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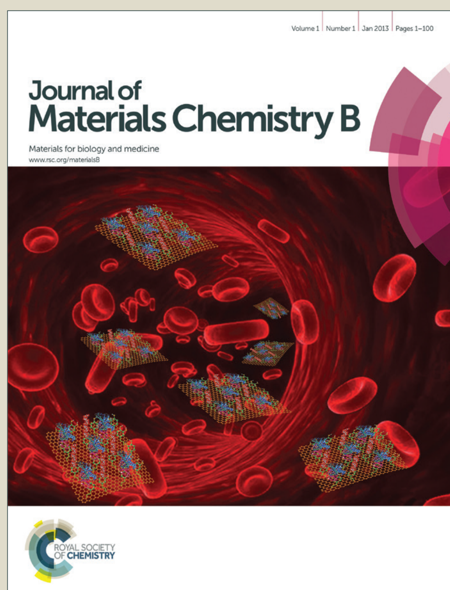

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Pluripotency maintenance of amniotic fluid-derived stem cells cultured on biomaterials with different elasticities and grafted with ECM-derived oligopeptides

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The stem cell fates of pluripotency and differentiation are regulated by not only soluble biological cues but also insoluble biochecmial cues (i.e., extracellular matrix (ECM)) and the physical cues of cell culture biomaterials (i.e., elasticity). We investigated the maintenance of pluripotency and the differentiation lineages of human amniotic fluid-derived stem cells (hAFSCs) cultured on poly(vinyl alcohol-co-itaconic acid) (PVA) hydrogels grafted with several types of ECM and corresponding oligopeptides in expansion medium. hAFSCs cultured on soft PVA hydrogels (12.2 kPa) that were grafted with oligopeptides derived from fibronectin and vitronectin showed high pluripotency, which was evaluated by *Oct4*, *Sox2* and *Nanog* expression. The hAFSCs grown on soft PVA hydrogels (12.2 kPa) grafted with each oligopeptide showed higher pluripotency, as assessed by *Oct4* and *Nanog* expression, than hAFSCs grown on stiff PVA hydrogels (25.3 kPa) grafted with the same oligopeptides and a much higher pluripotency than those grown on rigid tissue-culture polystyrene dishes. Soft biomaterials appeared to be adequate to maintain the pluripotency of hAFSCs. Surprisingly, hAFSCs that showed higher pluripotency on PVA hydrogels grafted with oligopeptides derived from fibronectin and vitronectin also expressed higher levels of early differentiation markers for three germ layers in expansion medium. This result suggests that hAFSCs are heterogeneous and that this population contains highly pluripotent stem cells and stem cells that can be easily differentiated.

1. Introduction

Human adult stem cells, such as adipose-derived stem cells (hADSCs), bone marrow-derived stem cells (hBMSCs) and amniotic fluid-derived stem cells (hAFSCs), are an attractive source of cells for tissue engineering and cell therapy.[1-7] The use of human adult stem cells avoids the ethical concerns raised by human embryonic stem cells (hESCs). Furthermore, no tumor generation has been reported to result from the transplantation of human adult stem cells into animals, whereas hESCs and human induced pluripotent stem cells (hiPSCs) generate tumors in transplanted animals due to their high differentiation ability.[8] Although human adult stem cells have a more limited differentiation ability than hESCs and hiPSCs, human adult stem cells are able to differentiate into typical mesoderm lineages such as osteoblasts, chondrocytes, adipocytes, and cardiomyocytes. Additionally, some researchers have reported that human adult stem cells can differentiate into neural cells (ectoderm) and endoderm cells (beta cells and hepatocytes).[9-16] It is less expensive to maintain human adult stem cells than to maintain hESC and hiPSC cultures; hESCs and hiPSCs also need to be cultured on a specific culture surface (e.g., mouse embryonic fibroblasts, Matrigel, or biomaterials immobilized with extracellular matrix (ECM) proteins or ECM-derived oligopeptides) in culture medium containing an expensive cocktail of several growth factors.[17,18] However, one of the drawbacks of human adult stem cells is their limited expansion period and the decrease in pluripotency of the stem cells with increasing time in culture.[19] In typical culture conditions, human adult stem cells can survive for no more than 8-12 passages. It is therefore necessary to develop culture conditions able to maintain the pluripotency of human adult stem cells.[19]

Stem cell characteristics, such as maintenance of pluripotency, are regulated not only by the stem cells themselves but also by the microenvironment.[1] Therefore, mimicking stem cell microenvironments (niches) using biopolymers should be important to keep pluripotency of stem cells. Several factors in the microenvironment of stem cells influence their fate: (1) biological cues, such as growth factors and bioactive molecules; (2) cell-cell interactions; (3) biochemical cues of stem cell-biomacromolecule (or biomaterial) interactions; and (4) physical cues of cell culture

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biomaterials, such as the elasticity of the biomaterials.[1] As for stem cell-biomacromolecule interactions, we selected several kinds of ECMs (collagen type 1, fibronectin, and vitronectin) and their ECM-derived oligopeptides, which were grafted onto the biomaterials for the culture of human adult stem cells.

Human adult stem cells maintain pluripotency with the aid of the soluble growth factor FGF-2 in the culture medium, which is similar to the growth conditions of hESCs and hiPSCs. Recently, in addition to biological cues such as growth factors, physical cues such as biomaterials have been recognized to affect the stem cell fate of differentiation.[8,20] Although there are several distinct studies describing the effect of the physical cues of biomaterials on human adult stem cell differentiation, there are few reports investigating the effect of the physical cues of biomaterials on the maintenance of pluripotency.[19,21-23]

Higuchi et al. investigated the expansion of hematopoietic stem cells (HSCs) on biomaterials grafted with fibronectin-derived oligopeptides possessing varying degrees of stiffness. HSCs can expand and maintain pluripotency on surfaces with intermediate stiffnesses ranging from 12.2 kPa to 30.4 kPa.[24]

Chowdhury et al. reported that the pluripotency of mouse ESCs (mESCs) could be maintained by culture on soft hydrogels coated with collagen I (0.6 kPa) matching the intrinsic stiffness of mESCs. By contrast, mESCs were not able to maintain self-renewal and pluripotency on hydrogels with much more rigid moduli.[8,21] It has been suggested that the pluripotency of mESCs can be maintained on soft cell-culture biomaterials via the biophysical mechanism of facilitating the production of low cell-matrix traction.[8,21] However, there is a contradictory study that rigid (stiff) biomaterials favor the maintenance of hESC pluripotency.[22] Sun et al. created elastomeric polydimethylsiloxane (PDMS) micropost arrays in which the height of the PDMS microposts regulated substrate stiffness (elasticity).[8,22] hESCs were cultivated on oxygen plasma-treated micropost arrays immobilized with vitronectin and were found to maintain pluripotency on stiff substrates. Furthermore, hESCs were found to be mechano-sensitive and increased their cytoskeleton contractility with increased matrix stiffness.[22] Currently, the optimal elasticity of biomaterials to maintain the pluripotency of adult stem cells such as hAFSCs remains unknown.

In this study, we selected second-trimester hAFSCs as the cell source of human adult stem cells. hAFSCs are known to be fetal stem cells and are expected to possess higher pluripotency than hADSCs and hBMSCs. However, hAFSCs also have an aging problem similar to that of hADSCs and hBMSCs. The goal of this study was to investigate (a) which ECM proteins or ECM-derived oligopeptides grafted onto the biomaterials are effective at maintaining the pluripotency of hAFSCs and (b) the optimal elasticity of the biomaterials to maintain hAFSC pluripotency.

2. Experimental

2.1 Materials

Poly(vinyl alcohol-co-vinyl acetate-co-itaconic acid) (PVA-IA; AF-17) was obtained as a gift from Japan VAM & Poval Co., Ltd. (Sakai, Osaka, Japan). The oligopeptides COLA (GTPGPQGIAGQRGVV), COLB ($(RADA)_4$GGDGEA), oligoFN (KGGAVTGRGDSPASS) and oligoVN (KGGPQVTRGDVFTMP) were obtained from MDBio, Inc. (Piscataway, NJ, USA). Tissue culture polystyrene (TCPS) dishes (diameter = 35 mm; 35-3001) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; 03450), N-hydroxysuccinimide (NHS; 13062), and glutaraldehyde (25% in water; G5882) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human fibronectin (FN; 356008) and collagen type I (Col; 354231) were obtained from BD Biosciences (San Jose, CA, USA). Recombinant human vitronectin (rVN) was purchased from Pepro Tech (140-09; Rocky Hill, NJ, USA). Fetal bovine serum (FBS; 04-001-1, lot 551035) was purchased from Biological Industries Ltd., Kibbutz Beit Haemek, Israel). Dulbecco's modified Eagle's medium (DMEM; D5648) and MCDB 201 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). FGF-2 (CYT-218) was obtained from ProSpec-Tany Technogene (Ness-Ziona, Israel). Trypsin-EDTA solution was purchased from Invitrogen Corporation (25200-056; Carlsbad, CA). RNAspin Mini RNA isolation kit was obtained from GE Healthcare (25-0500-72; Pittsburgh, PA, USA). SuperScript III First-Strand synthesis system (11904-018) and TaqMAN Real-Time Master Mixes were purchased from Life Technologies (Carlsbad, CA, USA). PCR probes for *Oct4* (Hs01895061_u1), *Sox2* (Hs00602736_s1), *Nanog* (Hs02387400_g1), *Nestin* (Hs04187831_g1), *Sox17* (Hs00751752_s1), *Runx2* (Hs00231692_m1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs03929097_g1) were obtained from Life Technologies (Carlsbad, CA, USA). Mouse anti-human SSEA4 antibody (ab16287) was obtained from Abcam (Cambridge, MA, USA). Rabbit anti-human Sox2 antibody (ab5603) was purchased from Millipore (Merck KGaA, Darmstadt, Germany). Mouse anti-human β-III tubulin antibody (MA1-118) and rabbit anti-human alpha-fetoprotein (AFP) antibody (PA5-21004) were obtained from Thermo Fischer Scientific (Rockford, IL, USA). CELLstart, Alexa Fluor 488-anti-rabbit IgG (A11008), Alexa Fluor 488-anti-mouse IgG (A11001), Alexa Fluor 594-anti-mouse IgG (A21203) and Alexa Fluor 594-anti-rabbit IgG (A21207) were purchased from Life Technologies (Carlsbad, CA, USA). Hoechst 33342 was obtained from Lonza (PA-3014; Allendale, NJ).

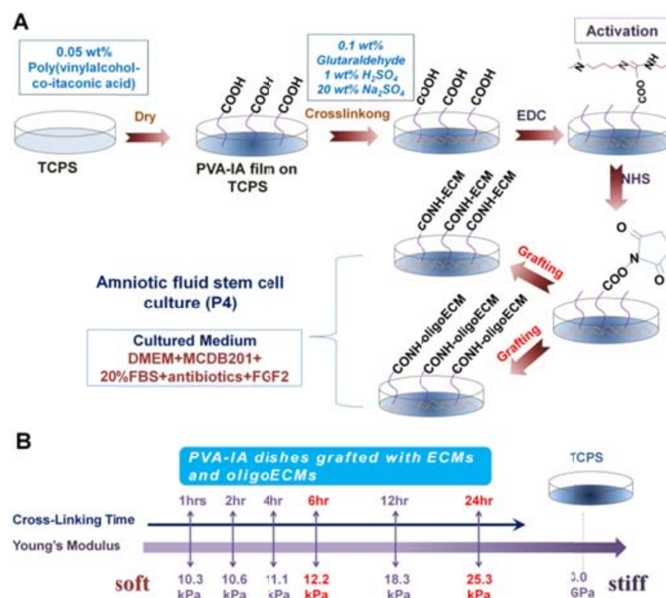

Fig. 1 Preparation of PVA dishes grafted with ECM proteins and ECM-derived oligopeptides. (A) Reaction scheme for PVA dish grafting with ECM proteins and ECM-derived oligopeptides. (B) Elasticity of PVA hydrogels prepared by different periods of crosslinking.

The other chemicals employed were of reagent grade, were used without further purification, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water produced by a Milli-Q system (Millipore Corporation, Billerica, MA, USA) was used

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throughout the experiments.

2.2 Preparation of crosslinked PVA hydrogel dishes

PVA-IA with 1.3 mol% itaconic acid with a degree of hydrolysis of 97.2% and 4 wt% viscocity = 30 mPa·sec was dissolved to 0.05 wt% for cell culture experiments or 0.5 wt% for rheometer measurements in ultrapure water, then agitated for two days and subsequently maintained at room temperature for one day to ensure that no air bubbles were present in the solution.[24] A 3 mL aliquot of the PVA-IA solution was then added to a 35-mm TCPS dish and dried for a week on a clean bench to produce a film. The PVA-IA (hereafter denoted as PVA) films were immersed in an aqueous crosslinking solution composed of 1 wt% glutaraldehyde, 20 wt% sodium sulfate, and 1 wt% sulfuric acid for 2, 4, 6, 12 and 24 h (Fig. 1). The naming convention 'PVA-*X*' (e.g., PVA-2) indicates PVA-IA hydrogels crosslinked for *X* hours (e.g., 2 h). After crosslinking, the PVA hydrogels were washed with ultrapure water and then immersed in ultrapure water. The ultrapure water was changed twice daily before ECM proteins or ECM-derived oligopeptides were grafted onto the dishes, which were then used for cell culture. The PVA hydrogels were sterilized via immersion in a 75% (v/v) ethanol solution overnight, subsequently washed in ultrapure water and maintained in ultrapure water until use for cell culture.

2.3 Preparation of PVA hydrogel dishes grafted with ECM proteins and ECM-derived oligopeptides

Following the preparation of PVA hydrogels with different elasticities, these hydrogels were activated via immersion in an aqueous solution containing 10 mg/ml EDC and 10 mg/ml NHS for 6 h at 4 °C.[24] Subsequently, the PVA hydrogels were washed with phosphate-buffered saline (PBS; pH 7.2) and immersed in a PBS solution containing 50 μg/mL of ECM protein (COL, FN or rVN) or ECM-derived oligopeptide (COLA, COLB, oligoFN or oligoVN) for 24 h at 4 °C (Fig. 1). After the grafting of ECM and ECM-derived oligopeptides, the PVA hydrogels were washed with ultrapure water for 12 h to remove the residual ECM and ECM-derived oligopeptide. The PVA hydrogels grafted with ECM and ECM-derived oligopeptides are hereafter referred to as PVA-*X*-ECM and PVA-*X*-oligoECM, respectively, where *X* indicates the crosslinking time (h) and oligoECM is an ECM-derived oligopeptide.

Water content of PVA-6 and PVA-24 hydrogels was measured to be 53% and 36%, respectively. No change of water content was observed on PVA-6 and PVA-24 hydrogels grafted with or without ECM and ECM-derived oligopeptide.

2.4 Characterization of surface-grafted PVA hydrogel dishes

The chemical composition of the surface-grafted PVA hydrogel dishes was measured using X-ray photoelectron spectroscopy (XPS; K-Alpha spectrometer; Thermal Scientific, Inc., Amarillo, TX, USA) equipped with a monochromatic Al-K X-ray source (1,486.6 eV photons). The energy of the emitted electrons was measured using a hemispherical energy analyzer at pass energies ranging from 50 to 150 eV. Data were collected at a photoelectron takeoff angle of 45 degrees with respect to the sample surface. The binding energy (BE) scale was referenced by setting the peak maximum in the C1s spectrum to 284.6 eV. The obtained high-resolution C1s spectra were fitted using Shirley background subtraction and a series of Gaussian peaks.

The storage modulus (E') of the PVA hydrogels prepared from a 5 wt% PVA-IA solution and crosslinked for 0.5-24 h was quantified using a rheometer (Physica MCR 101; Anton Pars Co. Ltd.) with a 5% strain at 1 Hz.[24]

2.5 Preparation and culture of hAFSCs

The experiments in this study were approved by the ethics committees of the National Central University, the Taiwan Landseed Hospital (IRB-13-05), and the Cathay Medical Research Institute. Fresh second-trimester amniotic fluid was centrifuged at 1200 rpm for 5 min, and the supernatant was removed. The cell solution after centrifugation was dissolved in DMEM/MCDM 201 (40%/60%) supplemented with 20% FBS and 10 ng/ml FGF-2 and was cultured in a CO_2 incubator at 37 °C.[25] After reaching approximately 80% confluence, the cells (i.e., hAFSCs) were harvested with a 0.25% trypsin-EDTA solution, centrifuged and seeded into TCPS dishes as a conventional passage procedure. hAFSCs at passage 4 were cultured on PVA hydrogel dishes grafted with ECM or ECM-derived oligopeptide, TCPS coated with CELLstart or TCPS dishes in a CO_2 incubator at 37 °C for 7 days.

2.6 Analysis of pluripotency and differentiation gene expression in hAFSCs

The expression levels of the pluripotency genes *Oct4*, *Sox2* and *Nanog* and the differentiation genes *Nestin*, *Sox17* and *Runx2* were analyzed by qRT-PCR using conventional methods.[26] Briefly, after the cells were harvested, RNA was extracted using the RNAspin Mini RNA isolation kit according to the manufacturer's instructions. The isolated RNA was treated with DNase to remove any traces of contaminating DNA.[27] RNA (2 μg) was reverse-transcribed by reverse transcriptase to produce cDNA using the SuperScript III First-Strand synthesis system. This cDNA was then used as a template for polymerase chain reaction (PCR) amplification with probes for *Oct4* (Hs01895061_u1), *Sox2* (Hs00602736_s1), *Nanog* (Hs02387400_g1), *Nestin* (Hs04187831_g1), *Sox17* (Hs00751752_s1), *Runx2* (Hs00231692_m1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase; Hs03929097_g1) using Taq DNA polymerase and a qRT-PCR instrument (StepOne Plus^{TM} Real-Time PCR System; Applied Biosystems, Foster, CA, USA). Each sample (n = 3) was tested in duplicate (totally n = 6), and the expression level of the *GAPDH* housekeeping gene was used as a control to normalize the results.

2.7 Immunostaining of proteins involved in pluripotency and differentiation in hAFSCs

Poly-immunostaining of Sox2, SSEA4, β-III tubulin and AFP in the cells was performed following the conventional protocol.[28] Cells on dishes were fixed with paraformaldehyde and then incubated with the primary antibody (e.g., anti-human Sox2 (1:100), SSEA4 (1:100), β-III tubulin (1:100) or AFP (1:100)). Subsequently, the cells were washed with PBS and incubated with the secondary antibody (Alexa Fluor 488-anti-rabbit IgG, Alexa Fluor 488-anti-mouse IgG, Alexa Fluor 594-anti-mouse IgG or Alexa Fluor 594-anti-rabbit IgG) at a 1:50 dilution. The cells were also incubated with Hoechst 33342 (1:50). The stained cells were analyzed using fluorescence microscopy with the appropriate filters (Eclipse Ti-U fluorescence inverted microscope; Nikon Instruments, Inc., Tokyo, Japan).

2.8 Statistical analysis

All of the quantitative results were obtained from three samples. The data are expressed as the mean ± SD. Statistical analyses were performed using an unpaired Student's t-test in Excel (Microsoft Corporation). Probability values (p) less than 0.05 were considered statistically significant.

3. Results and discussion

Journal of Materials Chemistry B Accepted Manuscript

3.1 Physical characteristics of PVA hydrogels grafted with ECM proteins and ECM-derived oligopeptides

PVA hydrogels grafted with ECM and ECM-derived oligopeptides develop different elasticities by controlling crosslinking intensity (time). The storage modulus, *E*', of self-standing PVA hydrogels with an approximately 22-35 μm thickness was evaluated using a rheometer, and the values are shown in Fig. 1B. The softest PVA hydrogel in this study, PVA-2, showed 10.6 kPa of *E*', whereas the *E*' of the hardest PVA hydrogel in this study, PVA-24, was 25.3 kPa. The *E*' of TCPS dishes is reported to be 12 GPa of elastic modulus.[24] The storage moduli of PVA hydrogels grafted with ECM (PVA-*X*-ECM) and ECM-derived oligopeptides (PVA-*X*-oligoECM) were found to be approximately the same as those of the unmodified PVA hydrogels (PVA-*X*) within the range of experimental error. This is because the layer of ECM and ECM-derived oligopeptide grafted onto the PVA hydrogels is too thin to contribute to the E' of bulk PVA hydrogels.

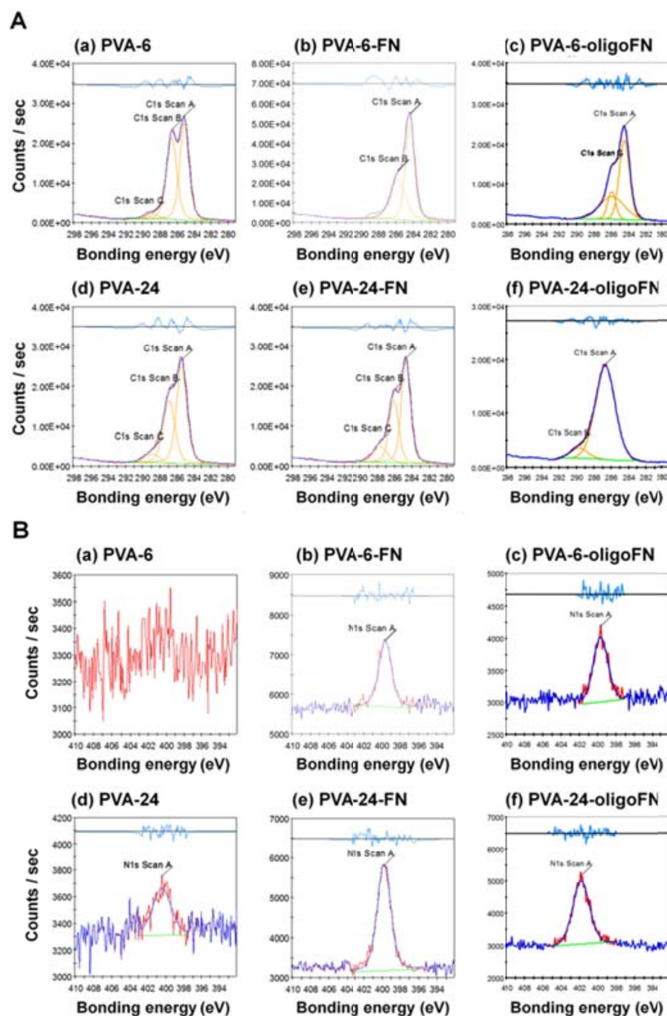

Fig. 2 Characterization of PVA hydrogel dishes grafted with ECM and ECM-derived oligopeptides. (A) High-resolution XPS spectra of the C1s peaks obtained from unmodified PVA-6 (a), PVA-6-FN (b), PVA-6-oligoFN (c), PVA-24 (d), PVA-24-FN (e) and PVA-24-oligoFN (f) dishes. (B) High-resolution XPS spectra of the N1s peaks obtained from unmodified PVA-6 (a), PVA-6-FN (b), PVA-6-oligoFN (c), PVA-24 (d), PVA-24-FN (e) and PVA-24-oligoFN (f) dishes.

3.2 Analysis of nanosegments grafted onto PVA hydrogel dishes by XPS

It is valuable to analyze the existence of nanosegments (i.e., ECM and ECM-derived oligopeptides) and the surface density of the nanosegments on PVA hydrogel dishes grafted with ECM and ECM-derived oligopeptides with different elasticities. We were not able to detect the absolute quantity of the nanosegments grafted onto PVA hydrogel dishes via colorimetric (e.g., microBCA) or other chemical titration and reaction methods in this study. Furthermore, the absolute quantity of nanosegments grafted on PVA hydrogel dishes could not be obtained through an enzyme-linked absorbent method. Therefore, we utilized XPS to detect ECM and ECM-derived oligopeptides on the surface of PVA hydrogel dishes. Fig. 2 shows the high-resolution XPS spectra of C1s peaks (Fig. 2A) and N1s peaks (Fig. 2B).

C–N bonding (285.9 eV), O–C=O bonding (289.3 eV), and C–C and C–H bonding (285.0 eV) were observed in the XPS spectra obtained for the PVA-6-FN, PVA-6-oligoFN, PVA-24-FN and PVA-24-oligoFN dishes in Fig. 2A. However, it was difficult to confirm the presence of ECM proteins and ECM-derived oligopeptides on the PVA hydrogels solely from the C1s spectra analyzed by XPS. Therefore, the high-resolution XPS spectra of the N1s peaks were evaluated on unmodified PVA, PVA-6-FN, PVA-6-oligoFN, PVA-24-FN and PVA-24-oligoFN dishes and are shown in Fig. 2B. An N1s peak at 399 eV was clearly observed in the PVA-6h-FN, PVA-6h-oligoFN, PVA-24h-FN and PVA-24h-oligoFN dishes, whereas only a faint N1s peak at 399 eV was found on the unmodified PVA (PVA-6 and PVA-24) dishes because PVA does not initially include molecules containing nitrogen atoms, whereas the nitrogen atoms can be derived from proteins and oligopeptides in PVA-ECM and PVA-oligoECM dishes (i.e., ECMs and ECM-derived oligopeptides).

The N1S peak on the surface of PVA-6-ECM, PVA-24-ECM, PVA-6-oligoECM and PVA-24-oligoECM dishes was evaluated by XPS, where ECM corresponds to COL, FN or

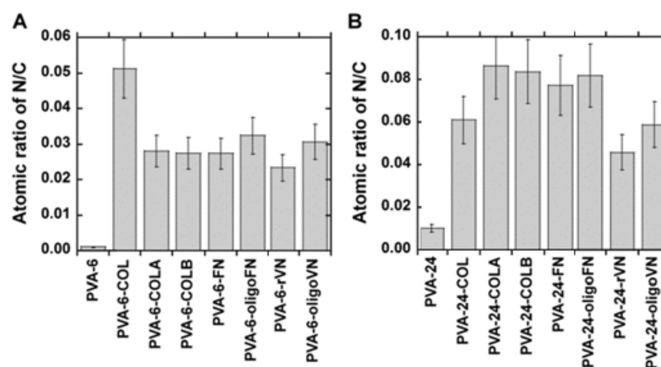

Fig. 3 The atomic ratios of nitrogen to carbon (N/C) on the surface of PVA-6 and PVA-24 dishes grafted with ECM proteins or ECM-derived oligopeptides. (A) The atomic N/C ratio on the surface of PVA-6, PVA-6-COL, PVA-6-COLA, PVA-6-COLB, PVA-6-FN, PVA-6-oligoFN, PVA-6-rVN and PVA-6-oligoVN dishes. (B) The atomic N/C ratio on the surface of PVA-24, PVA-24-COL, PVA-24-COLA, PVA-24-COLB, PVA-24-FN, PVA-24-oligoFN, PVA-24-rVN and PVA-24-oligoVN dishes. The data are expressed as the mean ±S.D. of three independent measurements.

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rVN and oligoECM is COLA, COLB, oligoFN or oligoVN. The atomic ratios of N/C on the surface of the hydrogel dishes were calculated and are shown in Fig. 3. Similar N/C ratios of approximately 0.028 were found on the surface of all PVA-6-ECM and PVA-6-oligoECM dishes ($p<0.05$), except for PVA-6-COL dishes, for which the N/C ratio was higher than that of PVA-6-ECM and PVA-6-oligoECM dishes. The surface on PVA-24-ECM and PVA-24-oligoECM dishes showed N/C ratios of 0.05-0.09, which is 2-3 times higher than the N/C ratio of the surface of PVA-6-ECM and PVA-6-oligoECM dishes. This is because the high water content of PVA-6-ECM and PVA-6-oligoECM hydrogels indicates a lower density of carboxylic acid in a unit volume (surface), which led to a lower degree of grafting of ECM or ECM-derived oligopeptides in a unit volume (surface). No significant difference in N/C was found among the surfaces of PVA-24-ECM and PVA-24-oligoECM, except for PVA-24-rVN, which was slightly reduced compared with that of PVA-24-COLA, PVA-24-COLB, PVA-24-FN and PVA-24-oligoFN. In summary, (1) the amount of COL grafted on the surface of PVA-6-COL and PVA-24-COL was approximately the same ($p>0.05$), and (2) the amount of ECM and ECM-derived oligopeptides grafted on the surface of PVA-24-ECM and PVA-24-oligoECM was 2-3 times higher than that of PVA-6-ECM and PVA-6-oligoECM, except for PVA-6-COL, PVA-24-COL and PVA-24-rVN.

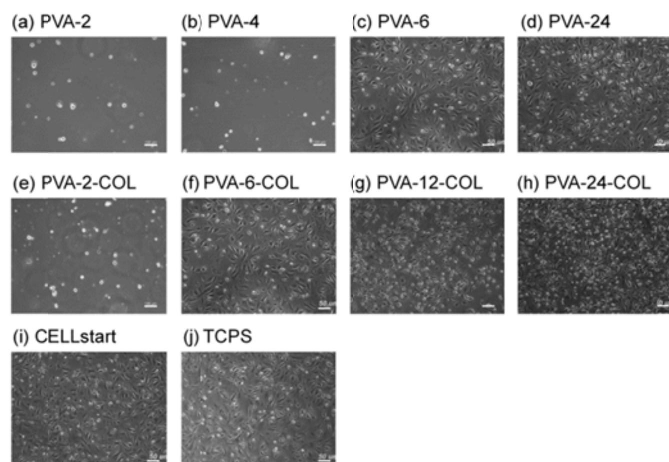

Fig. 4 The morphology of hAFSCs cultured on PVA-2 (a), PVA-4 (b), PVA-6 (c), PVA-24 (d), PVA-2-COL (e), PVA-6-COL (f), PVA-12-COL (g), PVA-24-COL (h), TCPS dishes coated with CELLstart (i) and TCPS (j) dishes for 7 days. The bar indicates 50 μm.

3.3 Culture of hAFSCs on PVA-ECM and PVA-oligoECM dishes

hAFSCs were cultured on non-modified PVA, PVA-ECM, PVA-oligoECM, TCPS and TCPS dishes coated with CELLstart in expansion medium. Fig. 4 shows the morphology of hAFSCs cultured for 7 days on PVA and PVA-COL with elasticities of *E*'= 10.6 kPa (2 h crosslinking time), 11.1 kPa (4 h crosslinking time), 12.2 kPa (6 h crosslinking time) and 25.3 kPa (24 h crosslinking time), as well as TCPS and TCPS dishes coated with CELLstart with 12 GPa of elasticity. hAFSCs could not be expanded on PVA dishes with or without grafting of COL when the PVA dishes had an elasticity less than 12 kPa (PVA-2, PVA-4 and PVA-2-COL), whereas hAFSCs were able to proliferate and expand on PVA dishes with or without grafting of COL when the PVA dishes had $E' > 12$

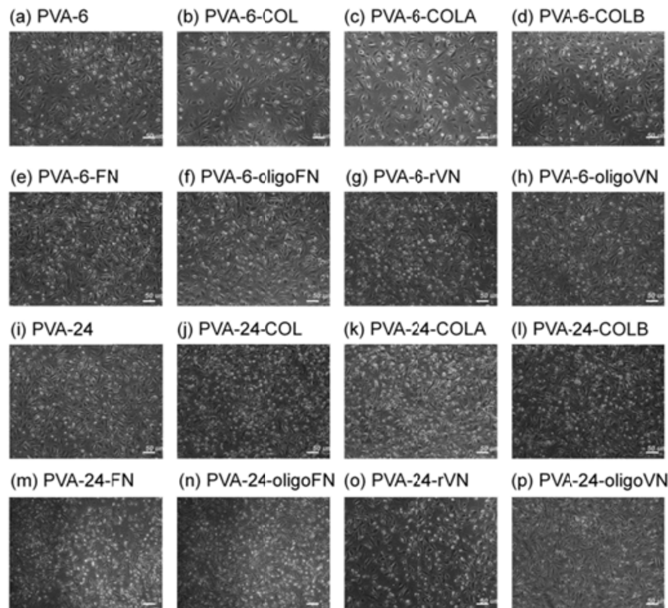

Fig. 5 The morphology of hAFSCs cultured on PVA-6 (a), PVA-6-COL (b), PVA-6-COLA (c), PVA-6-COLB (d), PVA-6-FN (e), PVA-6-oligoFN (f), PVA-6-rVN (g), PVA-6-oligoVN (h), PVA-24 (i), PVA-24-COL (j), PVA-24-COLA (k), PVA-24-COLB (l), PVA-24-FN (m), PVA-24-oligoFN (n), PVA-24-rVN (o) and PVA-6-oligoVN (p) dishes after 7 days of cultivation. The bar indicates 50 μm.

kPa (i.e., PVA-6, PVA-6-COL, PVA-12-COL, PVA-24 and PVA-24-COL). The stiffest surface, TCPS and TCPS dishes coated with CELLstart (12 GPa elasticity), were able to support hAFSC proliferation and expansion. Therefore, soft PVA dishes (PVA-6-ECM and PVA-6-oligoECM) and relatively stiff PVA dishes (PVA-24-ECM and PVA-24-oligoECM) grafted with several ECM and ECM-derived oligopeptides, as well as the stiffest dishes (TCPS and TCPS dishes coated with CELLstart), were selected to investigate the doubling time and expression of pluripotency and differentiation genes in hAFSCs in this study.

Fig. 5 shows the morphology of hAFSCs cultured for 7 days on relatively soft (PVA-6) and stiff (PVA-24) PVA hydrogel dishes grafted with several ECM proteins (COL, FN and rVN) or ECM-derived oligopeptides (COLA, COLB, oligoFN and oligoVN) in expansion medium. hAFSCs were able to proliferate and expand on any of these PVA hydrogel dishes. The cell density on each dish was analyzed at days 0-7, and the doubling time of hAFSCs cultured on these PVA hydrogel dishes and on TCPS and TCPS dishes coated with CELLstart was evaluated and is shown in Fig. 6.

The doubling time of hAFSCs cultured on relatively stiff PVA-24 hydrogel dishes grafted with and without ECM or ECM-derived oligopeptides or cultured on the stiff surface of TCPS and TCPS dishes coated with CELLstart was found to be approximately 6 days, which is shorter than that of cells cultured on the relatively soft PVA-6 hydrogel dishes grafted with and without ECM or ECM-derived oligopeptides in hAFSC culture conditions. The effect of ECM or ECM-derived oligopeptide grafting on the doubling time of hAFSCs was not found to be significant in this study. The soft surface of the PVA-6 hydrogel slowed the expansion of hAFSCs. Several other studies also reported a decreased expansion speed of stem cells cultured on soft biomaterials.[8,23] For example, Winer et al. cultured human mesenchymal stem cells (hMSCs) on 250-Pa crosslinked polyacrylamide (PAAm) hydrogels coated with

Journal of Materials Chemistry B Accepted Manuscript

fibronectin and collagen I. hMSCs cultured on a soft substrate halted progression through the cell cycle.[8,23] These nonproliferative hMSCs began to expand when hMSCs were re-seeded on a stiff substrate. Therefore, hMSCs on soft (250-Pa) PAAm hydrogels are quiescent but competent to begin proliferation or differentiation.[8,23]

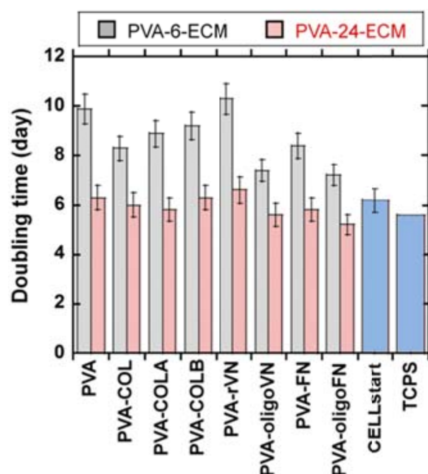

Fig. 6 The doubling time of hAFSCs cultured on soft PVA-6 (gray bar) and stiff PVA-24 (red bar) dishes grafted with ECM (COL, rVN and FN) or ECM-derived oligopeptides (COLA, COLB, oligoVN and oligoVN), TCPS (blue bar) dishes and TCPS dishes coated with CELLstart (blue bar). The data are expressed as the mean ±S.D. of three independent measurements.

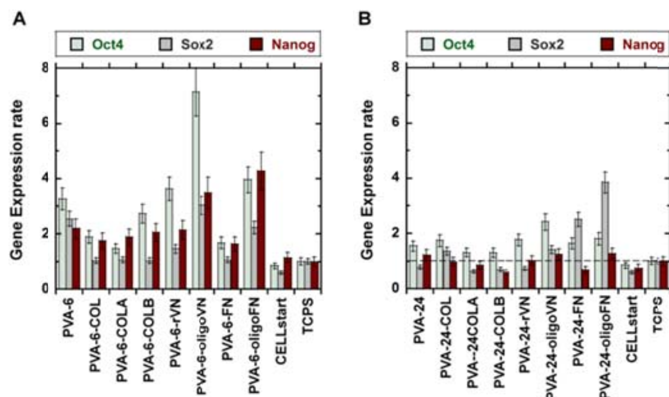

Fig. 7 The expression of pluripotency genes (green bar, *Oct4*; black bar, *Sox2*; and red bar, *Nanog*) in hAFSCs cultured on soft PVA-6 (A) and stiff PVA-24 (B) hydrogel dishes grafted with ECM (COL, rVN and FN) or ECM-derived oligopeptides (COLA, COLB, oligoVN and oligoVN) or TCPS and TCPS dishes coated with CELLstart after 7 days of cultivation. The data are expressed as the mean ±S.D. of three independent measurements.

3.4 The expression of pluripotency and differentiation genes in hAFSCs on PVA-ECM and PVA-oligoECM dishes

It has not been clear whether the slow expansion of hAFSCs maintains the pluripotency of adult stem cells or promotes their differentiation. Therefore, the expression of genes related to pluripotency and differentiation was evaluated in hAFSCs cultured on soft and stiff PVA hydrogels grafted with and without ECM or ECM-derived oligopeptides in expansion medium.

Fig. 7 shows the expression of the pluripotency genes *Oct4*, *Sox2* and *Nanog* in hAFSCs cultured on PVA-6-ECM (Fig. 7A), PVA-6-oligoECM (Fig. 7A), PVA-24-ECM (Fig. 7B), PVA-24-oligoECM (Fig. 7B), TCPS and TCPS dishes coated with CELLstart (Figs. 7A and 7B). The expression of the pluripotency genes in cells cultured on the stiffest surfaces of TCPS and TCPS dishes coated with CELLstart was found to be similar and was the lowest expression level observed in this study. The expression of the pluripotency genes *Oct4* and *Nanog* in hAFSCs cultured on the relatively soft hydrogels of PVA-6-ECM and PVA-6-oligoECM dishes was found to be higher than that of cells cultured on the stiffest surfaces of TCPS and TCPS dishes coated with CELLstart (p<0.05). Furthermore, the soft hydrogels of PVA-6-ECM and PVA-6-oligoECM dishes appeared to lead to higher expression of pluripotency genes in hAFSCs than the relatively stiff hydrogels of PVA-24-ECM and PVA-24-oligoECM.

When we compared the pluropotency gene expression of hAFSCs cultured on PVA hydrogels grafted with ECM and ECM-derived oligopeptides, no significant difference was found on PVA hydrogels grafted with collagen type 1 and collagen-derived oligopeptides (COLA and COLB). However, each pluripotency gene expression (*Oct4*, *Sox2* and *Nanog*) of hAFSCs cultured on PVA hydrogels grafted with vitronectin-derived oligopeptide (oligoVN) and fibronectin-derived oligopeptide (oligoFN) was found to be higher than that on PVA hydrogels grafted with vitronectin and fibronectin, respectively in this study (p<0.05). These results indicate that ECM-derived oligopeptides of oligoVN and oligoFN contribute to work for the maintenance of pluripotency of hAFSCs on PVA hydrogels grafted with ECM-derived oligopeptides.

In this study, we introduce the index of pluripotency gene expression ($I_{pluripotency}$) as the sum of the expression of pluripotency genes in hAFSCs, which is relative to that of cells cultured on TCPS dishes:

$$I_{pluripotency} = I_{Oct4} + I_{Sox2} + I_{Nanog} \quad (1)$$

where $I_{pluripotency}$ is the index of pluripotency gene expression. I_{Oct4}, I_{Sox2} and I_{Nanog} are the expression of *Oct4*, *Sox2* and *Nanog*, respectively, relative to the expression of the same genes in cells cultured on TCPS dishes. Fig. 8 shows the index of pluripotency gene expression ($I_{pluripotency}$) of hAFSCs cultured on PVA-6 and PVA-24 hydrogel dishes grafted with and without ECM or ECM-derived oligopeptides. hAFSCs cultured on the soft PVA-6 hydrogel dishes grafted with oligoVN (PVA-6-oligoVN) and oligFN (PVA-6-oligoFN) showed higher $I_{pluripotency}$ values than cells cultured on the stiff PVA-24 hydrogel dishes grafted with ECM or ECM-derived oligopeptides and cells grown on PVA-6 dishes grafted with ECM or ECM-derived oligopeptides other than oligoVN and oligFN. It is interesting to note that the $I_{pluripotency}$ of hAFSCs cultured on non-modified PVA-6 hydrogel was relatively high among those of the biomaterials evaluated in this study, although the $I_{pluripotency}$ of hAFSCs on PVA-6-oligoVN dishes was higher than that of cells cultured on PVA-6 dishes (p<0.05).

The expression of typical genes that mark differentiation into the three germ layers, *Nestin* (ectoderm), *Sox17* (endoderm) and *Runx2* (mesoderm) by hAFSCs cultured on PVA-6-ECM, PVA-6-oligoECM, PVA-24-ECM, PVA-24-oligoECM, TCPS and TCPS dishes coated with CELLstart was investigated in hAFSCs cultured in expansion medium, and the results are shown in Fig. 9A for PVA-6 hydrogel dishes and in Fig. 9B for PVA-24 hydrogel dishes. hAFSCs cultured on soft PVA-6 hydrogel dishes with or without ECM and ECM-derived oligopeptides showed more than 2.5-fold higher expression of *Nestin* compared with cells cultured on TCPS dishes. It appears that soft PVA-6-COL, PVA-6-oligoVN and PVA-6-FN hydrogel dishes are preferable for hAFSCs to induce

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spontaneous early differentiation into neural cells, which is evaluated by the expression of *Nestin*.

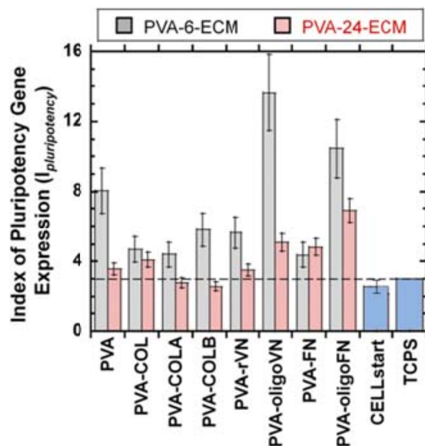

Fig. 8 Index of pluripotency gene expression ($I_{pluripotency}$) of hAFSCs cultured on soft PVA-6 (black bar) and stiff PVA-24 (red bar) hydrogel dishes grafted with ECM (COL, rVN and FN) or ECM-derived oligopeptides (COLA, COLB, oligoVN and oligoVN), TCPS dishes (blue bar) and TCPS dishes coated with CELLstart (blue bar) after 7 days of cultivation. The data are expressed as the mean ±S.D. of three independent measurements.

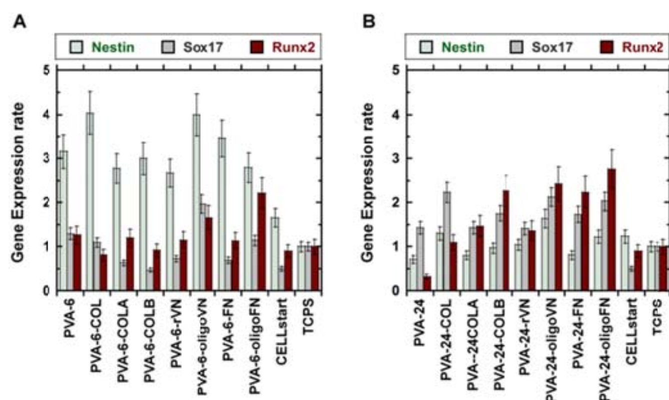

Fig. 9. The expression of differentiation genes (green bar, *Nestin*; black bar, *Sox17*; and red bar, *Runx2*) in hAFSCs cultured on soft PVA-6 (A) and stiff PVA-24 (B) hydrogel dishes grafted with ECM (COL, rVN and FN) or ECM-derived oligopeptides (COLA, COLB, oligoVN and oligoVN), TCPS dishes and TCPS dishes coated with CELLstart after 7 days of cultivation. The data are expressed as the mean ±S.D. of three independent measurements.

hAFSCs cultured on PVA-6-oligoVN expressed increased levels of *Sox17* compared with hAFSCs grown on PVA grafted with or without ECM and ECM-derived oligopeptides, except oligoVN, or compared with hAFSCs cultured on TCPS and TCPS dishes coated with CELLstart. hAFSCs grown on PVA-6-oligoVN and PVA-6-oligoFN showed higher *Runx2* expression than cells cultured on PVA-6 grafted with or without ECM and ECM-derived oligopeptides other than oligoVN and oligoFN. However, hAFSCs cultured on the stiff PVA-24-ECM and PVA-24 oligoECM dishes showed higher *Sox17* and *Runx2* expression than cells grown on soft PVA-6-ECM and PVA-6-oligoECM or stiff TCPS and TCPS dishes coated with CELLstart, except for PVA-6-oligoVN and PVA-6-oligoFN. PVA-24-COLB, PVA-24-oligoVN, PVA-24-FN and PVA-oligoFN dishes, as well as PVA-6-oligoFN dishes, appeared to be preferable cell culture dishes to promote the spontaneous early differentiation into osteoblasts, which is evaluated by *Runx2* gene expression. Furthermore, PVA-24-COL, PVA-24-oligoVN and PVA-24-oligoFN dishes, as well as PVA-6-oligoVN dishes, are the preferable cell culture dishes for promoting spontaneous early differentiation into endoderm, which is evaluated by *Sox17* gene expression.

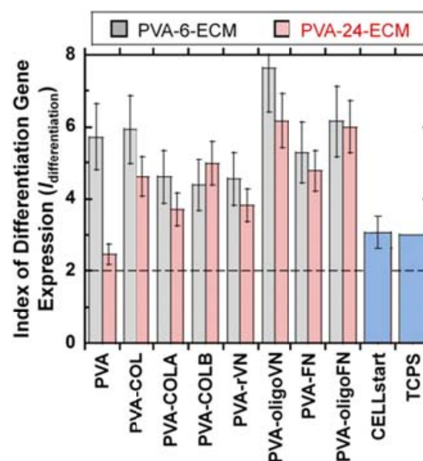

Fig. 10 Index of differentiation gene expression ($I_{differentiation}$) of hAFSCs cultured on soft PVA-6 (black bar) and stiff PVA-24 (red bar) hydrogel dishes grafted with ECM (COL, rVN and FN) or ECM-derived oligopeptides (COLA, COLB, oligoVN and oligoVN), TCPS dishes (blue bar) and TCPS dishes coated with CELLstart (blue bar) after 7 days of cultivation. The data are expressed as the mean ±S.D. of three independent measurements.

In this study, we also introduce the index of differentiation gene expression ($I_{differentiation}$) as the sum of differentiation gene expression of hAFSCs, which is relative to the expression of the same genes in cells cultured on TCPS:

$$I_{differentiation} = I_{Nestin} + I_{Sox17} + I_{Runx2} \quad (2)$$

where $I_{differentiation}$ is the index of differentiation gene expression. I_{Nestin}, I_{Sox17} and I_{Runx2} are the expression of *Nestin*, *Sox17* and *Runx2*, respectively, relative to the expression of the same genes in cells cultured on TCPS dishes. Fig. 10 shows the index of differentiation gene expression ($I_{differentiation}$) of hAFSCs cultured on PVA-6 and PVA-24 hydrogel dishes grafted with and without ECM or ECM-derived oligopeptides. hAFSCs cultured on soft and stiff PVA-ECM and PVA-oligoECM hydrogel dishes showed higher $I_{differentiation}$ values than those of cells grown on TCPS, TCPS coated with CELLstart, and PVA-24 dishes. The $I_{differentiation}$ of hAFSCs cultured on PVA-6-ECM and PVA-6-oligoECM dishes was not significantly different from the $I_{differentiation}$ of hAFSCs cultured on PVA-24-ECM and PVA-24-oligoECM dishes on each ECM or ECM-derived oligopeptide within experimental error (p>0.05). This is likely due to compensation by the expression of different genes, as soft PVA-6-ECM and PVA-6-oligoECM dishes preferentially promote spontaneous neural differentiation of hAFSCs (*Nestin* expression) and, in contrast, stiff PVA-24-ECM and PVA-24-oligoECM dishes preferentially promote the spontaneous osteogenic (*Runx2* expression) and endoderm (S*ox17* expression) differentiation of hAFSCs.

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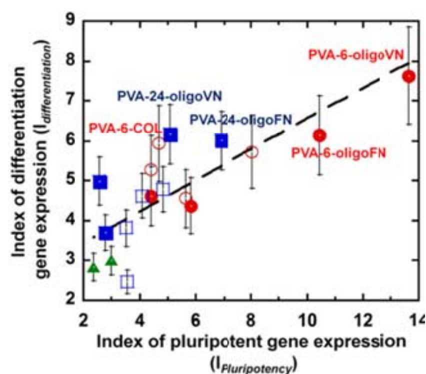

Fig. 11. The relationship between $I_{pluripotency}$ and $I_{differentiation}$ for hAFSCs cultured on soft PVA-6 (red circle) and stiff PVA-24 (blue square) hydrogel dishes grafted with ECM (open symbol, COL, rVN and FN) or ECM-derived oligopeptides (closed symbol, COLA, COLB, oligoVN and oligoVN), TCPS dishes (green triangle) and TCPS dishes coated with CELLstart (green triangle) after 7 days of cultivation. The data are expressed as the mean ±S.D. of three independent measurements.

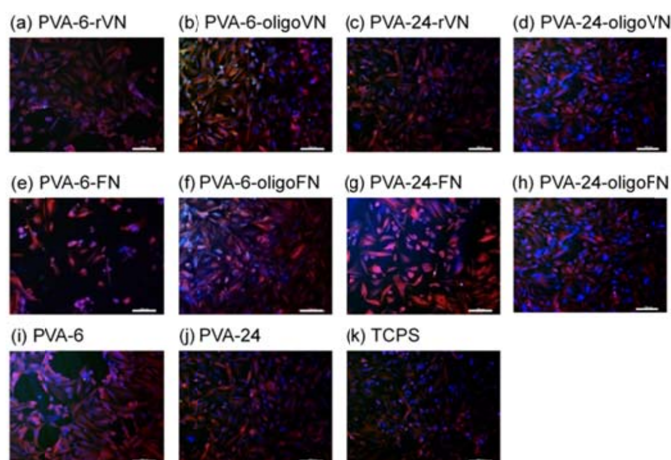

Fig. 12 Immunostaining of the pluripotency marker protein Sox2 (green) and the differentiation marker protein β-III tubulin (red) in hAFSCs cultured on PVA-6-rVN (a), PVA-6-oligoVN (b), PVA-24-rVN (c), PVA-24-oligoVN (d), PVA-6-FN (e), PVA-6-oligoFN (f), PVA-24-FN (g), PVA-24-oligoFN (h), PVA-6 (i), PVA-24 (j) and TCPS (k) dishes after 7 days of cultivation. The nucleus was stained with Hoechst 33342 (blue).

The relationship between $I_{differentiation}$ and $I_{pluripotency}$ was investigated for hAFSCs cultured on PVA-ECM and PVA-oligoECM dishes in expansion medium, and the results are shown in Fig. 11. $I_{differentiation}$ was found to increase with an increase in $I_{pluripotency}$ with a linearity of 0.796. These results were unexpected because the expression of pluripotency genes in hESCs and hiPSCs is reported to be downregulated when differentiation of the stem cells is induced.[29,30] The unexpected results found in this study may be explained as follows. The stem cells used in this study are not hESCs or hiPSCs but hAFSCs, which contain a heterogeneous cell population. hAFSCs, which maintain the stem cells with high pluripotency on specific biomaterials, also contain differentiated cells derived from the stem cells with high pluripotency. This tendency is only observed for the culture of heterogeneous population of stem cells on biomaterials such as hAFSCs in this study as well as human adipose-derived stem cells (unpublished data).

3.5 The expression of pluripotency and differentiation proteins in hAFSCs cultured on PVA-ECM and PVA-oligoECM dishes

In the previous sections, pluripotency and differentiation into specific lineages of stem cells were evaluated by gene expression (Figs. 7-11). We further evaluated the expression of pluripotency proteins and specific differentiation markers in the stem cells to evaluate pluripotency and/or the differentiation lineages of the stem cells.

Figs. 12 and 13 show immunostaining of the pluripotency protein markers Sox2 and SSEA4 and the differentiation protein markers β-III tubulin (neural cells, ectoderm) and AFP (hepatocytes, endoderm) in hAFSCs cultured on relatively soft PVA-6 hydrogel dishes (PVA-6, PVA-6-rVN, PVA-6-oligoVN, PVA-6-FN, and PVA-6-oligoFN), stiff PVA-24 hydrogel dishes (PVA-24, PVA-24-rVN, PVA-24-oligoVN, PVA-24-FN, and PVA-24-oligoFN) and the most stiff TCPS dishes; these conditions were tested because oligoVN and oligoFN promoted pluripotency as well as the spontaneous differentiation of hAFSCs when grafted as nanosegments on PVA hydrogel dishes.

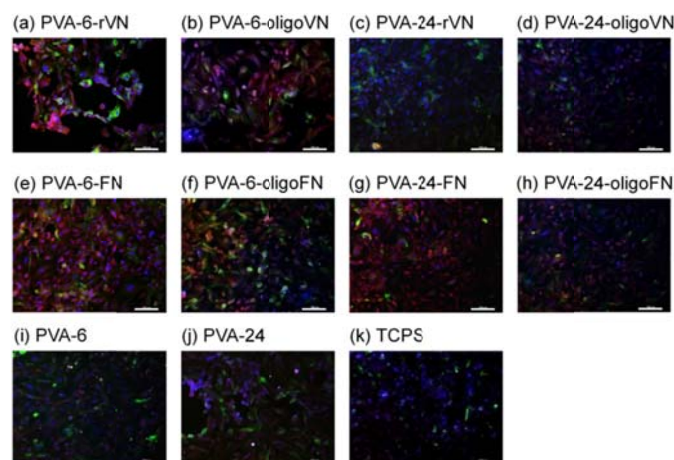

Fig. 13 Immunostaining of the pluripotency marker protein SSEA4 (green) and the differentiation marker protein AFP (red) in hAFSCs cultured on PVA-6-rVN (a), PVA-6-oligoVN (b), PVA-24-rVN (c), PVA-24-oligoVN (d), PVA-6-FN (e), PVA-6-oligoFN (f), PVA-24-FN (g), PVA-24-oligoFN (h), PVA-6 (i), PVA-24 (j) and TCPS (k) dishes after 7 days of cultivation. The nucleus was stained with Hoechst 33342 (blue).

We evaluated the expression of pluripotency proteins in hAFSCs cultured on soft and stiff PVA hydrogel dishes grafted with and without ECM proteins and ECM-derived oligoECMs. Relatively high levels of Sox2 immunostaining were found in hAFSCs cultured on soft PVA-6-oligoVN and PVA-6-oligoFN dishes (Fig. 12), which was consistent with the trend observed for Sox 2 gene expression in hAFSCs (Fig. 7A). We also found that hAFSCs cultured on soft PVA-6-FN, PVA-6-oligoFN, PVA-6-rVN and PVA-6-oligoVN dishes showed high immunostaining for SSEA4 (Fig. 13). hAFSCs cultured on the stiffest TCPS dishes exhibited the faintest immunostaining for Sox2 and SSEA4 in this study. From these results, soft PVA-6 dishes grafted with oligoFN and oligoVN appear

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to be preferable for culturing hAFSCs to maintain pluripotency, as indicated by immunostaining results of Sox2 and SSEA4, which were consistent with the results obtained for the gene expression of *Oct4*, *Sox2* and *Nanog* in hAFSCs shown in Fig. 7A.

hAFSCs cultured on soft and stiff PVA hydrogel dishes with and without ECM proteins or ECM-derived oligoECMs showed relatively similar immunostaining intensity for β-III tubulin (Fig. 12). However, high immunoreactivity of AFP was observed in hAFSCs cultured on soft PVA-6-FN, PVA-6-oligoFN, PVA-6-rVN, and PVA-6-oligoVN dishes, as well as PVA-6 dishes, whereas only faint staining of AFP was observed in hAFSCs cultured on stiff PVA-24 dishes grafted with and without ECM and ECM-derived oligopeptides and those grown on TCPS dishes (Fig. 13). Soft PVA-6-oligoVN dishes may be the preferable cell culture dishes for the spontaneous differentiation of hAFSCs into early hepatocytes because hAFSCs showed high immunostaining of AFP (Fig. 13) and because high expression of the endoderm differentiation gene Sox17 (Fig. 9A) was observed in cells cultured on PVA-6-oligoVN dishes.

3.6 Comparison of the present work with previously published work

Discher and Engler et al. proposed a general model for the effect of stiffness of cell culture biomaterials on the spontaneous differentiation of human mesenchymal stem cells (hMSCs) into several specific lineages depending on the stiffness of the biomaterials[20]; when hMSCs were cultured in expansion medium and not in induction medium to promote differentiation, softer materials with elasticities similar to that of the brain (0.3 kPa) tended to induce hMSCs to differentiate into early lineages of neural cells, whereas stiffer materials (10 kPa) mimicking muscle caused hMSCs to spontaneously differentiate into early myoblasts in Engler's work.[8,20] Rigid materials similar to collagenous bone were also reported to guide hMSCs toward early differentiation into osteoblasts. In this study, we examined the effect of stiffness on the hAFSC fates of pluripotency and differentiation. The early neural cell differentiation of hAFSCs occurred more readily on soft PVA-6 hydrogel dishes grafted with ECM or ECM-derived oligopeptides, as assessed by *Nestin* gene expression, whereas stiff PVA-24 hydrogel dishes grafted with ECMs or ECM-derived oligopeptides guided hAFSCs to differentiate into early osteoblasts, as measured by *Runx2* gene expression, similar to what was observed by Engler.[8,20] However, hAFSCs cultured on stiff TCPS dishes did not preferentially differentiate into osteoblasts and instead showed the lowest pluripotency and the lowest differentiation as measured by both gene expression and immunostaining results in this study.

Tse and Engler prepared crosslinked polyacrylamide (PAAm) hydrogels with radial elastic modulus gradients of 1 kPa/mm in an elastic module from 1.0 to 14 kPa by photopolymerization using a gradient-patterned photomask.[8,31] In expansion medium, hMSCs tended to migrate into the stiffer hydrogel and then to differentiate into a more contractile myogenic cell on the PAAm hydrogels immobilized with collagen. In contrast, hMSCs expressing a neuronal marker tended to reside on soft regions of the gradient PAAm hydrogels.[31] It was suggested that soft cell-culture biomaterials drive hMSCs toward neuronal differentiation lineages when hMSCs are cultivated in expansion medium or induction (differentiation) medium.[8,20,32-42] These studies are consistent with the results in this study that hAFSCs cultured on soft PVA-6 hydrogels grafted with ECM or ECM-derived oligopeptides better support pluripotency and the high expression of *Nestin* compared with cells cultured on stiff PVA-24 hydrogels or TCPS dishes. However, PVA hydrogels that are too soft (less than 11 kPa of *E*') could not support hAFSC proliferation in this study when grafted with and without ECM or ECM-derived oligopeptides (Fig. 4).

Gilbert et al. investigated whether the elastic modulus of the cell culture biomaterials plays a critical role in the self-renewal and differentiation of stem cells during muscle regeneration.[8,43] These authors created crosslinked PEG (poly(ethylene glycol)) hydrogels immobilized with laminin with different elastic moduli (2, 12, and 42 kPa). It was found that the number of muscle stem cells expanded on soft PEG hydrogels increased twofold compared with cells that were cultured on stiff TCPS dishes.[43] This result suggests that the muscle stem cells exhibit enhanced cell survival when cultured on soft PEG hydrogels. Furthermore, the muscle stem cells cultured on soft PEG hydrogels were found to express lower levels of a differentiation marker (myogenin) compared with cells cultured on rigid TCPS dishes.[8,43] In Gilbert's study, the soft cell culture biomaterials appeared to enhance cell numbers by increasing cell viability and by preventing the differentiation of muscle stem cells in vitro,[43] whereas hAFSCs in this study exhibited high expression of pluripotency genes and the expression of several differentiation-induced genes when cultured on soft PVA-6 hydrogel dishes grafted with ECM or ECM-derived oligopeptides (Figs. 7A and 9A). This conflicting phenomenon is likely due to the heterogeneous population of stem cells found in hAFSCs, whereas the muscle stem cells appear to be a homogeneous cell population.

An interfacial hydrogel was created by preparing an interpenetrating polymer network with an oligopeptide containing RGD (arginine-glycine-aspartic acid) sequences on the surface with a stiffness ranging from 10 to 10 000 Pa by Saha and Healy et al.[8,36] Rat neural stem cells expanded when cultivated on the hydrogels with elastic moduli greater than 100 Pa. The highest expression of a neural marker (β-III tubulin) in rat neural stem cells was observed on the hydrogels with a 500 Pa elastic modulus, which is close to the physiological stiffness of brain tissue.[8,36] It was found that neuronal differentiation preferentially occurred on softer hydrogels in their mixed glial and neuronal differentiation medium. By contrast, glial differentiation occurred preferentially on stiffer hydrogels in the same medium.[8,36] This study demonstrates that physical (elasticity of the cell culture biomaterials) and biochemical (ECM and ECM-derived oligopeptide) factors are important regulators of self-renewal and specific lineage differentiation of stem cells, as observed in the pluripotency and spontaneous differentiation of hAFSCs cultured on hydrogels with different elasticities grafted with ECM and ECM-derived oligopeptides in this study.

We found that soft PVA-6-oligoVN dishes can maintain pluripotency of hAFSCs from high expression of pluripotency genes (Figs. 7 and 8) and proteins (Figs. 12 and 13). The amino acid sequence of oligoVN selected in this study was also used in Melkoumian's investigation[44] where they developed polyacrylate dishes grafted with oligoVN for culture of hESCs and hiPSC maintaining their pluripotency. hESCs can be cultured on the dishes grafted with oligoVN without differentiation of hESCs for over 10 passages, whereas hESCs cannot be cultured on the dishes grafted with oligopeptides derived from laminin or fibronectin because of easy differentiation of hESCs on the dishes. Therefore, the specific sequence of oligoVN seems to be an optimal sequence to keep pluripotency of stem cells including hAFSCs as well as hESCs and hiPSCs. Not only biochemical factor (amino acid sequence of oligoVN) but also physical factor (low elasticity) of PVA-6-oligoVN should be the reason why PVA-6-oligoVN dishes can keep pluripotency of hAFSCs compared to other dishes investigated in this study.

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Conclusions

hAFSCs were cultured on soft and rigid PVA hydrogels grafted with and without ECM or ECM-derived oligopeptides to evaluate the regulation of pluripotency and spontaneous differentiation of the cells in expansion medium. It is necessary for the PVA hydrogels to have a minimum storage modulus (E'>12 kPa) to support hAFSC proliferation and expansion on the PVA hydrogels grafted with and without ECM or ECM-derived oligopeptides. The soft PVA-6-ECM and PVA-6-oligoECM hydrogel dishes (E'=12.2 kPa) are favorable for culturing hAFSCs that maintain high pluripotency, as assessed by pluripotency gene and protein expression, compared with the relatively stiff PVA-24-ECM and PVA-24-oligoECM hydrogel dishes (E'=25.3 kPa) or TCPS and TCPS dishes coated with CELLstart (12 GPa of elastic modulus). Soft PVA-6-oligoFN and PVA-6-oligoVN dishes are the most favorable cell culture dishes for hAFSCs to maintain high pluripotency and to induce spontaneous differentiation into early neural cells (i.e., high expression of *Nestin*). Relatively stiff PVA-24-COLB, PVA-24-oligoVN, PVA-24-FN and PVA-24-oligoFN dishes but not the stiffest TCPS dishes promote spontaneous differentiation of hAFSCs into early osteoblasts (i.e., high expression of *Runx2*). hAFSCs expressing higher levels of pluripotency genes (*Oct4*, *Sox2* and *Nanog*) on PVA-ECM and PVA-oligoECM hydrogel dishes also showed higher expression of three early germ layer marker genes (*Sox17*, *Runx2* and *Nestin*) in expansion medium. This result suggests that the hAFSCs contain a heterogeneous stem cell population and, therefore, contain highly pluripotent stem cells and stem cells that can easily differentiate. It is concluded that both insoluble biochemical cues (ECM-derived oligopeptides) and physical cues (stiffness) of cell culture biomaterials are important factors to maintain the pluripotency and spontaneous differentiation ability of stem cells.

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Notes and references

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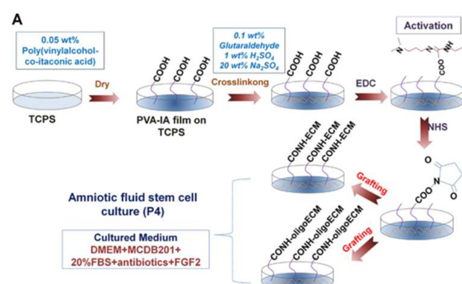

Human amniotic fluid-derived stem cells can keep their pluripotency cultured on soft polyvinylalcohol hydrogels grafted with several oligopeptides derived from extracellular matrices.

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