on the purpose. For example, the soft P-6h-PLL-rVN hydrogels are preferable cell culture materials for the usage to maintain hPSC as the stock cells, which can save the usage of expensive culture medium of hPSCs, whereas P-24h-PLL-rVN and P-48h-PLL-rVN hydrogels are preferable cell culture materials for the rapid expansion of hPSCs for research usage and clinical application of hPSCs. Therefore, biological cues (rVN) and the elasticity of cell culture biomaterials are important to control the differentiation fate of hPSCs into a specific lineage of the cells, such as cardiomyocytes.

P-*X*h-PLL-rVN hydrogels could support proliferation of hPSCs and showed characteristics of dishes that sort cardiomyocytes during the differentiation of hPSCs into cardiomyocytes. In contrast, a very low differentiation rate of hPSCs into cardiomyocytes was observed on conventional rVN-TCP dishes. This is because rVN can binds to $\alpha_V\beta_5$ integrin receptor of undifferentiated hPSCs, whereas the differentiated cells such as hPSC-derived cardiomyocytes seem not to express $\alpha_V\beta_5$ integrin extensively. The attachment of hPSC-derived cardiomyocytes on P-*X*h-PLL-rVN hydrogels could be supported by nonspecific adhesion mechanism between positively charged PLL and negatively charged hPSC-differentiated cardiomyocytes on P-*X*h-PLL-rVN hydrogels.

Several cell culture biomaterials have been studied for hPSC differentiation into cardiomyocytes. These include agarose hydrogels,[87] gelatin, fibronectin, or collagen IV-coated dishes,[88] fibrin patch and fibrin coated dishes,[89] vitronectin-derived oligopeptide-grafted dishes,[37] laminin-coating microcarriers,[90] and Matrigel-coated dishes.[91] However, the differentiation rate analyzed by cTnT reported in these studies was typically <65%, which was much less than that on P-24h-PLL-rVN hydrogels in this study. Approximately 80% of the differentiated cells from

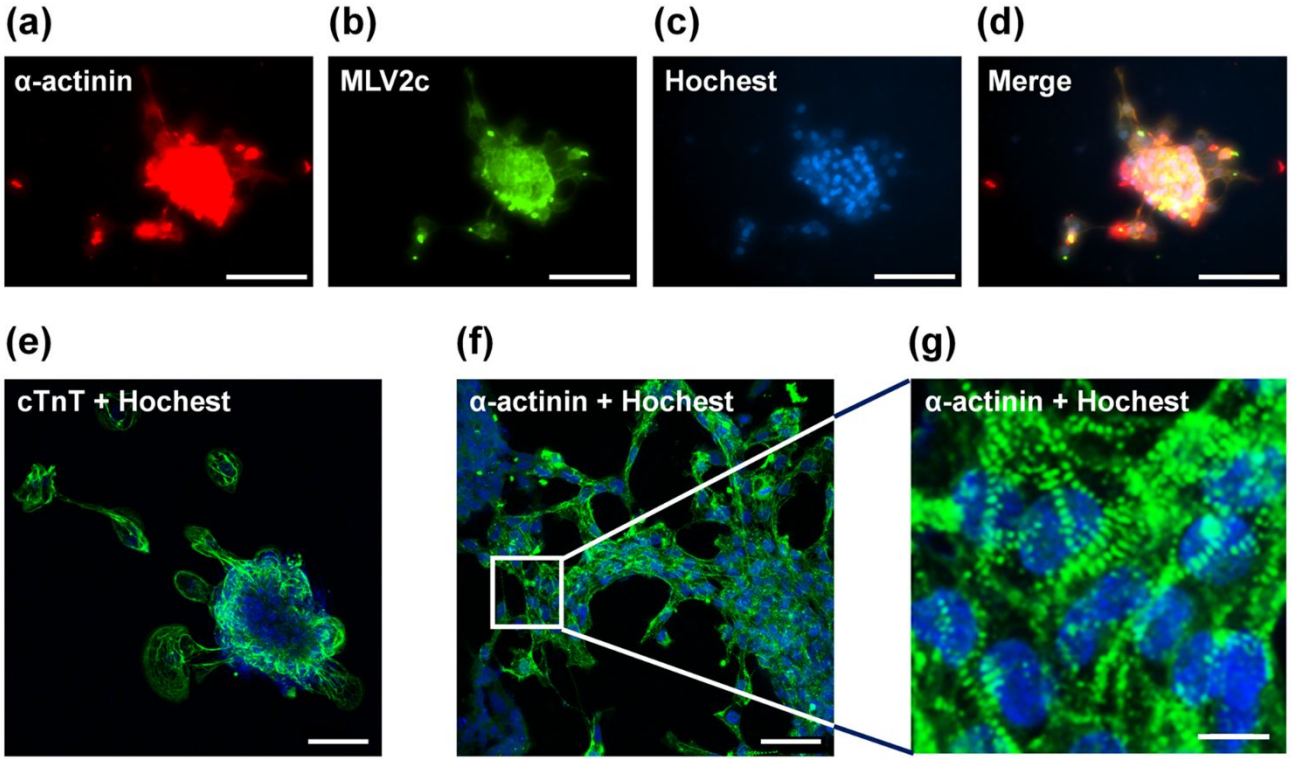


**Fig. 10** Immunostaining analysis of hESC-derived cardiomyocytes differentiated on P-24h-PLL-rVN hydrogels after culture on P-24h-PLL-rVN hydrogels for ten passages. (a-d) Expression of α-actinin (a) and MLV2c (b) on hESC-derived cardiomyocytes as analyzed by an immunostaining method. Nuclei were stained with Hoechst 33342 (c) and the merged picture is also shown (d). (e-g) Expression of cTnT (e) and α-actinin (f,g) on hESC-derived cardiomyocytes as analyzed by confocal laser microscopy. Nuclei were stained with Hoechst 33342. Scale bar indicates 100 μm (a-d), 50 μm (e,f), and 10 μm (g).

hPSCs on P-24h-PLL-rVN hydrogels expressed the cardiomyocyte marker, cTnT, within 12 days of the induction of differentiation. Further refinements of the process will aim to achieve >98% expression.

Specific biomaterials to guide hPSC differentiation into the specific lineage of the cells are needed. Laminin-related biomaterials can guide hPSCs into neural and myogenic cell lineages. Collagen and hydroxyapatite-related biomaterials promote hPSC differentiation into osteoblasts. However, a combination of biological cues (ECMs or ECM-derived oligopeptides) and physical cues (hydrophilicity, elasticity, topography, and surface potential) should be useful for the optimal design of cell culture biomaterials that guide hPSC differentiation into a specific cell lineage. P-24h-PLL-rVN hydrogels were developed with a combination of optimal elasticity of the biomaterials grafted with specific cell binding site of rVN by adjusting surface charge by PLL. These hydrogels promoted the differentiation of hPSCs into cardiomyocytes.

## Conclusions

PVI hydrogels grafted with rVN were developed in this study. PVI hydrogels grafted with rVN (P-*X*h-rVN) did not support the proliferation and attachment of hPSCs because the zeta potential of P-*X*h-rVN hydrogels was too negative. Therefore, PVI hydrogels were grafted with the positively charged polymer of PLL as well as rVN to produce an optimal zeta potential (P-*X*h-PLL-rVN) similar to that of rVN-TCP dishes in the aspect of surface potential. The hPSCs proliferated on P-*X*h-PLL-rVN hydrogels in feeder-free and xeno-free culture condition for over 10 passages. Furthermore, hPSCs efficiently differentiated into cardiomyocytes (approximately 80% at 12 days of induction) than those cultured on conventional rVN-TCP dishes. The survival rate of the cells on P-24h-PLL-rVN hydrogels was less than that on rVN-TCP dishes. These P-24h-PLL-rVN hydrogels displayed cell sorting characteristics, in which cardiomyocytes survived on PVI-24h-PLL-rVN hydrogels but undifferentiated cells did not survive in cardiomyocyte differentiation medium. Our results suggest that cell culture biomaterial designs including biological cues (optimal cell binding domain and surface density) and physical cues (surface charge and elasticity)[76,92-94] are important for facilitating the proliferation and differentiation of hPSCs into specific cell lineages in feeder-free and xeno-free culture conditions.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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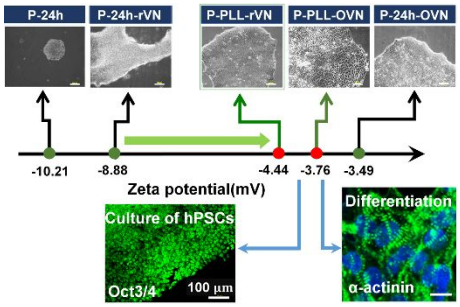


Xeno-free culture and cardiomyocyte differentiation of human embryonic stem cells on vitronectin-grafted hydrogels by adjusting surface charge and elasticity