Chapter 14
Stem Cell Culture on Polymer Hydrogels

Akon Higuchi, Hsing-Fen Li, S. Suresh Kumar, Abdullah A. Alarfaj and Murugan A. Munusamy

Abstract The fate of stem cell differentiation is guided by several different factors of the stem cell microenvironment, such as cell culture biomaterial elasticity (physical cues) and cell–biomaterial interactions (biological cues). Mimicking the stem cell microenvironment using polymer hydrogels with optimal elasticities is an excellent strategy for stem cell expansion and differentiation. This chapter describes poly(vinyl alcohol) (PVA) hydrogels grafted with several nanosegments that are designed for the culture and differentiation of human hematopoietic and progenitor cells (hHSPCs), human amniotic fluid stem cells (hAFSCs), and human pluripotent stem cells (hPSCs). The elasticity of the cell culture hydrogels can regulate stem cell adhesion overall, as well as cell phenotype, focal adhesions, and morphology, especially in 2-D culture conditions. The mechano-sensing of cell culture biomaterials by stem cells is typically regulated by integrin-mediated focal adhesion signaling. PVA hydrogels having a storage modulus (E') of 12–30 kPa were found to be efficient materials for ex vivo hHSPC expansion. We also developed PVA hydrogels grafted with oligopeptides derived from vitronectin (PVA-oligoVN hydrogels), which can be produced to have a variety of stiffnesses, for the xeno-free culture of hPSCs. The ideal stiffness of the PVA-oligoVN hydrogels for hPSC culture was found to be 25.3 kPa. A high concentration of oligoVN (500–1500 μg/mL) should be used to prepare the PVA-oligoVN hydrogels to achieve a sufficient

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oligoVN surface density to maintain hPSC pluripotency. Optimized stiffness (physical cues) and cell-binding moiety surface density (biological cues) are the key factors for designing hydrogel-based cell culture materials for supporting hPSC pluripotency in xeno-free culture conditions.

Keywords Hydrogels · Stem cells · Elasticity · Differentiation
Pluripotency · Nanosegment · Oligopeptide · Poly(vinyl alcohol)

1 Introduction

Humans can sustain injuries and lose organs and tissue because of diseases, birth defects, and accidents. Stem cells, such as human mesenchymal stem cells (hMSCs), human induced pluripotent stem cells (hiPSCs), and human embryonic stem cells (hESCs), are valuable cell sources in translational medicine.

The stem cell characteristics and differentiation fates are governed by the microenvironment of the stem cells. Therefore, it is a reasonable strategy to develop biomaterials, such as hydrogels, nanofibers, and microcarriers, to mimic the stem cell microenvironments; these materials could be used to maintain pluripotency for stem cell expansion or to promote stem cell differentiation into specific desired lineages in vitro.

Several biological cues, such as soluble factors (i.e., growth factors, hormones, inhibitors, and agonists) and extracellular matrices (ECMs), strongly affect stem cell pluripotency and differentiation fate (Fig. 1) (Higuchi et al. 2011b, 2012, 2013). However, recent research suggests that the physical cues provided by cell culture biomaterials (i.e., topography, elasticity, and cyclic biomaterial stretching) also affect stem cell differentiation (Fig. 1) (Higuchi et al. 2015b; Engler et al. 2006; Murphy et al. 2014; Wen et al. 2014).

It was reported that human bone marrow stem cell (hBMSC) differentiation into specific tissue lineages could be efficiently induced when the hBMSCs were cultivated on collagen-coated polyacrylamide hydrogels with a stiffness that was similar to the tissue of interest (Wen et al. 2014; Murphy et al. 2014; Engler et al. 2006). Softer hydrogels, with an elasticity close to that of the brain (e.g., 0.3 kPa) promoted cells to display neuronal morphologies and early markers of neural cells (P-NFH, β-III tubulin), whereas stiffer hydrogels (10 kPa) tended to promote the differentiation of cells into muscle-guided hBMSCs expressing myogenic markers (MyoD) (Engler et al. 2006). Furthermore, even stiffer hydrogels (approximately 35 kPa), which were similar in elasticity to collagenous bones, tended to induce hBMSCs into cells expressing early markers (Runx2) of osteoblasts (Engler et al. 2006).

Several other investigators also noted that cell culture biomaterial (hydrogel) elasticity is an important factor in the differentiation of hMSCs in two-dimensional (2-D) culture conditions (Lanniel et al. 2011; Park et al. 2011a, b), as the elasticity of the cell culture biomaterial guides hMSC differentiation fate. It should be noted

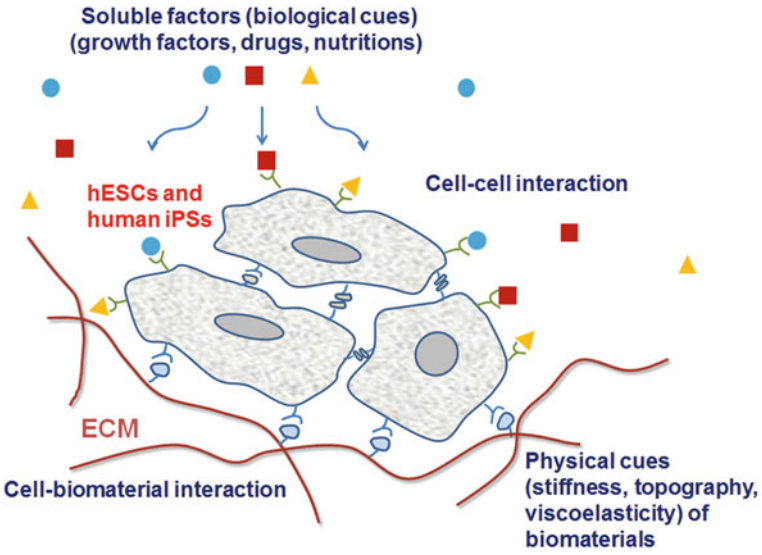


Fig. 1 Microenvironment of human stem cells is regulated by the following factors: (1) physical factors, e.g., stiffness, of the tissues or cell culture matrices, (2) interactions among molecules biologically active with cells, e.g., ECMs and biomaterials; (3) cell–cell interactions; and (4) several soluble factors, such as growth factors, inhibitors, nutrients, and other bioactive molecules (Higuchi et al. 2011a, b). Copyright 2011. Adapted with permission from the American Chemical Society

that some studies reported conflicting results and have offered different, but intriguing, ideas for explaining the effect of cell culture biomaterial elasticity on hMSC differentiation fate (Higuchi et al. 2013; Trappmann et al. 2012; Huebsch et al. 2010). While Engler's landmark report (Engler et al. 2006) showed that the elasticity of cell culture hydrogels guides the differentiation fate of hBMSCs, this was true only under limited conditions, in which hBMSCs were cultured on collagen-coated polyacrylamide hydrogels in 2-D culture conditions. Collagen type I can diffuse into soft polyacrylamide hydrogels, whereas it cannot diffuse into stiff hydrogels deeply. Hydrogels with different elasticities yield different ECM anchoring densities on the hydrogels; subsequently, different mechanical feedback is provided from the ECM to the stem cells.

The mechanical feedback seems to allow stem cells to either differentiate into specific cell lineages or maintain their stemness. The differentiation fate and pluripotency of ESCs has also been shown to be dictated by the cell culture microenvironment (Higuchi et al. 2011b). Even without leukemia inhibitory factor (LIF) in the culture medium, mouse ESCs (mESCs) maintained their pluripotency when they were cultivated on soft hydrogels (0.6 kPa) with an elasticity similar to the intrinsic elasticity of the mESCs; in contrast, the mESCs could not maintain their pluripotency when they were cultured on stiff tissue culture polystyrene plates (TCPS, 3 GPa) coated with collagen type I (Chowdhury et al. 2010).

Valuable research investigating the effects of cell culture biomaterial elasticity on the differentiation fate and pluripotency of stem cells has been conducted (Higuchi et al. 2015a, b; Engler et al. 2006; Murphy et al. 2014; Wen et al. 2014). However, little is known of how cell culture hydrogel elasticity affects the pluripotency and differentiation fate of (a) human hematopoietic stem and progenitor cells (hHSPCs) (Kumar et al. 2013), (b) human amniotic fluid stem cells (hAFSCs) (Wang et al. 2015), and (c) human pluripotent stem cells (hPSCs) (Higuchi et al. 2015a). In this chapter, we discuss (1) the culture of hHSPCs on hydrogels having optimal elasticity, (2) the maintenance of hAFSC pluripotency on hydrogels having optimal elasticity, and (3) the xeno-free culture of hPSCs on hydrogels having optimal elasticity.

2 hHSPCs Expansion on Hydrogels Having Optimal Elasticity

hHSPCs are multipotent cells that have the potential to generate all types of mature blood cells as well as the capacity for self-renewal (Mendez-Ferrer et al. 2010; Xie et al. 2009; Higuchi et al. 2009). Intravenous infusion of hHSPCs has been performed to cure patients having malignant diseases or hematological disorders after radiation and/or chemotherapy (Remberger et al. 2011; Copelan 2006; Doran et al. 2009). Transplantation of hHPSCs into patients has been performed via transplanting bone marrow, umbilical cord blood (UCB), and hHSPCs isolated from peripheral blood (Chua et al. 2007; Yamamoto et al. 2011).

In particular, UCB is considered a valuable source of hHPSCs because UCB transplantation carries a lower risk of graft-versus-host disease (GVHD) compared with that of other hHPSC sources, such as peripheral blood and bone marrow (Park et al. 2011a; Rocha et al. 2004; Kishore et al. 2011). In addition, donors do not have any risks for side effects when UCB is collected to obtain hHPSCs. However, side effects in donors are reported in some cases when hHSPCs are obtained from bone marrow or peripheral blood, and death occurs in approximately one out of every 4,000 bone marrow donors (Copelan 2006). The small volume (60–180 mL) that is generally obtained from a single donor of UCB limits the UCB transplantation treatment to pediatric patients (Higuchi et al. 2009). Approximately 1.7×10^7 $CD34^+$ cells/kg is expected to be used for transplanting UCB into a patient (Kumar et al. 2013). Therefore, the transplantation of UCB must be restricted to children weighing less than or equal to 20 kg (Fujimoto et al. 2007; Franke et al. 2007). The disadvantage of UCB transplantation is the low dose of hHSPCs, which leads to slower engraftment and higher chances of engraftment failure than those resulting from bone marrow transplantation. Therefore, several efforts have been directed toward the expansion of hHSPCs ex vivo not only to improve engraftment time and to reduce graft failure but also to develop this therapy for adult patients (Fujimoto et al. 2007; Franke et al. 2007).

In this chapter, we discuss on the ex vivo expansion of hHSPCs from UCB through culture on hydrogels grafted with various nanosegments. Several researchers have already developed cell culture materials for the ex vivo expansion of hHSPCs by mimicking the microenvironment of bone marrow (Mendez-Ferrer et al. 2010; Chen et al. 2012; Kumar et al. 2013; Calvi et al. 2003; Di Maggio et al. 2011).

One idea is to develop surface-modified electrospun nanofibers for the expansion of hHSPCs. Chua et al. investigated hHSPC expansion on aminated, hydroxylated, carboxylated, or nonmodified films and nanofibers (Chua et al. 2006). The aminated films and nanofibers led to the highest hHSPC expansion (180- and 200-fold, respectively). In addition, while the aminated films led to less hHSPC expansion than the aminated nanofibers, it should be noted that the difference did not appear to be significant (Chua et al. 2006).

Bone marrow microenvironments contain several ECM components that are crucial for maintaining the stemness of hHSPCs. ECMs mediate the bonds between cell adhesion molecules (CAMs) and hHSPCs, which are extensively important for the signal transduction involved in hematopoiesis and other interactions of hHSPCs in the bone marrow (Higuchi et al. 2009; Feng et al. 2006). Feng et al. reported the ex vivo expansion of hHSPCs on polyethylene terephthalate (PET) mesh with immobilized fibronectin (FN) and collagen (COL) (Feng et al. 2006). The hHSPCs cultivated on the PET mesh with immobilized FN expanded at the highest rate. FN is reported to be the preferred ECM component compared with other ECM components for the ex vivo expansion of hHSPCs (Feng et al. 2006).

CS1 (connecting segment-1, EILDVPST) and arginine-glycine-aspartic acid (RGD) motifs are known to be valuable adhesion domains in fibronectin that bind to surface receptors of hematopoietic progenitors. hHSPCs derived from UCB were expanded on PET films grafted with CS1 and RGD (GRGDSPC) oligopeptides, as reported by Jiang et al. (2006). The hHPSCs on the CS1 peptide-grafted PET films expanded the most, and hHPSCs (600 $CD34^+$ cells) expanded ex vivo for 10 days on the CS1 peptide-grafted PET films were successively engrafted into mice. However, the hHSPCs cultured on the PET film grafted with the RGD peptide expanded less efficiently. These results suggested that having optimal adhesion peptides (e.g., CS-1) on cell culture biomaterials could extensively influence the expansion of hHSPCs isolated from UCB (Jiang et al. 2006).

The effect of nanosegment species (e.g., RGDS, RGES, CS1, FN, and polyamine) immobilized on polystyrene (PSt) plates on the ex vivo expansion of hHSPCs isolated from UCB has been systematically investigated by our group (Chen et al. 2012).

hHSPCs cultivated on a PSt surface with immobilized CS1 exhibited higher-fold expansion and more pluripotent colony-forming units (CFUs) (i.e., CFU-granulocytes, erythroid cells, macrophages, and megakaryocytes [CFU-GEMM]) than did hHSPCs cultured on a PSt surface with immobilized polyamine, RGD, and FN (Chua et al. 2006). However, the elasticity of the cell culture biomaterials used in this study was approximately 3 GPa, and this

investigation did not study the effect of the biomaterial stiffness on the expansion of the cultured hHSPCs.

In this chapter, we discuss the physical effect of cell culture hydrogel elasticity on the stemness, proliferation, and fate of hHSPCs cultured on synthetic hydrogels consisting of poly(vinyl alcohol-co-itaconic acid) (PVA) grafted with CS1 and FN. These hydrogels were selected because the elasticity of the hydrogels could be easily controlled by crosslinking time (intensity) with glutaraldehyde. The stiffness of the PVA hydrogels could be changed using the same polymeric main chain with different degrees of crosslinking. CS1 and FN were grafted with the carboxylic acid group of PVA via NHS (carbodiimide reaction using N-hydroxysuccinimide) and EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) in an aqueous solution. The PVA hydrogels were transparent with or without immobilized CS1 and FN, which allowed observation of hHSPCs on the PVA hydrogels via optical microscopy, much as cells are observed on conventional TCPS dishes. The goal of this study was to discuss the optimal stiffness of PVA hydrogels with immobilized CS1 and FN for the ex vivo expansion of hHSPCs isolated from UCB (Kumar et al. 2013).

2.1 *Characterization of PVA Hydrogels Immobilized CS1 and FN*

PVA hydrogels with immobilized CS1 and FN and different elasticities were prepared by varying the crosslinking time in the applied glutaraldehyde solution. These gels were used to investigate the optimal hydrogel elasticity for the expansion of hHSPCs when they were cultured on hydrogels containing specific immobilized cell-binding domains or proteins (e.g., CS1 and FN) (Fig. 2) (Kumar et al. 2013). PVA hydrogels with a thickness of 1.4–1.6 μm under dry conditions were prepared in this study. The thickness of the PVA hydrogels in aqueous conditions was also measured (2.2–8.9 μm thick) and is summarized in Table 1. Thick PVA hydrogels (approximately 25 μm thick) were prepared to measure the E' (storage modulus) of the gels using a rheometer, each of which is also listed in Table 1 (Kumar et al. 2013). The stiffest PVA hydrogel (PVA-48) had an E'= 30.4 kPa, whereas the softest PVA hydrogel (PVA-0.5) had an E'= 3.7 kPa, nearly eightfold less than that of PVA-48. PVA-*X* (e.g., PVA-0.5) indicates a PVA hydrogel crosslinked for *X* hours (e.g., 0.5 h). The PVA hydrogels with immobilized CS1 (PVA-*X*-CS1) and FN (PVA-*X*-FN) were measured to have E' values identical to those of the corresponding PVA hydrogels (PVA-*X*), within the experimental errors. This was because the amount of immobilized CS1 and FN was small; therefore, the grafted CS1 and FN segments did not contribute to the storage moduli of the bulk PVA hydrogels.

We attempted to quantify the number of immobilized nanosegments (i.e., CS1 and FN) on the PVA hydrogels with immobilized CS1 and FN and different

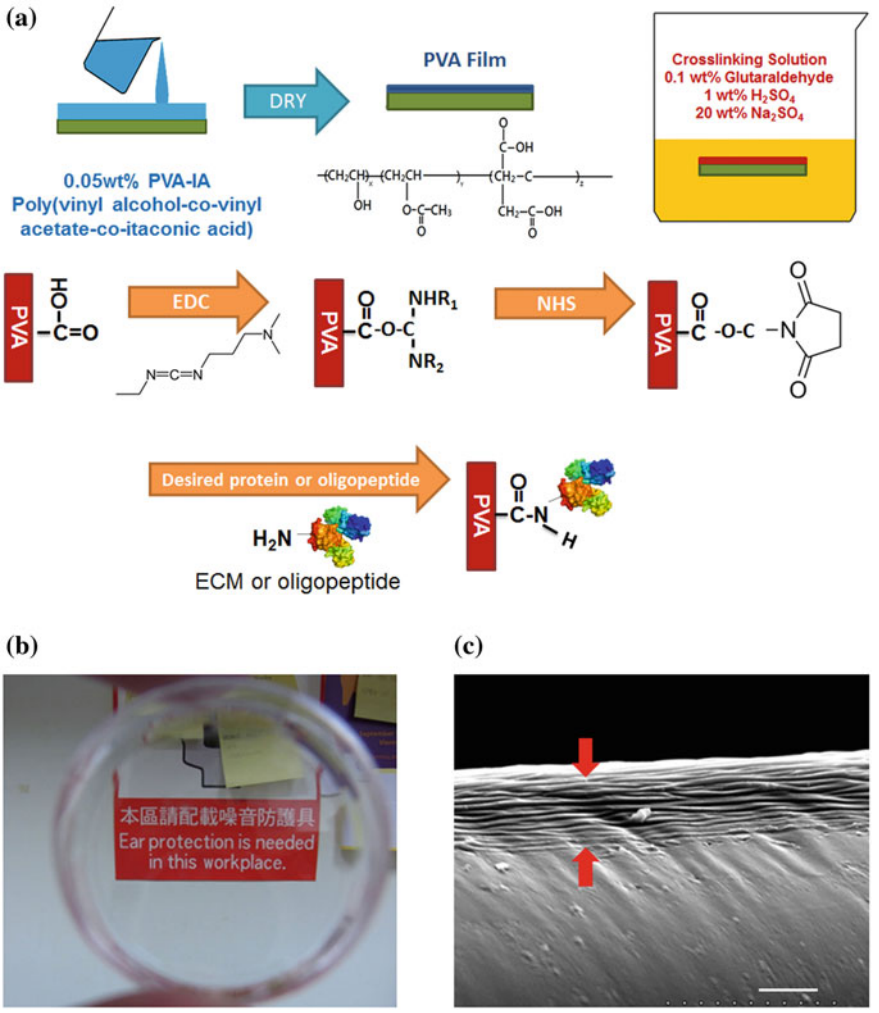


Fig. 2 Preparation of PVA hydrogels with immobilized CS1 and FN. **a** Reaction scheme of PVA hydrogels with immobilized CS1 and FN. **b** Macroscopic image of PVA-6-CS1 hydrogels. **c** Cross-sectional SEM image of PVA-6-CS1 hydrogels. The bar indicates 10 μm (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

elasticities. However, we failed to measure the absolute quantity of immobilized CS1 and FN on the PVA hydrogels using colorimetric (e.g., micro-BCA), chemical reaction, and titration methods in this study. Furthermore, it is known that the enzyme-linked immunosorbent assay (ELISA) cannot measure the absolute quantity of nanosegments immobilized on the biomaterials. Therefore, XPS analysis was performed to quantify the CS1 and FN nanosegments present on the PVA

Table 1 Characteristics of PVA hydrogels (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

Dishes	Thickness on dry condition (μm)	Thickness on wet condition (μm)	E' (kPa)	H (%)
PVA-0.5	1.4–1.6	8.9	3.7	83
PVA-1	1.4–1.6	5.1	10.3	71
PVA-6	1.4–1.6	3.6	12.2	59
PVA-24	1.4–1.6	2.3	25.3	36
PVA-48	1.4–1.6	2.2	30.4	31
TCPS	2000	2000	1,200.0	0.2

hydrogels. The C1 s peaks of the XPS spectra obtained from PVA hydrogels with and without immobilized CS1 or FN are shown in Fig. 3 (Kumar et al. 2013).

C–H and C–C bonding (285 eV), O–C=O bonding (289 eV), and C–N bonding (286 eV) were clearly identified in the XPS spectra obtained from the PVA-*X*-CS1 and PVA-*X*-FN hydrogels (excluding PVA-0.5-CS1), whereas C–H and C–C

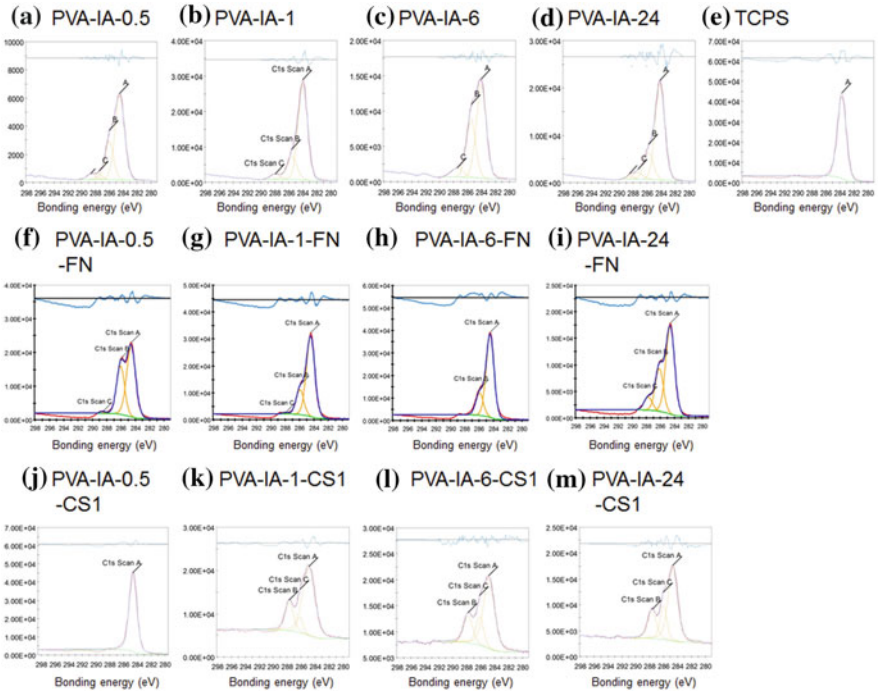


Fig. 3 XPS analysis of PVA hydrogels with immobilized CS1 and FN. C1 s spectra of *a-d* nonmodified PVA-*X* hydrogels, *f-i* PVA-*X*-FN hydrogels, *j-m* PVA-*X*-CS1 hydrogels, and **e** TCPS plates, where *X* is the reaction time *h* (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

bonding (285.0 eV) were mainly detected in the XPS spectra from TCPS and nonmodified PVA-*X* hydrogels (Fig. 3) (Kumar et al. 2013). These results indicated that CS1 and FN are securely bound to the PVA hydrogels, except for PVA-0.5-CS1.

The N1s peaks of the XPS spectra obtained from PVA-*X*-CS1, PVA-*X*-FN, and nonmodified PVA hydrogels were measured and are shown in Fig. 4 (Kumar et al. 2013). An N1s peak at 399 eV was broadly detected in the PVA-*X*-CS1 and PVA-*X*-FN hydrogel spectra, whereas only a trace N1s peak was observed in the nonmodified PVA hydrogel spectra. This is because PVA hydrogels do not contain nitrogen molecules, and the nitrogen atom can be attributed to the CS1 oligopeptides or FN protein. The atomic ratios of N/C of the PVA-*X*-CS1, PVA-*X*-FN, and PVA hydrogels after having reacted with EDC/NHS (PVA-EDC) were analyzed using the XPS spectra and are summarized in Fig. 5 (Kumar et al. 2013). A higher density of CS1 than FN was immobilized on the PVA hydrogels when the same crosslinking time (*X*) was used ($p < 0.01$), except for PVA-0.5-CS1 and PVA-0.5-FN. Approximately 2.5- to 3-fold more CS1 than FN was immobilized on the PVA-24-CS1 and PVA-48-CS1 hydrogels. Furthermore, the atomic ratios of N/C on the PVA-24-FN and PVA-48-FN gels were higher than those of the

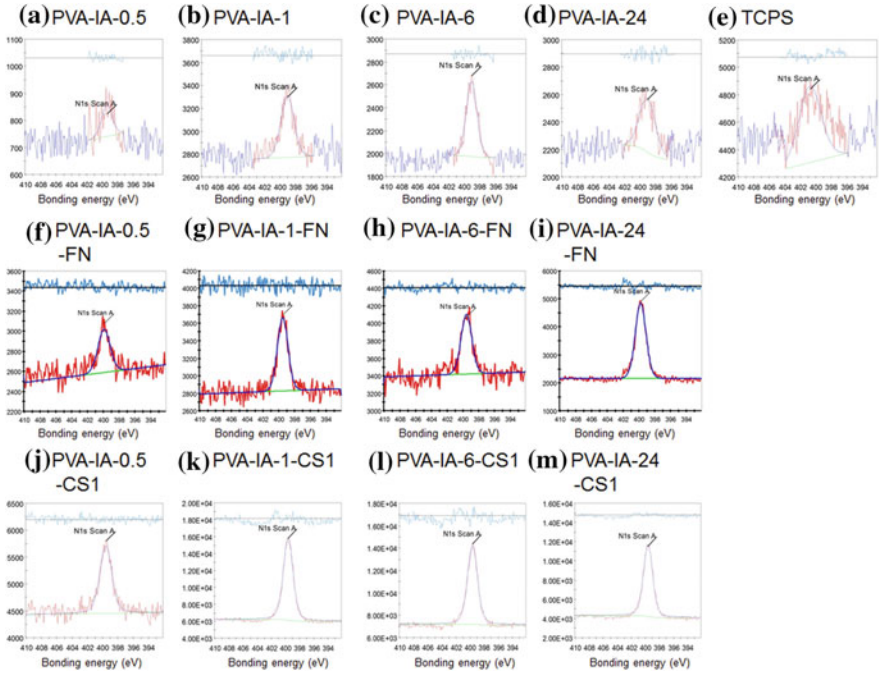


Fig. 4 XPS analysis of PVA hydrogels with immobilized CS1 and FN. N1s spectra of **a**-**d** nonmodified PVA-*X* hydrogels, **f**-**i** PVA-*X*-FN hydrogels, **j**-**m** PVA-*X*-CS1 hydrogels, and **e** TCPS plates, where *X* is the reaction time **h** (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

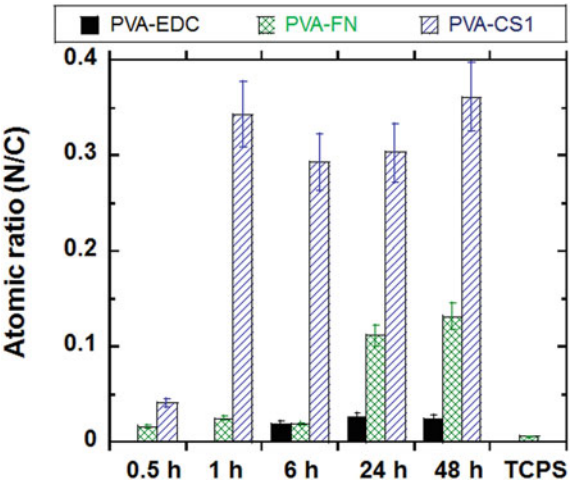


Fig. 5 XPS analysis of PVA hydrogels with immobilized CS1 and FN. The atomic ratios of nitrogen to carbon (N/C) in PVA hydrogels activated by EDC/NHS (PVA-*X*-EDC, black column, left), PVA-*X*-FN hydrogels (green column, middle), and PVA-*X*-CS1 hydrogels (blue column, right), where *X* is the reaction time (h) (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

PVA-0.5-FN, PVA-1-FN, and PVA-6-FN gels ($p < 0.01$). These results indicated that grafting FN onto PVA hydrogels is more unmanageable than grafting CS1 onto PVA hydrogels. This is because the FN molecule is larger than CS1, which leads to a less efficient reaction. The amounts of CS1 immobilized on the surface of PVA-1-CS1, PVA-6-CS1, PVA-24-CS1, and PVA-48-CS1 hydrogels appeared to be almost the same, corresponding to 2.5–3 times the surface density of FN immobilized on the PVA-24-FN and PVA-48-FN hydrogels (Kumar et al. 2013).

2.2 *Ex Vivo Expansion of hHSPCs in Nonmodified and Surface-Modified PVA Hydrogels*

hHSPCs purified from UCB were expanded for ten days on TCPS and PVA hydrogels with and without immobilized CS1 and FN, and the morphologies of the cultivated cells are shown in Fig. 6 (Kumar et al. 2013). The cell numbers increased after ten days of cultivation. The numbers of two hHSPC populations were analyzed using flow cytometry before and after hHSPC expansion, including $CD34^{+}CD45^{+}CD^{SS}$ cells that were analyzed using the method described by ISHAGE guidelines (Keeney et al. 1998; Gori et al. 2012; Higuchi et al. 2010), and $CD34^{+}CD38^{-}$ cells (Mortera-Blanco et al. 2011; Gori et al. 2012; Flores-Guzman et al. 2013; Rodriguez-Pardo and Vernot 2013; Salati et al. 2013; Roy et al. 2012; Holmes et al. 2012). The cells measured using the ISHAGE guidelines were determined to be hematopoietic progenitor cells, whereas the $CD34^{+}CD38^{-}$ cells were considered hematopoietic stem cells (Keeney et al. 1998). The flow cytometry scattergrams of the $CD34^{+}$ cells in UCB, which correspond to $CD34^{+}CD38^{-}$ cells (a), and the flow cytometry scattergrams of the UCB cells analyzed using ISHAGE guidelines (b and c) are shown in Fig. 7A (Kumar et al. 2013). The fold expansion

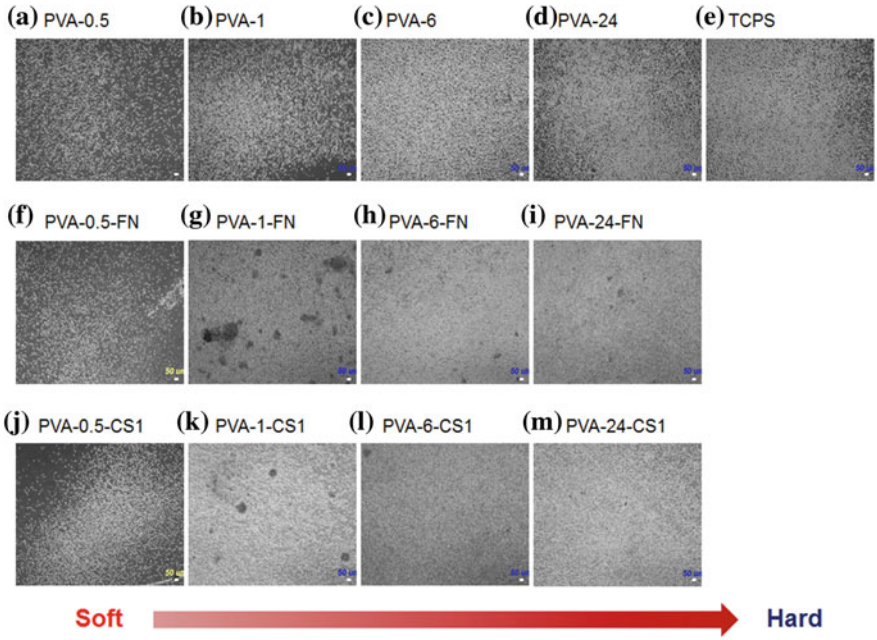


Fig. 6 Morphologies of cells cultivated on **a** PVA-0.5 hydrogels, **b** PVA-1 hydrogels, **c** PVA-6 hydrogels, **d** PVA-24 hydrogels, **e** TCPS plates, **f** PVA-0.5-FN hydrogels, **g** PVA-1-FN hydrogels, **h** PVA-6-FN hydrogels, **i** PVA-24-FN hydrogels, **j** PVA-0.5-CS1 hydrogels, **k** PVA-1-CS1 hydrogels, **l** PVA-6-CS1 hydrogels, and **m** PVA-24-CS1 hydrogels after 10 days of cultivation. The bar indicates 50 μm (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

of hHSPCs was analyzed using the protocol shown in Fig. 7a(a–c); these cells were cultured on TCPS dishes and nonmodified PVA hydrogels with different elasticities for ten days (Fig. 7b) (Kumar et al. 2013). The fold expansions of the analyzed $CD34^+CD38^-$ hHSPCs cells and those analyzed using the ISHAGE guidelines (Keeney et al. 1998) were observed to be the same, within experimental error. Therefore, $CD34^+CD38^-$ cell numbers were used in subsequent experiments to analyze the fold expansion of hHSPCs after $CD34^+$ cells isolated from UCB were cultured on TCPS and PVA hydrogels with and without immobilized CS1 and FN; the results are shown in Fig. 7b, c (Kumar et al. 2013). The expansion of hHSPCs cultured on nonmodified PVA hydrogels was found to increase with increasing PVA hydrogel elasticity (Fig. 7b). Although the E' of the PVA-48 hydrogels was 30.4 kPa, the fold expansion of hHSPCs on PVA-48 hydrogels was found to be almost equal to that of hHSPCs cultured on TCPS, which has the stiffest Young's modulus, i.e., 3 GPa.

The viability of hHSPCs cultured on PVA hydrogels with or without immobilized CS1 and FN was 60–75%, as determined by flow cytometry using a dye (7-AAD) exclusion method. There were no significant differences in the viability of

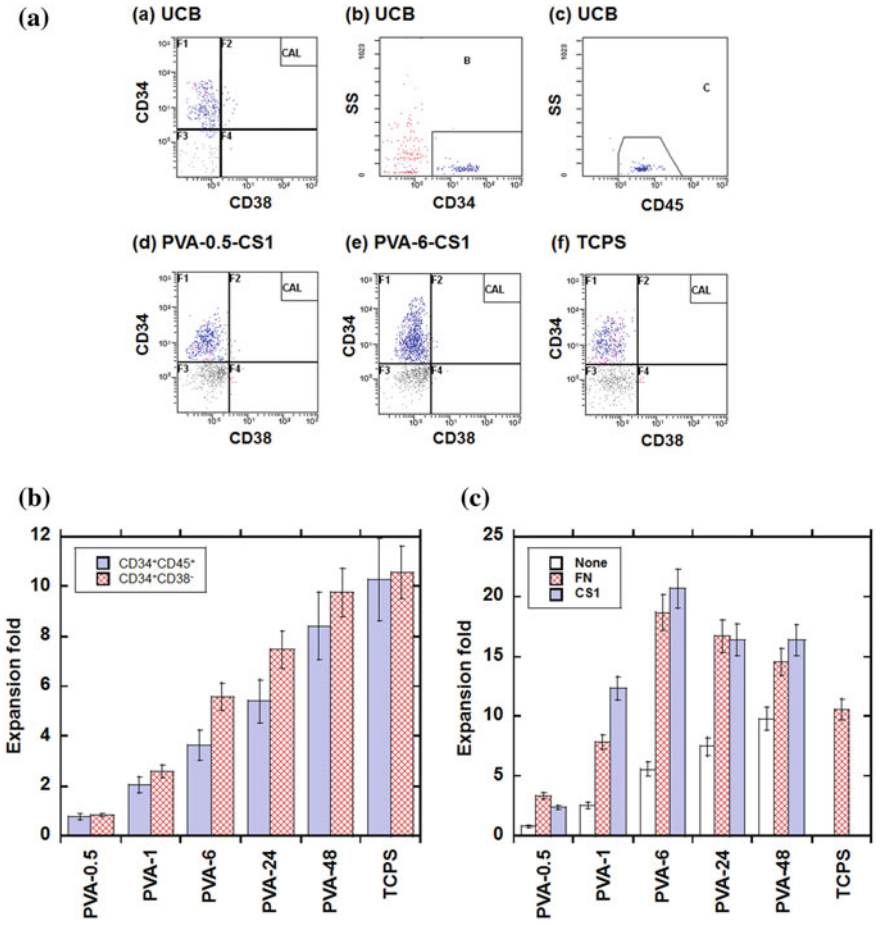


Fig. 7 hHSPC culture on PVA hydrogels with immobilized CS1 and FN. **a** (*a*, *d*, *e*, *f*) CD38 and CD34 expression analyzed by flow cytometry, (*b*) CD34 and side-scattering (SS) expression, and (*c*) CD45 and SS expression, obtained from hHSPCs prior to expansion (*a*, *b*, *c*) and after ten days of cultivation on (*d*) PVA-0.5-CS1 hydrogels, (*e*) PVA-6-CS1 hydrogels, and (*f*) TCPS plates. **b** Expansion (fold) of hHSPCs analyzed by $CD34^{+}CD45^{+}$ cells (blue column, left) and by $CD34^{+}CD38^{-}$ cells (red column, right) after the expansion of hHSPCs for ten days on several PVA hydrogels. **c** Expansion (fold) of hHSPCs ($CD34^{+}CD38^{-}$ cells) after the expansion of hHSPCs for ten days on PVA hydrogels (white column, left), PVA-FN hydrogels (red column, middle), PVA-CS1 hydrogels (blue column, right), and TCPS plates (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

hHSPCs cultured on nonmodified and surface-modified PVA hydrogels ($p > 0.05$). Only the number of viable hHSPCs was evaluated in this study.

The expansion of hHSPCs cultivated on PVA-6-CS1, PVA-FN-6, PVA-24-CS1, PVA-FN24, PVA-48-CS1, and PVA-48 hydrogels was higher than the expansion of hHSPCs cultured on TCPS ($p < 0.05$). In particular, although there was no

statistically significant difference, the expansion of hHSPCs cultured on PVA-*X*-CS1 hydrogels (X = 6–48) was found to be higher than that of hHSPCs cultured on PVA-*X*-FN hydrogels that had been prepared with the same crosslinking times ($p > 0.05$). PVA-6-CS1 and PVA-6-FN hydrogels, with an E' of 12.2 kPa, were selected as the optimal hydrogels for high-expansion hHSPC cultures in this study.

From Engler's hypothesis, it is generally believed that the optimal method of differentiating stem cells into specific lineages in vitro is to culture the stem cells on materials having the same elasticity as the tissue of interest (Engler et al. 2006). If we follow Engler's hypothesis, the optimal elasticity of hydrogels cultured for hHSPC expansion should coincide with the elasticity of bone marrow, which was reported to be 150 Pa (Higuchi et al. 2013). However, our present results indicate that using softer PVA hydrogels grafted with CS1 and FN (i.e., PVA-1-CS1 and PVA-1-FN, having E' values of 10.3 kPa, and PVA-0.5-CS1 and PVA-0.5-FN, having E' values of 3.7 kPa) for hHSPC expansion is less effective than using moderately stiff PVA hydrogels with immobilized CS1 and FN (i.e., PVA-6-CS1 and PVA-6-FN, having an E' of 12.2 kPa). In summary, we noted that Engler's hypothesis is not applicable for hHSPC expansion on PVA hydrogels with and without immobilized CS1 and FN.

XPS analysis indicated that the surface densities of CS1 on PVA-1-CS1, PVA-6-CS1, PVA-24-CS1, and PVA-48-CS1 were nearly equal. It should be mentioned that the surface density of FN on PVA-6-FN was much less than that on PVA-24-FN and PVA-48-FN. This result suggests that the key factor rendering the PVA-6-CS1 and PVA-6-FN hydrogels optimal for hHSPC expansion was the elasticity of these hydrogels rather than the surface densities of FN and CS1 (Kumar et al. 2013).

2.3 *Analysis of hHSPCs Expanded on Nonmodified and Surface-Modified PVA Hydrogels via Colony-Forming Assays*

The quality of the hHSPCs was characterized through colony-forming unit (CFU) assays after the hHSPCs were expanded on TCPS and PVA hydrogels with and without immobilized CS1 and FN. The macroscopic [(a–e)] and microscopic [(f–j)] morphologies of the colonies cultured on the methylcellulose gels for 14 days using the same hHSPC seeding densities (450 cells/dish) are shown in Fig. 8 (Kumar et al. 2013). Colonies originating from the hHSPCs could be clearly observed in each dish. The number of each type of colony (i.e., CFU-GEMM, CFU-GM, CFU-M, CFU-G, BFU-E, and CFU-E) in each dish was analyzed for hHSPCs cultured on TCPS and PVA hydrogels with and without immobilized CS1 and FN (Fig. 9) (Kumar et al. 2013). It was found that the total numbers of colonies generated from hHSPCs cultured on each PVA hydrogel were higher than the total numbers of colonies generated from hHSPCs cultured on TCPS ($p < 0.05$).

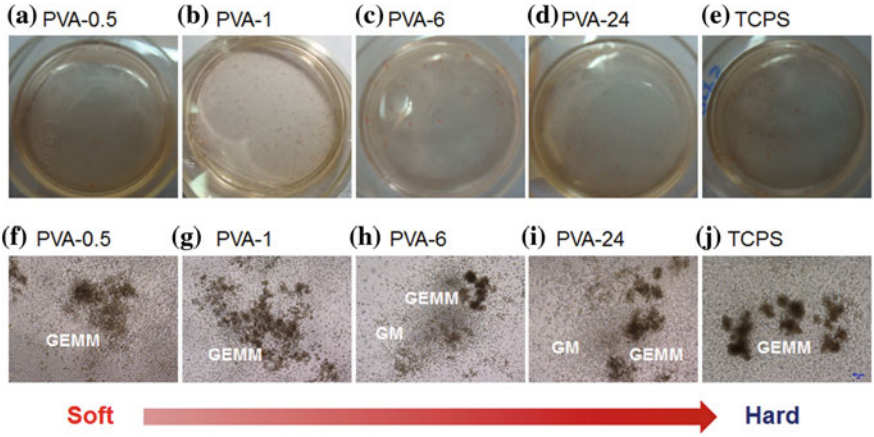


Fig. 8 Macroscopic and microscopic images of colonies on methylcellulose gels generated from hHSPCs after expansion for 14 days on PVA-0.5 hydrogels **a** and **f**, PVA-1 hydrogels **b** and **g**, PVA-6 hydrogels **c** and **h**, PVA-24 hydrogels **d** and **i**, and TCPS plates **e** and **j** at a seeding density of 450 hHSPCs/dish (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

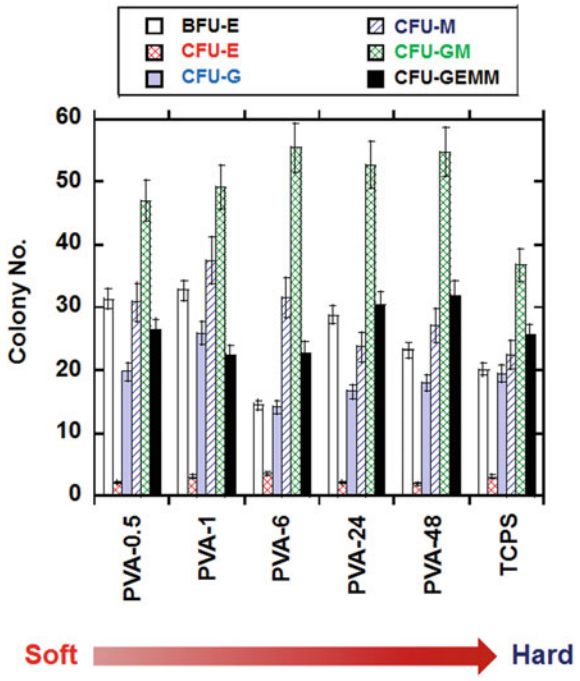


Fig. 9 CFU numbers (CFU-GEMM, CFU-GM, CFU-M, CFU-G, CFU-E, and BFU-E colonies from the right column to left column) originating from hHSPCs after expansion for 14 days on PVA hydrogels and TCPS plates at a seeding density of 450 hHSPCs/dish (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

CFU-GEMMs and CFU-GMs, the numbers of pluripotent colonies, are valuable indices for evaluating hHSPC expansion efficiency. Figure 10 represents the numbers of CFU-GEMMs and CFU-GMs produced by hHSPCs cultivated on TCPS and PVA hydrogels with and without immobilized CS1 and FN (Kumar et al. 2013). The numbers of both CFU-GEMMs and CFU-GMs generated from the hHSPCs cultured on PVA-6-FN, PVA-6-CS1, PVA-24-FN, PVA-24-CS1, PVA-48-FN, and PVA-48-CS1 hydrogels were higher than those cultivated on TCPS ($p < 0.05$), which was similar to the trend observed in the fold expansion of the hHSPCs. The number of CFU-GMs arising from hHSPCs cultivated on PVA-6-CS1 hydrogels was found to be the highest among all PVA-*X*-CS1 hydrogels evaluated in this study (<0.05), whereas the numbers of CFU-GEMMs and CFU-GMs produced from hHSPCs cultivated on PVA-6-FN hydrogels were slightly less than those produced from hHSPCs cultivated on PVA-24-FN and PVA-48-FN hydrogels ($p < 0.05$). These results can probably be attributed to the low surface density of FN on PVA-6-FN hydrogels compared with that on PVA-24-FN and PVA-48-FN hydrogels (Fig. 5), whereas the surface density of CS1 on PVA-6-CA1 hydrogels was almost the same as that on PVA-24-CS1 and PVA-48-CS1 hydrogels. We were not able to enhance the surface density of CS1 or FN on the soft PVA hydrogels (e.g., CS1 immobilized on PVA-0.5 hydrogels and FN immobilized on PVA-0.5, PVA-1, and PVA-6 hydrogels), even though we increased the concentration of CS1 and FN in the reaction solution as well as the reaction time (e.g., 48–60 h). It was found that the physical properties of appropriate cell culture materials (e.g., hydrogels) guided the expansion of hHSPCs and the formation of pluripotent colonies when the elasticity (E') ranges from 12 kPa (PVA-6-CS1 and PVA-6-FN) to 25 kPa (PVA-24-CS1 and PVA-24-FN) and when

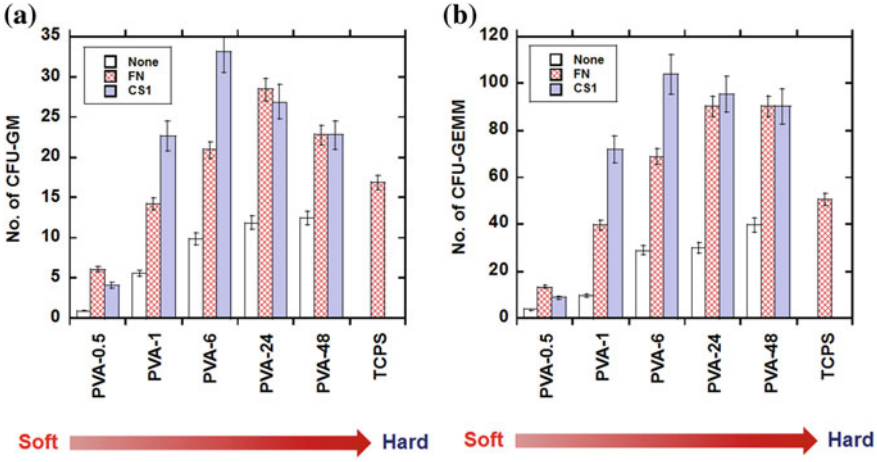


Fig. 10 Numbers of CFU-GEMM and CFU-GM colonies originating from hHSPCs after expansion for 14 days on PVA hydrogels (white column, left), PVA-FN hydrogels (red column, middle), PVA-CS1 hydrogels (blue column, right), and TCPS plates at a seeding density of 450 hHSPCs/dish (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

the cell culture materials have the optimal surface density of cell-binding molecules, such as CS1 and FN (Kumar et al. 2013).

2.4 hHSPC Culture on Hydrogels Having Optimal Elasticity

CS1 is one of the most valuable domains of FN and bind to surface receptors on hematopoietic progenitors in early stages (Kerst et al. 1993). The VLA-4 integrin receptor, $\alpha_4\beta_1$, is known to bind to CS1 and appears on hHSPCs in early stages (Kerst et al. 1993). The signal transduction pathways generated by the binding of FN and CS1 to the VLA-4 integrin receptor on hHSPCs exert key effects on the expansion (Figs. 7B, C) and stemness maintenance of HSPCs (Fig. 10).

The total number of hHSPC colonies produced after hHSPC expansion is calculated from both the fold expansion of hHSPCs (Fig. 7B, C) and the number of colonies per the initial seeding density (Fig. 10, 450 HSPCs/dish). Total CFU expansion is defined as follows:

$$\begin{aligned}\text{Total CFU expansion} = &(\text{hHSPC fold expansion}) \\ &\times (\text{Number of CFUs per 450 hHSPCs}) \end{aligned} \quad (1)$$

If the total CFU expansion is higher than that of UCB without expansion, then the hHSPCs generated after expansion were more able to produce CFUs than the initial UCB hHSPCs before expansion, indicating the utility of the hHSPC expansion.

Total CFU expansion calculated via hHSPC expansion (Fig. 7) and the CFU assay (Fig. 10) of hHSPCs after expansion on PVA hydrogels with and without immobilized CS1 and FN are summarized in Fig. 11 (Kumar et al. 2013). The hHSPCs cultivated on the hydrogels, except the PVA-0.5, PVA-0.5-FN, PVA-0.5-CS1, and PVA-1 hydrogels, exhibited a higher expansion than that of UCB ($p < 0.01$). In particular, the hHSPCs cultivated on the PVA-6-FN, PVA-24-FN, PVA-48-FN, PVA-6-CS1, PVA-24-CS1, and PVA-48-CS1 hydrogels expressed a greater total CFU expansion than hHSPCs cultivated on TCPS ($p < 0.01$).

UCB is considered to be an important source of hHSPCs because much lower risk of graft-versus-host disease (GVHD) has been reported to be associated with UCB transplantation than with peripheral blood or bone marrow transplantation (Chua et al. 2006; Higuchi et al. 2009; Feng et al. 2006; Jiang et al. 2006). In addition, donors have no risk for side effects when UCB is collected for hHSPC transplantation, whereas several side effects for donors have been reported when hHSPCs are obtained from bone marrow or peripheral blood (Copelan 2006). A total of 1.7×10^7 $CD34^+$ cells/kg are reportedly used for UCB transplantation (Chua et al. 2006). As such, UCB transplantation is restricted to use in children weighing less than or equal to 20 kg (Fujimoto et al. 2007; Franke et al. 2007). The main disadvantage of UCB transplantation is the low cell number involved,

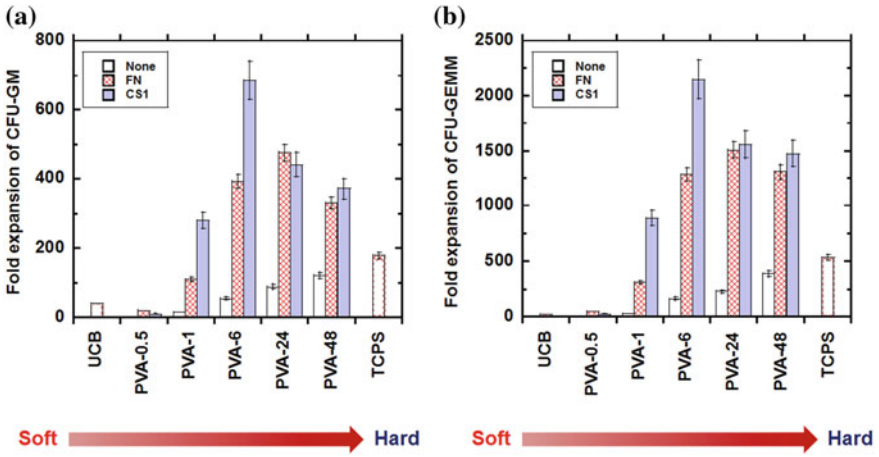


Fig. 11 CFU fold expansion (CFU-GEMM and CFU-GM colonies) of fresh hHSPCs isolated from UCB using the Ficoll–Paque method followed by MACS (UCB), or hHSPCs after expansion for 14 days on PVA-*X* hydrogels (white column, left), PVA-*X*-FN hydrogels (red column, middle), PVA-*X*-CS1 hydrogels (blue column, left), and TCPS plates at a seeding density of 450 hHSPCs/dish, where *X* is the reaction time (h) (Kumar et al. 2013). Copyright 2013. Adapted with permission from Elsevier Ltd.

which leads to prolonged engraftment times and higher percentages of engraftment failure compared with those of the bone marrow transplantation. Several ideas have been considered for hHSPC expansion to reduce the required engraftment time and decrease the engraftment failure rate, particularly for the development of UCB transplantation for adult patients (Fujimoto et al. 2007; Franke et al. 2007). Toward this goal, several investigators have studied the development of cell culture materials for ex vivo hHSPC expansion through mimicking the microenvironment of bone marrow (Chua et al. 2006; Feng et al. 2006; Jiang et al. 2006; Mendez-Ferrer et al. 2010; Di Maggio et al. 2011). However, the effect of and the optimal stiffness of cell culture materials have not yet been investigated in the context of hHSPC expansion. Therefore, the effect of the elasticity of PVA hydrogels with immobilized CS1 and FN on hHSPC expansion was investigated in this study. The elasticity of the bone marrow microenvironment is reported to be 150 kPa (Higuchi et al. 2013), whereas the optimal elasticity of PVA hydrogels for hHSPC expansion was determined to be 12–30 kPa in this study. Engler's idea (Engler et al. 2006) that stem cells tend to differentiate into specific tissue lineages most efficiently when the stem cells are cultivated on materials having an elasticity similar to the tissue of interest does not explain the ex vivo hHSPC expansion observed in this study. Furthermore, the hHSPCs cultivated on PVA hydrogels with immobilized CS1 and FN were found to expand more efficiently and produce higher numbers of multipotent CFU-GEMMs and CFU-GMs than hHSPCs cultivated on nonmodified PVA hydrogels (Kumar et al. 2013).

3 Pluripotent Maintenance of HAFSCs on Hydrogels Having Optimal Elasticity

MSCs are easily obtainable stem cells that can be used for clinical applications. hAFSCs, which are stem cells obtained from amniotic fluid, are pluripotent fetal cells. hAFSCs are capable of differentiating into cells representing the three embryonic germ layers (Zheng et al. 2008; De Coppi et al. 2007; Higuchi et al. 2011a, b). hAFSCs should be a more promising stem cell source for translational medicine than hESCs and hiPSCs because there are no ethical concerns about using hAFSCs, as there are for hESCs. Furthermore, hAFSCs do not raise concerns of xenogeneic contamination generated from the use of a feeder layer (e.g., mouse embryonic fibroblasts, MEFs), as do hESCs and hiPSCs. Stem cell properties, such as pluripotency maintenance and appropriate differentiation into desired lineages, are known to be controlled by their microenvironment. Therefore, mimicking the microenvironments of the stem cells using natural polymers, such as ECMs, should be a good strategy. This strategy may promote the production of the large numbers of stem cells and specifically differentiated cells that are in demand for translational medicine (Dellatore et al. 2008). The goal of this chapter is to discuss (a) which ECM-derived oligopeptides or ECMs immobilized onto the hydrogels are efficient for the maintenance of hAFSC pluripotency and (b) the optimal hydrogel elasticity for the maintenance of hAFSC pluripotency.

3.1 Culture on hAFSCs on Hydrogels Having Optimal Elasticity

hAFSCs were cultivated on (a) TCPS, (b) TCPS plates coated with CELLstart, (c) PVA hydrogels grafted with several oligopeptides derived from ECMs (PVA-oligoECM), (d) PVA hydrogels grafted with several ECMs (PVA-ECM) and nonmodified PVA in expansion medium. The ECMs used in this chapter are vitronectin (VN), fibronectin (FN), and collagen type I (COL). The oligoECMs used in this chapter are oligoVN (KGGPQVTRGDVFTMP), oligoFN (KGGAVTGRGDSPASS), COL-A (GTPGPQGIAGQRGVV), and COL-B ($(RADA)_4$GGDGEA). Figure 12 shows the morphology of hAFSCs cultivated for seven days on (a) PVA hydrogels and PVA-COL hydrogels having elasticities of E' = 25 kPa (24 h crosslinking time), 12 kPa (6 h crosslinking time), 11 kPa (4 h crosslinking time), and 10 kPa (2 h crosslinking time) and (b) TCPS and TCPS dishes coated with CELLstart with an elasticity of 3 GPa (Wang et al. 2015). hAFSCs were not able to be cultivated on PVA hydrogels with and without immobilized COL when the PVA hydrogels had an E' less than 12 kPa (PVA-2-COL, PVA-2 and PVA-4), whereas hAFSCs could be cultured and expanded on PVA hydrogels with and without immobilized COL when the E' of the PVA hydrogels was more than 12 kPa (i.e., PVA-24-COL, PVA-24, PVA-12-COL,

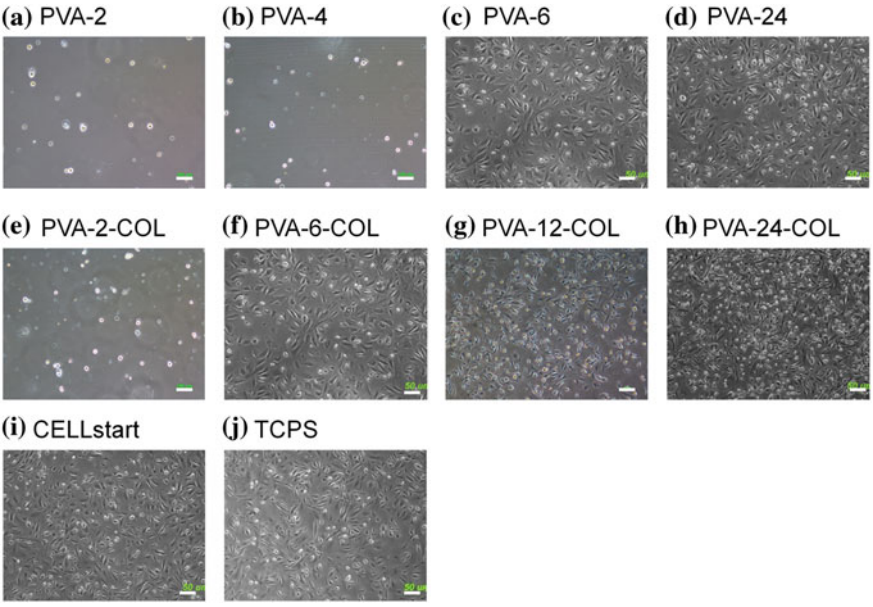


Fig. 12 Morphologies of hAFSCs cultivated on **a** PVA-2 hydrogels, **b** PVA-4 hydrogels, **c** PVA-6 hydrogels, **d** PVA-24 hydrogels, **e** PVA-2-COL hydrogels, **f** PVA-6-COL hydrogels, **g** PVA-12-COL hydrogels, **h** PVA-24-COL hydrogels, **i** TCPS plates coated with CELLstart, and **j** TCPS plates for seven days. The bar represents 50 μm (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

PVA-6-COL, and PVA-6). The stiffest surface, TCPS plates coated with CELLstart (3 GPa elasticity) and TCPS, allowed the culture and expansion of hAFSCs. Therefore, relatively stiff PVA hydrogels with several immobilized ECM-derived oligopeptides and ECMs (PVA-24-oligoECM and PVA-24-ECM), relatively soft PVA hydrogels with several immobilized ECM-derived oligopeptides and ECMs (PVA-6-oligoECM and PVA-6-ECM), and the stiffest plates (TCPS dishes coated with CELLstart and TCPS) were chosen to study the doubling time, expression of differentiation genes, and expression of pluripotent genes or proteins of hAFSCs in this study.

Figure 13 depicts the morphology of hAFSCs cultivated for seven days on relatively stiff (PVA-24) and soft (PVA-6) PVA hydrogels with several immobilized ECM-derived oligopeptides (oligoVN, oligoFN, COL-A, and COL-B) and ECMs (VN, FN, and COL) in expansion medium (Wang et al. 2015). hAFSCs could expand and proliferate on any of these PVA hydrogels with and without immobilized ECM-derived oligopeptides and ECMs.

Figure 14 shows the doubling time of hAFSCs cultured on TCPS dishes coated with CELLstart, TCPS, and PVA hydrogels with and without immobilized ECM-derived oligopeptides and ECMs (Wang et al. 2015). The doubling time of the hAFSCs was found to be approximately 6 days when the hAFSCs were

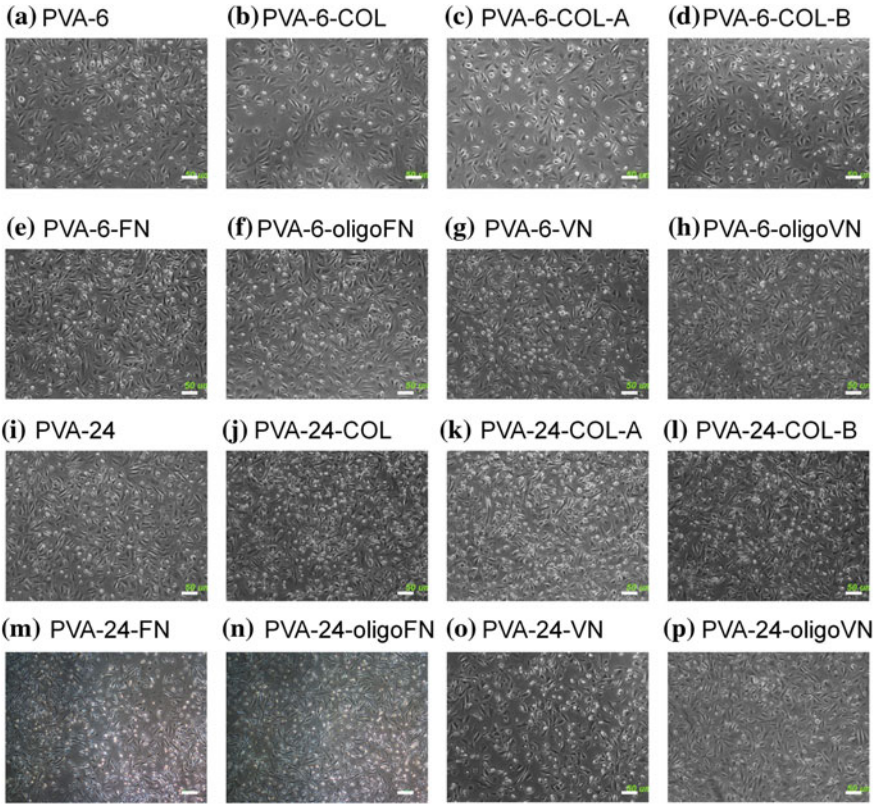


Fig. 13 Morphologies of hAFSCs cultivated on **a** PVA-6 hydrogels, **b** PVA-6-COL hydrogels, **c** PVA-6-COL-A hydrogels, **d** PVA-6-COL-B hydrogels, **e** PVA-6-FN hydrogels, **f** PVA-6-oligoFN hydrogels, **g** PVA-6-VN hydrogels, **h** PVA-6-oligoVN hydrogels, **i** PVA-24 hydrogels, **j** PVA-24-COL hydrogels, **k** PVA-24-COL-A hydrogels, **l** PVA-24-COL-B hydrogels, **m** PVA-24-FN hydrogels, **n** PVA-24-oligoFN hydrogels, **o** PVA-24-VN hydrogels, and **p** PVA-6-oligoVN hydrogels for seven days. The bar represents 50 μm (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

cultivated on relatively stiff PVA-24 hydrogels with and without immobilized ECM-derived oligopeptides and ECMs or on the stiff surface of TCPS and TCPS dishes coated with CELLstart. This doubling time is shorter than that of cells cultivated on the relatively soft PVA-6 hydrogels with and without immobilized ECM-derived oligopeptides and ECMs in expansion medium. The effect of different ECM-derived oligopeptides and ECMs present on PVA hydrogels on the doubling time of the hAFSCs was not clear. The soft surface of the PVA-6 hydrogels led to slow hAFSC growth. Other researchers have also observed slower stem cell growth when the cells were cultivated on softer cell culture materials (Higuchi et al. 2013; Winer et al. 2009). For example, hMSCs cultivated on soft polyacrylamide hydrogels (PAAm, 250 Pa) stopped growing, as reported by Winter

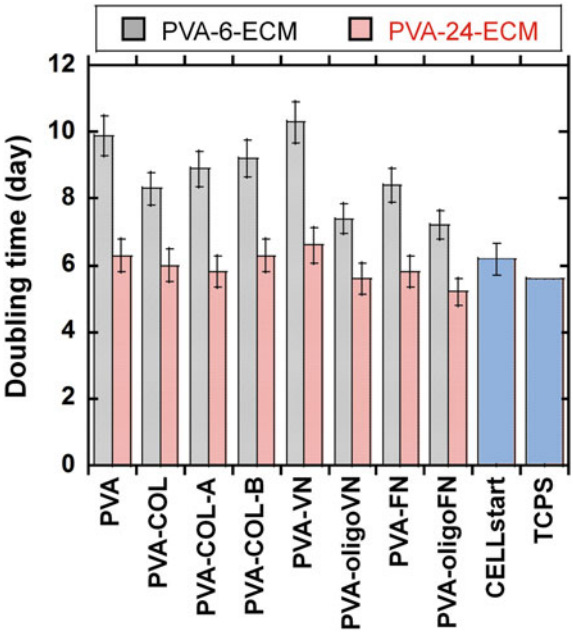


Fig. 14 Doubling time of hAFSCs cultivated on stiff PVA-24 hydrogels (red column) and soft PVA-6 hydrogels (gray column) with immobilized ECMs (FN, VN, and COL) or ECM-derived oligopeptides (oligoFN, oligoVN, COL-A, and COL-B), on TCPS plates coated with CELLstart (blue bar), and on TCPS plates (blue bar) (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

et al. (Higuchi et al. 2013; Winer et al. 2009). These resting hMSCs began to grow again when they were shifted onto stiff hydrogels. Therefore, hMSCs on soft PAAm hydrogels remain quiescent but have the potential to reinitiate growth or to differentiate upon transfer to stiffer materials (Wang et al. 2015; Higuchi et al. 2013; Winer et al. 2009).

3.2 *The Gene Expression of Pluripotency and Differentiation in hAFSCs Cultured on PVA Hydrogels*

We investigated whether slow or fast stem cell growth maintains their pluripotency or facilitates their differentiation. Therefore, hAFSC gene expression relating to differentiation and pluripotency was investigated when hAFSCs were cultivated on stiff and soft PVA hydrogels with and without immobilized ECM-derived oligopeptides and ECMs in expansion medium.

Figure 15 describes the expression of genes related to pluripotency, *Nanog, Sox2,* and *Oct4*, in hAFSCs cultivated on PVA-6-oligoECM (Fig. 15a), PVA-6-ECM (Fig. 15a), PVA-24-oligoECM (Fig. 15b), and PVA-24-ECM (Fig. 15b) hydrogels, as well as TCPS plates coated with CELLstart and TCPS plates (Fig. 15a, b) (Wang et al. 2015). The pluripotency genes of hAFSCs cultivated on the stiffest TCPS plates coated with and without CELLstart expressed the

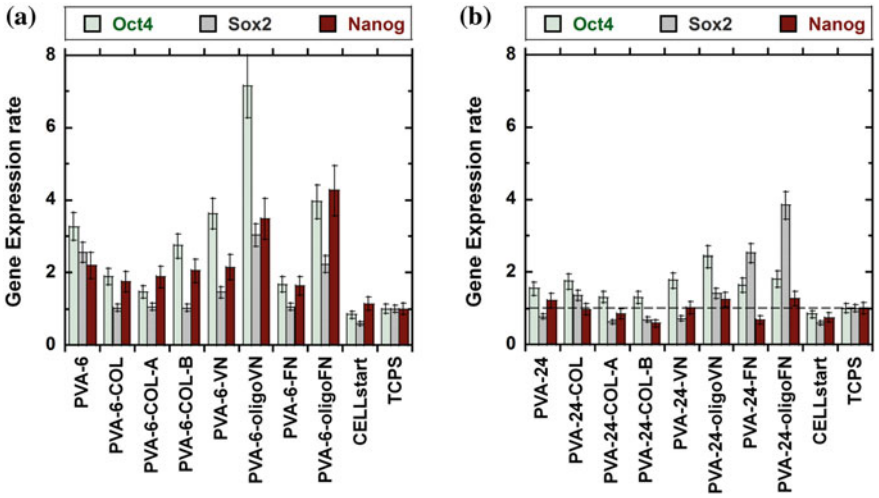


Fig. 15 Pluripotency gene expression (red bar, *Nanog*; black bar, *Sox2*; green bar, *Oct4*) in hAFSCs cultivated on soft PVA-6 hydrogels **a** and stiff PVA-24 hydrogels **b** with immobilized ECMs (FN, VN, and COL) or ECM-derived oligopeptides (oligoFN, oligoVN, COL-A, and COL-B) and on TCPS plates coated with CELLstart and TCPS plates after seven days of culture (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

lowest levels of the genes investigated in this study. The hAFSCs cultivated on the relatively soft PVA-6-oligoECM and PVA-6-ECM hydrogels showed higher gene expression of *Nanog* and *Oct4* (pluripotency genes) compared with that of hAFSCs cultivated on the stiffest surfaces, TCPS dishes coated with CELLstart and TCPS plates ($p < 0.05$). Furthermore, hAFSCs cultivated on the soft PVA-6-oligoECM hydrogels and PVA-6-ECM hydrogels expressed higher levels of pluripotency genes (*Nanog* and *Oct4*) than hAFSCs on the stiff PVA-24-oligoECM and PVA-24-ECM hydrogels.

When the pluripotency gene expression of hAFSCs cultivated on PVA hydrogels immobilized with ECM-derived oligopeptides and ECMs was analyzed, we found no significant differences in the gene expression of hAFSCs on PVA hydrogels with immobilized collagen-derived oligopeptides (COL-A and COL-B) and collagen type 1. However, hAFSCs cultivated on PVA hydrogels with immobilized oligoFN and oligoVN were found to express higher levels of pluripotency genes (*Nanog, Sox2*, and *Oct4*) than hAFSCs cultivated on PVA hydrogels with immobilized fibronectin and vitronectin, respectively ($p < 0.05$). These results suggest that ECM-derived oligopeptides, such as oligo FN and oligoVN, efficiently act as bioactive nanosegments immobilized on PVA hydrogels to maintain hAFSC pluripotency.

We proposed $I_{\text{pluripotency}}$, the index of pluripotency gene expression, which is defined as the sum of the pluripotency gene expression levels of hAFSCs, calculated from the relative gene expression of hAFSCs cultivated on TCPS plates:

$$I_{\text{pluripotency}} = I_{\text{Nanog}} + I_{\text{Sox2}} + I_{\text{Oct4}} \tag{2}$$

where I_{Nanog}, I_{Sox2}, and I_{Oct4}, are the expression levels of *Nanog*, *Sox2*, and *Oct4*, respectively, which are calculated to be relative to the expression of the same genes of hAFSCs cultivated on TCPS plates. Figure 16 depicts $I_{\text{pluripotency}}$ of hAFSCs cultivated on PVA-24 and PVA-6 hydrogels with and without immobilized ECM-derived oligopeptides and ECMs (Wang et al. 2015). hAFSCs cultivated on the soft PVA-6 hydrogels with immobilized oligoFN (PVA-6-oligoFN) and oligoVN (PVA-6-oligoVN) expressed higher $I_{\text{pluripotency}}$ values than hAFSCs cultivated on the stiff PVA-24 hydrogels with and without immobilized ECM-derived oligopeptides or ECMs. Furthermore, hAFSCs grown on the stiff PVA-6-oligoFN and PVA-6-oligoVN showed higher $I_{\text{pluripotency}}$ values than those grown on PVA-6 hydrogels with immobilized ECM or ECM-derived oligopeptides other than oligoVN and oligoFN. It should be noted that hAFSCs cultivated on nonmodified PVA-6 hydrogels expressed relatively high $I_{\text{pluripotency}}$ values among the PVA hydrogels investigated in this study, although hAFSCs cultivated on PVA-6-oligoVN hydrogels showed higher the $I_{\text{pluripotency}}$ values compared to hAFSCs cultivated on nonmodified PVA-6 hydrogels ($p < 0.05$).

The expression of typical differentiation genes of the three germ layers, *Runx2* (mesoderm), *Sox17* (endoderm), and *Nestin* (ectoderm), in hAFSCs was evaluated

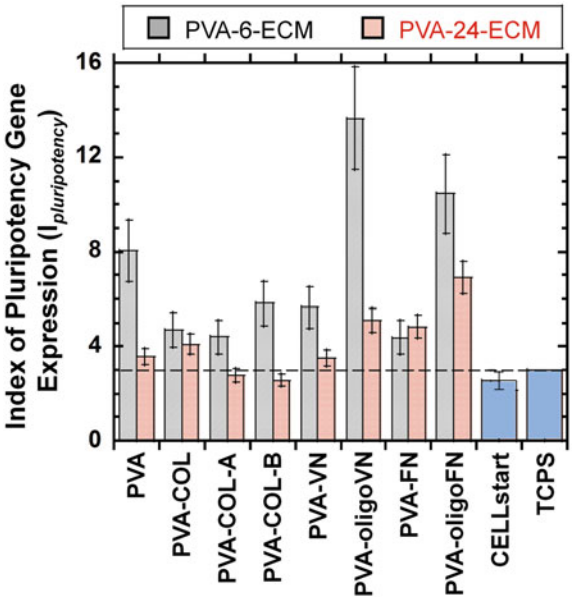


Fig. 16 $I_{\text{pluripotency}}$ (index of pluripotency gene expression) of hAFSCs cultivated on stiff PVA-24 hydrogels (red column) and soft PVA-6 hydrogels (black column) with immobilized ECMs (FN, VN, and COL) or ECM-derived oligopeptides (oligoFN, oligoVN, COL-A, and COL-B) and on TCPS plates coated with CELLstart and TCPS plates for seven days (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

when hAFSCs were cultivated on CELLstart-coated TCPS plates, TCPS, PVA-24 hydrogels, and PVA-6 hydrogels in expansion medium; the results are shown in Fig. 17 (Wang et al. 2015). hAFSCs grown on soft PVA-6 hydrogels with or without ECM-derived oligopeptides and ECMs exhibited more than two-fold higher expression of *Nestin* than hAFSCs grown on TCPS plates. Soft PVA-6-FN, PVA-6-oligoVN, and PVA-6-COL hydrogels seem to be preferable for hAFSCs to spontaneously differentiate into early neural cells, indicated by the high levels of *Nestin* expressed by the hAFSCs.

hAFSCs grown on PVA-6-oligoVN showed high *Sox17* expression compared with that of hAFSCs cultured on PVA with or without immobilized ECM-derived oligopeptides and ECMs and compared with hAFSCs cultured on CELLstart-coated TCPS plates and TCPS. hAFSCs cultured on PVA-6-oligoFN and PVA-6-oligoVN showed higher expression of *Runx2* than did hAFSCs grown on PVA-6 with and without immobilized ECM-derived oligopeptides and ECMs. However, hAFSCs cultivated on the stiff PVA-24-oligoECM and PVA-24-ECM hydrogels exhibited higher expression of *Sox17* and *Runx2* than did hAFSCs cultured on soft PVA-6-oligoECM and PVA-6-ECM hydrogels or stiff TCPS dishes coated with CELLstart and TCPS plates. PVA-oligoFN, PVA-24-FN, PVA-24-oligoVN, and PVA-24-COL-B hydrogels, as well as PVA-6-oligoFN hydrogels, seem to be preferable cell culture materials for spontaneously inducing early stages of osteoblast differentiation, as indicated by the expression of *Runx2*. Furthermore, PVA-24-oligoFN, PVA-24-oligoVN, and PVA-24-COL hydrogels, as well as

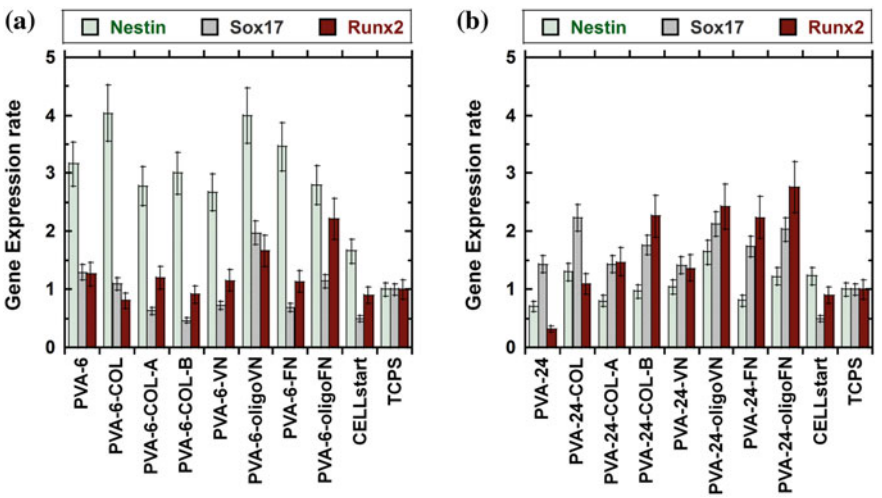


Fig. 17 Expression of differentiation genes (red bar, *Runx2*; black bar, *Sox17*; green bar, *Nestin*) in hAFSCs cultivated on soft PVA-6 hydrogels **a** and stiff PVA-24 hydrogels **b** with immobilized ECMs (FN, VN, and COL) or ECM-derived oligopeptides (oligoFN, oligoVN, COL-A, and COL-B) and on TCPS plates coated with CELLstart and TCPS plates for seven days (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

PVA-6-oligoVN hydrogels, seem to be preferable cell culture materials for spontaneously inducing early stages of endoderm differentiation, as indicated by the expression of *Sox17*.

Here, we propose $I_{\text{differentiation}}$, the index of differentiation gene expression, which is defined as the sum of the differentiation gene expression levels of hAFSCs, calculated from the relative gene expression of hAFSCs cultivated on TCPS plates:

$$I_{\text{differentiation}} = I_{\text{Runx2}} + I_{\text{Sox17}} + I_{\text{Nestin}} \tag{3}$$

where I_{Runx2}, I_{Sox17}, and I_{Nestin} are the expression levels of *Runx2*, *Sox17,* and *Nestin,* respectively, which are calculated to be relative to the expression of the same genes of hAFSCs cultivated on TCPS plates. Figure 18 depicts $I_{\text{differentiation}}$ of hAFSCs cultivated on PVA-24 and PVA-6 hydrogels with and without immobilized ECM-derived oligopeptides and ECMs. hAFSCs grown on stiff and soft PVA-oligoECM and PVA-ECM hydrogels exhibited higher values of $I_{\text{differentiation}}$ than hAFSCs cultured on PVA-24 hydrogels, TCPS plates coated with CELLstart, and TCPS plates (Wang et al. 2015). The $I_{\text{differentiation}}$ of hAFSCs cultivated on PVA-24-oligoECM and PVA-24-ECM hydrogels was almost equal to the $I_{\text{differentiation}}$ of hAFSCs cultured on PVA-6-oligoECM and PVA-6-ECM hydrogels with each ECM-derived oligopeptide or ECM, within experimental error ($p > 0.05$). This is because of compensation via the expression of different genes. Stiff PVA-24-oligoECM and PVA-24-ECM hydrogels extensively induce the spontaneous differentiation of hAFSCs into early osteoblasts (expression of *Runx2*) and early endoderm cells (expression of S*ox17*); in contrast, soft PVA-6-oligoECM

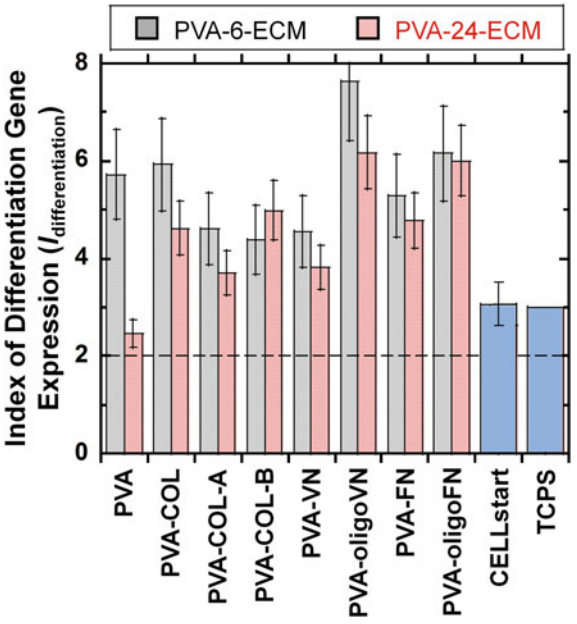


Fig. 18 $I_{\text{differentiation}}$ (index of differentiation gene expression) of hAFSCs cultivated on stiff PVA-24 hydrogels (red column) and soft PVA-6 hydrogels (black column) with immobilized ECMs (FN, VN, and COL) or ECM-derived oligopeptides (oligoFN, oligoVN, COL-A, and COL-B) and on TCPS plates coated with CELLstart and TCPS plates for seven days (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

and PVA-6-ECM hydrogels extensively induce the spontaneous differentiation of hAFSCs into early neural cells (expression of *Nestin*).

The relationship between $I_{\text{pluripotency}}$ and $I_{\text{differentiation}}$ was examined for hAFSCs grown on PVA-oligoECM and PVA-ECM hydrogels in expansion medium (Fig. 19) (Wang et al. 2015). $I_{\text{differentiation}}$ increased with increasing $I_{\text{pluripotency}}$, with a linearity of 0.80. This result may be unexpected because the pluripotency gene expression of hiPSCs and hESCs would be downregulated upon the induction of differentiation (Takahashi et al. 2007). The unexpected result shown in Fig. 19 is considered as follows: The cells used in the experiments were hAFSCs, not hiPSCs or hESCs; hAFSCs, which tend to maintain high stemness on optimal hydrogels having nanosegments, are a heterogeneous population of cells that includes cells that spontaneously differentiated from the hAFSCs. This phenomenon is generally found in cultures of heterogeneous stem cell populations, such as hAFSCs, in expansion medium (Wang et al. 2015).

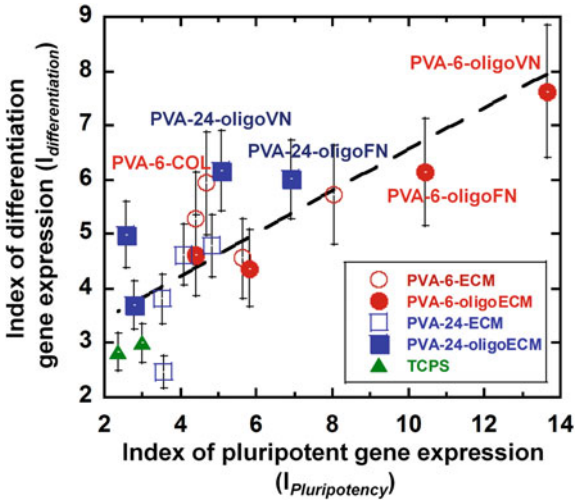


Fig. 19 Relationship between $I_{\text{differentiation}}$ and $I_{\text{pluripotency}}$ for hAFSCs cultivated on stiff PVA-24 hydrogels (blue square) and soft PVA-6 hydrogels (red circle) with immobilized ECMs (open symbols, FN, VN, and COL) or ECM-derived oligopeptides (closed symbols, oligoFN, oligoVN, COL-A, and COL-B) and on TCPS plates coated with CELLstart (green triangle) and TCPS plates (green triangle) for seven days (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

3.3 *The Differentiation and Pluripotency Proteins Expressed on HAFSCs Cultivated on PVA-OligoECM and PVA-ECM Hydrogels*

The differentiation and pluripotency of hAFSCs cultivated on PVA-oligoECM and PVA-ECM hydrogels were investigated from gene expression levels determined in the previous sections (Figs. 15, 16, 17, 18, and 19). Here, we investigated the expression of pluripotency and differentiation surface markers of hAFSCs grown on PVA-oligoECM and PVA-ECM hydrogels.

Figures 20 and 21 represent the expression levels of the surface markers SSEA4 and Sox2 (pluripotency markers), the differentiation protein marker AFP (hepatocytes, endoderm), and β-III tubulin (neural cells, ectoderm) on hAFSCs cultivated on the TCPS plates, stiff PVA-24 hydrogels (PVA-24-oligoFN, PVA-24-oligoVN, PVA-24-FN, PVA-24-VN, and PVA-24), and soft PVA-6 hydrogels (PVA-6-oligoFN, PVA-6-oligoVN, PVA-6-FN, PVA-6-VN, and PVA-6) (Wang et al. 2015). These surface markers were evaluated because oligoFN and oligoVN

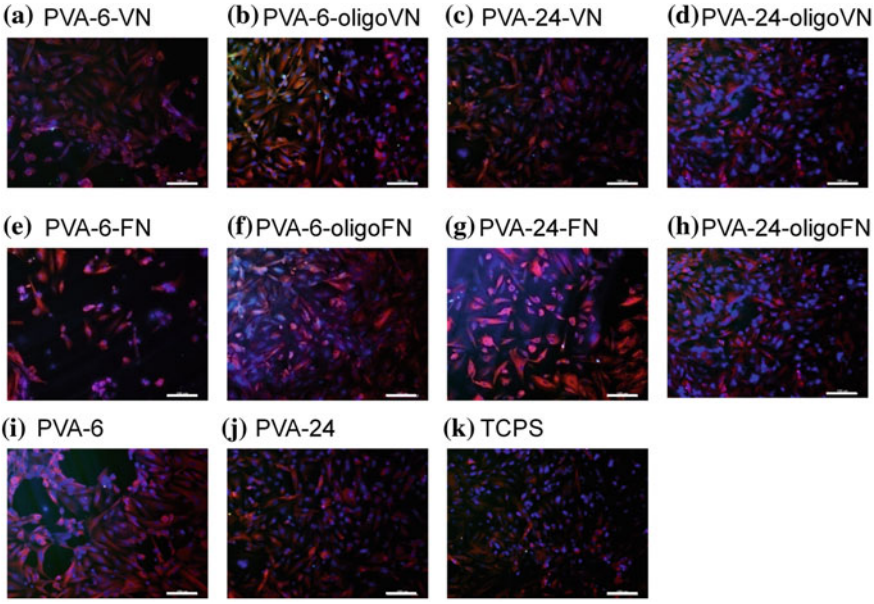


Fig. 20 Differentiation marker expression of β-III tubulin (red) and pluripotency marker expression of Sox2 (green) in hAFSCs cultivated on **a** PVA-6-VN hydrogels, **b** PVA-6-oligoVN hydrogels, **c** PVA-24-VN hydrogels, **d** PVA-24-oligoVN hydrogels, **e** PVA-6-FN hydrogels, **f** PVA-6-oligoFN hydrogels, **g** PVA-24-FN hydrogels, **h** PVA-24-oligoFN hydrogels, **i** PVA-6 hydrogels, **j** PVA-24 hydrogels, and TCPS plates **k** after seven days of culture. Nuclei were stained with Hoechst 33,342 (blue) (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

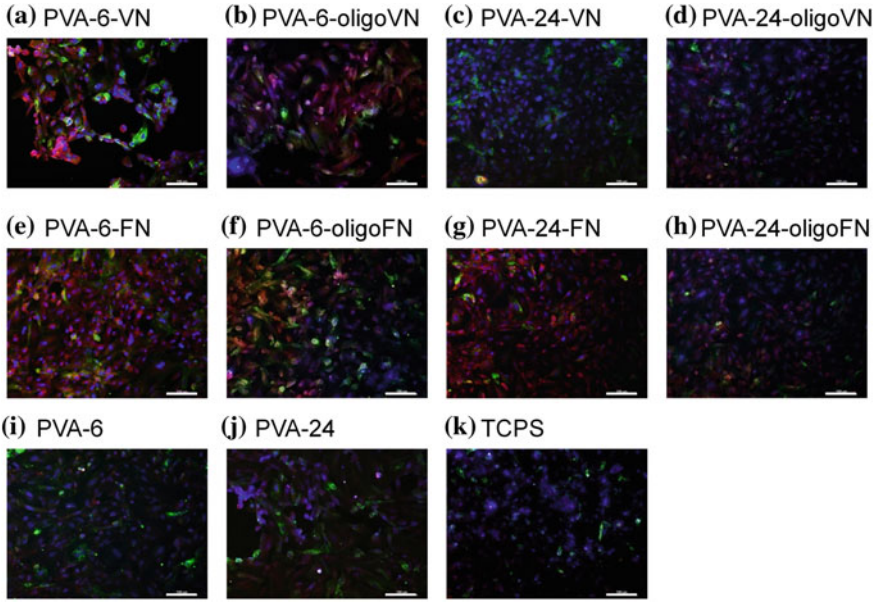


Fig. 21 Differentiation marker expression of AFP (red) and pluripotency marker expression of SSEA4 (green) in hAFSCs cultivated on (**a**) PVA-6-VN hydrogels, (**b**) PVA-6-oligoVN hydrogels, (**c**) PVA-24-VN hydrogels, (**d**) PVA-24-oligoVN hydrogels, (**e**) PVA-6-FN hydrogels, (**f**) PVA-6-oligoFN hydrogels, (**g**) PVA-24-FN hydrogels, (**h**) PVA-24-oligoFN hydrogels, (**i**) PVA-6 hydrogels, (**j**) PVA-24 hydrogels, and TCPS plates (**k**) for seven days. Nuclei were stained with Hoechst 33,342 (blue) (Wang et al. 2015). Copyright 2015. Adapted with permission from the Royal Society of Chemistry

tend to maintain hAFSC pluripotency as well as trigger the spontaneous differentiation of hAFSCs when immobilized on PVA hydrogels.

Slightly high expression of Sox2 was observed in hAFSCs cultivated on soft PVA-6-oligoFN and PVA-6-oligoVN hydrogels (Fig. 20), which was similar to the earlier trend in *Sox2* expression observed in hAFSCs (Fig. 15a). We also noticed that hAFSCs cultivated on soft PVA-6-oligoVN, PVA-6-oligoFN, PVA-6-VN, and PVA-6-FN hydrogels exhibited high expression of SSEA4 (Fig. 21). hAFSCs grown on the stiffest materials, the TCPS plates, showed only the faintest expression of SSEA4 and Sox2. The soft PVA-6 hydrogels with immobilized oligoVN and oligoFN were found to be favorable for supporting hAFSC pluripotency, as indicated by the surface marker expression of SSEA4 and Sox 2; these results showed a trend similar to that observed in the gene expression of *Nanog, Sox2*, and *Oct4* in hAFSCs (Fig. 15a).

hAFSCs grown on stiff and soft PVA hydrogels with and without immobilized oligoECMs and ECMs exhibited similar expression of β-III tubulin (Fig. 20). High expression of AFP was found in hAFSCs grown on soft PVA-6, PVA-6-oligoVN, PVA-6-oligoFN, PVA-6-VN, and PVA-6-FN hydrogels, whereas hAFSCs

displayed only faint expression of AFP when they were cultivated on stiff TCPS plates and PVA-24 hydrogels with and without immobilized oligoECMs and ECMs (Fig. 21). Soft PVA-6-oligoVN hydrogels seem to be favorable cell culture biomaterials for the induction of spontaneous hAFSC differentiation into early-stage hepatocytes due to the high expression of AFP (Fig. 21) as well as the high expression of Sox17 (Fig. 17a) in the hAFSCs (Wang et al. 2015).

3.4 *Comparison of the Present Study with Previously Published Studies*

Engler et al. presented a hypothesis for the effect of elasticity of cell culture matrices on the spontaneous differentiation of hBMSCs into some cell lineages (i.e., early-stage neural cells, muscle cells, and osteoblasts) depending on the elasticities of the matrices (Engler et al. 2006); when hBMSCs were cultivated in expansion medium containing low serum concentrations, stiff matrices with elasticity similar to that of collagenous bone induced hBMSCs toward early osteoblast differentiation. Softer matrices having elasticity similar to that of the brain (0.3 kPa) induced the differentiation of hBMSCs into early neural cell lineages, whereas stiffer matrices (10 kPa) mimicking muscles spontaneously induced hBMSCs to differentiate into early myoblasts (Engler et al. 2006; Higuchi et al. 2013; 2015b). The effect of PVA hydrogel elasticity on the differentiation and pluripotency of hAFSCs was investigated in this study. hAFSCs spontaneously differentiated into early neural cells on soft PVA-6 hydrogels with immobilized oligoECMs and ECMs, which were identified by the expression of *Nestin*, whereas hAFSCs grown on stiff PVA-24 hydrogels with immobilized oligoECMs and ECMs spontaneously differentiated into early-stage osteoblasts, which were identified by the expression of *Runx2*, similar to the results reported by Engler (Engler et al. 2006; Higuchi et al. 2013; 2015b). However, hAFSCs grown on the stiffest surface, TCPS plates, did not spontaneously differentiate into osteoblasts, but instead exhibited the lowest differentiation and pluripotency in this study, as indicated by the evaluation of both gene and surface marker expression.

Tse and Engler made PAAm hydrogels using photopolymerization and a gradient-patterned photomask, which had radial elastic modulus gradients from 1 to 14 kPa and 1 kPa/mm (Higuchi et al. 2013; 2015b; Tse and Engler 2011). hBMSCs were observed to migrate from softer hydrogels into stiffer hydrogels in expansion medium and subsequently differentiate into contractile myogenic cells. In contrast, hBMSCs expressing neuronal markers tended to stay on soft sites of the PAAm hydrogels (Tse and Engler 2011). The soft cell culture hydrogels were suggested to induce hBMSCs to differentiate toward neuronal lineages in expansion medium (Engler et al. 2006; Higuchi et al. 2013; 2015b). These results are consistent with those found in this study; hAFSCs grown on soft PVA-6 hydrogels with immobilized oligoECMs and ECMs preferred to maintain pluripotency and to express

Nestin compared with hAFSCs cultivated on stiff TCPS plates and PVA-24 hydrogels. However, PVA hydrogels having an E' of less than 11 kPa were found to be too soft to support hAFSC proliferation in this study, despite having several immobilized oligoECMs and ECMs (Fig. 12).

Gilbert et al. evaluated whether the elastic modulus of cell culture materials serves an important factor in the differentiation or self-renewal of muscle stem cells (MuSCs) during muscle regeneration (Gilbert et al. 2010; Higuchi et al. 2013; 2015b). They prepared crosslinked poly(ethylene glycol) (PEG) hydrogels with immobilized laminin that had different elastic moduli (2–42 kPa). The MuSCs were found to expand more on soft PEG hydrogels than on stiff TCPS plates (Gilbert et al. 2010). In addition, the MuSCs showed higher cell survival when grown on soft PEG hydrogels. Furthermore, it was found that the MuSCs grown on soft PEG hydrogels expressed less myogenin (differentiation marker) than the cells grown on stiff TCPS plates (Gilbert et al. 2010; Higuchi et al. 2013; 2015b). Soft cell culture materials seemed to increase the number of MuSCs by restricting MuSC differentiation as well as by enhancing cell viability (Gilbert et al. 2010). In contrast, our studies showed that hAFSCs had high pluripotent gene expression and expressed several differentiation genes when grown on soft PVA-6 hydrogel dishes grafted with oligoECMs and ECMs (Figs. 15A and 17A) (Wang et al. 2015). These conflicting results may be related to the heterogeneous population of hAFSCs, as MuSCs are not a heterogeneous cell population.

Saha et al. prepared hydrogels having an interpenetrating polymer network with oligopeptides containing the cell-binding sequence RGD and elasticity ranging from 10 Pa to 10 kPa (Higuchi et al. 2013; Saha et al. 2008). The hydrogels required elastic moduli higher than 0.1 kPa for the expansion of rat neural stem cells. The expression of the neural marker β-III tubulin was found to be the highest in the rat neural stem cells cultured on the hydrogels with an elastic modulus of 0.5 kPa, which is similar to the physiological elasticity of brain tissue (Higuchi et al. 2013; Saha et al. 2008). The differentiation of rat neural stem cells into neurons was preferentially induced on softer hydrogels in the differentiation medium. On the contrary, differentiation into glial cells was preferentially induced on stiffer hydrogels (Higuchi et al. 2013; Saha et al. 2008). These results indicated that physical cues (i.e., hydrogel stiffness) are key factors involved in the differentiation and proliferation of stem cells, which was also demonstrated by the spontaneous differentiation and pluripotency of hAFSCs grown on PVA hydrogels of different stiffness in this study.

The soft PVA-6-oligoVN hydrogels could support hAFSC pluripotency, as indicated by high pluripotency gene expression (Figs. 15 and 16) and high surface marker expression (Figs. 20 and 21). The amino acid sequence of the oligoVN chosen in this study has been used for cultivating hESCs and hiPSCs that maintain their pluripotency, as reported in the literature (Higuchi et al. 2015a; Melkoumian et al. 2010; Higuchi et al. 2014a). hPSCs were reported to have been expanded for over ten passages on the plates with immobilized oligoVN without differentiating, whereas hPSCs could not be expanded on plates with immobilized peptides obtained from fibronectin and laminin because they differentiated (Higuchi et al.

2014a; 2015a; Melkoumian et al. 2010) Therefore, oligoVN tends to maintain stem cell pluripotency, for both hPSCs and hAFSCs. Therefore, both the physical cues (e.g., low elasticity) and biochemical cues (e.g., specific sequence of oligopeptides such as oligoVN) of PVA-6-oligoVN seem to contribute to better maintenance of hAFSC pluripotency than the other cell culture hydrogels evaluated in this study (Wang et al. 2015).

4 Xeno-Free Culture of HPSCs on Hydrogels with Optimal Elasticity

hPSCs should be cultured on biomaterials in chemically defined and xeno-free conditions if they are to be considered for use in regenerative medicine. Currently, hPSC cultures for maintaining pluripotency are typically performed using the following conditions: (a) cultivation on feeder layers of MEFs or human fibroblasts or (b) cultivation on Geltrex or Matrigel (Higuchi et al. 2011b, 2014b); Geltrex and Matrigel are produced from an extract of Engelbreth-Holm-Swarm mice. Therefore, the above typical culture methods of hPSCs are undefined and contain components of xeno origin, which hinder the clinical usage of hPSCs.

There is an increasing demand for the development of cell culture materials for hPSC culture on xeno-free and feeder-free conditions for the clinical usage of hPSCs. Some cell culture biomaterials have been proposed for culturing hPSCs and maintaining their pluripotency under xeno-free conditions. These biomaterial designs rely on the immobilization of biological cues, such as oligoECMs (Park et al. 2015; Chen et al. 2014; Wu et al. 2014; Fan et al. 2014; Deng et al. 2013; Pennington et al. 2015; Lin et al. 2014; Higuchi et al. 2014a), ECMs (Jonas et al. 2013; Liu et al. 2014; Lu et al. 2014; Rodin et al. 2010; Miyazaki et al. 2012; Tsutsui et al. 2011), and heparin-mimicking polymers (Brafman et al. 2010; Zhang et al. 2013; Villa-Diaz et al. 2010; Qian et al. 2014; Nandivada et al. 2011), on cell culture plates.

Cell culture plates with immobilized ECMs, such as fibronectin (CELLstart), laminin (laminin-322, laminin-521, and laminin-511), and vitronectin, have resulted in good hPSC proliferation in chemically defined media (Jonas et al. 2013; Liu et al. 2014; Lu et al. 2014; Rodin et al. 2010; Miyazaki et al. 2012; Tsutsui et al. 2011). Cell culture biomaterials grafted with oligoECM have also been found to support the pluripotency of hPSCs in chemically defined media (Park et al. 2015; Chen et al. 2014; Wu et al. 2014; Fan et al. 2014; Deng et al. 2013; Pennington et al. 2015; Lin et al. 2014; Higuchi et al. 2014a, b). Synthetic polymer plates, such as (a) copoly[2-(acryloyloxyethyl)] trimethylammonium-co-2-(diethylamino)ethyl acrylate] (Zhang et al. 2013), (b) aminopropylmethacrylamide (Irwin et al. 2011), (c) poly[2-(methacryloyloxy](ethyldimethyl-(3-sulfopropyl) ammonium hydroxide (Nandivada et al. 2011; Qian et al. 2014; Villa-Diaz et al. 2010), and (d) poly (methylvinylether-alt-maleic anhydride) (Brafman et al. 2010), have also been

reported to be feasible cell culture plates for hPSC culture in chemically defined medium.

The biological cues and physical cues, such as stiffness of the cell culture materials, have been suggested to guide stem cell pluripotency and differentiation fate (Engler et al. 2006; Higuchi et al. 2013). Several previous studies investigated the effect of the cell culture hydrogel stiffness on the differentiation fate and pluripotency of mouse ESCs and hBMSCs (Engler et al. 2006; Higuchi et al. 2013; Kumar et al. 2013; Chowdhury et al. 2010). However, the effect of the cell culture hydrogel stiffness on the expansion of hESCs has not yet been reported, aside from our study (Higuchi et al. 2015a). Therefore, we prepared PVA hydrogels with immobilized oligoVN to investigate the physical effect of hydrogel stiffness on the expansion of hiPSCs and hESCs. The PVA hydrogels were controlled to have different stiffnesses by regulating the crosslinking time. OligoVN was covalently bound to the carboxylic acid site of the PVA hydrogels using an EDC/NHS reaction. The goal of this study was to determine the optimal stiffness of PVA-oligoVN hydrogels for the long-term (10–20 passages) culture of hESCs and hiPSCs in xeno-free conditions (Higuchi et al. 2015a).

4.1 Cultivation of hESCs and hiPSCs on PVA hydrogels Having Optimal Stiffness

hPSCs were cultivated on PVA-oligoVN hydrogels with different stiffnesses to investigate the effect of PVA-oligoVN hydrogel stiffness on hPSC proliferation. For the preliminary screening experiments, we investigated hESC attachment and morphology on PVA-oligoVN hydrogels prepared using different conditions; hESCs exhibit high attachment ratios and well-formed colonies when they are cultured on adequate materials. The morphology of WA09 cells (hESCs) cultivated on PVA-oligoVN hydrogels having different stiffnesses (PVA-48-500, PVA-24-500, PVA-12-500, PVA-6-500, and PVA-1-500 hydrogels) as well as on plates coated with Synthemax II (Synthemax II plates) at passage 1 is shown in Fig. 22 (Higuchi et al. 2015a). PVA-*X*-*Y* hydrogels are defined as PVA hydrogels crosslinked for *X* hours and grafted with oligoVN using a reaction solution containing *Y* μg/mL of oligoVN. While the WA09 cells could not adhere well to the soft PVA-1-500 hydrogels, WA09 cells could attach to the PVA hydrogels with stiffnesses higher than 12–15 kPa (i.e., the PVA-48-500, PVA-24-500, PVA-12-500, and PVA-6-500 hydrogels). It is found that PVA-oligoVN hydrogels need to have a minimum stiffness to allow hESCs to attach to the surface.

The attachment ratio of hPSCs cultivated on Matrigels, Synthemax II plates, and PVA-oligoVN hydrogels was investigated at passages 1–5, and the attachment ratios at passage 3 are described in Fig. 21 (Higuchi et al. 2015a). High attachment ratios of HPS0077 (hiPSCs) and WA09 cells (hESCs) were observed on PVA-24-500 hydrogels and Matrigels. Low or moderate attachment ratios of hPSCs

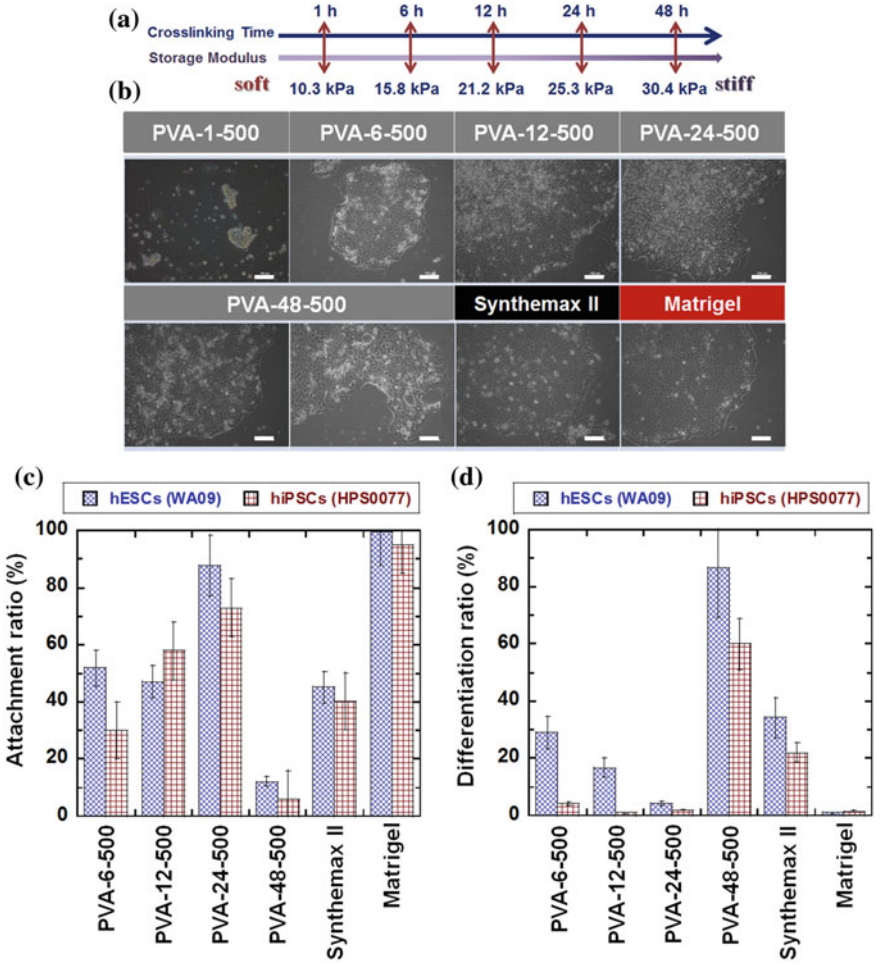


Fig. 22 hPSC cultivation on PVA-oligoVN hydrogels having optimal elasticity. **a** Stiffness (storage modulus) can be controlled by the crosslinking intensity (time) of PVA-oligoVN hydrogels. **b** Picture of WA09 cells (hESCs) cultivated on PVA-oligoVN hydrogels having different stiffnesses (PVA-1-500, PVA-6-500, PVA-12-500, PVA-24-500, and PVA-48-500 hydrogels), Matrigels, and Synthemax II plates at passage 1. The bar represents 100 μm. **c** Attachment ratio of hPSCs (red column, HPS0077 cells [hiPSCs] and blue column, WA09 cells [hESCs]) on PVA-oligoVN hydrogels with different stiffnesses, Matrigels, and Synthemax II plates at passage 3. **d** Differentiation ratio of hPSCs (red column, HPS0077 cells [hiPSCs] and blue column, WA09 cells [hESCs]) on PVA-oligoVN hydrogels with different stiffnesses, Matrigels, and Synthemax II plates at passage 3 (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

were observed on the Synthemax II plates and PVA-48-500, PVA-12-500, and PVA-6-500 hydrogels. Those PVA-oligoVN hydrogels that provided the optimal elasticity (25 kPa, PVA-24-500 hydrogels) resulted in the highest hPSC attachment

ratios among the PVA-oligoVN hydrogels. hPSCs cultured on Synthemax II plates exhibited a lower attachment ratio than hPSCs cultured on PVA-24-500 hydrogels ($p < 0.05$) and an approximately equal attachment ratio compared to hPSCs cultured on the PVA-12-500 hydrogels ($p > 0.05$).

The pluripotency of hESCs and hiPSCs was investigated using colony morphology and live staining of alkaline phosphatase. Figure 23 displays the morphology of (a) completely differentiated, (b) partially differentiated, and (c) pluripotent WA09 cells (c) (Higuchi et al. 2015a). The completely differentiated cells did not show alkaline phosphatase (ALP) activity. On the other hand, alkaline phosphatase activity of the partially differentiated cells was observed in the colony center, while cells on the colony edge did not show ALP activity. The pluripotent cells showed excellent colony morphology and ALP activity. The differentiation ratio was investigated for whole hPSC colonies on the plates and evaluated using the following equation:

$$\begin{aligned}\text{Differentiation ratio}\,(\%) = {} & \%\ \text{of partially differentiated cells} \times 0.5\,(\%) \\ & + \%\ \text{of differentiated cells} \times 1.0\,(\%)\end{aligned} \tag{4}$$

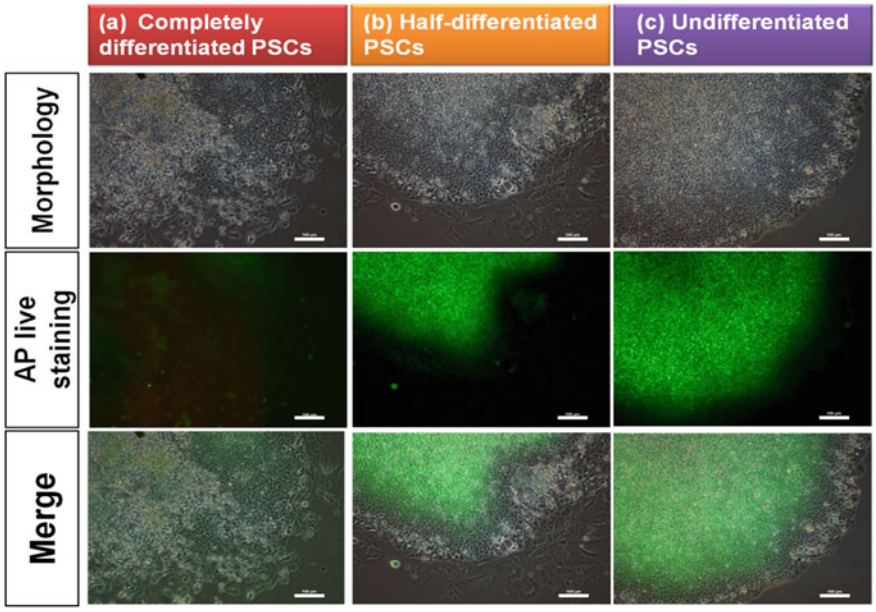


Fig. 23 Definition of completely differentiated hPSCs **a**, partially differentiated hPSCs **b**, and undifferentiated (pluripotent) hPSCs **c**. **a** No ALP (alkali phosphatase) activity was observed in completely differentiated cells. **b** ALP activity was observed in the center of the colonies, whereas the edges of the partially differentiated cell colonies did not show ALP activity. **c** The undifferentiated (pluripotent) cells displayed good colony morphologies and ALP activity in whole cells (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

The differentiation ratios of hPSCs cultivated on Matrigels, Synthemax II plates, and PVA-oligoVN hydrogels were investigated at passages 1–5, and the results at passage 3 are described in Fig. 22. hPSCs cultured on Matrigels and PVA-24-500 hydrogels showed low differentiation ratios (Higuchi et al. 2015a). On the other hand, hPSCs cultured on Synthemax II plates and PVA-48-500 hydrogels exhibited high differentiation ratios. These results indicate that the cell culture hydrogels should have an optimal stiffness (e.g., 25.3 kPa) to maintain hPSC pluripotency when they are cultivated on PVA-oligoVN hydrogels. PVA-24-500 hydrogels provided higher hPSC pluripotency (lower differentiation ratio) and attachment ratios than the Synthemax II plates (commercial dishes) ($p < 0.05$) (Higuchi et al. 2015a).

4.2 *Effect of Surface Density of Biological Cues (OligoVN) on HPSC Proliferation*

PVA-oligoVN hydrogels with a stiffness of 25.3 kPa (crosslinking time = 24 h) were selected as the optimal cell culture material having optimal stiffness of those described in the previous section. In the following experiments, we evaluated the effect of the oligoVN surface density of PVA-oligoVN hydrogels on hPSC proliferation. The concentration of oligoVN in the reaction solution can control the surface density of oligoVN on PVA-24-EDC hydrogels. Figure 24a shows the morphologies of WA09 cells cultured on PVA-oligoVN gels having different oligoVN surface densities (PVA-24-1500, PVA-24-1000, PVA-24-500, PVA-24-250, PVA-24 h-100, and PVA-24 h-50 hydrogels), as well as on Matrigels and Synthemax II plates at passage 1 (Higuchi et al. 2015a). WA09 cells detached easily from the PVA-oligoVN hydrogels with an oligoVN concentration < 500 μg/mL (PVA-24-250, PVA-24-100, and PVA-24-50 hydrogels). WA09 cells could not be cultivated on PVA-24-50 gels after two passages. Figure 24b shows the attachment ratio of HPS0077 cells (hiPSCs) and WA09 cells (hESCs) on PVA-oligoVN hydrogels prepared with 100–1500 μg/mL of oligoVN in the reaction solution, as well as on Matrigels and Synthemax II plates at passage 3 (Higuchi et al. 2015a). The attachment ratios of HPS007 and WA09 cells increased with increasing oligoVN concentrations up to 500 μg/mL. The attachment ratios of HPS007 and WA09 cells on the PVA-24-500, PVA-24-1000, and PVA-24-1500 hydrogels showed no significant differences ($p > 0.05$). Furthermore, HPS007 and WA09 cells cultured on the PVA-24-1500 hydrogels showed higher attachment ratios than the cells cultured on the Synthemax II plates ($p < 0.05$), but slightly less than the cells cultured on Matrigels ($p < 0.05$).

We evaluated the differentiation ratios of HPS007 and WA09 cells grown on PVA-oligoVN hydrogels prepared with 100–1500 μg/mL of oligoVN in the reaction solution, as well as on Matrigels and Synthemax II plates at passage 3 to investigate the hPSC pluripotency; the results are displayed in Fig. 24c

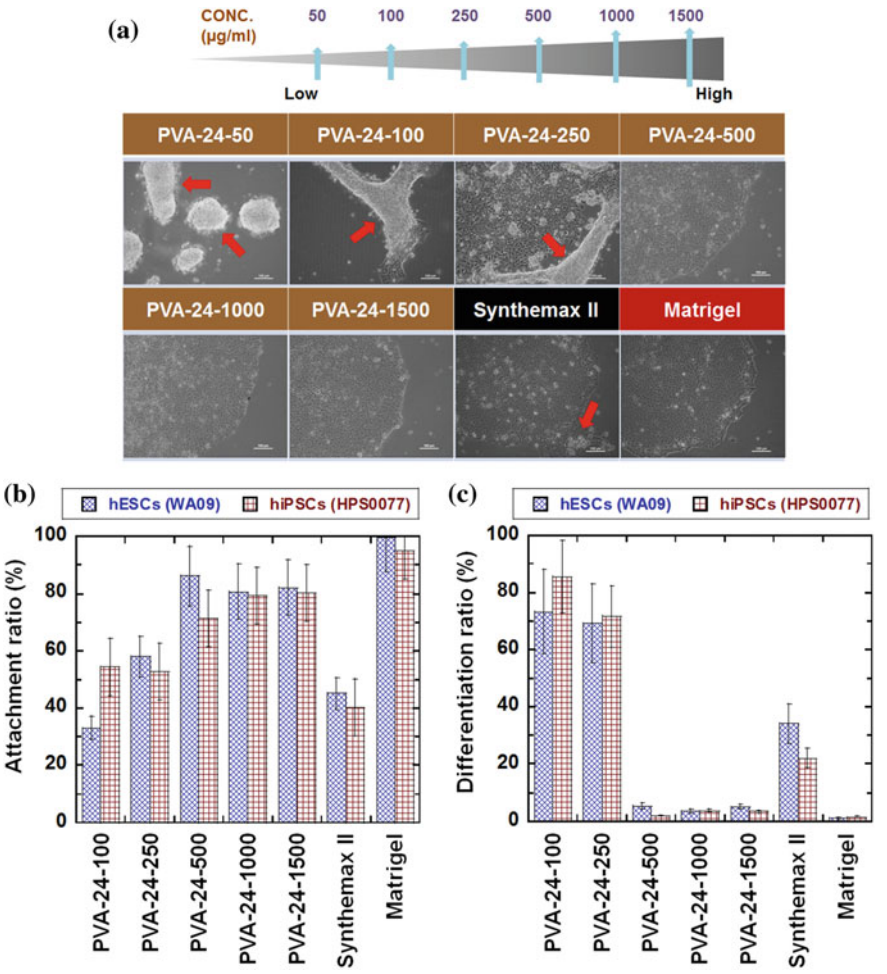


Fig. 24 hPSC cultivation on PVA-oligoVN hydrogels having several surface densities of oligoVN. **a** Pictures of WA09 (hESCs) cultivated on PVA-oligoVN hydrogels having different surface densities of oligoVN (PVA-24-50, PVA-24-100, PVA-24-250, PVA-24-500, PVA-24-1000, and PVA-24-1500 hydrogels), Matrigels, and Synthemax II plates at passage 1. The red arrows indicate detached cells. The bar represents 100 μm. **b** Attachment ratio of hPSCs (red column, HPS0077 cells [hiPSCs] and blue column, WA09 cells [hESCs]) on PVA-oligoVN hydrogels having different surface densities of oligoVN, Matrigels, and Synthemax II plates at passage 3. **c** Differentiation ratio of hPSCs (red column, HPS0077 cells [hiPSCs] and blue column, WA09 cells [hESCs]) on PVA-oligoVN hydrogels having different surface densities of oligoVN, Matrigels, and Synthemax II plates at passage 3 (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

(Higuchi et al. 2015a). hPSCs on the PVA-24-500, PVA-24-1000, and PVA-24-1500 hydrogels, as well as on the Matrigels, showed very low differentiation ratios, whereas hPSCs on PVA-24-100 and PVA-24-250 hydrogels, as well as on

Synthemax II plates, displayed high differentiation ratios. It was found that there is a minimum surface density of oligoVN (500 μg/mL) on the PVA-oligoVN hydrogels required to maintain hPSC pluripotency, with a low differentiation ratio, as well as to achieve a high attachment ratio. The active layer of Synthemax dishes is reported to be polyacrylate grafted with oligoVN of the same amino acid sequence as was prepared for the PVA-oligoVN hydrogels in this study (Melkoumian et al. 2010). High concentrations, such as 1 mM (1590 μg/mL), of oligoVN in the reaction solution was also necessary to produce the Synthemax plates (Melkoumian et al. 2010). Therefore, it is reasonable that high concentrations of oligoVN in the reaction solution are required to maintain hPSC pluripotency on the surface of PVA-oligoVN hydrogels (Higuchi et al. 2015a).

4.3 hPSC Culture for Long Period Under Xeno-Free Culture Conditions

In the previous section, PVA-24-500, PVA-24-1000, and PVA-24-1500 hydrogels were found to be good materials for hPSC culture; these materials have optimal biological cues (optimal surface density of oligoVN) and good physical cues (optimal stiffness). Based on these results, we performed long-term (20 passages) hPSC culture using PVA-24-1000 hydrogels, Synthemax II dishes and Matrigels in Essential 8 medium (xeno-free culture medium).

Figure 25 shows the differentiation ratios, attachment ratios, and expansion rates of HPS0077 (hiPSCs) and WA09 (hESCs) cells cultivated on PVA-24-1000 hydrogels, Matrigels and Synthemax II plates for 1–20 passages (Higuchi et al. 2015a). HPS0077 and WA09 cells on the PVA-24-1000 hydrogels showed almost the same fold expansion as the cells cultivated on commercial dishes (Synthemax II), but they exhibited slightly worse expansion than cells cultured on Matrigels ($p < 0.05$). HPS0077 and WA09 cells cultured on the PVA-24-1000 hydrogels exhibited slightly higher attachment ratios compared with the cells on Synthemax II plates over 20 passages, but the difference was not significant ($p > 0.05$). However, it should be noted that HPS0077 and WA09 cells on the PVA-24-1000 hydrogels showed significantly higher attachment ratios than cells on Synthemax II plates ($p < 0.05$) during the early passages (i.e., <5 passages). The attachment ratios of HPS0077 and WA09 cells cultured on Matrigels were more than 80% after 20 passages, which was extensively greater than the attachment ratios of cells on the Synthemax II plates and PVA-24-1000 hydrogels ($p < 0.05$). HPS0077 and WA09 cells on Matrigels and PVA-24-1000 hydrogels showed lower differentiation ratios than the cells on Synthemax II plates ($p < 0.05$), indicating that hPSCs could maintain pluripotency on the PVA-24-1000 hydrogels as well as on Matrigels for a long time (e.g., >20 passages).

These results suggest that hESCs and hiPSCs can be cultured on PVA-24-1000 hydrogels under xeno-free and feeder-free conditions with better performance than on commercially available plates (Synthemax II), although hPSCs cultivated on

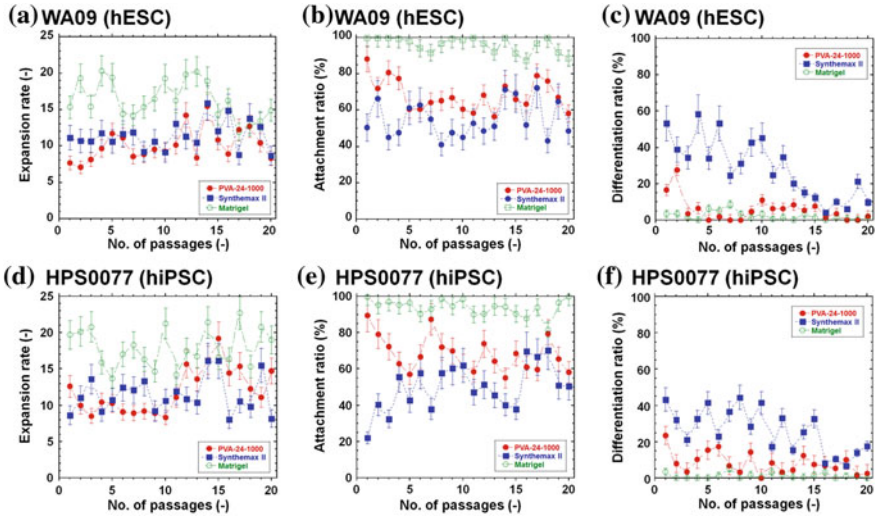


Fig. 25 hPSCs cultivated on PVA-oligoVN hydrogels having an optimal elasticity for long-term, xeno-free culturing. **a** Expansion rate, **b** attachment ratio, and **c** differentiation ratio of WA09 cells (hESCs) cultured on PVA-24-1000 hydrogels (closed red circle), Matrigels (open green circle), and Synthemax II plates (closed blue square) for 1–20 passages. **d** Expansion rate, **e** attachment ratio, and **f** differentiation ratio of HPS0077 cells (hiPSCs) cultured on PVA-24-1000 hydrogels (closed red circle), Matrigels (open green circle), and Synthemax II plates (closed blue square) for 1–20 passages. (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

Matrigels showed a slightly better attachment ratio and expansion rate. However, Matrigel is not a xeno-free material; hESC and hiPSC cultures on PVA-24-1000 hydrogels are xeno-free, which is required for clinical applications. The hESC and hiPSC cultures on PVA-24-1000 hydrogels showed better performance (i.e., lower differentiation ratio) than the commercial dishes, i.e., Synthemax II; thus, PVA-24 h-1000 hydrogels allow hPSCs to maintain higher levels of pluripotency than do Synthemax II plates. This finding was further evaluated in the experiments described below.

HPS0077 and WA09 cells used in the experiments shown in Figs. 22, 23, 24, and 25 were expanded on Matrigels for several passages in advance to allow them to adjust to feeder-free conditions. These procedures are necessary for the cells to transition from cell culture on MEFs to cell culture on the materials in feeder-free conditions. WA09 cells were also shifted from cell culture on MEFs to cell culture on Synthemax II and PVA-24-1000 hydrogels, starting with WA09 cells cultured on MEFs and not cultured on Matrigels in advance. The results are described in Fig. 26 (Higuchi et al. 2015a). The WA09 cells transferred directly from MEFs to the Synthemax II plates showed differentiated cells at passage 1, whereas the WA09 cells transferred from MEFs to PVA-24-1000 hydrogels were found to maintain pluripotency. These data show trends similar to those in Figs. 25c, f, where hPSCs tended to differentiate on Synthemax II plates compared to hPSCs on PVA-24-1000 hydrogels.

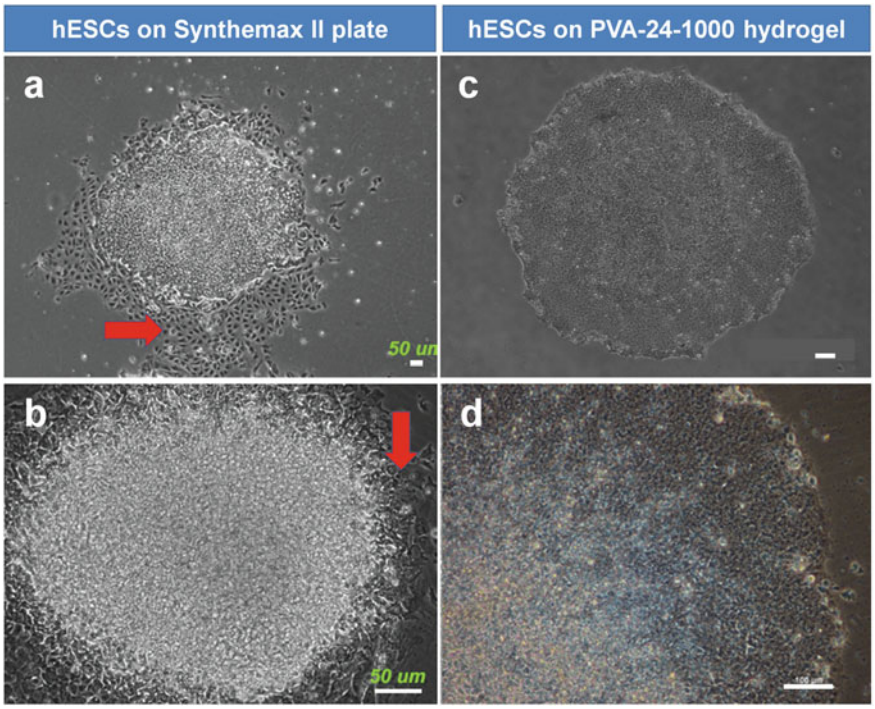


Fig. 26 Comparison of hESCs cultivated on PVA-oligoVN hydrogels and Synthemax II plates. The morphologies of WA09 cells (hESCs) cultivated on Synthemax II plates **a**, **b** and PVA-24-1000 hydrogels **c**, **d** at passage 1 after the WA09 cells were transferred from cultivation on MEFs to cultivation on Synthemax II plates or PVA-24-1000 dishes. Red arrows indicate differentiated cells. The bar represents 50 μm **a**, **b** and 100 μm **c**, **d** (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

The pluripotency of HPS0077 and WA09 cells was investigated by immunostaining for pluripotency proteins SSEA-4, Tra-1-81, Sox2, and Oct3/4 after the cells on PVA-24-1000 hydrogels were passaged 20 times; the results are shown in Fig. 27 (Higuchi et al. 2015a). The pluripotent proteins (SSEA-4, Tra-1-81, Sox2, and Oct3/4) were expressed on HPS0077 and WA09 cells cultivated on PVA-24-1000 hydrogels in Essential 8 xeno-free culture medium for 20 passages (Higuchi et al. 2015a).

4.4 Differentiation of hESCs and hiPSCs Cultured on PVA Hydrogels

We evaluated the ability of HPS0077 and WA09 cells to differentiate into cells derived from the three germ layers in vitro (embryoid body (EB) formation method) and in vivo (teratoma formation method) to evaluate their pluripotency after being

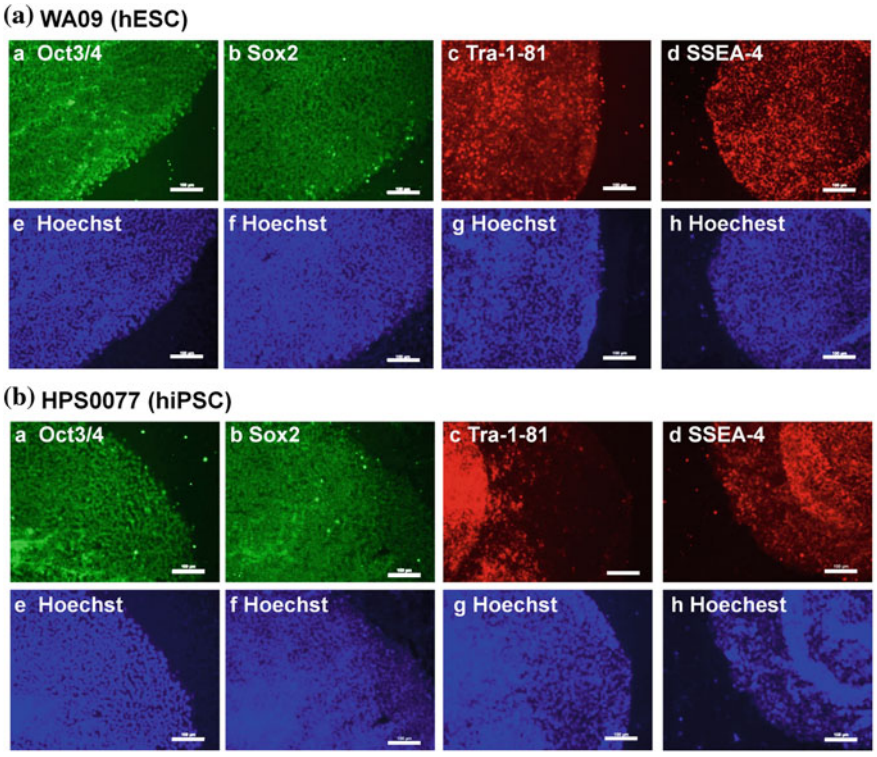
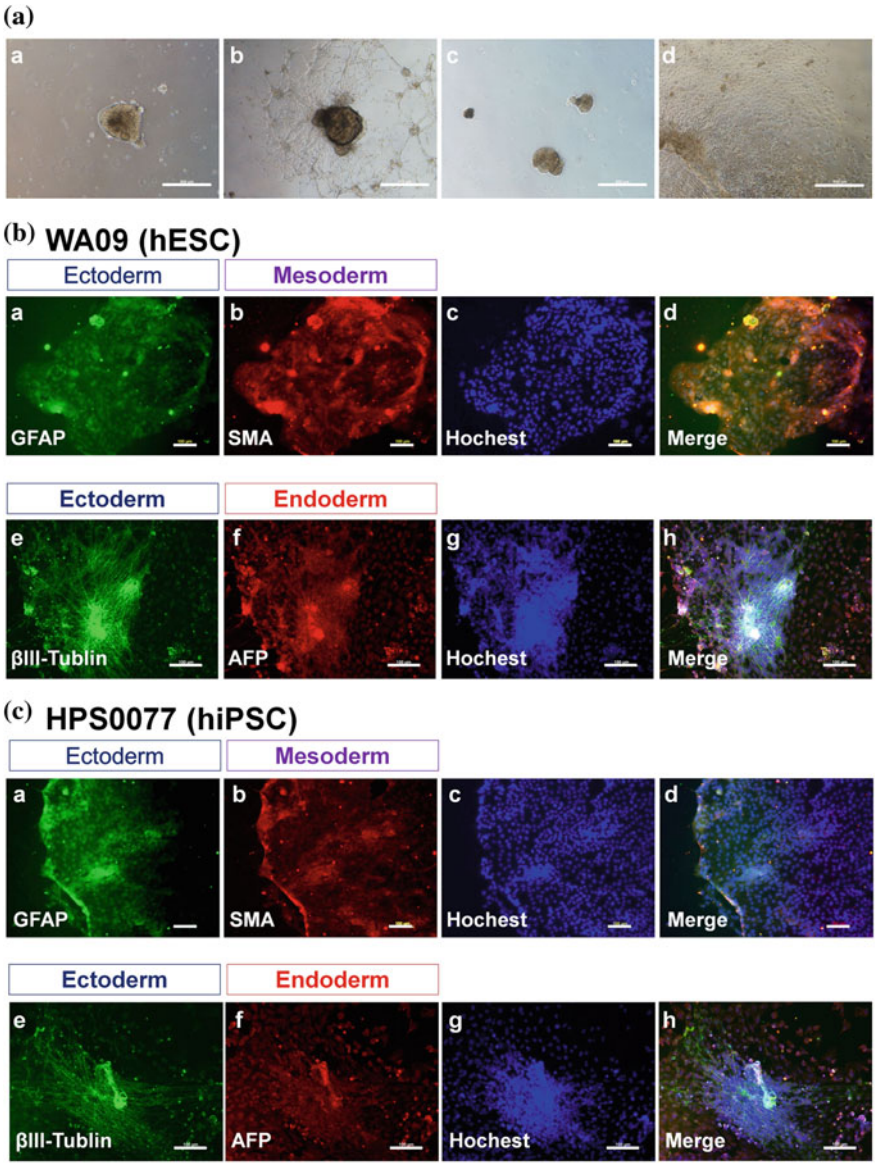


Fig. 27 Expression of pluripotency proteins of hiPSCs and hESCs cultivated on PVA-oligoVN hydrogels. **a** Expression of pluripotency proteins on WA09 cells (hESCs) measured by immunostaining after cultivation on PVA-24-1000 hydrogels in xeno-free culture conditions for 20 passages. (*a*) Oct3/4, (b) Sox2, (*c*) Tra-1-81, (*d*) SSEA-4, and (*e–h*) Hoechst staining of hESCs used in (*a–d*). The bar represents 100 μm. **b** Expression of pluripotency proteins on HPS0077 cells (hiPSCs) measured by immunostaining after cultivation on PVA-24-1000 hydrogels in xeno-free conditions for 20 passages. (*a*) Oct3/4, (*b*) Sox2, (*c*) Tra-1-81, (*d*) SSEA-4, and (*e–h*) Hoechst staining of hESCs used in (*a–d*). The bar represents 100 μm (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

cultured on PVA-24-1000 hydrogels in xeno-free conditions for 20 passages. Figure 28a shows EBs generated from WA09 and HPS0077 cells after a suspension culture of hPSCs on ultra-low-binding plates (Higuchi et al. 2015a). Figure 28b, c shows the cells from EBs prepared from WA09 to HPS0077 cells, respectively, and immunostained with glial fibrillary acidic protein (GFAP, ectoderm), β-III tubulin (ectoderm), smooth muscle actin (SMA, mesoderm), and alpha-fetoprotein (AFP, endoderm) (Higuchi et al. 2015a). It was found that both WA09 and HPS0077 cells could differentiate into cells derived from the three germ layers, as indicated by the expression of GFAP, β-III tubulin, SMA, and AFP. Therefore, the hiPSCs and hESCs not only maintained their pluripotency after being cultivated on



PVA-24-1000 hydrogels under xeno-free conditions but also maintain the ability to differentiate into cells derived from the three germ layers in vitro.

Using the teratoma formation assay, the ability of WA09 cells to differentiate into cells derived from the three germ layers was investigated in vivo. WA09 cells were transplanted into SCID (non-obese diabetic/severe combined immunodeficiency) mice to produce teratomas after being cultured on PVA-24-1000 hydrogels

◂**Fig. 28** Expression of differentiation proteins in embryoid bodies (EBs) generated from hPSCs after cultivation on PVA-oligoVN hydrogels. **a** Morphologies of EBs generated by WA09 cells (hESCs, a and b) and HPS0077 cells (hiPSCs, c and d) after cultivation on PVA-24-1000 hydrogels in xeno-free culture conditions for 20 passages. **b** Immunostaining of endoderm proteins (f, AFP), mesoderm proteins (b, SMA), and ectoderm proteins (a, GFAP; e, β-III tubulin) on WA09 cells (hESCs) after cultivation on PVA-24-1000 hydrogels in xeno-free culture conditions for 20 passages. (c) Hoechst nuclear staining of WA09 cells utilized in (*a*, *b*). (d) Merged images of (*a–c*). (*g*) Hoechst nuclear staining of WA09 cells utilized in (*e*) and (*f*). (*h*) Merged images of (*e–g*). The bar represents 100 μm. **c** Immunostaining of endoderm proteins (f, AFP), mesoderm proteins (b, SMA), and ectoderm proteins (a, GFAP; e, β-III tubulin) on HPS0077 cells (hiPSCs) after cultivation on PVA-24-1000 hydrogels in xeno-free culture conditions for 20 passages. (c) Hoechst nuclear staining of HPS0077 cells utilized in (*a*) and (*b*). (*d*) Merged images of (*a–c*). (*g*) Hoechst nuclear staining of HPS0077 cells utilized in (*e*) and (*f*). (*h*) Merged images of (*e–g*). The bar represents 100 μm (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

for ten passages (Fig. 29) (Higuchi et al. 2015a). The teratomas stained with H and E (hematoxylin and eosin) showed the presence of cells originating from the three germ layers (neurons (ectoderm), chondrocytes (mesoderm), osteoblasts (mesoderm), and enterocytes (endoderm)). Therefore, hESCs cultivated on PVA-24-1000 hydrogels for long periods (10–20 passages) are able to maintain their pluripotency and can differentiate into cells of the three germ layers in vivo and in vitro (Higuchi et al. 2015a).

4.5 Discussion of Cell Culture Materials for hESCs and hiPSCs

Various matrices for culturing hPSCs have been investigated in chemically defined and feeder-free conditions. Table 2 summarizes cell culture matrices for hPSCs reported by several other researchers (Higuchi et al. 2015a). Cell culture plates grafted or coated with ECMs (e.g., vitronectin, laminin-332, laminin-521, laminin-511, and fibronectin) (Jonas et al. 2013; Liu et al. 2014; Lu et al. 2014; Rodin et al. 2010; Miyazaki et al. 2012; Tsutsui et al. 2011) and ECM-derived oligopeptides (Park et al. 2015; Chen et al. 2014; Wu et al. 2014; Fan et al. 2014; Deng et al. 2013; Pennington et al. 2015; Lin et al. 2014; Higuchi et al. 2014a) have been extensively reported. Because generating human ECMs under xeno-free conditions while complying with good manufacturing practices (GMPs) is relatively expensive, the development of cell culture plates with immobilized ECM-derived oligopeptides could be a reasonable alternative. To date, totally synthetic polymeric materials have been prepared as hPSC culture substrates, including (a) poly(methyl vinyl ether-alt-maleic anhydride), or PMVE-alt-MA, (b) poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammoniumhydroxide], or PMEDSAH, and (c) aminopropylmethacrylamide, or APMAAm (Brafman et al.

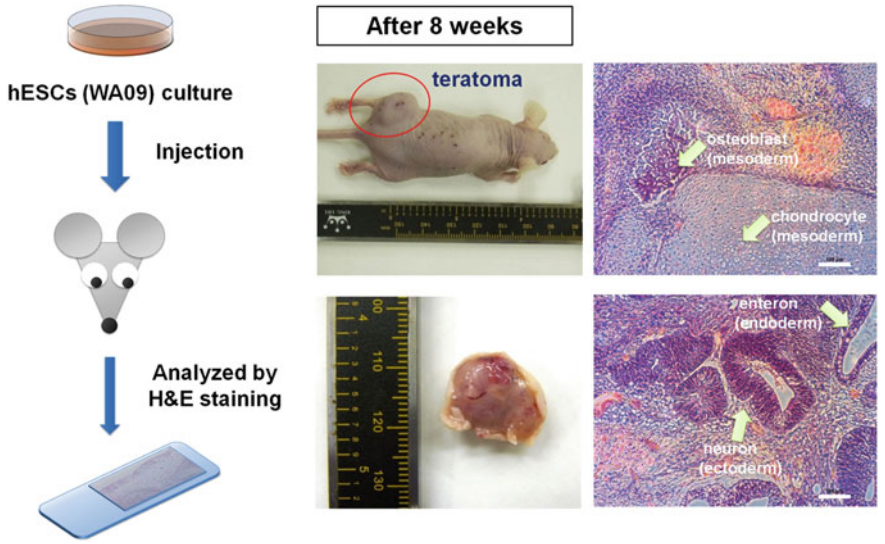


Fig. 29 Differentiation potential of hESCs (WA09) demonstrated by teratoma formation *in vivo* after cultivation on PVA-24-1000 hydrogels for 20 passages. Enterocytes (endoderm), neurons (ectoderm), chondrocytes (mesoderm), and osteoblasts (mesoderm) were generated in the teratomas. The bar represents 100 μm (Higuchi et al. 2015a). Adapted with permission from a Creative Commons Attribution License

Table 2 Coating and substrates for feeder-free hPSC culture in a defined medium[a] (Higuchi et al. 2015a). Adapted 2015. Reproduced with permission a Creative Commons Attribution License

(a) *Coating materials of ECMs*
Gelatin, fibronectin, laminin, **laminin-332**, **laminin-511**, **laminin-521**, **vitronectin**, **recombinant vitronectin**
(b) *Polysaccharide substrates*
Chitin/alginate, cellulose, positively charged cellulose
(c) *Oligopeptides for immobilization on substrates*
Poly-D-lysine, cyclic RGD, pronectin, **oligovitronectin**
(d) *Chimera protein for immobilization on substrates*
E-cadherin chimera
(e) *Synthetic polymer of substrates*[b]
MEASAH, PMVE-alt-MA[39], PMEDSAH[40–43], APMAAm[44], Copoly(AEtMA-co-DEAEA)[45], Poly-3,4-dihydroxy-L-phenyl-alanine

[a]Bold biomolecules are typically used
[b]PMVE-alt-MA, poly(methyl vinyl ether-alt-maleic anhydride); PMEDSAH, poly[2-(methacryloyloxy(ethyl dimethyl))-(3-sulfopropyl) ammoniumhydroxide]; APMAAm, aminopropylmethacrylamide; Copoly(AEtMA-co-DEAEA), copoly[2-(acryloyloxyethyl) trimethylammonium-co-2-(diethylamino)ethyl acrylate]

2010; Nandivada et al. 2011; Qian et al. 2014; Villa-Diaz et al. 2010; Irwin et al. 2011; Zhang et al. 2013). However, hPSCs are generally cultivated on Matrigels prior to being grown on completely synthetic polymer materials, and Matrigels are xeno-containing materials. The mechanism of successful hPSC culture on completely synthetic polymer materials is generally considered to involve heparin-mimicking polymers, although the exact mechanism of hPSC proliferation on completely synthetic polymer materials is still under investigation. The mechanism of how synthetic polymer materials enable hPSC proliferation and attachment should be systematically investigated in the future. Another drawback of using completely synthetic polymer materials is that currently these polymers are not commercially available, as only professional chemists can synthesize these polymers; in contrast, ECM-derived oligopeptides and ECMs are commercially available.

Currently, only coating materials can be obtained commercially for hPSC culture matrices; these materials are chimeric proteins, ECMs and matrices made of ECM-derived oligopeptides (Stephenson et al. 2012; Nagaoka et al. 2010). Synthetic polymer materials cannot be purchased commercially for hPSC culture. Therefore, the development of reliable synthetic polymer materials that can support hPSC expansion with high pluripotency and high reproducibility is required.

The PVA-24-1000 hydrogels include the hPSC-binding site of oligoVN that binds to integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$ (Higuchi et al. 2014b). This is why hPSCs grown on MEFs can be safely transferred onto PVA-24-1000 hydrogels without differentiating, whereas hPSCs shifted from MEFs onto Synthemax II plates will differentiate significantly (Fig. 26). However, it should be noted that the sequence of the oligoVN cell-binding domain on the Synthemax II plates is the same as that used on PVA-24-1000 hydrogels (Melkoumian et al. 2010).

The effect of cell culture material stiffness on the differentiation and pluripotency of stem cells (i.e., stem cell fate) has mainly been studied using adult stem cells, such as hBMSCs and hHSPCs (Engler et al. 2006; Higuchi et al. 2013; Kumar et al. 2013). However, we suggest that finely tuning the stiffness of the cell culture matrices is an extremely important factor for the expansion and attachment of hESCs and hiPSCs. hPSCs grown on the stiffest substrates (E'= 30 kPa) were induced to differentiate after 5 days of cultivation, whereas only a small number of hPSCs colonies attached to the softest substrates (E'= 10–20 kPa). hPSCs cultivated on the PVA-oligo hydrogels with adequate stiffness (E'= 25 kPa) could maintain their pluripotency and proliferate for over 10–20 passages under feeder-free and xeno-free conditions. It was reported that hPSCs could be cultured on Synthemax II plates for 10–20 passages under xeno-free conditions (Melkoumian et al. 2010). However, in this study, hPSCs were found to differentiate more on Synthemax II plates than on PVA-24 h-1000 hydrogels and Matrigels. This can probably be explained by the extremely stiff base of the Synthemax II cell culture plates, as Synthemax II is coated on TCPS (approximately 3 GPa modulus). If the base of the Synthemax II cell culture plates was selected to be a softer material than TCPS, then hPSCs should maintain more pluripotency on the Synthemax II coated on the soft material.

hPSCs can be cultured for a long period on Matrigels and maintain their pluripotency because Matrigels are composed of several growth factors and ECMs that support the pluripotency of hPSCs. Although Matrigels are loaded on the extremely stiff surface of TCPS, the top layer of Matrigel on the plate is expected to produce a somewhat soft hydrogel (in comparison with TCPS alone) for hPSCs, which enables hPSCs to grow and maintain their pluripotency during long-term culture.

hESCs were reported to be cultured on polyacrylamide hydrogels with immobilized glycosaminoglycan, which had elastic moduli of 0.7, 3, and 10 kPa (Musah et al. 2012). In this study, hESCs could be grown on only relatively stiff hydrogels (10 kPa), which is similar to the results of the present study indicating that very soft hydrogels (0.7–3 kPa) cannot allow hESCs to attach to the surface. Unfortunately, their study of hESCs cultured on glycosaminoglycan-binding polyacrylamide hydrogels did not investigate hESCs culture on hydrogels stiffer than 10–12 kPa; our results indicated that the optimal stiffness of the hydrogels was approximately 25 kPa. However, their study suggests that hPSCs are able to receive mechanical information from cell culture hydrogels using glycosaminoglycan engagement that may activate specific signaling pathways relating to pluripotency.

Hydrogels with heparin-mimicking sites and several different moduli were prepared by Chang et al. by changing the crosslinking intensity (55, 140, and 345 kPa) (Chang et al. 2013). hESCs could attach slightly to the hydrogels with a low stiffness (55 kPa), whereas hESCs could attach to the hydrogels with a moderate stiffness (140 kPa); these cells would spontaneously differentiate. hESCs showed excellent attachment and could maintain their pluripotency on the most rigid hydrogels (345 kPa) for more than 20 passages. Although these hydrogels do not possess binding sites for hPSCs, FGF-2-binding moieties originating from heparin-mimicking moieties may contribute to the hPSCs binding to the hydrogels. In particular, FGF-2 could bind to a lesser extent to their softer hydrogels due to the lower density of heparin-mimicking sites. The minimum stiffness requirement of hydrogels for the attachment of hPSCs indicates the existence of a minimum density of FGF-2-binding moieties for hPSC binding. In our study, PVA-24-1000 hydrogels having a modulus of $E' = 25$ kPa were found to be the optimal materials for maintaining long-term cultures of pluripotent hPSCs. Both our present study and Chang's study showed that there is an optimal hydrogel stiffness that supports the long-term culture of hPSCs. The difference of optimal stiffness between our present study and Chang's study is probably due to the different hydrogel materials used.

We should consider optimizing the stiffness (physical cues) of the materials used for hPSC expansion and fine-tuning the surface density of cell-binding moieties (biological cues) to be important steps of designing cell culture materials for hPSC culture in feeder-free and xeno-free conditions. PVA hydrogels with concentrations of oligoVN higher than 500 μg/mL and an optimal stiffness of 25 kPa were the optimal cell culture materials for hPSC expansion in this study. The PVA-24-1500, PVA-24-1000, and PVA-24-500 hydrogels exhibited lower differentiation ratios and excellent attachment ratios for hPSCs compared with those of the Synthemax II

plates (commercial plates). Because Synthemax II plates are prepared by coating bioactive polymers on stiff TCPS plates, Synthemax II plates do not possess the optimal stiffness for hPSC culture, whereas the PVA-oligoVN hydrogels can easily be made with the optimal stiffness for hPSC culture by controlling the crosslinking intensity. hPSCs could be cultivated on PVA-24-1000 hydrogels for many passages (10–20 passages) while maintaining their pluripotency and their ability to differentiate into cells of the three germ layers both in vitro and in vivo (Higuchi et al. 2015a).

5 Conclusion and Future Perspective

This research studied the effect of hydrogel elasticity on the culture of hHSPCs, hAFSCs, and hPSCs. PVA hydrogels having an E' of 12–30 kPa were found to be efficient cell culture materials for hHSPC expansion ex vivo. Among the PVA hydrogels and TCPS plates investigated in this study, the PVA-6-FN and PVA-6-CS1 hydrogels were found to be the best cell culture materials for hHSPCs exhibiting high expansion and capable of generating many multipotent CFU-GMs and CFU-GEMMs. The XPS analysis suggested that the surface density of FN on PVA-6-FN gels was extensively less than that on PVA-24-FN and PVA-48-FN hydrogels. Furthermore, hHSPCs cultured on PVA-1-CS1 hydrogels expansion slightly less and generated fewer CFU-GEMMs and CFU-GMs than hHSPCs cultured on PVA-6-CS1, PVA-24-CS1, and PVA-48-CS1 hydrogels, although the surface density of CS1 on PVA-1-CS1 was almost equal to that on PVA-6-CS1, PVA-24-CS1, and PVA-48-CS1 hydrogels, as determined via XPS analysis. The presentation of the specific CS1 cell-binding domains to hHSPCs at high concentrations relative to the density of FN should be an essential point for the signal transduction pathways promoting hHSPC expansion and maintaining hHSPC stemness. Therefore, both biological cues (i.e., surface density of CS1 and FN) and physical cues (i.e., cell culture material elasticity) are key factors in determining the success of efficient hHSPC expansion and stemness maintenance ex vivo.

hAFSCs were cultivated on stiff and soft PVA hydrogels with and without immobilized ECM-derived oligopeptides and ECMs to investigate the spontaneous differentiation and pluripotency of stem cells in expansion medium. It is found to be important for the PVA hydrogels with and without immobilized ECM-derived oligopeptides and ECMs to possess a minimum storage modulus ($E' > 12$ kPa) to support hAFSC expansion. The soft PVA-6-oligoECM and PVA-6-ECM hydrogels ($E' = 12$ kPa) were found to be optimal for culturing hAFSCs with high pluripotency, as evaluated by protein and gene expression analyses, compared with the relatively stiff PVA-24-oligoECM and PVA-24-ECM hydrogels ($E' = 25$ kPa), as well as with TCPS coated with CELLstart (3 GPa of elastic modulus) and TCPS plates. Soft PVA-6-oligoVN and PVA-6-oligoFN hydrogels were found to be the most optimal cell culture plates for supporting hAFSC pluripotency and spontaneous differentiation into early-stage neural cells, as determined from high *Nestin*

expression. hAFSCs cultured on relatively stiff PVA-24-oligoFN, PVA-24-FN, PVA-24-oligoVN, and PVA-24-COL-B hydrogels spontaneously differentiated into early osteoblasts, as determined from high *Runx2* expression. We found that hAFSCs showing high levels of pluripotency genes (*Nanog*, *Sox2,* and *Oct4*) on PVA-oligoECM and PVA-ECM hydrogels also expressed high levels of genes relating to the three early germ layers (*Nestin, Runx2*, and *Sox17*) in expansion medium. This evidence indicates that the hAFSC population of stem cells is inhomogeneous. Therefore, hAFSCs contain stem cells that spontaneously differentiate as well as those with high pluripotency. Both physical cues (stiffness) and biochemical cues (cell adhesive oligopeptides) of cell culture materials are key factors for supporting the stemness of hAFSCs.

We also developed PVA-oligoVN hydrogels with a variety of stiffnesses for the xeno-free culture of hESCs and hiPSCs, supporting their pluripotency for more than 20 passages. The best stiffness of the PVA-oligoVN hydrogels was found to be 25.3 kPa. Furthermore, high concentrations of oligoVN (500–1500 μg/mL) should be used in the preparation of PVA-oligoVN hydrogels to ensure a sufficient surface density of oligoVN on the hydrogels for maintaining hPSC pluripotency. Optimizing the stiffness (physical cues) and fine-tuning the surface density of the cell-binding moieties (biological cues) of hydrogels are the key factors for designing hPSC culture materials that will support pluripotency in xeno-free conditions.

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