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Complete List of Authors:	Gao, Yan; Wenzhou Medical University Ku, Nien-Ju; National Central University Sung, Tzu-Cheng; National Central University Higuchi, Akon; National Central University Hung, Chi-Sheng; National Central University Lee, Henry; Cathay General Hospital Hsinchu Branch Ling, Qing-Dong; Cathay General Hospital Cheng, Nai-Chen; National Taiwan University Hospital Umezawae, Akihiro; National Center for Child Health and Development Barro, Lassina ; Taipei Medical University Burnouf, Thierry; Taipei Medical University, Ye, Qingsong; University of Queensland Chen, Hao; Wenzhou Medical University Eye Hospital

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Yan Gao,†[a] Nien-Ju Ku,†[b] Tzu-Cheng Sung,†[b] Akon Higuchi,*[a,b,c,d] Chi-Sheng Hung,[b] Henry Hsin-Chung Lee,[e,f] Qing-Dong Ling,[g] Nai-Chen Cheng,[h] Akihiro Umezawa,[i] Lassina Barro,[j] Thierry Burnouf,[j,k] Qingsong Ye[l] and Hao Chen*[a]

Human mesenchymal stem cells (hMSCs), such as human adipose-derived stem cells (hADSCs), present heterogeneous characteristics, including varying differentiation abilities and genotypes. hADSCs isolated under different conditions exhibit differences in stemness. We isolated hADSCs from human fat tissues via culture on different cell culture biomaterials including tissue culture polystyrene (TCPS) dishes and extracellular matrix protein (ECM)-coated dishes in medium supplemented with 5% or 10% serum-converted human platelet lysate (hPL) or 10% fetal bovine serum (FBS) as a control. Currently, it is not clear whether xeno-free hPL in the cell culture medium promotes the ability of hMSCs such as hADSCs to differentiate into several cell lineages compared to the xenomaterial FBS. We investigated whether a synchronized effect of ECM (Matrigel, fibronectin, and recombinant vitronectin) coating on TCPS dishes for efficient hADSC differentiation could be observed when hADSCs were cultured in hPL medium. We found that Matrigel-coated dishes promoted hADSC differentiation into osteoblasts and suppressed differentiation into chondrocytes in 10% hPL medium. Recombinant vitronectin- and fibronectin-coated dishes greatly promoted hADSC differentiation into osteoblasts and chondrocytes in 5% and 10% hPL media. hPL promoted hADSC differentiation into osteoblasts and chondrocytes compared to FBS on fibronectin-coated surface and recombinant vitronectin-coated surface.

Introduction

Human fat tissue contains a type of human mesenchymal stem cells (hMSCs) known as human adipose-derived stem cells (hADSCs), which are expected to be useful in several clinical therapies.[1-7] Human ADSCs seem to be a more practical cell source than hPSCs (human pluripotent stem cells) such as hESCs (human embryonic stem cells)[8-15] and hiPSCs (human induced pluripotent stem cells)[16-22] because they present fewer ethical concerns and less likelihood of tumor generation.[23,24] hADSCs exhibit multipotency and show the ability to differentiate into cells derived mainly from mesoderm lineages, such as chondrocytes, cardiomyocytes, osteoblasts and adipocytes, thus providing patient-derived renewable sources to support tissue regeneration.[6,25-34] Therefore, hADSCs have been utilized in several clinical trials,[35-37] whereas there have been only a few clinical trials utilizing hPSCs.[2,9,38-41] However, hADSCs exhibit heterogeneous characteristics and present differences in stemness such as varying differentiation abilities and pluripotency depending on the applied isolation, purification, and cultivation methods.[24] Therefore, these stem cell characteristics are especially dependent on the cultivation conditions of hADSCs, which are cultured on different substrates and in specific cell culture media during their expansion.

Typically, hMSCs such as hADSCs and bone marrow-derived stem cells (hBMSCs) are cultivated in medium containing fetal bovine serum (FBS, or fetal calf serum (FCS)).[42,43] FBS are xeno-derived materials, and the usage of FBS should, therefore, be

[a] School of Biomedical Engineering, The Eye Hospital of Wenzhou Medical University, No. 270, Xueyuan Road, Wenzhou, Zhejiang, 325027, China
E-mail: higuchi@wmu.edu.cn & chenhao@mail.eye.ac.cn; Fax: +86-577-88824115; Tel: +86-577-88824116
[b] Department of Chemical and Materials Engineering, National Central University, No. 300, Jhongda RD., Jhongli, Taoyuan, 32001 Taiwan.
[c] Center for Emergent Matter Science, Riken, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[d] Wenzhou Institute, University of Chinese Academy of Science, No. 16, Xinsan Road, Hi-tech Industry Park, Wenzhou, Zhejiang, China
[e] Department of Surgery, Hsinchu Cathay General Hospital, No. 678, Sec 2, Zhonghua Rd., Hsinchu, 30060, Taiwan
[f] Graduate Institute of Translational and Interdisciplinary Medicine, National Central University, No. 300, Jhongda RD., Jhongli, Taoyuan, 32001 Taiwan
[g] Cathay Medical Research Institute, Cathay General Hospital, No. 32, Ln 160, Jian-Cheng Road, Hsi-Chi City, Taipei, 221, Taiwan
[h] Department of Surgery, National Taiwan University Hospital and College of Medicine, 7 Chung-Shan S. Rd., Taipei 100, Taiwan
[i] Department of Reproduction, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[j] International PhD Program in Biomedical Engineering, College of Biomedical Engineering, Taipei Medical University, No. 250 Wu-Xing Street, Taipei 11031, Taiwan
[k] Graduate Institute of Biomedical Materials and Tissue Engineering, College of Biomedical Engineering, Taipei Medical University, No. 250 Wu-Xing Street, Taipei 11031, Taiwan
[l] Regenerative Dentistry Group, School of Dentistry, The University of Queensland, 288 Herston Road, Herston Qld, Brisbane 4006, Australia
† These authors contributed equally to this work.
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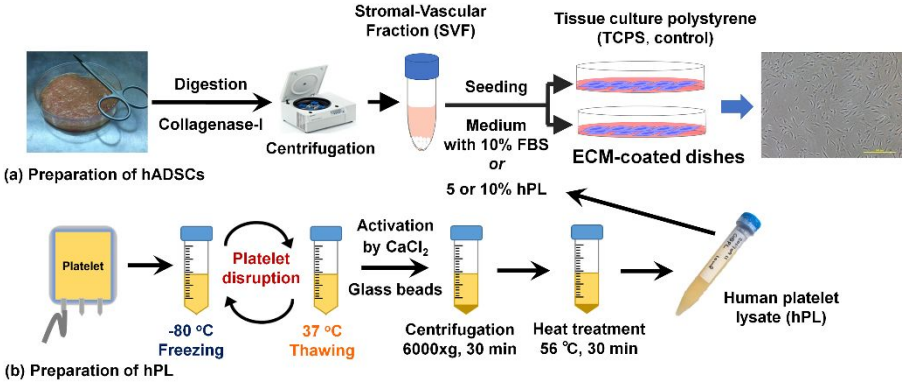

Fig. 1 Outline of hADSC isolation and cultivation. (a) Procedure for the isolation of hADSCs. The primary adipose tissue cell solution (SVF) was obtained from fat tissue by collagenase digestion. The SVF was cultivated in uncoated TCPS dishes and ECM-coated dishes to isolate hADSCs via the conventional culture method in media containing FBS or hPL. (b) Procedure for the preparation of hPL from platelet-rich plasma. Platelet-rich plasma was subjected to three freezing and thawing cycles (-80 °C/37 °C) followed by centrifugation to prepare hPL.

avoided in clinical-grade hMSC cultivation. Although several serum-free media have been developed, cell culture media supplemented with human platelet lysate (hPL) are particularly promising media for clinical-grade hMSC cultivation.[44-47] This is because platelets are known to show several functions during tissue repair and release multiple cytokines and growth factors.[48,49] Platelets consist of intracellular granules that are abundant in potent mitogenic and reparative substances.[50,51] The bioactive components are released from platelets into a resultant clot and exert an important influence on the regeneration of tissue by triggering stromal cell migration, proliferation, and differentiation.[25,52]

Several researchers have reported that hMSCs (mainly hBMSCs) that are cultivated in media supplemented with hPL can proliferate and maintain the ability to differentiate into cardiomyocytes, chondrocytes, adipocytes, hepatocytes, ligament cells, endothelial cells, and/or osteoblasts, similar to hMSCs cultured in media supplemented with FBS.[25,45,48,53-57]

Chevallier *et al.* compared the osteogenic differentiation ability of hBMSCs cultivated in media supplemented with FBS and hPL.[58] *In vivo*, ectopic bone formation was only detected on hydroxyapatite-beta tricalcium phosphate scaffolds seeded with hBMSCs that were cultivated in medium supplemented with hPL when the scaffolds were implanted under the skin of immunodeficient mice. Therefore, the authors concluded that hPL accelerated hBMSC expansion and promoted hBMSC differentiation into osteoblasts, as reported by other researchers.[58-61] Hildner *et al.* also reported that hADSCs cultivated in media supplemented with hPL presented an increased chondrogenic differentiation ability compared with hADSCs cultivated in media supplemented with FBS.[62] Shirzad *et al.* found that human peripheral blood-derived platelet lysate (PB-hPL) promoted the osteoblastic differentiation of hBMSCs to a greater extent than FBS when used as a supplement in the cell culture medium and that human umbilical cord-derived platelet lysate (UCB-hPL) induced more chondrogenic differentiation than FBS.[63] Re *et al.* prepared a gelatin–chitosan hybrid hydrogel (chitosan content 8% or 15%) in which hADSCs and hBMSCs were cultured. They reported that the culture of both types of stem cells in medium supplemented with hPL significantly promoted their proliferation and osteogenic differentiation compared with culture with FBS.[59]

On the other hand, Kolle *et al.* reported that the use of hPL as a supplement in the cell culture medium greatly increased the proliferation speed of hADSCs compared to FBS supplementation, whereas there was no difference in the hADSC differentiation into adipocytes, osteoblasts, or chondrocytes between culture with hPL or FBS as a supplement in the cell culture medium,[64] as reported by other researchers.[48,65,66] Xia *et al.* found that hBMSCs cultured in medium containing 7.5% hPL exhibited increased osteogenic differentiation compared to hBMSCs cultured in 10% FBS, whereas the adipogenic differentiation of hBMSCs in the hPL group was decreased compared to hBMSCs in the FBS group.[61] On the other hand, Warnke *et al.* reported that hBMSCs cultured in medium containing hPL showed less adipogenic differentiation than those in medium containing FBS.[67]

Therefore, it is not clear whether hPL in the cell culture medium promotes the ability of hMSCs to differentiate into several lineages (e.g., to undergo adipogenic, osteogenic, or chondrogenic differentiation) compared to FBS, especially hMSCs are cultured on specific biomaterials. In this study, we investigated and compared the differentiation ability of hADSCs cultured in conventional 10% FBS medium and medium supplemented with 5% or 10% hPL when the cells were cultured in tissue culture polystyrene (TCPS) dishes coated with or without several extracellular matrix proteins (ECMs; Matrigel, fibronectin (FN), and recombinant vitronectin (rVN)). This approach was adopted to investigate whether a synchronized effect of ECM coatings on the cell culture dishes used for hADSC differentiation can be observed when hADSCs are cultured in medium supplemented with hPL. Therefore, the effect of cell culture substrates coated with ECMs on the differentiation of hADSCs cultured in media supplemented with FBS and hPL is discussed in detail in this study.

Materials and Methods

The experiments performed in this project were approved by the ethics committees of the Cathay Medical Research Institute (CT099012), National Central University (NCU-106-007), Taipei Medical University (TMU-JIRB N201905059) and Taiwan Landseed Hospital (LSHIRB-13-05). All experiments were performed in accordance with any relevant and applicable institutional and governmental guidelines and/or regulations. We followed the declaration of Helsinki.

Materials

The biomaterials used in this study are shown in Supplementary Table 1. The other chemicals that were used in this research were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of stromal vascular fraction (SVF) solution

Human adipose tissue was obtained from the fat pads of human patients from the omentum (40-80 years old, eight people) after the patient provided his or her informed consent in writing. The adipose tissue cell solution (stromal vascular fraction, SVF) was obtained following a conventional method (Fig. 1).[24] The fat tissues were cleaned with PBS (phosphate-buffered saline) to remove impurities and blood. The fat tissues were cut and minced into small pieces (approximately 0.5-1.5 mm^3) and subsequently treated with 2.4 mg/mL type I collagenase at 36-38 °C for 55-65 min. The digested solution was centrifuged at 1210-1220 x g for five min, followed by the removal of erythrocytes utilizing erythrocyte lysis buffer (154 mM/L NH_4Cl in 20 mM/L Tris, pH 7.3-7.5). The resulting cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% hPL, or 10% hPL and then seeded into TCPS dishes that were either coated or not coated with ECMs (ECMs; Matrigel, fibronectin (FN), and recombinant vitronectin (rVN)) to yield the SVF solution (Fig. 1). The number of hADSCs in the solution was characterized utilizing flow cytometry (BD Accuri™ C6, BD Biosciences, Franklin Lakes, NJ, USA) with antibodies against CD105, CD90, CD73, CD44, and CD34 as well as appropriate isotype antibodies. The total cell number in the solution was also analyzed by flow cytometry after staining with 7-AAD. When the cells reached approximately 80-85% confluence (7-10 days), the cells were passaged utilizing a conventional cell passage method.

Preparation of ECM-coated TCPS dishes

Solutions of 5 μg/mL of rVN and fibronectin were prepared by diluting each ECM stock solution with PBS, and the ECM solution was then added to the TCPS dishes, followed by incubation for 120 min at 24-26 °C. After aspirating the solution, the 6 cm dishes were used for cell cultivation. A 1 mL aliquot of Matrigel at 5-7 °C was diluted with DMEM to produce 80 mL of Matrigel solution. Then, the diluted Matrigel solution was added to TCPS dishes, followed by incubation for 120 min at 24-26 °C. After aspirating the solution, the TCPS dishes were washed with DMEM once and utilized for cell cultivation.

Preparation of hPL

Therapeutic-grade human platelet concentrates were obtained from the Taipei Blood Center (Guandu, Taiwan Blood Services Foundation, Taiwan) upon IRB approval. Each donation was tested at the blood center, and only those specimens that were nonreactive for markers of blood-borne viruses (HIV, HBV, HCV) were used. Upon receipt, the blood cell count was evaluated using an ABC Vet hemocytometer (ABX Diagnostics, Thermo Fisher Scientific, Waltham, MA, USA).[68] Platelet concentrates were frozen at -80°C. A homogeneous pool of at least 15 donors was prepared to decrease the impact of donor variability and provide a sufficient amount. All procedures were conducted under aseptic conditions in a closed system. Our dedicated hPL developed for use as a supplement for hADSC propagation was prepared by performing a total of three freezing and thawing cycles (-80 °C/37 °C) and centrifugation (3000 x g for 30 min at 20 °C) as recommended to induce complete lysis of platelets and release of growth factors.[69] The thawed platelet concentrates were activated with calcium chloride ($CaCl_2$, final concentration of 23 mM/L) in the presence of glass beads (5 g glass beads with 0.3 mL 1 M/L $CaCl_2$ per 10 mL of hPL).[70] The mixture was shaken for 1 h at 24-26 °C to activate the coagulation cascade and to convert fibrinogen into fibrin. The fibrin clots that adhered to the glass beads were removed. Subsequently, the suspension was centrifuged at 6000 xg for half an hour at 20 °C. The resulting clear supernatant was heat treated at 56 °C for 30 min and then centrifuged, and the clear supernatant was isolated to remove insoluble proteins. The supernatant was sterile filtered (0.2 μm pore size, MF-Millipore membrane filter, Merck KGaA, Darmstadt, Germany), separated into small amount of volume (10 mL), and stored frozen at -20 °C. This serum-converted hPL has the advantage of not requiring the addition of porcine heparin (which is usually necessary to avoid gelation of the growth medium during cell culture).[47]

Pluripotency assay of hADSCs

The expression levels of the pluripotency genes *Nanog, Sox2*, and *Oct4* were evaluated by qRT-PCR utilizing conventional methods. PCR amplification was performed with Taq DNA polymerase in a thermocycler (TPersonal, Biometra, GmbH, Goettingen, Germany). Each sample was evaluated in duplicate, and the expression level of the GAPDH housekeeping gene was utilized as a control to normalize the results. The hADSCs were also immunostained for Sox2 and SSEA-4 to characterize pluripotency by following a standard protocol. The stained cells were observed using fluorescence microscopy (Eclipse Ti-U fluorescence inverted microscope; Nikon Instruments, Inc., Tokyo, Japan).

Osteogenic induction of hADSCs

Human ADSCs in SVF were inoculated into TCPS dishes that were coated or not coated with ECMs in DMEM containing 10% FBS, 5% hPL, or 10% hPL and cultured for three passage before the medium was exchanged with a commercially available induction medium for osteoblasts.

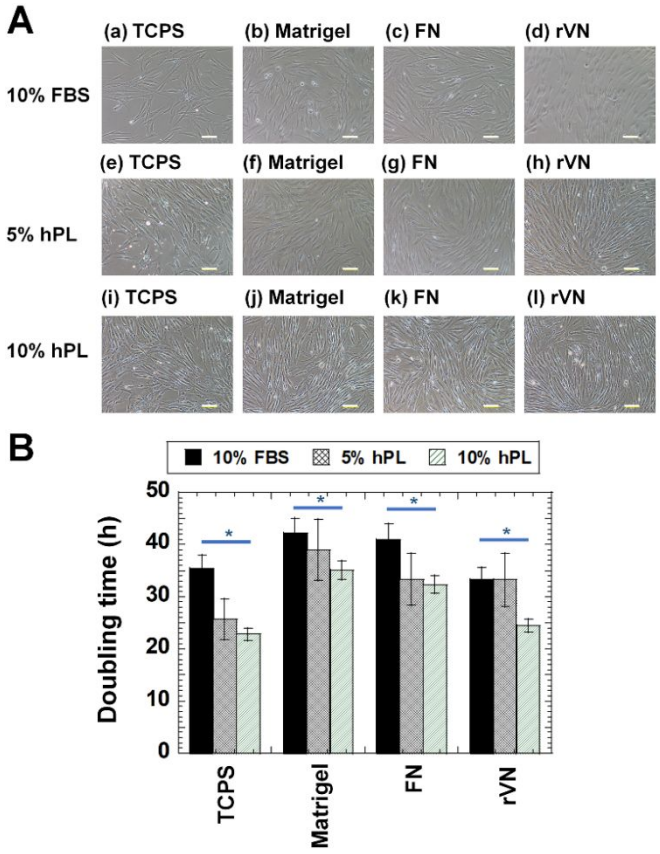

Fig. 2 Primary culture of hADSCs in uncoated TCPS and ECM-coated dishes in FBS and hPL media. (A) Cell morphologies of primary cultures of hADSCs in uncoated TCPS dishes (a, e, i), Matrigel-coated dishes (b, f, j), FN-coated dishes (c, g, k), and rVN-coated dishes (d, h, l) in 10% FBS medium (a-d), 5% hPL medium (e-h), and 10% hPL medium (i-l) at day 7. Scale bar indicates 100 μm. (B) Doubling time of hADSCs at passage 0 in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media. *$p < 0.05$.

hADSCs were cultured in the osteogenic medium with the supplements for up to 28 days. The medium was changed once every three days.

The alkaline phosphate activity (ALP) of the osteogenically induced cells was evaluated after a two-week osteogenic induction period using an Alkaline Phosphatase Assay Kit according to the manufacturer's manual.

The differentiated cells were subjected to von Kossa staining to evaluate calcium phosphate deposition. Both ALP activity and calcium phosphate deposition serve as indexes of differentiation into osteoblasts. Micrographs of the stained cells were captured using an inverted phase microscope (Eclipse Ti, Nikon Co., Tokyo, Japan). The images were evaluated utilizing ImageJ software (http://rsb.info.nih.gov/ij/) to characterize the numbers of stained and unstained cells.

The osteogenic differentiation rate was obtained from the following equation:

Osteogenic differentiation rate (%) = (Stained cell number)/(Unstained cell number + Stained cell number) x 100. (1)

Chondrogenic induction of hADSCs

Human ADSCs in SVF were inoculated into TCPS dishes that were coated or not coated with ECMs in DMEM containing 10% FBS, 5% hPL, or 10% hPL and cultured for three passages before chondrogenic induction. For their chondrogenic induction, hADSCs on TCPS dishes that were coated or not coated with ECMs were cultured in commercially available chondrogenic differentiation medium. The medium was exchanged once every two days taking care not to destroy the cell pellets.

After 28 days of chondrogenic differentiation, the cells were stained with a 1% Alcian blue working solution at room temperature for 30 min. Subsequently, the staining solution was discarded, and the cells were rinsed with a 0.1 mol/L HCl solution. Finally, the cells were washed three times with ultrapure water, and the stained cells were examined under a microscope. Quantitative analysis of the Alcian blue staining area of differentiated hADSCs was performed for six different places of each dish with ImageJ software.

Statistical analysis

Quantitative data were obtained from four different adipose tissue samples. The data are shown as the mean ± SD. Statistical analyses were performed using the unpaired Student's t-test in Excel (Microsoft Corporation). Probability values (p) of less than 0.05 were considered statistically significant.

Results

Effect of hPL and ECM-coated surfaces on the proliferation of primary hADSCs

Stem cells in primary tissue solutions such as SVF (primary adipose tissue cell) solution present highly adhesive characteristics in conventional TCPS dishes.[24] Therefore, hADSCs as well as hBMSCs and human amniotic fluid stem cells (hAFSCs) have typically been isolated through the cultivation of primary tissue solutions such as SVF, bone marrow solution, or amniotic fluid solutions, which is an established culture method.[24] Typically, TCPS dishes have been used for the selection of adhesive cells and non-adhesive cells.[24]

Human MSCs such as hADSCs and hBMSCs and fetal stem cells such as hAFSCs are characterized by heterogeneous populations. Therefore, stem cell characteristics such as proliferation speed (doubling time), stemness (stem cell marker expression and pluripotency gene expression), and differentiation potential (mainly differentiated cells derived from mesoderm lineages such as osteoblasts and chondrocytes) should be different in hADSCs isolated through purification methods as well as under fat tissue sample conditions. Therefore, we isolated adhesive hADSCs in cell culture dishes using not only conventional TCPS dishes that are generally used for this purpose but also several types of ECM-coated TCPS dishes, where the ECMs selected in this study were Matrigel, fibronectin (FN), and recombinant vitronectin (rVN). Matrigel, which is derived from Engelbreth-Holm-Swarm mouse sarcomacells, was selected because it is typically utilized as a coating material for cell culture surfaces for the proliferation of hPSCs.[71,72] rVN is also used as a xeno-free coating material for

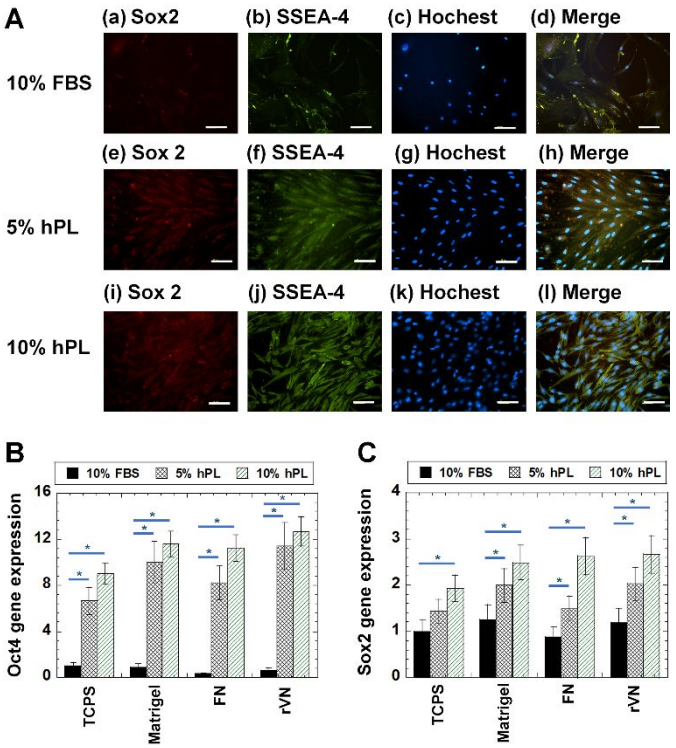

Fig. 3 Expression of pluripotency proteins and genes on hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media after 3 passages. (A) Pluripotency protein (Sox2 (red; a, e, i) and SSEA-4 (green; b, f, j)) expression on hADSCs after culture in rVN-coated dishes in 10% FBS (a-d), 5% hPL (e-h), and 10% hPL (i-l) for 3 passages. Nuclear staining is performed with Hoechst 33342 (c, g, k). Merged images of a-c, e-g, and i-k are shown in d, h, and l, respectively. Scale bar indicates 100 μm. (B) *Oct4* expression in hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media for 3 passages. (C) *Sox2* expression in hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media for 3 passages. *$p < 0.05$.

the cultivation of hPSCs. FN is used as a typical coating material for stem cell cultivation as well, and CellStart, which is a typical coating material used for cell cultivation, is composed of FN and serum albumin.[71]

SVF was inoculated into TCPS and ECM-coated dishes using medium supplemented with (a) conventional 10% FBS, (b) 5% hPL, or (c) 10% hPL. Three days after the inoculation of SVF, the cells attached to each dish. Then, the cell numbers in each dish were counted, and the cell culture was maintained until day 7. The cell morphologies of the primary cultures of hADSCs at day 7 are shown in Fig. 2A. Spindle-shaped hADSCs were present in all of the TCPS and ECM-coated dishes in the media supplemented with 10% FBS, 5% hPL, and 10% hPL (hereafter, these media will be denoted as 10% FBS medium, 5% hPL medium, and 10% hPL medium, respectively). From the cell numbers observed at days 3 and 7 of primary cell culture, the doubling time of hADSCs in uncoated TCPS and ECM-coated dishes in 10% FBS medium, 5% hPL medium, and 10% hPL medium was calculated, and the results are shown in Fig. 2B. The doubling time of the hADSCs cultured in the 10% hPL medium was shorter (higher expansion speed) than that in the 10% FBS medium when the cells were cultured in TCPS dishes with any of the ECM coatings tested in this study ($p<0.05$). The doubling time of the hADSCs cultured in the uncoated TCPS and ECM-coated dishes in the 5% hPL medium was statistically no different from the doubling time of hADSCs cultured in the 10% FBS medium ($p>0.05$). Therefore, the 5% hPL medium seemed to be equivalent to the 10% FBS medium with respect to hADSC expansion. 10% hPL medium promotes the expansion of hADSCs significantly more than 10% FBS according to the shorter doubling times of hADSCs cultured in 10% hPL ($p<0.05$).

hADSCs cultured in rVN-coated dishes and uncoated TCPS dishes in 10% hPL medium showed the shortest doubling time (approximately 22-25 h) in this study. It was found that both the uncoated TCPS dishes and rVN-coated dishes provided the preferable surfaces for primary hADSC expansion in this study.

We analyzed hMSC surface marker (CD44, CD73, CD90, and CD105) expression in hADSCs cultured in uncoated TCPS dishes in 10% FBS medium and 10% hPL medium using flow cytometry, and the results are shown in Supplementary Fig. 1. More than 80% of the hADSCs expressed MSC markers (CD44, CD70, CD93, and CD105) in uncoated TCPS dishes in 10% FBS medium and 10% hPL medium. These results indicate that hADSCs can be isolated in both FBS and hPL media. The pluripotency and differentiation ability of hADSCs isolated under different conditions using different cell culture dishes and different cell culture media in this study are evaluated in the following sections.

Pluripotency of hADSCs cultivated in uncoated TCPS and ECM-coated dishes in FBS and hPL media

The pluripotency of hADSCs isolated and cultured on different surfaces in different media was investigated based on the expression of pluripotent marker proteins (Sox2 and SSEA-4) on the cells detected by immunostaining after 3 passages in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media, where the nuclei of hADSCs were stained with Hoechst 33342, and the results are shown in Fig. 3A and Supplementary Figs. 2-4. We observed Sox2 expression on hADSCs in all conditions. However, the signals were found to be extremely weak. The expression of SSEA-4 on hADSCs could be found in all conditions, and the signals were relatively stronger than those of the expression of Sox2. It was also found that the expression of Sox2 and SSEA-4 in all types of cell culture dishes in 5% hPL medium and 10% hPL medium seemed to be higher than that in 10% FBS medium. The gene expression of *Oct4, Sox2*, and *Nanog* in hADSCs cultured in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media after 3 passages was therefore evaluated using the qRT-PCR to evaluate pluripotency gene expression on hADSCs quantitatively, and the results are shown in Fig. 3B and 3C and Supplementary Fig. 5.

It was found that the hADSCs expressed pluripotency genes such as *Oct4*, *Sox2*, and *Nanog* when they were cultured in any of the cell culture dishes and any of the cell culture media tested in this study. Pluripotency gene expression on hADSCs

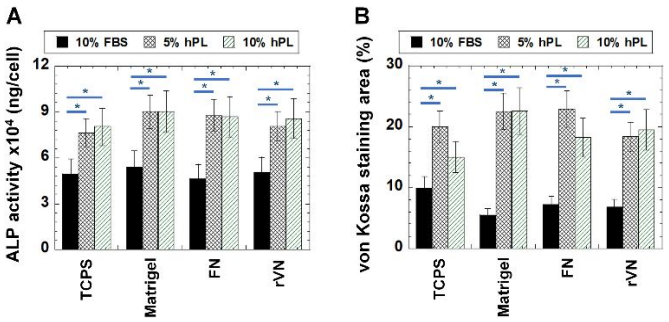

Fig. 4 Osteogenic differentiation of hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media for 3 passages. (A) ALP activity of the cells after osteogenic induction of hADSCs for 14 days. (B) von Kossa staining area of the cells (calcium phosphate deposition) determined using ImageJ software after osteogenic induction of hADSCs for 28 days. *$p < 0.05$.

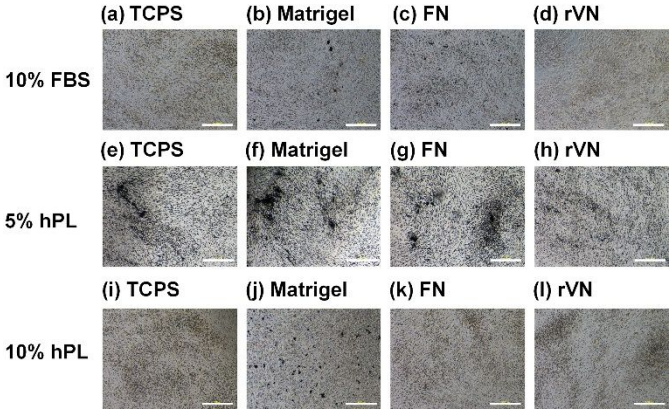

Fig. 5 Osteogenic differentiation of hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media for 3 passages. Micrograph of cells after osteogenic differentiation evaluated by von Kossa staining after hADSCs were cultivated in uncoated TCPS dishes (a, e, i), Matrigel-coated dishes (b, f, j), FN-coated dishes (c, g, k), and rVN-coated dishes (d, h, l) in 10% FBS medium (a-d), 5% hPL medium (e-h) and 10% hPL medium (i-l) for 3 passages. The cells were cultivated for 28 days in osteogenic induction media. The scale bar represents 500 μm.

cultured in 5% hPL or 10% hPL was higher than that on ADSCs cultured in 10% FBS in the uncoated TCPS, Matrigel-coated TCPS, FN-coated TCPS, or rVN-coated dishes (p<0.05), except for *Sox2* expression of hADSCs cultured in the uncoated TCPS dishes in 5% hPL. These results indicated that hADSCs cultured in hPL medium, especially in 10% hPL medium, can express higher levels of pluripotency genes than hADSC culture in 10% FBS medium. Furthermore, hADSCs cultured in rVN-coated and Matrigel-coated dishes in 5% hPL medium showed higher expression of the *Oct4, Sox2*, and *Nanog* pluripotency genes than hADSCs in TCPS dishes in 5% hPL, indicating that hADSCs cultured in rVN-coated and Matrigel-coated dishes in 5% hPL medium maintained pluripotency gene expression in this study.

Osteogenic differentiation potential of hADSCs cultivated in uncoated TCPS and ECM-coated dishes in FBS and hPL media

The potential of hMSCs such as hADSCs to differentiate into cells derived from mesoderm, such as osteoblasts, chondrocytes, myocytes, and adipocytes, is one of the most important characteristics of hMSCs. Therefore, the potential of hADSCs to differentiate into osteogenic and chondrogenic cells was investigated for evaluation of the purity and quality of hADSCs isolated and cultured on several types of cell culture biomaterials (uncoated TCPS and ECM-coated dishes) in several different cell culture media (10% FBS, 5% hPL, and 10% hPL media) in this study.

Osteogenic induction was first evaluated in hADSCs after hADSCs were cultivated in uncoated TCPS and ECM-coated dishes in several types of cell culture media (10% FBS, 5% hPL, and 10% hPL media) for three passages. Fig. 4A shows the expression of alkaline phosphatase (ALP) activity in hADSCs differentiated into osteoblasts; ALP is an early marker of osteogenesis and was evaluated on day 14 of the induction of hADSCs to undergo osteogenic differentiation. The ALP activity of hADSCs after culturing in 5% hPL or 10% hPL for three passages was found to be much higher than that after culturing in 10% FBS (p<0.05), whereas no significant difference in the ALP activity produced by hADSCs was obtained (p>>0.05) after hADSCs were cultured in 5% hPL and 10% hPL for three passages. No effect of the cell culture materials used for hADSC culture on osteogenic differentiation was found from the evaluation of the activity of the early osteogenesis marker ALP.

The evaluation of mineralization by von Kossa staining showed more extensive differences between the hADSCs cultured in FBS medium and hPL medium. Figs. 4B and 5 show the results of von Kossa staining in differentiated hADSCs under each condition. The black spots showing calcium phosphate deposition detected by von Kossa staining were extensively observed under each condition, as shown in Fig. 5. Quantitative analysis of the von Kossa staining areas of differentiated hADSCs suggested that calcium phosphate deposition in differentiated hADSCs after culturing in any of the dishes in 5% or 10% hPL medium was much higher than that observed after culturing in 10% FBS (p<0.05). The calcium phosphate deposition evaluated by von Kossa staining of differentiated hADSCs after the cells were cultivated in uncoated TCPS dishes, Matrigel-coated dishes, FN-coated dishes, and rVN-coated dishes for 3 passages was not significantly different from that observed when the same culture media (10% FBS, 5% hPL, and 10% hPL medium) were used for hADSC culture for 3 passages in this study.

Chondrogenic differentiation of hADSCs cultivated in uncoated TCPS and ECM-coated dishes in FBS and hPL media

The chondrogenic differentiation of hADSCs was also evaluated after hADSCs were cultivated in uncoated TCPS dishes or ECM-coated TCPS dishes in 10% FBS, 5% hPL or 10% hPL media for 3 passages. The chondrogenic differentiation intensity was evaluated on the basis of the staining intensity of Alcian blue, which stains acidic polysaccharides such as glycosaminoglycans. Fig. 6A shows images of the Alcian blue staining of differentiated hADSCs after the chondrogenic induction of hADSCs at 28 days. Alcian blue-stained cells were extensively

observed among the differentiated hADSCs after the hADSCs were cultured in each type of dish in each medium for 3 passages. Quantitative analysis of the Alcian blue staining area of differentiated hADSCs was also performed with ImageJ software, and the results are shown in Fig. 6B. The Alcian blue staining area of differentiated hADSCs after they were cultivated in uncoated TCPS dishes and any of the ECM-coated dishes in 5% hPL medium or on an FN-coated surface or rVN-coated surface in 10% hPL medium was higher than that associated with each surface in 10% FBS medium, which indicates greater chondrogenic differentiation of hADSCs. The results suggested that the cultivation of hADSCs in 5% and 10% hPL medium promotes the chondrogenic differentiation of hADSCs on FN-coated surface and rVN-coated surface when they are induced to differentiate into chondrocytes.

Table 1 Effect of hPL and FBS on differentiation potential of hMSCs into osteogenic, chondrogenic, and adipogenic lineages.[a]

Cell source	Differentiation into Osteoblasts			Differentiation into Chondrocytes		Differentiation into Adipocytes		Ref
	Supplement of Medium	Medium containing FCS or FBS	Medium containing hPL	Medium containing FCS or FBS	Medium containing hPL	Medium containing FCS or FBS	Medium containing hPL	
hBMSCs	10% FBS, 5% hPL	Similar diff.		Similar diff.		Similar diff.		48
hBMSCs	10% FBS, 5%hPL	Less diff.	Better diff.					58
hBMSCs	5% hPL, 10% hPL, or 10% FBS	Similar diff.		Similar diff.		Similar diff.		53
hADSCs	10% FBS, 2.5% hPL, 10% hPL	Similar diff.				Similar diff.		54
hADSCs	10% FBS, 10% hPL	Similar diff.		Similar diff.		Similar diff.		64
hBMSCs	10% hPL, 10% FBS	Less diff.	Better diff.					60
hBMSCs	10% FBS, 7.5% hPL	Less diff.	Better diff.			Better diff.	Less diff.	61
hBMSCs	10% FBS, 10% hPL	Similar diff.				Better diff.	Less diff.	67
hADSCs	10% FBS, 5% & 10% hPL,			Less diff.	Better diff.			62
hDPSCs, hAPSCs	1%, 5%, 10% hPL, 10% FBS	Similar diff.						73
hPLSCs	10% FBS, 10% hPL, 5%hPL+5% FBS	Similar diff.						74
hBMSCs	0-15% FBS, 0-15% hPL	Similar diff.				Similar diff.		65
hADSCs	10% FBS, 10% hPL	Similar diff.		Similar diff.		Similar diff.		66
hBMSCs	10% FBS, 5% or 10% UCB-hPL, 5% or 10% hPL	Less diff.	Better diff. (PB-hPL)	Less diff.	Better diff. (UCB-hPL)			63
hBMSCs, hADSCs	10% FBS, 5% hPL	Less diff.	Better diff.					59

[a] FBS, fetal bovine serum; hADSCs, human adipose-derived stem cells; hAPSCs, human apical papilla stem cells; hBMSCs, human bone marrow-derived stem cells; hDPSCs, Human dental pulp stem cells; hPL, human platelet lysate; hPLSCs, human periodontal ligament stem cells; PB-hPL, pheripheral blood-derived hPL; UCB-hPL, umbilical cord-derived hPL.

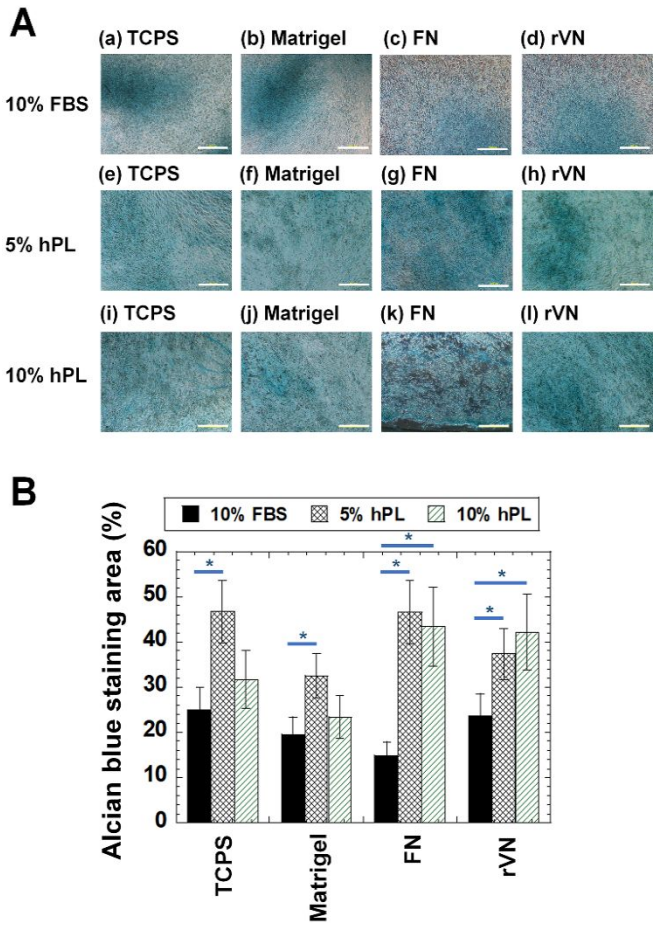

Fig. 6 Chondrogenic differentiation of hADSCs after hADSC culture in uncoated TCPS and ECM-coated dishes in 10% FBS, 5% hPL, and 10% hPL media for 3 passages. (A) Micrograph of cells after chondrogenic differentiation analyzed by Alcian blue staining after hADSCs were cultivated in uncoated TCPS dishes (a, e, i), Matrigel-coated dishes (b, f, j), FN-coated dishes (c, g, k), and rVN-coated dishes (d, h, l) in 10% FBS medium (a-d), 5% hPL medium (e-h) and 10% hPL medium (i-l) for 3 passages. The cells were cultivated for 28 days in chondrogenic differentiation media. The scale bar represents 500 μm. (B) Alcian blue staining area of the cells (polysaccharide and glycosaminoglycan deposition) determined using ImageJ software after chondrogenic induction of hADSCs for 28 days. *$p < 0.05$.

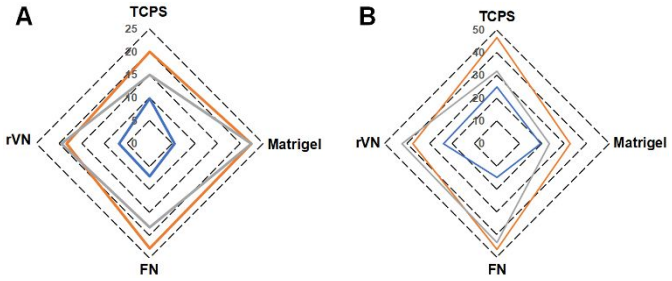

Fig. 7 Effects of cell culture biomaterials on the ability of hADSCs to differentiate into osteoblasts (A) and chondrocytes (B) after hADSCs were cultivated in uncoated TCPS and ECM-coated dishes in 10% FBS medium (blue line), 5% hPL medium (orange line), and 10% hPL medium (gray line) for 3 passages. Osteogenic differentiation percentages were analyzed according to the percentage of the von Kossa staining area, and chondrogenic differentiation percentages were evaluated on the basis the percentage of the Alcian blue staining area.

Discussion

Human MSCs derived from human tissues, such as hADSCs, are known to present heterogeneous characteristics, including various differentiation abilities and genotypes. hADSCs isolated under different conditions present differences in stemness and purity levels. The isolation and cultivation of hADSCs in uncoated TCPS and rVN-coated dishes in 10% hPL medium led to a shorter doubling time (higher proliferation speed) of hADSCs (Fig. 2). Furthermore, hADSCs cultured in both 5% hPL and 10% hPL showed higher pluripotency gene (Oxt4, Sox2, and Nanog) expression than hADSCs cultivated in 10% FBS in this study.

Several researchers have reported that the supplementation of medium using hPL increases the differentiation potential of hMSCs such as hBMSCs and hADSCs,[58-63] whereas other researchers have found that there is no significant difference in the differentiation potential of hMSCs in FBS medium and hPL medium or that the differentiation potential of hMSCs is suppressed in hPL medium compared to that in FBS medium.[48,64-67] Table 1 summarizes the effect of hPL on the differentiation potential of hMSCs into osteogenic, chondrogenic, and adipogenic lineages, which is compared to cells cultured with FBS.[48,53,54,58-67,73,74] Regarding the osteogenic differentiation of hMSCs, 14 articles report a comparison of

hMSC differentiation using hPL medium versus FBS medium, among which five articles indicate that cultivation in hPL medium facilitates the osteogenic differentiation of hMSCs compared to that in FBS medium, whereas nine articles report no difference in the differentiation potential of hMSCs cultivated in hPL medium versus FBS medium (Table 1).

Regarding the chondrogenic differentiation of hMSCs, six articles report a comparison of hMSC differentiation using hPL medium versus FBS medium, among which two articles indicate that cultivation in hPL medium facilitates the chondrogenic differentiation of hMSCs compared to cultivation in FBS medium, whereas four articles report no difference in the differentiation potential of hMSCs cultivated in hPL medium versus FBS medium (Table 1).

Regarding the adipogenic differentiation of hMSCs, eight articles report a comparison of hMSC differentiation using hPL medium versus FBS medium, among which two articles indicate that cultivation in hPL medium suppresses the adipogenic differentiation of hMSCs compared to that in FBS medium, whereas six articles report no difference in the differentiation potential of hMSCs cultivated in hPL medium versus FBS medium (Table 1).

Human PL medium at least does not suppress the differentiation of hMSCs into osteoblasts and chondrocytes compared to FBS medium because no decrease in the differentiation of hMSCs into osteoblasts or chondrocytes when using hPL medium has yet been reported, although hPL medium does sometimes seem to suppress the differentiation of hMSCs into adipocytes. The present research suggests an advantage of using hPL medium for the promotion of the osteogenic and chondrogenic differentiation of hADSCs, as several researchers have reported.[58-63] The isolation and cultivation of hADSCs using hPL medium will contribute to the xeno-free isolation and cultivation of hADSCs for future clinical use.

A summary of the effects of the tested cell culture biomaterials on the differentiation of hADSCs into osteoblasts and chondrocytes cultivated in 10% FBS medium, 5% hPL medium, and 10% hPL medium is shown in Fig. 7, where the osteogenic differentiation index was evaluated from the von Kossa staining area, and the chondrogenic differentiation index was characterized from the Alcian blue staining area. Matrigel-coated dishes promote hADSC differentiation into osteoblasts and suppress differentiation into chondrocytes, especially in 10% hPL medium. The rVN-coated dishes and FN-coated dishes strongly promote hADSC differentiation into both osteoblasts and chondrocytes in both 5% and 10% hPL media. Uncoated TCPS dishes also promote hADSC differentiation into both osteoblasts and chondrocytes, especially in 5% hPL media. Therefore, it was found that the selection of biomaterials for cell cultivation such as specific ECM-coated dishes and the contents of cell culture media such as 5% or 10% hPL medium or FBS medium are significantly important for hADSC isolation, culture, and differentiation into specific lineages of the cells.

Conclusions

Human ADSCs were isolated and cultured in uncoated TCPS and ECM-coated dishes in 10% FBS medium, 5% hPL medium and 10% hPL medium, where the hPL medium consisted of xeno-free components. A high proliferation rate of hADSCs was observed in TCPS dishes and rVN-coated dishes in 10% hPL medium. High pluripotency gene expression in hADSCs was observed when hADSCs were cultured on all of the tested cell culture biomaterials in 5% or 10% hPL medium compared to hADSCs cultured in 10% FBS medium. Synchronized effects of cell culture materials and cell culture medium were observed in this study: (a) Matrigel-coated dishes promoted hADSC differentiation into osteoblasts and suppressed differentiation into chondrocytes in 10% hPL medium. (b) The recombinant vitronectin-coated dishes and fibronectin-coated dishes strongly promoted hADSC differentiation into osteoblasts and chondrocytes in 5% and 10% hPL media. Overall, it was found that 5% and 10% hPL media promoted hADSC differentiation into osteoblasts and chondrocytes compared to 10% FBS medium on all of the tested cell culture biomaterials.

Conflicts of interest

There are no conflicts to declare.

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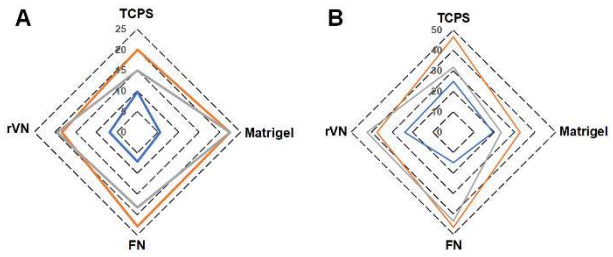

Synchronized effects of cell culture materials and cell culture medium on osteoblast (left) and chondrocyte (right) differentiation were observed.