

Figure 1. Schematic representation of the microenvironment and niches of hESCs and human iPSCs and their regulation by the following factors: (1) several soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (2) cell–cell interaction; (3) cell–biomacromolecule (or biomaterial) interaction; and (4) physical factors, such as rigidity, of the environment.

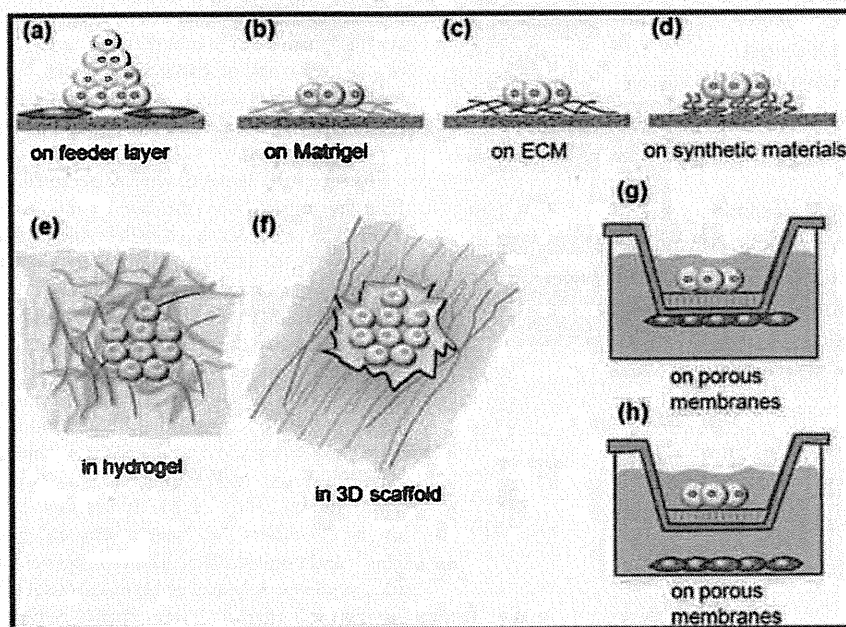


Figure 2. Schematic representation of different culture methods for hESCs and human iPSCs. hESCs and human iPSCs have been cultured (a) on MEF, (b) on Matrigel, (c) on 2D materials coated with ECM or other biomacromolecules, (d) on 2D materials prepared from synthetic materials, (e) in hydrogels made from glycosaminoglycan or other biomacromolecules, (f) on a 3D scaffold, and (g,h) on porous polymeric membranes.

using biomacromolecules and/or synthetic materials will facilitate the production of large numbers of stem cells and specifically differentiated cells needed for *in vitro* regenerative medicine.²¹

Tissue-specific stem cell niches provide crucial cell–cell contacts and paracrine signaling.^{21,22} The extracellular matrix (ECM) keeps stem cells in the niche and serves to initiate signal transduction,^{23,24} while locally concentrated glycosaminoglycans (GAGs) provide soluble growth factors or cytokines. Both

in vitro and *in vivo*, the niche is established by supportive cells, the ECM and soluble factors, which regulate stem cell fate *via* complementary mechanisms, including the presentation of immobilized signaling molecules, the modulation of matrix rigidity, and the creation of cytokine gradients.²¹ Thus, it would be highly beneficial to design, construct, and reproduce the microenvironment and niche of pluripotent stem cells *in vitro* with biomacromolecules and synthetic polymers.

Table 1. Characterization of Pluripotent ESCs and iPSCs^a

characterization	specification (examples)	ref
1. morphology		
cell morphology	colony formation	2, 14, 31, 34, 39, 45, 47, 50, 76
2. protein level		
surface marker analysis	Oct-4 , Oct-3/4 , Nanog , TRA-1-60 , Tra-1-81 , SSEA-3 , and SSEA-4	6, 28–30, 37, 38, 40, 50, 72, 75, 95
immunohistochemical analysis	Oct3/4 , Oct-4 , Sox-2 , SSEA-3 , SSEA-4 , TRA-1-60 , TRA-1-81 , and Nanog alkaline phosphatase (AP) SSEA-1 (negative staining)	28–35, 38, 46, 48–50, 68, 71
3. gene level	Oct3/4 , Oct-4 , Sox-2 , Nanog , TGDF-1 , UTF-1 , REX1 , hTERT , ABCG2 , DPPA5 , CRIPTO , FOXD3 , Tert1 , Rex2 , and DPPA5	31, 37, 39, 40, 44, 46, 48, 68
4. differentiation ability	embryonic body formation in vitro (EB) teratoma formation In Vivo	2, 5, 31, 34, 45, 47, 68, 72, 75, 96, 127

^a Bold genes and proteins are frequently analyzed for the characterization of hESCs and human iPSCs.

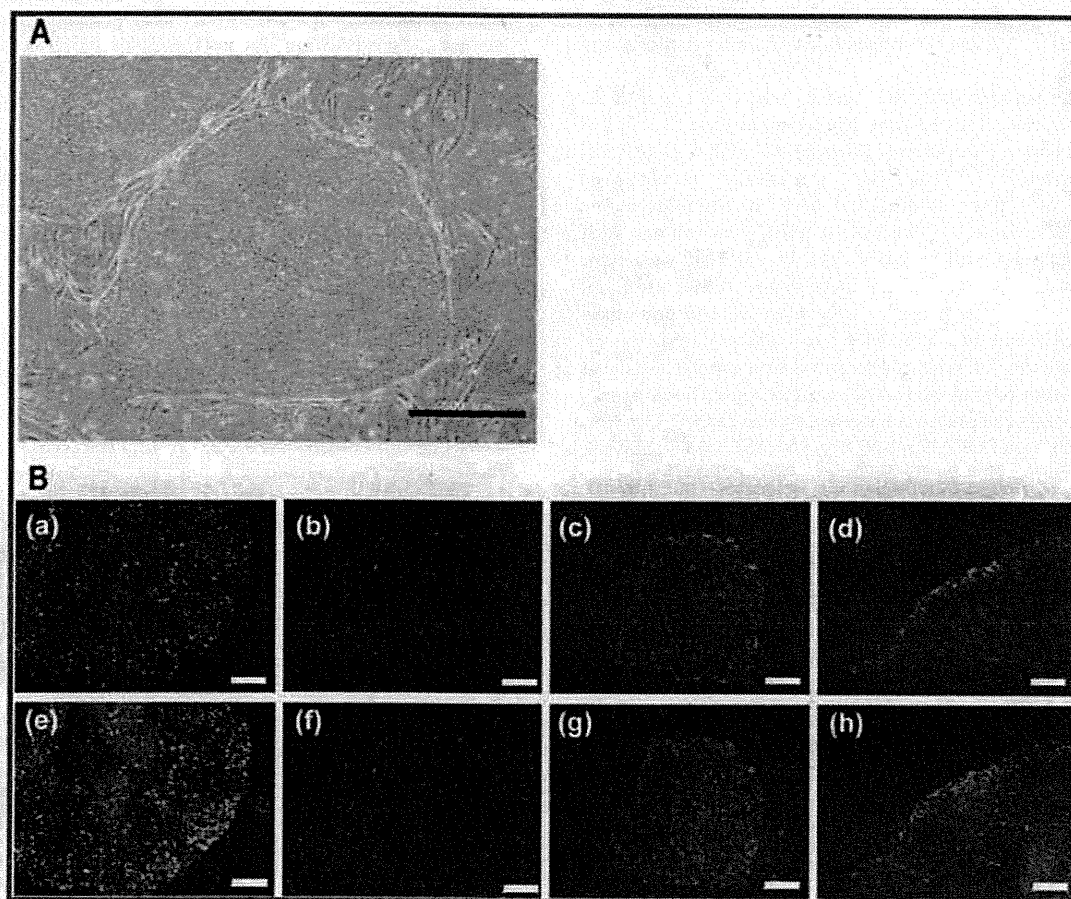


Figure 3. Morphology and expression of pluripotent markers in human iPSCs grown on a MEF feeder layer. Human iPSCs were derived from MRC-5 cells injected with Oct-4, Sox2, Klf4, and c-Myc using a retroviral vector. (A) A human iPSC colony grown on MEF. (B) Human iPSCs stained with antibodies and/or dye for (a) Oct3/4 (green), (b) Sox2 (red), (c) SSEA-4 (red), (d) TRA-1-60 (green), (e) Oct3/4 (green) + Nanog (red) + DAPI (blue), (f) Sox2 (red) + DAPI (blue), (g) SSEA-4 (red) + DAPI (blue), and (h) TRA-1-60 (green) + DAPI (blue).

Recently, several articles from both material scientists and molecular biologists have discussed the effect of culture materials on the fate of stem cells.^{14,25–27} This review describes and discusses the use of culture materials derived from biomacromolecules and synthetic polymers that support the propagation of hESCs and human iPSCs while maintaining their pluripotency.

Figure 2 shows a schematic representation of the culture methods discussed in detail in this review: (a) cells cultured on two-dimensional (2D) materials coated with ECM or other biomacromolecules, (b) cells cultured on 2D materials prepared from synthetic materials, (c) cells cultured in hydrogels from glycosaminoglycan or other biomacromolecules, (d) cells cultured

on porous polymeric membranes, and (e) cells cultured on three-dimensional (3D) materials. In addition, this review discusses the design and importance of cell culture materials that maintain the pluripotency of hESCs and iPSCs.

2. ANALYSIS OF THE PLURIPOTENCY OF HESCS AND HUMAN iPSCS

hESCs and human iPSCs display high telomerase activity and express several pluripotency surface markers, such as glycolipid stage-specific embryonic antigen 4 (SSEA-4), [28–30] tumor rejection antigen 1–60 (Tra-1–60), keratan sulfate-related antigen,^{28,31–33} and tumor rejection antigen 1–81 (Tra-1–81),^{31,33–35} but not glycolipid stage-specific embryonic antigen 1 (SSEA-1). SSEA-1 is expressed on mouse ESCs.^{1,2,6,31} hESCs also show high expression levels of specific pluripotency genes, such as Oct3/4,^{32,36,37} Oct-4 (POU5F1, POU domain transcription factor),^{28,38,39} Nanog,^{39,40} Sox-2,^{40,41} Rex-1,^{39,42} and hTERT, the catalytic component of telomerase.^{39,43,44} Table 1 summarizes the characteristics of pluripotent hESCs.

The pluripotency of hESCs and human iPSCs is evaluated based on (a) the colony morphology by microscopy,^{14,31,34,45} (b) the expression of pluripotency genes by RT-PCR and qRT-PCR measurements,^{31,40,44,46} and (c) the expression of pluripotent proteins by flow cytometry⁶ and immunofluorescence analyses^{31,38} (Table 1). Pluripotent hESCs and human iPSCs generate colonies with spherical cells. Figure 3 shows a typical example of a colony of iPSCs. Differentiated hESCs and human iPSCs have small, coagulated, or fibroblast-like morphologies.⁴⁷ Once hESCs and human iPSCs have differentiated, the cells expand from the differentiated stem cells and cannot be used as a source of stem cells in clinical or research applications.

The expression of pluripotency genes, such as Oct3/4, Oct-4, Sox-2, Nanog, Rex-1, hTERT, Tra-1–60, Tra-1–81, SSEA-3, and SSEA-4, is generally analyzed by RT-PCR and/or qRT-PCR methods (Table 1). The expression of pluripotency proteins, including Oct-4, alkaline phosphatase (AP),^{48–50} SSEA-4, Tra-1–60, and Tra-1–81, is analyzed by immunofluorescence or flow cytometry analysis with specific antibodies. The mRNA expression level of a pluripotency gene does not directly relate to the expression level of the corresponding pluripotency protein due to regulation by interference RNA, such as microRNA (miRNA).^{51,52} Figure 3 also shows the typical expression patterns of pluripotency proteins in human iPSCs.

The analysis of pluripotency genes and proteins through RT-PCR and/or qRT-PCR analysis and immunofluorescence and/or flow cytometry analysis, respectively, is important to verify the pluripotency of hESCs and human iPSCs. The difference between hESCs and human iPSCs and adult or fetal stem cells, such as bone marrow-derived stem cells (mesenchymal stem cells),^{53,55} adipose-derived stem cells,^{56,57} and amniotic fluid stem cells,^{58,59} is the ability to differentiate into cells of all three germ layers (endoderm, mesoderm, and ectoderm).^{30,47,60,61} Mesenchymal stem cells and other adult and fetal stem cells primarily differentiate into cells from the mesoderm, such as osteoblasts, chondrocytes, and adipocytes, although several exceptions have been reported.^{62–65} Therefore, the ability to differentiate into cells from all three germ layers is also used to evaluate the pluripotency of hESCs and human iPSCs. hESCs and human iPSCs can generate embryonic bodies (EB) when cultured on untreated polystyrene dishes in differentiation medium, which includes three germ layers or tissue.^{35,45,66} The formation of teratomas that include all three germ layers is also

used to evaluate the pluripotency of hESCs and human iPSCs by injecting hESCs and human iPSCs into immunodeficient mice, such as mice with severe combined immunodeficiency (SCID).^{2,45,67} Table 2 summarizes the characterization methods used to analyze the ability of cells to differentiate into all three germ layers in both EB and teratomas.

The ability to differentiate into cells from all three germ layers in EB and teratomas is analyzed in several ways: (1) observation of tissue that includes all three germ layers [the epithelial component (endoderm (E)), renal tissue (E), intestinal mucosa (E), cartilage (mesoderm (M)), bone (M), muscle (M), chondrocyte (M), mesenchymal tissue (M), the neural component (ectoderm (EC)),²⁰¹ and the epidermal component (EC)],^{2,5,31,34,47,68} (2) expression of differentiated genes, including endoderm genes [α -fetoprotein (AFT), SOX17, amylase, albumin, FOXA1 (HNF3 α), GATA6, and PDX1], mesoderm genes (Brachyury T, β -globin, MIX-LIKE-1, Hand1, and Msx1), and ectoderm genes [β III-tubulin, SOX1, neurofilament heavy chain (NFH), keratin 15, neural progenitor markers PAX6 and NeuroD, and Nestin)], by RT-PCR and/or qRT-PCR,^{38,40,49,50,69,70} and (3) the expression of differentiated proteins, including endoderm-related proteins [AFP, cytokeratin 19 (CK19), glucagons, and albumin], mesoderm-related proteins (actin, α -actinin, cTnI, and Brachyury T), and ectoderm-related proteins [β -III tubulin, enolase, nestin, and glial fibrillary acidic protein (GFAP)].^{30,38,40,71,72} Figure 4 shows typical examples of the histochemical analysis of teratomas with cells from all three germ layers.

hESCs proliferate continuously under the appropriate conditions and are able to differentiate into all types of somatic cells from all three germ layers *in vivo* and *in vitro*.²

3. CELL-FREE CULTURE OF HESCS ON BIOMATERIALS MAINTAINS PLURIPOTENCY

hESCs and human iPSCs are currently cultured on MEFs as a feeder layer to maintain the pluripotency and self-renewing characteristics. hESCs and human iPSCs can be cocultured with MEFs for extended periods of time without undergoing differentiation. However, if the MEFs are removed and hESCs and human iPSCs are cultured under normal culture conditions, differentiation into many somatic cell types is triggered.^{2,14,73}

However, concerns over the cross-species transfer of viruses^{14,28,35,74} and immunogenic epitopes, such as N-glycolylneuraminic acid (Neu5Gc),^{28,74} have prompted the investigation of xeno-free culture and cell-free culture in recent years. As an alternative to cocultures of hESCs with MEFs, several isolated ECM or cell adhesion molecules that support hESC attachment and proliferation have been evaluated. Tables 3 and 4 summarize the feeder layer-free culture of hESCs and human iPSCs on biomacromolecules and synthetic polymers. The addition of a high concentration of basic FGF (bFGF, FGF-2) is necessary for the culture of hESCs and human iPSCs in the absence of a feeder layer and/or without a conditioned medium from MEFs. It has also been suggested that inhibition of the BMP signaling pathway plays a significant role in the molecular mechanism of hESC self-renewal.^{14,75,76} FGF-2 signaling is critical for the self-renewal of hESCs, and the transforming growth factor beta (TGF- β) signaling pathways are necessary for preventing differentiation.⁷⁷ Therefore, hESCs and human iPSCs require FGF-2 for self-renewal. At the same time, it is necessary to block BMP signaling to maintain the phenotype.⁷⁷ The addition of FGF-2 and Activin A/Nodal to serum-free media increases the expression of pluripotency markers compared with Activin A/Nodal alone, while FGF-2 alone is

Table 2. Characterization of Differentiation Ability to Three Germ Layers in EB and Teratoma^a

characterization	specification (examples)	ref
1. morphology	Von Kossa staining (calcification)	30, 68
	Picrosirius staining for collagen, blood vessels, etc.	
	Alizarin Red staining (calcification)	
	Alcian blue/Van Giesson's staining	
	haematoxylin and eosin staining	
	(a) endoderm differentiation	
	columnar epithelia with goblet cell, primitive epithelium stained with cytokeratin 18 antibody, respiratory epithelium, gut epithelium, epithelial, intestinal mucosa, intestinal epithelium, pigmented epithelium, and renal tissue	2, 5, 31, 34, 45, 47, 68, 75, 96
	(b) mesoderm differentiation	
	hyaline cartilage, muscle, cartilage, bone, smooth muscle, striated muscle, mesenchymal tissue, smooth muscle stained with actin antibody, and chondrocytes	2, 5, 31, 34, 45, 47, 68, 75, 127
	(c) ectoderm differentiation	
neural rosettes, neural epithelium, neuroectoderm, neuronal tissue stained with neurofilament 200 K antibody, peripheral Schwann cells, embryonic ganglia, stratified squamous epithelium, epithelium, and neural tubes	2, 5, 31, 34, 47, 68, 72, 75, 127	
2. protein level immunohisto chemical analysis	(a) endoderm differentiation	
	AFP, Glucagon, pdx-1, HNF3 β , CK19, glucagon, NFH, GFAP, IFABP, albumin, Tif1, TTF-1, and FOXA2	28, 30, 31, 40, 71–73, 75, 88
	(b) mesoderm differentiation	
	FOXA2, cTnI, α -SMA, brachyury, vimentin, α -actin, α -actinin, muscle actin, actin, BMP-4, and cTnI	28, 30, 38, 71, 72, 88, 127
	(c) ectoderm differentiation	
	NCAM, Tuj1, neurofilament, β III-tubulin, GFAP, enolase, and nestin	28, 30, 38, 40, 48, 71, 72, 75, 128
	surface marker	
	VEGFR2 (mesoderm), PDGFR α (mesoderm), and CXCR4 (endoderm)	128
	3. gene level	
	(a) ectoderm gene expression	
SOX-1, PAX6, Nestin, NES, Tuj1, MAP2, NeuroG1, TUBB3, β III-tubulin, NeuroD, NOG, NEFL, keratin, Keratin 8, Keratin 18, Keratin 15, NFH, and neurofilament (NF)-68	28, 32, 33, 33, 38, 40, 46, 48, 50, 69, 70, 76, 88	
(b) endoderm gene expression		
AFP, cerberus, GATA3, GATA4, GATA6, SOX17 (G3, G16, A17, A14, A1), ONECUT1, FOXA2 (A17G101), IPF1, FOXA1, PROX1, HHEX, ALB, HNF3b, HNF4a, Albumin, PDX1, amylase, TTF-1, IFABP, and Tif1	28, 33, 38, 46, 48, 50, 69, 70, 73, 76, 88	
(c) mesoderm gene expression		
brachyury T, Hand1, IGF2, FLK1, MIXL1, MESP1, EOMES, PAX3, MYOD1, PECAM1, NKX2, GATA1, GATA2, GATA4, KDR, BMP4, SIL, HOXB4, MyoD, Msx1, C-actin, β -globin, α -cardiac actin, cardiac actin, VE-cadherin, enolase, MtdD, and CD31	28, 32, 33, 38, 46, 48, 50, 69, 70, 88	
(d) cardiomyocyte differentiation		
Nkx2.5, GATA-4, MYH-6, TNNT2, TBX-5, Mlc2a, MLC-2 V, tropomyosin, cTnI, ANP, desmin, α -MHC, β -MHC, cTnT, Isl-1, and Mef2c	33, 40, 47	
(e) hepatocyte differentiation		
AFP, albumin, and TAT		
(f) neural differentiation		
Nestin, Musashi 1, Tuj1, astrocytes (GFAP), and oligodendrocytes (myelin basic protein)		

^a NCAM, neural cell adhesion molecule; cTnI, cardiac Troponin-T; FOXA2, forkhead box 2; α -SMA, alpha smooth muscle actin (K15); pdx-1, pancreatic marker; Tuj1, β -III-tubulin (neuronal marker); AFP, α -fetoprotein; NFH, neuro-filament heavy chain; GFAP, glial fibrillary acidic protein; cTnI, cardiac troponin I.

insufficient to maintain pluripotency marker expression.⁷⁷ FGF-2 induces the expression of hESC supportive factors, and Smad2/3

activation (TGF- β pathway) is required for hESCs to maintain pluripotency.⁷⁷ In addition to soluble factors in the culture medium,

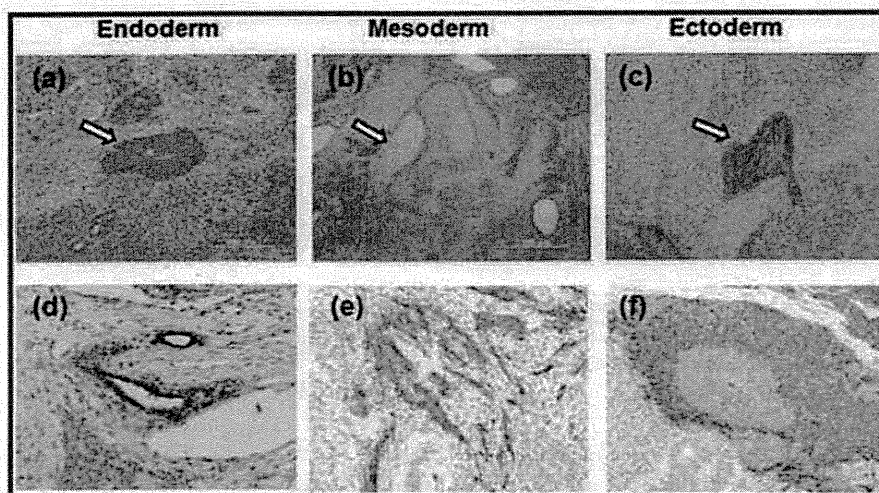


Figure 4. *In vivo* differentiation (teratoma formation) of human iPSCs, and histological and immunochemistry analysis of teratomas. The arrow indicates (a) hepatocytes (endoderm), (b) cartilage (mesoderm), and (c) retinal pigment epithelium (ectoderm). Differentiation markers representing the three germ layers, (d) AFP (endoderm), (e) smooth muscle actin (mesoderm), and (f) Tuj1 (ectoderm) were also observed.

the interaction between cells and their environment is also important and can influence the pluripotency of hESCs and human iPSCs. Figure 5 summarizes literature reports of different culture materials (substrates) in the feeder layer-free culture of hESCs.

3.1. hESC Culture on Matrigel

Matrigel is typically used as a substrate in feeder layer-free culture of many hESC lines. Matrigel is composed of isolated components from the sarcomas of Engelbreth–Holm–Swarm mice,^{78,79} including laminin, collagen IV, heparan sulfate proteoglycans, enactin, and growth factors (e.g., TGF- β , EGF, and FGF),^{14,80} which have been reported to support the pluripotency of many hESC lines.²⁸

The effect of Matrigel and its individual components, such as collagen IV, laminin, and fibronectin, on the self-renewing capacity of hESCs has been investigated.⁴⁵ Collagen IV, one of the components of Matrigel, could not maintain the pluripotency and self-renewing capability of hESCs, while laminin, which is also a component of Matrigel, successfully maintained and supported long-term hESC culture in undifferentiated states using MEF-conditioned medium (MEF-CM).⁴⁵ MEF-CM is a culture medium that has been used to culture MEFs and contains several growth factors, extracellular matrix (ECM) and cell binding molecules, which are secreted by MEFs. It has been reported that the successful maintenance of hESCs without MEF-CM requires a culture medium supplemented with high concentrations of FGF-2, TGF- β 1, and LIF and the use of fibronectin-coated dishes. In this culture system, FGF-2 maintains hESC culture,⁸¹ TGF- β 1 supports hESC proliferation,⁴⁵ fibronectin promotes cell adhesion,⁸² and LIF activates the JAK/STAT3 pathway and supports the self-renewal of hESCs.^{35,83} However, there are several reports that neither LIF nor activation of the STAT3 pathway contributes to the self-renewal of hESCs.^{2,84}

The first feeder cell-free culture was reported by Xu et al.⁴⁵ They reported that hESCs cultured on Matrigel attached and formed small colonies that were less compact than hESC colonies on MEF feeder layers.⁴⁵ Differentiated hESCs appeared between colonies after a few days. hESCs on Matrigels were, therefore, reported to be dense, undifferentiated colonies surrounded by differentiated cells. This group was able to culture hESCs on Matrigel in MEF-CM and

maintained the undifferentiated hESCs for over 130 population doublings (>180 days).⁴⁵ In contrast, hESCs seeded onto gelatin in MEF-CM had a low survival rate, and the cells tended to differentiate within the first passage.⁴⁵ Furthermore, it was observed that only a few appropriate hESC colonies existed in cultures on Matrigels with conditioned media from STO (an immortal mouse embryonic fibroblast cell line) or BJSta (a human foreskin fibroblast cell line immortalized with telomerase) cells after passage 39. Only conditioned medium from specific cells seems to support hESC growth. hESCs on Matrigels in MEF-CM maintained a normal karyotype and a stable proliferation rate (a doubling time of 31–33 h, similar to that for hESCs grown on a MEF feeder layer) and displayed high telomerase activity.⁴⁵

The hESCs on Matrigels showed successful expression of pluripotency genes, including Oct-4 and hTERT, alkaline phosphatase activity (AP), and the surface markers of pluripotency proteins, including SSEA-4, Tra-1-60, and Tra-1-81, after 53 passages.⁴⁵ hESCs generated embryonic bodies (EB) with heterogeneous morphologies, including beating cells *in vitro* and teratomas in SCID/beige mice, which differentiated into cells from all three germ layers.⁴⁵ It should be noted that hESCs can be maintained on Matrigel in MEF-CM, but hESCs on Matrigel in nonconditioned hESC medium completely differentiated after two passages. Thus, culture on Matrigels is not sufficient to maintain the pluripotency of hESCs. Several soluble factors, such as growth factors and ECM components secreted by MEFs, are also required.

Ullmann et al. reported the successful culture of hESCs on Matrigel-coated plates using MEF-CM and conditioned medium from human fetal skin fibroblasts.³⁴ They were unable to maintain the pluripotency of feeder-free hESCs on Matrigel for more than 37 passages, whereas the maintenance of hESCs on MEFs maintained pluripotency for more than 100 passages.^{34,85} Immunohistochemistry analysis showed that cells at the periphery of the hESC colonies in the feeder-free culture on Matrigel were negative for E-cadherin expression and positive for vimentin expression, which is indicative of the epithelial–mesenchymal transition (EMT). It was suggested that the feeder-free culture conditions using Matrigels forced the hESC colonies to undergo early differentiation into an EMT process.^{34,60}

Table 3. Feeder Layer-free Culture of hESCs and Human iPSCs Keeping Their Pluripotency on Biomaterials^a

hESCs (cell lines)	cell culture substrates	culture medium	longest time in culture	pluripotency evaluation	ref (year)
H1, H7, H9, H14	matrigel	MEF-CM	6 months	proteins, genes, EB, teratoma	45 (2001)
H1, H7, H9, H14	matrigel	MEF-CM	88 passages	proteins, genes	37 (2003)
BG03	matrigel	MEF-CM	24 passages	proteins, genes, EB	46 (2004)
H1, H7, H9	matrigel	MEF-CM	70 passages	proteins, genes, teratoma	72 (2004)
H1	matrigel	HEF1-CM	12 passages	proteins, genes, EB	86 (2004)
H7, H9	matrigel	SFM	15 passages	proteins, EB, teratoma	44 (2005)
H1, H9	matrigel	SFM	10 passages	genes, EB, teratoma	76 (2005)
H1	matrigel	MEF-CM or SFM	5 passages	proteins, genes, Diff	88 (2005)
H1, BGN1, BGN2	matrigel	MEF-CM or SFM	5 passages	proteins, genes, EB	87 (2005)
SA002, AS038, SA121	matrigel	SFM	35 passages	proteins, teratoma	68 (2005)
H14	matrigel	SFM	35 passages	morphology	60 (2006)
VUB01, VUB03_DM1, VUB04_CF	matrigel	MEF-CM	37 passages	protein	34 (2007)
VUB01, VUB03_DM1, VUB04_CF	matrigel	hF-CM	37 passages	proteins	34 (2007)
HUES7, NOTT-1, HESC-NL-1	matrigel	MEF-CM	20 passages	proteins, genes	89 (2008)
HS401	matrigel	SFM	30 passages	proteins, genes, EB	29 (2009)
H1, H7, H9, H14	laminin	MEF-CM	6 passages	morphology, genes	45 (2001)
H1	human laminin	SFM	11 passages	proteins, genes, EB, teratomas	75 (2005)
HSF6	laminin	SFM	20 passages	proteins, genes, teratomas	96 (2005)
H9, H13	laminin	SFM	15 passages	protein, EB	95 (2006)
KhES-1, KhES-2, KhES-3	laminin	MEF-CM	10 passages	protein, EB	50 (2008)
BG03	fibronectin	MEF-CM	24 passages	proteins, genes, EB	46 (2004)
I-3, I-6, H-9	fibronectin	SFM	30 passages	proteins, EB, teratoma	35 (2004)
HS360	fibronectin	SFM	2 passages	proteins, genes, EB	29 (2009)
MAN1, HUES7, HUES1	fibronectin	SFM	10 passages	proteins, genes, EB	38 (2009)
HUES1, HES2, HESC-NL3	vitronectin	SFM	8 passages	proteins	71 (2008)
HUES-1, Shefl	collagen (type 1)	SFM	24 passages	proteins, genes, EB	32 (2008)
H1, H7, H9, H14	gelatin	MEF-CM	few passages	morphology	45 (2001)
H9, ACT-14	MEF-ECM	SFM	30 passages	proteins, EB, teratoma	127 (2005)
hES2, hES3, hES7	hMSC-derived matrix	hMSC-CM	30 passages	proteins	49 (2008)
HS360, HS401	hECM mixture	SFM	6–7 passages	proteins, genes, EB	29 (2009)
H9	recombinant E-cadherin	SFM	35 passages	proteins, genes, teratoma	103 (2010)
HS360	FBS	SFM	10 passages	proteins, genes, EB	29 (2009)
HS360	human serum	SFM	2 passages	proteins, genes, EB	29 (2009)
H1, hES-NCL1	human serum	hES-dF-CM	27 passages	proteins, genes, EB, teratoma	39 (2005)
hESCs	hyaluronic acid hydrogels	MEF-CM	20 days	proteins, EB	113 (2007)

^a MEF-CM, mouse embryonic fibroblast-conditioned medium; SFM, serum free medium; hMSC-CM, human mesenchymal stem cell-conditioned medium; hMSC-derived matrix, extracellular matrix derived from human mesenchymal stem cell; hECM mixture, mixture of human extracellular matrix, proteins, surface marker analysis, and immunohistochemical analysis of pluripotency of hESCs; genes, gene expression analysis of pluripotency of hESCs; EB, embryonic body analysis; teratoma, teratoma analysis; Diff, differentiation analysis.

3.2. hESC Culture on Serum-Coated Dishes

Matrigel^{37,45,86–89} has been frequently used for the feeder-free growth of hESCs in undifferentiated states because it supports the attachment and growth of undifferentiated hESCs in MEF-CM. The use of Matrigel is, however, not ideal for potential medical applications of hESCs due to the risk of xenogenic pathogens. Stojkovic et al. reported the maintenance of undifferentiated hESC cultures on human serum-coated dishes for several passages using conditioned medium from fibroblasts derived from differentiated hESCs (hES-dF-CM).³⁹ hESCs grown on human serum-coated dishes under these conditions maintained undifferentiated characteristics after prolonged culture (>27 passages), while hESC cultured on uncoated dishes formed embryoid bodies or attached to the plates, leading to spontaneous differentiation. hESCs cultured on human serum-coated

dishes expressed cell surface and intracellular hESC markers typical of undifferentiated cells: SSEA-4, Oct-4, TRA-1-60, TRA-1-81, and alkaline phosphatase.³⁹ When hESCs were cultured on human serum-coated dishes in the absence of hES-dF-CM, hESCs tended to differentiate spontaneously within 48 h, indicating that hES-dF-CM is a key factor for the maintenance of undifferentiated hESCs in this system. Furthermore, hESCs cultured on human serum-coated dishes had the potential to differentiate into tissues from all three embryonic germ layers *in vivo* and *in vitro* (e.g., cartilage, muscle, primitive neuroectoderm, neural ganglia, kidney, secretory epithelia, connective tissues, etc.) and maintained a normal karyotype.³⁹ It has been reported that hESCs can maintain pluripotency when cultured on dishes coated with different types of human serum from different batches or prepared from patients with type I diabetes.³⁹

Table 4. Feeder Layer-free Culture of hESCs and Human iPSCs Keeping Their Pluripotency on Synthetic Polymers^a

hESCs (cell lines)	cell culture substrates	culture medium	longest time in culture	pluripotency evaluation	ref (year)
CHA-hES3, H9	porous PET membranes	SFM +STO feeder cells	25 passages	protein, teratoma	¹¹⁴ (2007)
HUES7, NOTT1	oxygen plasma etched TCPS	MEF-CM	14 passages	proteins, genes, Diff.	²⁸ (2009)
hESCs	TMA-PSt microcarriers	SFM	6 passages	proteins, EB	⁴⁰ (2008)
HS237	PDTEC	hES-CM	1 passages	proteins, genes, EB	²⁹ (2009)
HS237	PLDLA	hES-CM	1 passages	proteins, genes, EB	²⁹ (2009)
HI	calcium alginate hydrogels	SFM	260 days	proteins, genes	⁴⁸ (2008)
BG01 V	chitosan and alginate 3D scaffolds	SFM	21 days	proteins, genes, teratoma	³⁰ (2010)
SA167, AS034.1	TCPS	hF-CM	43 passages	proteins, teratoma	³¹ (2008)
BG01, WIBR3	FBS-coated acrylate copolymer	MEF-CM	10 passages	proteins, genes, teratoma	¹¹⁷ (2010)
BG01, WIBR3	human serum-coated acrylate copolymer	SFM	5 passages	proteins, genes, teratoma	¹¹⁷ (2010)

^a MEF-CM; mouse embryonic fibroblast-conditioned medium, SFM; serum free medium; proteins; hES-CM; human foreskin fibroblast-conditioned medium, hF-CM; human fibroblasts-conditioned medium, FBS; fetal bovine serum, proteins; surface marker analysis and immunohistochemical analysis of pluripotency of hESCs, genes; gene expression analysis of pluripotency of hESCs, EB; embryonic body analysis, teratoma; teratoma analysis, Diff; differentiation analysis, PET, polyethylene terephthalate; TMA-PSt, trimethylammonium-coated polystyrene microcarriers; PDTEC; poly-(desaminotyrosyl-tyrosine-ethyl ester carbonate), and PLDLA; poly-L,D-lactide.

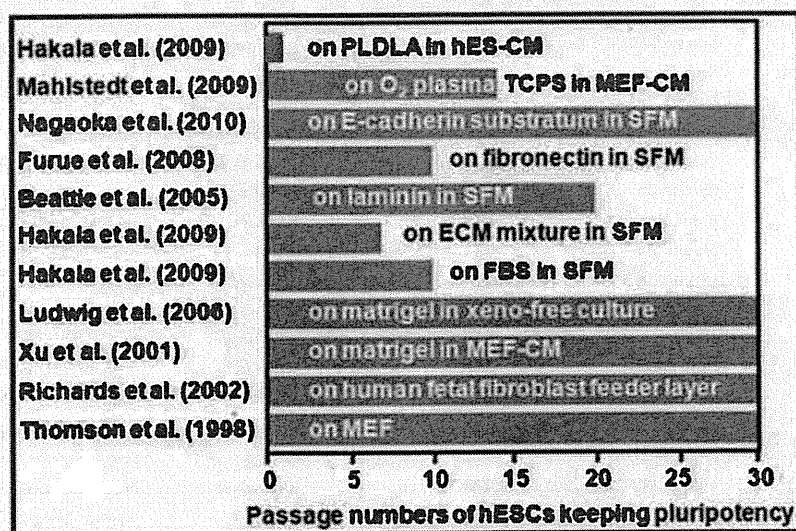


Figure 5. Use of different culture materials for the feeder layer-free culture of hESCs reported in the literature.

This indicates that different soluble growth factors, adhesion molecules, and ECM components that support the maintenance of undifferentiated hESCs are common and consistently present in different batches of human serum prepared from both normal donors and patients with type I diabetes. However, Hakala et al. reported that hESCs cultured on human serum-coated dishes could not maintain pluripotency in a chemically defined medium or conditioned medium prepared from human foreskin fibroblasts.²⁹ This indicates that the soluble factors in the culture medium of hESCs can also determine the fate of undifferentiated hESCs cultured on human serum-coated dishes.

3.3. hESC Culture on ECM-Coated Dishes

Recombinant or natural collagen IV, laminin, fibronectin, and vitronectin, which are components of the ECM, have been used instead of Matrigel or serum as coating materials for the feeder-free growth of undifferentiated hESCs and human iPSCs. Feeder cell-free and serum-free hESC culture on human fibronectin-coated dishes was investigated in a culture medium containing KnockOut Serum Replacement (Ko-SR) together with TGF- β 1 and FGF-2 (Table 3).^{29,90}

The feeder layer-free and serum-free culture of hESCs (I-3, I-6, and H-9) on fibronectin-coated dishes showed low differentiation percentages in medium containing Ko-SR, TGF- β 1, and FGF-2, while hESCs cultured on gelatin showed complete differentiation on day 8.³⁵ With regard to the growth rates of hESCs, the colony forming efficiency on human fibronectin was lower but similar to that on MEFs, while the colony forming efficiency of hESCs cultured on bovine fibronectin was dramatically lower than that on MEFs and on human fibronectin.³⁵ Amit et al. reported that more than 50 passages were possible for hESC culture on fibronectin without differentiation of the cells.³⁵ However, it should be noted that another study²⁹ reported that hESCs cultured on fibronectin-coated dishes did not maintain pluripotency under the same conditions described by Amit et al.³⁵ The hESCs quickly differentiated and attached poorly beyond the second passage. Furthermore, Xu et al. found that hESCs cultured on collagen IV and fibronectin in MEF-CM did not contain as many undifferentiated colonies as those cultured on Matrigels or laminin.⁴⁵

Gelatin is thermally denatured collagen derived from animal skin and bones. Mouse ESCs can be maintained on gelatin-coated

dishes in a culture medium supplemented with LIF for extended periods of time.^{91–93} The binding of the LIF receptor β /gp130 heterodimer and activation of the JAK/STAT3 signaling pathway have been implicated in the self-renewal of mouse ESCs.⁹⁴ However, gelatin was not able to support undifferentiated hESCs. hESCs seeded onto gelatin in MEF-CM had low survival rates, and the cells tended to differentiate within the first passage.⁴⁵

Laminin is the first extracellular matrix protein expressed in two- to four-cell stage mouse embryos and is a major component of the extracellular matrix of basal laminae in all vertebrates.^{45,95,96}

hESCs cultured on laminin could be maintained in an undifferentiated state for more than 42 days.⁴⁵ The hESCs cultured on laminin successfully expressed pluripotency genes (Oct-4 and hTERT) and had high telomerase activity. hESC culture on recombinant human laminin (rh laminin) in MEF-CM has also been reported.⁵⁰ Recombinant human ECM is abundantly available and a well-characterized source of human-derived proteins produced in an *in vitro* system. Laminin, which is a major component of Matrigel, plays an important role in cellular adhesion and consists of three distinct subunits: α_1 to α_3 , β_1 to β_3 , and γ_1 to γ_3 .^{50,97} More than 15 laminin isoforms have been identified, and the expression of these isoforms is specifically regulated by different types of cell surface receptors during embryonic development.⁹⁸ Four laminin-binding types ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_4$, and $\alpha_7\beta_1$) among 24 unique integrin isoforms have been identified.⁹⁸ Consistent with Xu et al.,⁴⁵ Miyazaki et al. found that hESCs primarily expressed integrin $\alpha_4\beta_1$, which binds predominantly to laminin-111, -332, and -511/-521.⁵⁰ When hESCs were cultured on rh laminin in MEF-CM, the cells adhered well to rh laminin-332-coated plates but did not adhere to rh laminin-511- and rh laminin-111-coated plates.⁵⁰ The hESCs proliferated on these three rh laminin-coated plates in MEF-CM for several passages while maintaining pluripotency.⁵⁰ These results show that rh laminin-111, -332, and -511 are able to expand undifferentiated hESCs due to their high affinity for integrin $\alpha_4\beta_1$, which is expressed on hESCs; however, it is unknown whether hESC pluripotency can be maintained for extended passages.⁵⁰ In addition, because the hESCs were cultured in MEF-CM, the hESC culture on rh laminin is feeder-free but not xeno-free.⁵⁰

hESC culture on laminin-coated dishes using serum-free medium that contains human-derived and recombinant proteins supplemented with recombinant growth factors has also been reported.⁷⁵ The hESCs maintained an undifferentiated morphology and expressed pluripotency genes (SSEA-4, Tra-1-60, and Cripto) similarly to cells cultured in MEF-CM. These hESCs were also able to generate teratomas in SCID/beige mice, indicating that the cells from all three germ layers differentiated when the hESCs were cultured for less than 14 passages.⁷⁵ hESCs cultured in xeno-free conditions must be evaluated for longer periods (>20–30 passages) for bioengineering applications.

Several reports have suggested that dishes coated with individual proteins from the ECM are inadequate for culturing and maintaining undifferentiated hESCs.^{29,60} Therefore, Ludwig et al. used a combination of four human ECM proteins (collagen IV, vitronectin, fibronectin, and laminin) and a defined, xenogenic component-free culture medium to investigate xeno-free hESC culture.⁹⁹ The derived hESC lines, however, were karyotypically abnormal.⁶⁰ Therefore, a modified, more reasonable xenogenic protein-containing medium (mTeSR1) combined with Matrigel was used for hESC culture and has been offered commercially by their group.⁶⁰ Hakala et al. also tried hESC (HS237, HS360, and HS401) culture on a mixture of human ECM components.²⁹ They were able to culture hESCs for a maximum of seven passages in

conditioned medium or xeno-free culture medium (TeSR1), after which all cells showed differentiated morphologies and lost the expression of Oct3/4, a marker of undifferentiated hESCs.²⁹ The human ECM mixture and xeno-free culture medium did not support maintenance of undifferentiated hESCs beyond the early passages and led to cell detachment and the loss of pluripotency markers.

These results suggest that it is difficult to culture several cell lines of hESCs in feeder layer-free conditions on ECM-coated dishes for multiple passages without using Matrigel containing undefined and animal-derived components.

3.4. hESC Culture on a Recombinant E-cadherin Substratum

E-cadherin is a Ca^{2+} -dependent cell–cell adhesion molecule^{100,101} and is essential for intercellular adhesion and colony formation of ESCs.^{34,102} Undifferentiated ESCs are expressing a high amount of E-cadherin. Nagaoka et al. prepared a fusion protein consisting of an E-cadherin extracellular domain and the IgG Fc domain (E-cad-Fc), and they investigated the hESC culture on the recombinant E-cadherin substratum in MEF-CM and in serum free medium (mTeSR1).¹⁰³ The hESCs thus cultured could maintain pluripotency for >35 passages and could generate embryonic body *in vitro* and teratoma *in vivo* where histological analysis revealed the presence of cells from all three germ layers.

Integrin-mediated cell-ECM interactions have been considered essential for maintenance of stem cell pluripotency and viability.^{104,105} Eventually, as found in previous sections, significant efforts have been devoted to finding a suitable ECM component that can maintain pluripotency of hESCs with interaction between hESCs and integrin receptors on ECMs. Integrin-ECM interactions activate signaling pathways of integrin-linked kinase (ILK) or focal adhesion kinase (FAK) as well as PI3K/Akt and MAPK pathways,¹⁰⁶ while E-cadherin-mediated adhesion of hESCs is typically associated with β -catenin signaling and also stimulates PI3K/Akt signaling.^{103,107,108} Especially Akt signaling pathways are considered to be important for maintenance of pluripotency of hESCs.^{109,110} It was suggested that trans-homodimerization between E-cadherin on hESCs and the E-cadherin domain presented on the recombinant E-cadherin substratum could promote and maintain the pluripotency of hESCs by activation of the PI3K/Akt signaling pathway.¹⁰³

Mouse ESCs on the recombinant E-cadherin substratum did not form aggregated colonies and were scattered with a spindle-like morphology.¹¹¹ In contrast, hESCs retained their ability to form the colonies, as is typically observed on MEF or Matrigels.¹⁰³ The pluripotency of mouse ESCs should be maintained by LIF signaling pathways under no aggregated colony formation, where the aggregated colony formation is important to maintain the pluripotency of human ESCs.

3.5. hESC Culture on Glycosaminoglycan

Hyaluronic acid (HA) is a linear polysaccharide of $\beta(1-4)$ -D-glucuronic acid and $\beta(1-3)$ -N-acetyl-D-glucosamine found in the ECM of undifferentiated cells during early embryogenesis. Differentiated cells have reduced expression of HA.^{14,112} A synthetic hydrogel matrix of HA has been used for the long-term culture of hESCs with self-renewing capabilities.¹¹³ To prepare hESCs encapsulated in HA gel, hESCs were added to a 2% methacrylated HA and 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone solution. The solution was poured into a mold to generate discs that were 3 mm in diameter and 2 mm thick. These discs were photopolymerized with 10 mW/cm² of ultraviolet light for 10 min.¹¹³ hESCs encapsulated in dextran gels were also prepared using a similar method. hESCs encapsulated within HA hydrogels and grown in MEF-CM remained undifferentiated for 20 days, while

hESCs cultured on a monolayer of HA or encapsulated in dextran hydrogels did not maintain pluripotency. Differentiation could be induced within HA hydrogels by addition of different soluble factors to the culture media. hESCs were released from the HA hydrogel by treatment with hyaluronidase to digest the HA hydrogels.^{14,113} The HA hydrogels provide a unique microenvironment for the self-renewal and differentiation of hESCs. However, it is difficult to keep the hESCs cultured on HA hydrogels undifferentiated for more than 10 passages.

3.6. hESC Culture on Synthetic Polymers

Biomacromolecules, such as Matrigel and several kinds of ECM, are costly and have limited shelf lives. The development of completely synthetic substrates is desirable for the culture of hESCs and human iPSCs.²⁸ The use of synthetic polymers that maintain pluripotency and the self-renewing capabilities of the cells has been reported (Table 4).^{28–31,40,48,114}

3.6.1. hESC Culture on 2D Synthetic Polymers. hESCs have been cultured on oxygen plasma etched tissue culture polystyrene (PE-TCPS) using MEF-CM. This synthetic culture surface was stable at room temperature for at least a year. hESCs (HUES7 and NOTT1) cultured on PE-TCPS expressed stem cell marker proteins (Oct-4, TRA1-60, and SSEA-4) and showed a stable karyotype after 10–14 passages.²⁸ The differentiation of HUES7 and NOTT1 cells cultured on PE-TCPS was evaluated by inducing the aggregation of defined numbers of hESCs into EB. qRT-PCR showed that markers of early germ layer formation, Brachyury T (mesoderm), SOX17 (endoderm), and SOX1 (ectoderm), were similarly expressed in hESCs cultured on PE-TCPS and Matrigels.²⁸ α -Actin (mesoderm), alkaline phosphatase (AFP, endoderm), and β -III tubulin (ectoderm), which are present during the late stage differentiation, were also detected by immunostaining analysis. NOTT1 cells were also induced to differentiate into cardiomyocytes. Beating outgrowths were mechanically isolated from the main body of EBs and seeded onto Matrigel-coated microelectrode arrays (MEAs). The extracellular field potentials of the cell clusters were analyzed with microelectrode arrays.²⁸ The cell clusters showed a beating rate of 100 per min at rest, while the beating rate increased significantly to 151 per min when the cell clusters were treated with 1 μ M isoprenaline, a β -adrenoceptor agonist that is known to have a positive chronotropic effect on the human heart. The pharmacological response observed suggested that the clusters of beating cells contained cardiomyocytes that differentiated from the hESCs because contraction of skeletal or smooth muscle cells would be inhibited or unaffected by isoprenaline.²⁸

Harding et al. investigated the use MEF-CM for the culture of hESC on PE-TCPS.²⁸ hESCs cultured solely on synthetic polymers without the use of xeno-derived biomacromolecules were attempted by Hakala et al.²⁹ In their study, poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTEC) and poly-L,D-lactide (PLDLA) were used for hESC culture.²⁹ PLDLA is commonly used for hESC differentiation,¹¹⁵ while PDTEC is used for guided bone regeneration in animal models.¹¹⁶ However, the hESCs did not attach to the synthetic polymers in a xeno-free and chemically defined medium. Furthermore, the hESCs did not attach to PLDLA in human foreskin fibroblast-conditioned medium, while some of the hESCs attached to PDTEC, as well as Ti, TiO₂, and ZrO₂ surfaces, in human foreskin fibroblast-conditioned medium.²⁹ The hESC colonies were very fragile on the synthetic polymers and the Ti, TiO₂, and ZrO₂ surfaces. PLDLA and PDTEC without ECM failed to support hESC culture and did not maintain undifferentiated hESCs, even in human foreskin fibroblast-conditioned medium. These

results demonstrate the difficulty of culturing hESCs solely on synthetic polymers in xeno-free cultures.

It is inefficient to evaluate synthetic polymers for the culture of hESCs without the concept of design of the biomaterials. Therefore, Mei et al. developed a combinatorial technique to evaluate the biomaterials using microarrays for the culture of hESC and iPSC maintaining their pluripotency.¹¹⁷ The microarrays were prepared from 22 acrylate monomers with diversified hydrophobicity–hydrophilicity (water contact angle) and cross-linking densities. The microarrays were prepared by copolymerization between each of 16 “major” monomers and each of six “minor” monomers at six different ratios [100:0, 90:10, 85:15, 80:20, 75:25, 70:30 (v/v)]. Therefore, microarrays with 496 [16 + (16 × 5 × 6)] different combinations of copolymers were evaluated, consisting of the major monomer (70–100%) and minor monomer (0–30%). Water contact angle, surface topography, surface chemistry [analysis of functional group by time-of-flight secondary-ion mass spectroscopy (ToF-SIMS) analysis], and indentation elastic modulus of polymeric substrates were quantified using high-throughput methods to develop structure–function relationships between material properties and pluripotency of hESCs cultured on the polymeric substrates coated with fetal bovine serum (FBS) in the microarray.¹¹⁷

Proteins such as ECM and growth factors, and glycosaminoglycans from FBS can adsorb onto the material surface used for cell culture.¹¹⁸ The surface properties of cell-culture substrates are regulated by both the amount and the conformation of adsorbed proteins and glycosaminoglycans, which interact with cell surface receptors to initiate signal transduction and alter cell behavior. Therefore, the synthetic polymeric materials having no specific binding sites for hESCs can be converted into the materials having specific binding sites after the materials were adsorbed with FBS and culture medium, because components and amount of proteins and glycosaminoglycans on the materials depend on the surface chemistry and physics of the materials.¹¹⁷

The colony-formation frequency was defined and investigated as the number of polymer spots on which hESC colonies (expressing Oct-4 and SSEA-4) formed divided by the total number of replicate spots of the same kind of polymer on each array. The surface roughness of the substrate in air, in PBS, and in culture medium after FBS adsorption did not correlate strongly with colony-formation frequency, although it was reported to affect the cell growth and attachment of adult somatic and stem cells.^{26,119} A positive correlation was observed between the indentation elastic modulus of hydrated polymeric substrate and colony-formation frequency. However, it was found that the polymeric substrate exhibiting a low indentation elastic modulus also exhibited a low water contact angle in their polymer substrates. The optimum wettability (65° < water contact angle < 80°) of copolymer showed high colony-formation frequency over a broad range of polymer stiffness. Especially, polymers with a moderate water contact angle generated from multiple-acrylate-group-containing monomers performed the best colony-formation frequency in their experiments.¹¹⁷

The hit arrays were further evaluated for their capacity to maintain the pluripotency of hESCs after more than 2 months of culture (>10 passages). hESCs were found to maintain an undifferentiated state with evidence from expression of pluripotent markers, Oct-4, Nanog, Tra-1-60, and SSEA-4 after prolonged culture. The differentiation of these hESCs into all three germ layer lineages was also confirmed.¹¹⁷

3.6.2. hESC Culture on Porous Polymeric Membranes. hESCs cultured on a conventional MEF-feeder layer must be

treated with enzymes, such as collagenase or Dispase, when the hESCs are transferred to new culture dishes for isolation or expansion. Continuous exposure to enzymes can cause cytogenetic aberrations in the hESCs, although enzyme treatment is advantageous for the large-scale bulk expansion of hESCs over a short time period with laborious and time-consuming steps. An interesting hESC culture method was reported by Kim et al.; in this study, porous polymeric membranes (1, 3, and 8 μm pore sizes) were used to separate hESCs and feeder cells.¹¹⁴ The feeder cells were seeded and attached to the bottom of the porous membranes of trans-well inserts. hESCs were then cultured on top of the membranes (Figure 2). This method allowed the hESCs to be successfully cultured and effectively separated from the feeder cell layer without enzyme treatment. hESCs were placed onto the feeder cells through the porous membrane barrier without displacing the feeder cells, while the hESCs seeded on the feeder cells began to push the feeder cells away and then attach and grow on the culture dish.¹¹⁴

hESCs on the membranes interacted with the feeder cells through the pores of the membranes. The interaction was dependent on the pore size of the porous membranes used. The number of attached hESC colonies was dependent on the cell density of the feeder cells on the bottom of the membranes. On the other hand, hESC colonies did not attach to the porous membranes when the feeder cells were located on the bottom of the culture dish instead of on the porous membrane.¹¹⁴

hESC attachment on 3- μm and 8- μm porous membranes was higher compared to that on the 1- μm porous membranes. However, porous membranes with greater than 3- μm pore sizes allowed feeder cells on the bottom of the porous membranes to migrate upward, which generated contamination of the hESC colonies. The 1- μm pore membranes rarely permitted migration of the feeder cells.¹¹⁴ hESCs cultured on the 1- μm pore size membrane failed to maintain the hESCs for more than 15 passages, while hESCs on the 3- μm pore size membranes sustained the culture for more than 25 passages.¹¹⁴

The hESCs cultured on the porous membranes not only exhibited the expression of several undifferentiated markers and a normal karyotype but also formed teratomas that consisted of all three germ layers *in vivo*. This indicates that cell-cell contacts through the membrane pore and/or a close distance between the hESCs and feeder cells are important for maintaining the undifferentiated states of hESCs. Although the hESCs were cultured under xeno-containing conditions, culturing hESCs on porous membranes would be a useful method to exclude enzyme treatment and prevent contamination from feeder cells.

3.7. hESC Culture on 3D Biomaterials

The culture of hESCs on a 3D porous polymeric scaffold composed of chitosan and alginate and without the support of feeder cells or conditioned medium has been reported.³⁰ The pluripotency of the hESCs was maintained in the serum-free medium for 21 days. The hESCs expressed the expected gene profile for undifferentiated hESCs, including Oct-4, Nanog, SSEA-4, TERT, and AFP. The hESCs also formed teratomas in SCID mice that included derivatives of all three germ layers. However, this study³⁰ did not determine whether the pluripotency of hESCs could be maintained for >30 days.

hESC culture and expansion on microcarriers has also been reported. Phillips et al. reported the successful feeder-free 3D suspension culture of hESCs (ESI-017) on trimethyl ammonium-coated polystyrene microcarriers in serum-free medium.⁴⁰ The hESCs were maintained through six passages with a 14-fold increase in cell number. The cells expressed several undifferentiated markers,

including Oct-4 and Tra-1-81,⁴⁰ showing that the suspension-based expansion of hESCs on microcarriers was possible under feeder layer-free conditions. Using directed differentiation protocols, it was possible to induce the hESCs cultured on the microcarriers after six passages to express the pancreatic marker, pdx-1, and neuronal marker, Tuj1 (β -III-tubulin). The hESCs expressed cardiomyocyte markers, such as α -actin, Nkx2.5, Mlc2a, and tropomyosin.⁴⁰ Thus, the hESCs retained their capacity to differentiate into the pancreatic (endoderm), neuronal (ectoderm), and cardiomyocyte (mesoderm) lineages. However, the pluripotency for the hESCs was not determined for passages >10.

One of the difficulties of 3D culture using microcarriers or porous materials is the detachment of hESCs during the passage of the cells.¹²⁰ In general, hESCs were tightly adhered in tortuous environment, and it was difficult to recover the hESCs efficiently, even with an enzymatic treatment.

In another report, hESCs were maintained in a feeder layer-free and xeno-free environment by encapsulation in hydrogels.⁴⁸ hESCs were encapsulated in calcium alginate hydrogels and grown in a serum-free medium for up to 260 days. The encapsulated hESCs formed aggregates that increased in number and size without loss of the cells from the hydrogel. The aggregates were tightly and homogeneously packed with defined spherical borders. The hESCs retained their pluripotency and differentiated into cells of all three germ layers when they were subsequently cultured in differentiation medium.⁴⁸ Immunohistochemistry and RT-PCR experiments showed that the hESC aggregates expressed pluripotent proteins and genes, including Oct-4, Nanog, SSEA-4, TRA-1-60, and TRA-1-81. The hESCs were arranged in closely packed clusters and showed no cytoplasmic organelles, suggesting that they were in an undifferentiated state.⁴⁸ This study showed that encapsulation in the appropriate hydrogels allows the hESCs to maintain an undifferentiated state without passaging, EB formation, or xenogenic contamination. Furthermore, hESCs encapsulated in alginate hydrogels were easily recovered from the hydrogels using a dissolution buffer.

Although hESCs encapsulated in HA maintained an undifferentiated state only for 10 passages,¹¹³ encapsulation (3D culture) of hESCs in the appropriate materials seems to support long-term maintenance in the undifferentiated state without the need for feeders or passaging.

The 3D microenvironments that hESCs encounter *in vivo* have a combination of biological, chemical, physical, and mechanical cues, which can be mimicked by hydrogels, while traditional 2D culture is conducted on flat and rigid substrates of tissue culture polystyrene dishes (TCPS).^{14,121} During embryogenesis, cells in the inner cell mass are embedded in a 3D matrix, which regulates both their self-renewal and differentiation.¹²²

It is important to establish a 3D culture system using hydrogels in which hESCs can be maintained as undifferentiated cells and then induced to differentiate by external signals, such as soluble growth factors or chemicals in the culture medium.

4. CONCLUSIONS

Human feeder cells, including human fetal fibroblasts and human bone marrow cells, have been developed for culturing hESCs.^{5,114,123-125} However, it is difficult to achieve high passage numbers and to produce sufficient hESCs for clinical therapy with human feeder cells because human feeder cells are unable to maintain continuous, undifferentiated hESCs as well as animal feeder cells, such as STO and MEF.^{114,123}

The development of feeder cell-free hESC culture would substantially reduce the labor and cost of hESC culture, and would increase

the production of hESCs for potential clinical applications.²⁹ Currently, no synthetic polymers, with or without ECM and growth factors, have been able to maintain the pluripotency and undifferentiated state of hESCs in a xeno-free culture medium for an extended period of time. Matrigel-coated plates combined with a chemically defined culture medium containing xenogenic proteins support long-term undifferentiated hESC culture, and this would be the most adequate conditions for feeder layer-free culture. Mouse ESC culture is simpler because the culture medium containing LIF and/or a LIF-immobilized surface sufficiently supports mouse ESCs in a feeder layer-free culture.^{6,126} The replacement of Matrigel with LIF and/or mixed mouse or human ECMs¹²⁷ is insufficient to maintain the pluripotency of hESCs. Matrigel is known to contain several soluble factors, including ECM and growth factors. Some currently unknown key growth factors or soluble factors might be required for the maintenance of undifferentiated hESCs, and identification of these factors would contribute to the development of feeder layer-free hESC culture in xeno-free and chemically defined culture medium.

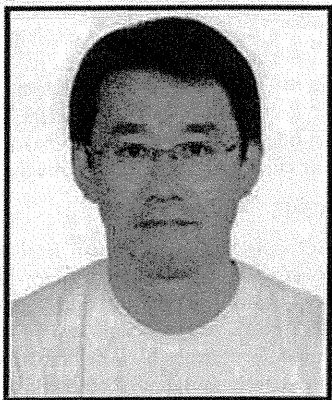
The relationship between the surface chemistry of culture materials and the maintenance of the pluripotency of hESCs and iPSCs is not still clear tentatively, although the surface roughness affects the cell growth and attachment of MSCs and also the elasticity of culture materials can direct MSCs into specific cell lineages (e.g., soft culture materials that mimic brain are neurogenic, stiffer culture materials that mimic muscle are myogenic, and rigid culture materials that mimic collagenous bone prove osteogenic).²⁶ It is only reported that the optimal wettability of the culture materials where FBS coated is maintaining the pluripotency of hESCs and iPSCs.¹¹⁷ If we design the culture dishes prepared with different synthetic polymers (having different roughness, elasticity, and wettability) and immobilized with ECMs, the culture dishes having different surface chemistry can be prepared where the same ECMs are immobilized. The combination of surface chemistry of the culture materials and specific interaction between human ECM proteins and hESCs or iPSCs would improve the maintenance of undifferentiated hESCs and iPSCs in a xeno-free culture for a longer time.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +866-34227151-34253. Fax: +866-3-2804271. E-mail: higuchi@ncu.edu.tw.

BIOGRAPHIES



Akon Higuchi is a Chair (Distinguished) Professor in the Department of Chemical and Materials Engineering, National Central

University. He is also joined to the Department of Reproduction, National Research Institute for Child Health and Development, and the Cathay Medical Research Institute, Cathay General Hospital, as a special researcher. He received his B.S. from Tokyo Institute of Technology in 1979 and his Ph.D. from Tokyo Institute of Technology in 1985. He was a Professor in the Department of Materials & Life Science at Seikei University from 1993 to 2007. He received the Sofue Memorial Award from the Society of Fiber Science, Japan, in 1994, and the Seikei Academic Award from the Seikei Alumni Association in 2003. He is interested in the development of materials for stem cell research. He established a purification method for hematopoietic stem cells and mesenchymal stem cells by a filtration method through polymeric porous membranes. He is also developing culture materials for stem cells.

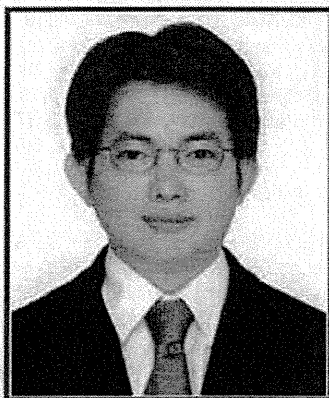


Qing-Dong Ling is a Senior Scientist and the director of the Cathay Medical Research Institute, Cathay General Hospital, in Taipei, Taiwan. In 2006, he was joined to the Graduate Institute of Systems Biology and Bioinformatics, the National Central University as an adjunct associate professor. He received his B.S. and DDS Degrees in Medical School, Zhejiang University, in 1979 and his Ph. D. Degree in Dental Medicine from Tokyo Dental College in 1996. He spent two and a half years at the National Institute of Health as a visiting fellow from 1996 to 1999. Dr. Ling's research interests include cellular and molecular mechanisms in neuronal plasticity following neonatal inflammation; gene expression in cancer and stem cells using microarray experiments; and the signal transduction and systems biology of stem cells.



Yi-An Ko was born in Taiwan in 1985. She received her B.S. degree in Food Science from National Taiwan Ocean University in 2007. In the same year, she joined the laboratory of Professor Qing-Dong Ling at National Central University,

where she acquired her M.Sc. degree in 2009. Now, she is a Ph.D. student under the guidance of Professor Ales Cvekl at Albert Einstein College of Medicine of Yeshiva University. Her research focuses on lens development in mouse ES cells.



Yung Chang was born March 7, 1976 in Tokyo, Japan. He received a B.E. degree from Chung Yuan Christian University in 1998 and a Ph.D. from National Taiwan University in 2004. He was a postdoctoral fellow in Prof. Shaoyi Jiang's group at University of Washington from 2004 to 2006. He joined the Department of Chemical Engineering of Chung Yuan Christian University in 2006 and is currently an Associate Professor. He is developing a new generation of antibiofouling materials, which are also very useful as membrane bioseparation agents, biomaterial coatings, tissue engineering scaffolds, drug-delivery carriers, and contact lenses, due to their super biocompatibility.



Akihiro Umezawa is a Department Head and Chairman in the Department of Reproductive Biology at the National Research Institute for Child Health and Development. He received his M.D. at Keio University School of Medicine in 1985 and his Ph.D. at Keio University Graduate School of Medicine in 1990. He served as an Associate Professor in the Department of Pathology at Keio University School of Medicine until 2002. He also served as an adjunct Professor at Keio University and Seikei University. He received the Henry Christian Memorial Award from the American Federation for Clinical Research Foundation in 1993 and the Kitasato Award from the School of Medicine at Keio University in 1997. Dr. Umezawa's research focuses on stem cell-based therapy using induced pluripotent stem cells, embryonic stem cells, and mesenchymal stem cells.

ACKNOWLEDGMENT

This research was partially supported by the National Science Council of Taiwan under Grant Nos. NSC97-2221-E-008-011-MY3, NSC97-2120-M-008-002, NSC98-2120-M-008-002, and NSC99-2120-M-008-002. This work was also supported by the VGHUST Joint Research Program, Tsou's Foundation (VGHUST97-P3-08 and VGHUST98-P3-11) and Cathay General Hospital Project (98CGH-NCU-B1 and 99CGH-NCU-B2). Grants-in-Aid for Scientific Research (No. 21500436) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan are also acknowledged.

REFERENCES

- (1) Reubinoff, B. E.; Pera, M. F.; Fong, C. Y.; Trounson, A.; Bongso, A. *Nat. Biotechnol.* **2000**, *18*, 399.
- (2) Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. *Science* **1998**, *282*, 1145.
- (3) Braam, S. R.; Denning, C.; van den Brink, S.; Kats, P.; Hochstenbach, R.; Passier, R.; Mummery, C. L. *Nat. Methods* **2008**, *5*, 389.
- (4) Osakada, F.; Ikeda, H.; Mandai, M.; Wataya, T.; Watanabe, K.; Yoshimura, N.; Akaike, A.; Sasai, Y.; Takahashi, M. *Nat. Biotechnol.* **2008**, *26*, 215.
- (5) Richards, M.; Fong, C. Y.; Chan, W. K.; Wong, P. C.; Bongso, A. *Nat. Biotechnol.* **2002**, *20*, 933.
- (6) Mallon, B. S.; Park, K. Y.; Chen, K. G.; Hamilton, R. S.; McKay, R. D. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1063.
- (7) Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. *Cell* **2007**, *131*, 861.
- (8) Okita, K.; Ichisaka, T.; Yamanaka, S. *Nature* **2007**, *448*, 313.
- (9) Takahashi, K.; Yamanaka, S. *Cell* **2006**, *126*, 663.
- (10) Belmonte, J. C.; Ellis, J.; Hochedlinger, K.; Yamanaka, S. *Nat. Rev. Genet.* **2009**, *10*, 878.
- (11) Rodriguez-Piza, I.; Richaud-Patin, Y.; Vassena, R.; Gonzalez, F.; Barrero, M. J.; Veiga, A.; Raya, A.; Belmonte, J. C. *Stem Cells* **2010**, *28*, 36.
- (12) Zhou, H.; Wu, S.; Joo, J. Y.; Zhu, S.; Han, D. W.; Lin, T.; Trauger, S.; Bien, G.; Yao, S.; Zhu, Y.; Siuzdak, G.; Scholer, H. R.; Duan, L.; Ding, S. *Cell Stem Cell* **2009**, *4*, 381.
- (13) Judson, R. L.; Babiarz, J. E.; Venere, M.; Brelloch, R. *Nat. Biotechnol.* **2009**, *27*, 459.
- (14) Abraham, S.; Eroshenko, N.; Rao, R. R. *Regen. Med.* **2009**, *4*, 561.
- (15) Allan, L. E.; Petit, G. H.; Brundin, P. *Curr. Opin. Neurol.* **2010**, *23*, 426.
- (16) Naegele, J. R.; Maisano, X.; Yang, J.; Royston, S.; Ribeiro, E. *Neuropharmacology* **2010**, *58*, 855.
- (17) Holden, C. *Science* **2009**, *326*, 358.
- (18) Sumi, S. *J. Hepatobiliary Pancreat. Sci.* **2010**, *18*, 6.
- (19) Kaestner, K. H. *Gastroenterology* **2010**, *138*, 2211.
- (20) Noguchi, H. *Curr. Diabetes Rev.* **2010**, *6*, 184.
- (21) Dellatore, S. M.; Garcia, A. S.; Miller, W. M. *Curr. Opin. Biotechnol.* **2008**, *19*, 534.
- (22) Little, L.; Healy, K. E.; Schaffer, D. *Chem. Rev.* **2008**, *108*, 1787.
- (23) Pierret, C.; Morrison, J. A.; Rath, P.; Zigler, R. E.; Engel, L. A.; Fairchild, C. L.; Shi, H.; Maruniak, J. A.; Kirk, M. D. *BMC Dev. Biol.* **2010**, *10*, 5.
- (24) Bendall, S. C.; Stewart, M. H.; Bhatia, M. *Regen. Med.* **2008**, *3*, 365.
- (25) Lutolf, M. P.; Gilbert, P. M.; Blau, H. M. *Nature* **2009**, *462*, 433.
- (26) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. *Cell* **2006**, *126*, 677.
- (27) Lee, M. R.; Kwon, K. W.; Jung, H.; Kim, H. N.; Suh, K. Y.; Kim, K.; Kim, K. S. *Biomaterials* **2010**, *31*, 4360.
- (28) Mahlstedt, M. M.; Anderson, D.; Sharp, J. S.; McGilvray, R.; Munoz, M. D.; Buttery, L. D.; Alexander, M. R.; Rose, F. R.; Denning, C. *Biotechnol. Bioeng.* **2010**, *105*, 130.

- (29) Hakala, H.; Rajala, K.; Ojala, M.; Panula, S.; Areva, S.; Kello-maki, M.; Suuronen, R.; Skottman, H. *Tissue Eng. Part A* **2009**, *15*, 1775.
- (30) Li, Z.; Leung, M.; Hopper, R.; Ellenbogen, R.; Zhang, M. *Biomaterials* **2010**, *31*, 404.
- (31) Bigdeli, N.; Andersson, M.; Strehl, R.; Emanuelsson, K.; Kilmare, E.; Hyllner, J.; Lindahl, A. *J. Biotechnol.* **2008**, *133*, 146.
- (32) Furue, M. K.; Na, J.; Jackson, J. P.; Okamoto, T.; Jones, M.; Baker, D.; Hata, R.; Moore, H. D.; Sato, J. D.; Andrews, P. W. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 13409.
- (33) Adler, S.; Pellizzer, C.; Hareng, L.; Hartung, T.; Bremer, S. *Toxicol. In Vitro* **2008**, *22*, 200.
- (34) Ullmann, U.; In't Veld, P.; Gilles, C.; Sermon, K.; De Rycke, M.; Van de Velde, H.; Van Steirteghem, A.; Liebaers, I. *Mol. Hum. Reprod.* **2007**, *13*, 21.
- (35) Amit, M.; Shariki, C.; Margulets, V.; Itskovitz-Eldor, J. *Biol. Reprod.* **2004**, *70*, 837.
- (36) Hong, H.; Takahashi, K.; Ichisaka, T.; Aoi, T.; Kanagawa, O.; Nakagawa, M.; Okita, K.; Yamanaka, S. *Nature* **2009**, *460*, 1132.
- (37) Carpenter, M. K.; Rosler, E. S.; Fisk, G. J.; Brandenberger, R.; Ares, X.; Miura, T.; Lucero, M.; Rao, M. S. *Dev. Dyn.* **2004**, *229*, 243.
- (38) Baxter, M. A.; Camarasa, M. V.; Bates, N.; Small, F.; Murray, P.; Edgar, D.; Kimber, S. J. *Stem Cell Res.* **2009**, *3*, 28.
- (39) Stojkovic, P.; Lako, M.; Przyborski, S.; Stewart, R.; Armstrong, L.; Evans, J.; Zhang, X.; Stojkovic, M. *Stem Cells* **2005**, *23*, 895.
- (40) Phillips, B. W.; Horne, R.; Lay, T. S.; Rust, W. L.; Teck, T. T.; Crook, J. M. *J. Biotechnol.* **2008**, *138*, 24.
- (41) Brevini, T. A.; Pennarossa, G.; Antonini, S.; Paffoni, A.; Tettamanti, G.; Montemurro, T.; Radaelli, E.; Lazzari, L.; Rebulli, P.; Scanziani, E.; de Eguileor, M.; Benvenisty, N.; Ragni, G.; Gandolfi, F. *Stem Cell Rev.* **2009**, *5*, 340.
- (42) Bhandari, D. R.; Seo, K. W.; Roh, K. H.; Jung, J. W.; Kang, S. K.; Kang, K. S. *PLoS One* **2010**, *5*, e10493.
- (43) Wang, S.; Hu, C.; Zhu, J. *Mol. Biol. Cell* **2010**, *21*, 821.
- (44) Xu, C.; Rosler, E.; Jiang, J.; Lebkowski, J. S.; Gold, J. D.; O'Sullivan, C.; Delavan-Boorsma, K.; Mok, M.; Bronstein, A.; Carpenter, M. K. *Stem Cells* **2005**, *23*, 315.
- (45) Xu, C.; Inokuma, M. S.; Denham, J.; Golds, K.; Kundu, P.; Gold, J. D.; Carpenter, M. K. *Nat. Biotechnol.* **2001**, *19*, 971.
- (46) Brimble, S. N.; Zeng, X.; Weiler, D. A.; Luo, Y.; Liu, Y.; Lyons, I. G.; Freed, W. J.; Robins, A. J.; Rao, M. S.; Schulz, T. C. *Stem Cells Dev.* **2004**, *13*, 585.
- (47) Ameen, C.; Strehl, R.; Bjorquist, P.; Lindahl, A.; Hyllner, J.; Sartipy, P. *Crit. Rev. Oncol. Hematol.* **2008**, *65*, 54.
- (48) Siti-Ismail, N.; Bishop, A. E.; Polak, J. M.; Mantalaris, A. *Biomaterials* **2008**, *29*, 3946.
- (49) Peiffer, I.; Barbet, R.; Zhou, Y. P.; Li, M. L.; Monier, M. N.; Hatzfeld, A.; Hatzfeld, J. A. *Stem Cells Dev.* **2008**, *17*, 519.
- (50) Miyazaki, T.; Futaki, S.; Hasegawa, K.; Kawasaki, M.; Sanzen, N.; Hayashi, M.; Kawase, E.; Sekiguchi, K.; Nakatsuji, N.; Suemori, H. *Biochem. Biophys. Res. Commun.* **2008**, *375*, 27.
- (51) Hansen, T. B.; Bramsen, J. B.; Kjems, J. *PLoS One* **2010**, *5*, e10961.
- (52) Letzen, B. S.; Liu, C.; Thakor, N. V.; Gearhart, J. D.; All, A. H.; Kerr, C. L. *PLoS One* **2010**, *5*, e10480.
- (53) Satija, N. K.; Singh, V. K.; Verma, Y. K.; Gupta, P.; Sharma, S.; Afrin, F.; Sharma, M.; Sharma, P.; Tripathi, R. P.; Gurudutta, G. U. *J. Cell Mol. Med.* **2009**, *13*, 4385.
- (54) Lim, T. Y.; Wang, W.; Shi, Z.; Poh, C. K.; Neoh, K. G. *J. Mater. Sci. Mater. Med.* **2009**, *20*, 1.
- (55) Boskey, A. L.; Roy, R. *Chem. Rev.* **2008**, *108*, 4716.
- (56) Mahmoudifar, N.; Doran, P. M. *Biomaterials* **2010**, *31*, 3858.
- (57) Flynn, L. E.; Prestwich, G. D.; Semple, J. L.; Woodhouse, K. A. *Biomaterials* **2008**, *29*, 1862.
- (58) Sun, H.; Feng, K.; Hu, J.; Soker, S.; Atala, A.; Ma, P. X. *Biomaterials* **2010**, *31*, 1133.
- (59) Zheng, Y. B.; Gao, Z. L.; Xie, C.; Zhu, H. P.; Peng, L.; Chen, J. H.; Chong, Y. T. *Cell Biol. Int.* **2008**, *32*, 1439.
- (60) Ludwig, T. E.; Bergendahl, V.; Levenstein, M. E.; Yu, J.; Probasco, M. D.; Thomson, J. A. *Nat. Methods* **2006**, *3*, 637.
- (61) Placzek, M. R.; Chung, I. M.; Macedo, H. M.; Ismail, S.; Mortera Blanco, T.; Lim, M.; Cha, J. M.; Fauzi, I.; Kang, Y.; Yeo, D. C.; Ma, C. Y.; Polak, J. M.; Panoskaltis, N.; Mantalaris, A. *J. R. Soc. Interface* **2009**, *6*, 209.
- (62) Furth, M. E.; Atala, A. *J. Cell Biochem.* **2009**, *106*, 507.
- (63) Jezierski, A.; Gruslin, A.; Tremblay, R.; Ly, D.; Smith, C.; Turksen, K.; Sikorska, M.; Bani-Yaghoub, M. *Stem Cell Rev.* **2010**, *6*, 199.
- (64) De Coppi, P.; Bartsch, G., Jr.; Siddiqui, M. M.; Xu, T.; Santos, C. C.; Perin, L.; Mostoslavsky, G.; Serre, A. C.; Snyder, E. Y.; Yoo, J. J.; Furth, M. E.; Soker, S.; Atala, A. *Nat. Biotechnol.* **2007**, *25*, 100.
- (65) Franco Lambert, A. P.; Fraga Zandonai, A.; Bonatto, D.; Cantarelli Machado, D.; Pegas Henriques, J. A. *Differentiation* **2009**, *77*, 221.
- (66) Yamanaka, S.; Li, J.; Kania, G.; Elliott, S.; Wersto, R. P.; Van Eyk, J.; Wobus, A. M.; Boheler, K. R. *Cell Tissue Res.* **2008**, *331*, 5.
- (67) Harkness, L.; Taipaleenmaki, H.; Mahmood, A.; Frandsen, U.; Saamanen, A. M.; Kassem, M.; Abdallah, B. M. *Stem Cell Rev.* **2009**, *5*, 353.
- (68) Sjogren-Jansson, E.; Zetterstrom, M.; Moya, K.; Lindqvist, J.; Strehl, R.; Eriksson, P. S. *Dev. Dyn.* **2005**, *233*, 1304.
- (69) Zhou, J.; Su, P.; Wang, L.; Chen, J.; Zimmermann, M.; Genbacev, O.; Afonja, O.; Horne, M. C.; Tanaka, T.; Duan, E.; Fisher, S. J.; Liao, J.; Wang, F. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 7840.
- (70) Su, Z.; Frye, C.; Bae, K. M.; Kelley, V.; Vieweg, J. *Clin. Cancer Res.* **2008**, *14*, 6207.
- (71) Braam, S. R.; Zeinstra, L.; Litjens, S.; Ward-van Oostwaard, D.; van den Brink, S.; van Laake, L.; Lebrin, F.; Kats, P.; Hochstenbach, R.; Passier, R.; Sonnenberg, A.; Mummery, C. L. *Stem Cells* **2008**, *26*, 2257.
- (72) Rosler, E. S.; Fisk, G. J.; Ares, X.; Irving, J.; Miura, T.; Rao, M. S.; Carpenter, M. K. *Dev. Dyn.* **2004**, *229*, 259.
- (73) Thomson, J. A.; Kalishman, J.; Golos, T. G.; Durning, M.; Harris, C. P.; Becker, R. A.; Hearn, J. P. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 7844.
- (74) Martin, M. J.; Muotri, A.; Gage, F.; Varki, A. *Nat. Med.* **2005**, *11*, 228.
- (75) Li, Y.; Powell, S.; Brunette, E.; Lebkowski, J.; Mandalam, R. *Biotechnol. Bioeng.* **2005**, *91*, 688.
- (76) Xu, R. H.; Peck, R. M.; Li, D. S.; Feng, X.; Ludwig, T.; Thomson, J. A. *Nat. Methods* **2005**, *2*, 185.
- (77) Oh, S. K.; Choo, A. B. *Cytotechnology* **2006**, *50*, 181.
- (78) Hughes, C. S.; Postovit, L. M.; Lajoie, G. A. *Proteomics* **2010**, *10*, 1886.
- (79) Uemura, M.; Refaat, M. M.; Shinoyama, M.; Hayashi, H.; Hashimoto, N.; Takahashi, J. *J. Neurosci. Res.* **2010**, *88*, 542.
- (80) Greenlee, A. R.; Kronenwetter-Koepel, T. A.; Kaiser, S. J.; Ellis, T. M.; Liu, K. *Toxicol. In Vitro* **2004**, *18*, 543.
- (81) Amit, M.; Carpenter, M. K.; Inokuma, M. S.; Chiu, C. P.; Harris, C. P.; Waknitz, M. A.; Itskovitz-Eldor, J.; Thomson, J. A. *Dev. Biol.* **2000**, *227*, 271.
- (82) Takashima, A.; Grinnell, F. *J. Invest. Dermatol.* **1984**, *83*, 352.
- (83) Yoshida, K.; Chambers, I.; Nichols, J.; Smith, A.; Saito, M.; Yasukawa, K.; Shoyab, M.; Taga, T.; Kishimoto, T. *Mech. Dev.* **1994**, *45*, 163.
- (84) Humphrey, R. K.; Beattie, G. M.; Lopez, A. D.; Bucay, N.; King, C. C.; Firpo, M. T.; Rose-John, S.; Hayek, A. *Stem Cells* **2004**, *22*, 522.
- (85) Mateizel, I.; De Temmerman, N.; Ullmann, U.; Cauffman, G.; Sermon, K.; Van de Velde, H.; De Rycke, M.; Degreef, E.; Devroey, P.; Liebaers, I.; Van Steirteghem, A. *Hum. Reprod.* **2006**, *21*, 503.
- (86) Xu, C.; Jiang, J.; Sottile, V.; McWhir, J.; Lebkowski, J.; Carpenter, M. K. *Stem Cells* **2004**, *22*, 972.
- (87) James, D.; Levine, A. J.; Besser, D.; Hemmati-Brivanlou, A. *Development* **2005**, *132*, 1273.
- (88) Wang, G.; Zhang, H.; Zhao, Y.; Li, J.; Cai, J.; Wang, P.; Meng, S.; Feng, J.; Miao, C.; Ding, M.; Li, D.; Deng, H. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 934.
- (89) Braam, S. R.; Denning, C.; Matsa, E.; Young, L. E.; Passier, R.; Mummery, C. L. *Nat. Protoc.* **2008**, *3*, 1435.

- (90) Amit, M.; Itskovitz-Eldor, J. *Methods Enzymol.* **2006**, *420*, 37.
- (91) Smith, A. G.; Heath, J. K.; Donaldson, D. D.; Wong, G. G.; Moreau, J.; Stahl, M.; Rogers, D. *Nature* **1988**, *336*, 688.
- (92) Nichols, J.; Evans, E. P.; Smith, A. G. *Development* **1990**, *110*, 1341.
- (93) Williams, R. L.; Hilton, D. J.; Pease, S.; Willson, T. A.; Stewart, C. L.; Gearing, D. P.; Wagner, E. F.; Metcalf, D.; Nicola, N. A.; Gough, N. M. *Nature* **1988**, *336*, 684.
- (94) Niwa, H.; Burdon, T.; Chambers, I.; Smith, A. *Genes Dev.* **1998**, *12*, 2048.
- (95) Darr, H.; Maysar, Y.; Benvenisty, N. *Development* **2006**, *133*, 1193.
- (96) Beattie, G. M.; Lopez, A. D.; Bucay, N.; Hinton, A.; Firpo, M. T.; King, C. C.; Hayek, A. *Stem Cells* **2005**, *23*, 489.
- (97) Miner, J. H.; Yurchenco, P. D. *Annu. Rev. Cell Dev. Biol.* **2004**, *20*, 255.
- (98) Hynes, R. O. *Cell* **2002**, *110*, 673.
- (99) Ludwig, T. E.; Levenstein, M. E.; Jones, J. M.; Berggren, W. T.; Mitchen, E. R.; Frane, J. L.; Crandall, L. J.; Daigh, C. A.; Conard, K. R.; Piekarczyk, M. S.; Llanas, R. A.; Thomson, J. A. *Nat. Biotechnol.* **2006**, *24*, 185.
- (100) Takeichi, M. *Curr. Opin. Cell Biol.* **1995**, *7*, 619.
- (101) Gumbiner, B. M. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 622.
- (102) Eastham, A. M.; Spencer, H.; Soncin, F.; Ritson, S.; Merry, C. L.; Stern, P. L.; Ward, C. M. *Cancer Res.* **2007**, *67*, 11254.
- (103) Nagaoka, M.; Si-Tayeb, K.; Akaike, T.; Duncan, S. A. *BMC Dev. Biol.* **2010**, *10*, 60.
- (104) Guilak, F.; Cohen, D. M.; Estes, B. T.; Gimble, J. M.; Liedtke, W.; Chen, C. S. *Cell Stem Cell* **2009**, *5*, 17.
- (105) Pakzad, M.; Totonchi, M.; Tasei, A.; Seifinejad, A.; Hassani, S. N.; Baharvand, H. *Stem Cell Rev.* **2010**, *6*, 96.
- (106) Dedhar, S. *Curr. Opin. Cell Biol.* **2000**, *12*, 250.
- (107) Zhang, X.; Groopman, J. E.; Wang, J. F. *J. Cell Physiol.* **2005**, *202*, 205.
- (108) De Santis, G.; Miotti, S.; Mazzi, M.; Canevari, S.; Tomassetti, A. *Oncogene* **2009**, *28*, 1206.
- (109) Watanabe, S.; Umehara, H.; Murayama, K.; Okabe, M.; Kimura, T.; Nakano, T. *Oncogene* **2006**, *25*, 2697.
- (110) Armstrong, L.; Hughes, O.; Yung, S.; Hyslop, L.; Stewart, R.; Wappler, I.; Peters, H.; Walter, T.; Stojkovic, P.; Evans, J.; Stojkovic, M.; Lako, M. *Hum. Mol. Genet.* **2006**, *15*, 1894.
- (111) Nagaoka, M.; Koshimizu, U.; Yuasa, S.; Hattori, F.; Chen, H.; Tanaka, T.; Okabe, M.; Fukuda, K.; Akaike, T. *PLoS One* **2006**, *1*, e15.
- (112) Toole, B. P. *J. Intern. Med.* **1997**, *242*, 35.
- (113) Gerecht, S.; Burdick, J. A.; Ferreira, L. S.; Townsend, S. A.; Langer, R.; Vunjak-Novakovic, G. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 11298.
- (114) Kim, S.; Ahn, S. E.; Lee, J. H.; Lim, D. S.; Kim, K. S.; Chung, H. M.; Lee, S. H. *Stem Cells* **2007**, *25*, 2601.
- (115) Levenberg, S.; Huang, N. F.; Lavik, E.; Rogers, A. B.; Itskovitz-Eldor, J.; Langer, R. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 12741.
- (116) Asikainen, A. J.; Noponen, J.; Lindqvist, C.; Pelto, M.; Kellomaki, M.; Juuti, H.; Pihlajamaki, H.; Suuronen, R. *J. R. Soc. Interface* **2006**, *3*, 629.
- (117) Mei, Y.; Saha, K.; Bogatyrev, S. R.; Yang, J.; Hook, A. L.; Kalcioğlu, Z. I.; Cho, S. W.; Mitalipova, M.; Pyzocha, N.; Rojas, F.; Van Vliet, K. J.; Davies, M. C.; Alexander, M. R.; Langer, R.; Jaenisch, R.; Anderson, D. G. *Nat. Mater.* **2010**, *9*, 768.
- (118) Higuchi, A.; Tamiya, S.; Tsubomura, T.; Katoh, A.; Cho, C. S.; Akaike, T.; Hara, M. *J. Biomater. Sci. Polym. Ed.* **2000**, *11*, 149.
- (119) Saha, K.; Keung, A. J.; Irwin, E. F.; Li, Y.; Little, L.; Schaffer, D. V.; Healy, K. E. *Biophys. J.* **2008**, *95*, 4426.
- (120) Kehoe, D. E.; Jing, D.; Lock, L. T.; Tzanakakis, E. S. *Tissue Eng. Part A* **2010**, *16*, 405.
- (121) Lee, K. Y.; Mooney, D. J. *Chem. Rev.* **2001**, *101*, 1869.
- (122) Abbott, A. *Nature* **2003**, *424*, 870.
- (123) Cheng, L.; Hammond, H.; Ye, Z.; Zhan, X.; Dravid, G. *Stem Cells* **2003**, *21*, 131.
- (124) Richards, M.; Tan, S.; Fong, C. Y.; Biswas, A.; Chan, W. K.; Bongso, A. *Stem Cells* **2003**, *21*, 546.
- (125) Amit, M.; Margulets, V.; Segev, H.; Shariki, K.; Laevsky, I.; Coleman, R.; Itskovitz-Eldor, J. *Biol. Reprod.* **2003**, *68*, 2150.
- (126) Cetinkaya, G.; Turkoglu, H.; Arat, S.; Odaman, H.; Onur, M. A.; Gumusderelioglu, M.; Tumer, A. *J. Biomed. Mater. Res. A* **2007**, *81*, 911.
- (127) Klimanskaya, I.; Chung, Y.; Meisner, L.; Johnson, J.; West, M. D.; Lanza, R. *Lancet* **2005**, *365*, 1636.
- (128) Takenaga, M.; Fukumoto, M.; Hori, Y. *J. Cell Sci.* **2007**, *120*, 2078.



Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

Analysis of cell characterization using cell surface markers in the dermis

Yuichi Hasebe^{a,*}, Seiji Hasegawa^{a,d}, Noriko Hashimoto^b, Masashi Toyoda^c,
Kenji Matsumoto^b, Akihiro Umezawa^c, Akiko Yagami^d, Kayoko Matsunaga^d,
Hiroshi Mizutani^a, Satoru Nakata^a, Hirohiko Akamatsu^e

^a Research Laboratories, Nippon Menard Cosmetic Co., Ltd., 2-7 Torimicho, Nishi-Ku, Nagoya, Aichi, 451-0071, Japan

^b Department of Allergy and Immunology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo, 157-8535, Japan

^c Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo, 157-8535, Japan

^d Department of Dermatology, Fujita Health University School of Medicine, 1-98 Kutsukakecho, Toyoake, Aichi 470-1192, Japan

^e Department of Applied Cell and Regenerative Medicine, Fujita Health University Institute for Comprehensive Medical Science, 1-98 Kutsukakecho, Toyoake, Aichi 470-1192, Japan

ARTICLE INFO

Article history:

Received 13 July 2010

Received in revised form 13 January 2011

Accepted 27 January 2011

Keywords:

Stem cell
Dermis
Differentiation
CD54
CD271

ABSTRACT

Background: In recent years, it has been reported that stem cells exist in the mesenchymal tissues of the bone marrow and adipose. These stem cells are thought to express specific cell surface markers such as CD44, CD54, CD105, CD90, and CD271 and have been confirmed to be pluripotent. Furthermore, although it has been reported that stem cells are also present in the dermis, their cell surface markers and characteristics are not fully understood.

Objective: To confirm the presence of stem cells in the dermis and their ability, employing the mesenchymal stem cell markers which have previously been reported as an indication.

Methods: We analyzed the percentages of CD44 (+), CD54 (+), CD90 (+), CD105 (+), and CD271 (+) cells in the dermis of neonatal mice (HR-1 mouse) by performing immunostaining and FACS. Secondly, we isolated each type of marker-positive and -negative cells from dermal tissues and evaluated their proliferation potential and their ability to differentiate into adipocytes, osteoblasts, and chondrocytes.

Results: According to the immunostaining and FACS results, we confirmed that stem cells that express CD44, CD54, CD90, CD105, and CD271 are present in the dermal tissues of neonatal mice. In addition, when we measured the proliferation and differentiation potentials of each type of marker-positive cells, it was revealed that cells expressing CD54 or CD271 have a high proliferation potential and are able to differentiate into adipocytes, osteoblasts, and chondrocytes.

Conclusions: These results indicated that dermal tissues contain stem cells that express CD44, CD54, CD90, CD105, and CD271 which are stem cell markers. More precisely, it was suggested that both CD54 (+) and CD271 (+) stem cells have high proliferation and differentiation potentials.

© 2011 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Somatic stem cells, which have high proliferation and differentiation potentials, are thought to play an important role in maintaining the homeostasis of organisms [1]. Previous studies have revealed that somatic stem cells can be found in various tissues, such as the bone marrow [2–4], skin epidermis [5,6], skeletal muscle [7,8], adipose [9], umbilical cord [10,11] and placenta [12].

The skin is the largest tissue in our body and is constantly exposed to the external environment. Thus, the skin has a higher regeneration capacity than other body tissues because it is

vulnerable to external stimuli such as UV irradiation and wounding, and it has long been believed that stem cells exist in the skin [13]. The skin is formed of the epidermis, dermis, and subcutaneous adipose tissues, each of which functions independently. Previous reports have demonstrated that stem cells are present in the skin and that these skin stem cells express specific surface marker proteins. For example, keratin19, integrin- α 6, and p63 were reported as cell surface markers of skin stem cells and are considered to be involved in maintaining the homeostasis of epidermal tissues [14]. The CD13 (+), 44 (+), 90 (+), 105 (+), and 271 (+) cells were reported as stem cells existing in subcutaneous adipose tissues [9,15], and other studies reported stem cells exist in hair follicles, in which CD34, keratin15, keratin19, CD200, Fzd, and integrin- β 1 were identified as cell surface markers of hair follicle stem cells [16–18]. These stem cells are reported to be pluripotent and are able to differentiate into cells of other tissues

* Corresponding author. Tel.: +81 52 531 6269; fax: +81 52 531 6277.
E-mail address: hasebe.yuichi@menard.co.jp (Y. Hasebe).

Table 1
Previously reported cell surface markers and the tissues in which these markers are expressed.

Cell surface marker	Tissue	Reference	Year	Reference no.
CD44 (+)	Adipose	Zuk	2002	[9]
	Bone marrow	Colter	2001	[4]
	Dermis	Chunmeng	2004	[34]
	Dermis	Lorenz	2008	[33]
CD54 (+)	Adipose	Zuk	2002	[9]
	Adipose	Strem	2005	[40]
	Dermis	Shi	2003	[41]
	Dermis	Chunmeng	2004	[34]
CD90 (+)	Adipose	Zuk	2002	[9]
	Bone marrow	Pittenger	1999	[3]
	Dermis	Chunmeng	2004	[34]
	Dermis	Lorenz	2008	[33]
CD105 (+)	Adipose	Zuk	2002	[9]
	Bone marrow	Dominici	2006	[35]
	Dermis	Chen	2007	[23]
	Dermis	Lorenz	2008	[33]
CD271 (+)	Adipose	Yamamoto	2007	[15]
	Bone marrow	Buhring	2007	[42]
	Bone marrow	Buttula	2009	[43]
	Dermis	Toma	2005	[21]

such as nerves, smooth muscles, and sebaceous gland cells as well as skin tissue cells [19].

There have only been a few studies on dermal stem cells, some of which are outlined below. Recently, Miller et al. found a type of dermal stem cell called SKP cells (skin-derived precursor cell) that showed pluripotency [20–22], and Chen et al. demonstrated the existence of multipotent stem cells in human dermal tissues, which were able to differentiate into adipocytes, osteoblasts, chondrocytes, and smooth muscle cells [23]. Furthermore, it was reported that some dermal stem cells were able to differentiate into nerves [22], liver cells [24], and pancreatic cells [25], without forming germ layers.

In recent studies, it has been reported that dermal stem cells express several cell surface markers (Table 1). However, the abilities of stem cells isolated by each cell surface marker are yet to be elucidated. Abilities of each stem cell may be clarified by isolating cells using cell surface markers. And the relationship between cell surface markers and properties of cells has not been clarified. In order to use dermal stem cells in regenerative medicine, it will be important for us to fully understand abilities and characteristics of dermal tissues. In this study, we sorted cells in dermal tissues according to cell surface markers and compared their proliferation and differentiation potentials.

2. Materials and methods

2.1. Animals

In our study, we used the skin of HR-1 mice which is used in various studies such as a study on effects of UV exposure on the skin [26], a skin stimulation study [27], and percutaneous absorption studies [28,29] since it is similar to human skin and their physiological function and pathological analysis have been well-conducted. The details are as follows. Male HR-1 hairless mice were purchased from Japan SLC (Shizuoka, Japan). Each experiment was performed with neonatal mice. The animals were cared for according to the International Guiding Principles for Biomedical Research Involving Animals published by the Council for the International Organization of Medical Science. The present experimental protocol was planned according to these guidelines and approved by the Nippon Menard Research Laboratories Subcommittee on Research Animal Care.

2.2. Isolation and culture of stem cells

Dermal stem cells were isolated according to the method used in the study by Toma et al. [20], in which they used neonatal mice. More specifically, skin tissues were collected from the backs of neonatal HR-1 mice and were reacted overnight in 200 U/mL of Dispase II (Godo Shusei, Tokyo, Japan) at 4 °C. On the following day, epithelial and adipose tissues were peeled off from the skin tissues and shredded. The shredded dermal tissues were reacted in 0.2% collagenase (Sigma, MO, USA) at 37 °C for 1 h, before being filtered through a 100 µm mesh. Then, the collagenase was removed by diluting the mixture with PBS (–) and centrifuging it twice for 5 min at 1500 rpm. The resultant pellet was suspended in high yield lysis (Invitrogen, NY, USA) and incubated to remove any contaminating red blood cells. The cells were then washed and centrifuged twice with PBS (–), before being dissolved in phenol red-free DMEM/F-12 (Invitrogen) containing the following primary antibodies: CD44 (Santa Cruz Biotechnology, CA, USA), CD54 (eBioscience, CA, USA), CD90 (Biolegend), CD105 (Beckman Coulter, CA, USA), and CD271 (Millipore, MA, USA) and then reacted on ice for 30 min. Then, the cells were dissolved in an Alexa Fluor 488-labeled secondary antibody (Invitrogen) and reacted on ice for 30 min. After being washed with PBS (–), the measurement of cell surface markers and the isolation of the cells were performed using FACS (FACS Aria, BD Biosciences, CA, USA). The marker-positive cells and marker-negative cells isolated by FACS were suspended in cell growth medium and cultured at a concentration of 2.0×10^4 cells/cm², before being seeded on a 24-well plate. The growth medium used contained DMEM (Invitrogen): α MEM (Invitrogen) in a 1:1 ratio supplemented with 2% FBS (Sigma), 15 mM HEPES (Sigma), 10 ng/mL basic FGF (PeproTech, NJ, USA), 100 \times Insulin–Transferrin–Selenium–A Supplement (Invitrogen), and 1000 \times ESGRO (Millipore). After 24 h of culture, the nonadhesive cells were removed, and the adherent cells were cultured in culture medium, which was replaced every four days. In the proliferation analysis and differentiation induction analysis, we compared the capabilities of the cells isolated by FACS and non-isolated cells.

2.3. Cell proliferation analysis

Cells attached to plastic dishes were harvested and seeded into 96-well plates at a cell density of 2×10^3 cells/well. Cell proliferation rates were measured using the modified MTT assay and Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

2.4. Immunohistochemistry

Skin tissues were excised from the backs of neonatal mice. The obtained tissues were fixed with 4% paraformaldehyde according to the usual method. These sections were then processed for H.E. staining and immunostaining using the following antibodies: anti-CD44 (Santa Cruz Biotechnology), anti-CD54 (eBioscience), anti-CD90 (Biolegend, CA, USA), anti-CD105 (Beckman Coulter), and anti-CD271 (Millipore). For the secondary antibodies, anti-rat IgG antibody labeled with Alexa Fluor 488 (Invitrogen) and anti-rabbit IgG antibody labeled with Alexa Fluor 594 (Invitrogen) were used. DAPI (Vectashield, CA, USA) was used for nuclear staining. A fluorescence microscope (Power BX-51, Olympus, Tokyo, Japan) was used for observation.

2.5. Cell differentiation

For the differentiation analysis, the cells were first grown to 100% confluence and then cultured in the following differentia-

tion media: DMEM supplemented with 10% FBS, 1 μ M dexamethasone, 500 μ M 3-isobutyl-1-methylxanthine, 200 μ M indomethacin, 10 μ g/mL insulin, and 33 μ M biotin (Sigma) for adipogenesis [30]; DMEM supplemented with 10% FBS, 10 mM μ -glycerophosphate, 50 μ M ascorbic acid 2-phosphate (Sigma) for osteogenesis [30]; and DMEM supplemented with 3% FBS, 0.5 \times ITS, 0.1 μ M dexamethasone, 50 μ M ascorbic acid 2-phosphate (Sigma), 5 ng/mL TGF- β 3, and 5 ng/mL BMP-2 (Peprotec) for chondrogenesis [30]. For chondrogenic differentiation, a cell pellet consisting of 2.5×10^5 cells was incubated at the bottom of a 15 mL conical tube containing 0.5 mL of chondrogenic medium. During the differentiation period, the medium was replaced every 2–3 days.

Adipogenesis was assayed by measuring the amount of lipid accumulation in the differentiated cells with Oil Red O staining. After staining, the Oil Red O was extracted with 100% isopropanol, and the optical density (OD) of the solution was measured at 520 nm.

Osteogenesis was assayed by matrix mineralization with Alizarin Red S staining, and the total calcium content was determined by a colorimetric assay using the Calcium E-Test (Wako, Osaka, Japan).

Chondrogenesis was assayed by measuring the sulfated glycosaminoglycan (GAG) content [31], which was normalized to the DNA content [32].

3. Results

Mesenchymal stem cells are isolated from bone marrow, adipose tissue, hair follicles, and the dermis and are known to express specific markers. Typical markers for the skin and mesenchymal stem cells are listed in Table 1. In this study, we used CD44, CD54, CD90, CD105, and CD271, which are considered to be typical of dermal stem cells, as markers for analysis. We isolated stem cells from dermal tissues of neonatal mice according to the study by Toma et al. [20].

3.1. Expression of stem cell surface markers in the dermis

Skin tissues were excised from the backs of neonatal mice. These sections were then processed for immunostaining. The cell nuclei were stained with DAPI, and the resultant tissue fluorescent images were analyzed (Fig. 1). As a result, all of the cell surface markers were found to be expressed in the dermis, and the expression of CD54 and CD271 was especially intensive. Secondly, we performed FACS to measure the percentage of cells that showed positivity for each surface cell marker. The results were as follows: CD44 (+): 2.5%, CD54 (+): 39.2%, CD90 (+): 2.7%, CD105 (+): 28.2%, and CD271 (+): 39.4%. Accordingly, it was revealed that high percentages of CD54 (+), CD105 (+), and CD271 (+) cells were present in dermal tissues.

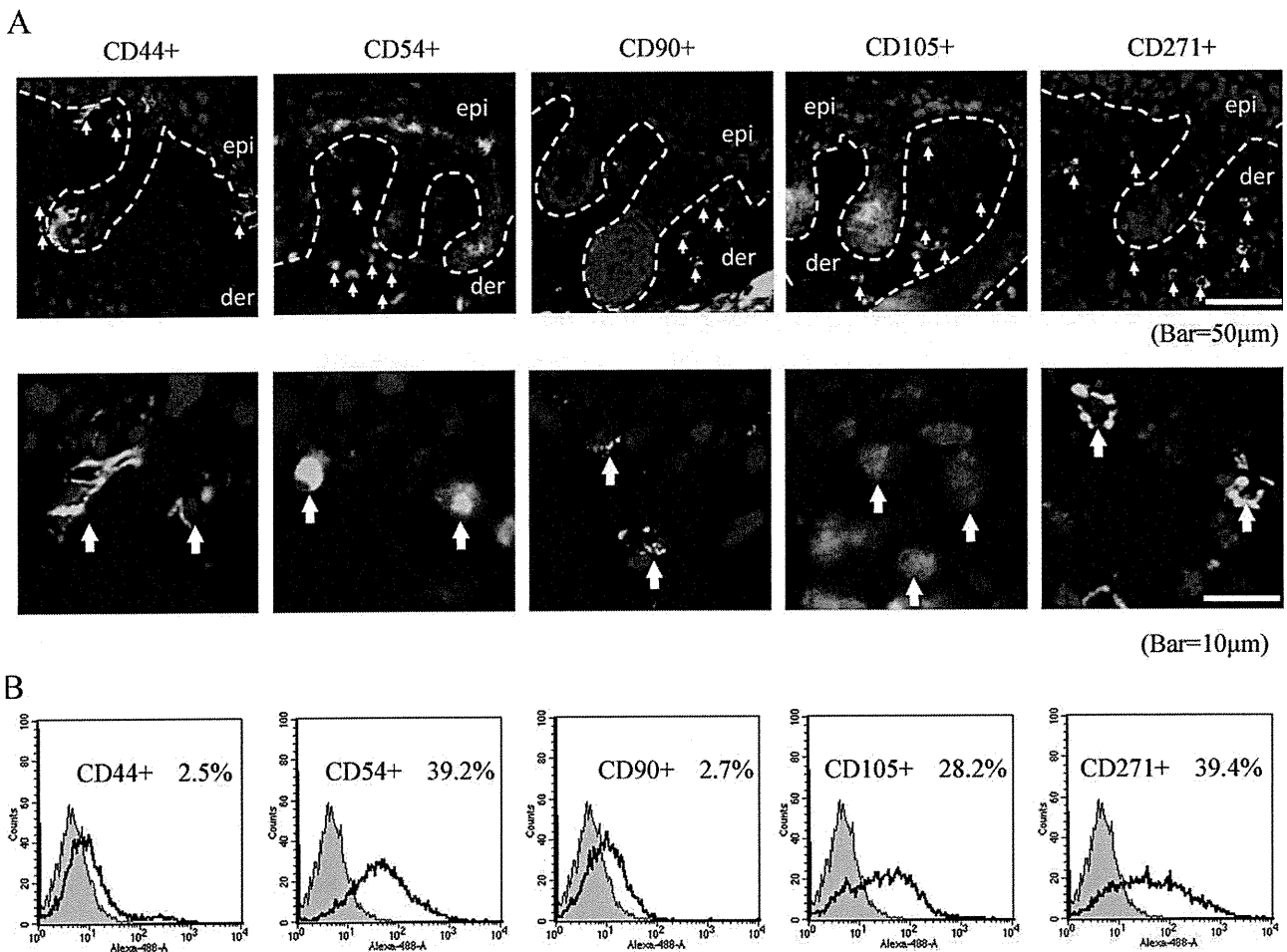


Fig. 1. (A) Neonatal HR-1 mouse dermal tissues were sectioned and immunostained with several antibodies (CD44, CD54, CD90, CD105, and CD271). Under panels are high magnification image. Dotted lines indicate the basement membrane. epi: epidermal layer, der: dermal layer. Arrows indicate the cells expressing cell surface markers in dermal tissues. (B) Analysis of cell surface marker expression by flow cytometry. Dermal cell suspensions were stained with anti mouse monoclonal several CD antibodies (solid line) as indicated in the histograms. The gray line represents the respective IgG isotype control.

3.2. Isolation of cells and a comparison of their proliferation potentials

Each type of marker-positive and -negative cells was isolated from the dermis of neonatal HR-1 mice for analysis by FACS. These cells showed a fibroblast-like morphology after 24 h of culture (Fig. 2A). The proliferation potentials of the sorted cells by each cell surface marker were compared to that of non-sorted cells used as control. The results revealed that the proliferation potential of CD54 (+) and CD271 (+) cells was significantly higher than non-sorted cells while those of CD44 (+), CD54 (-), CD90 (+) and CD105 (-) cells were significantly lower than control (Fig. 2B). As a result, it was suggested that CD54 (+) and CD271 (+) cells have a high proliferation potential in dermal tissues.

3.3. Differentiation into adipocytes

It was confirmed that neonatal dermal cells are able to differentiate into adipocytes, osteoblasts, and chondrocytes.

Therefore, we examined the potential of sorted cells to differentiate into these three cell types. First, we investigated which cells have the potential to differentiate into adipocytes. After two weeks of inducing adipocyte differentiation, fat droplets were detected in the cytoplasm of all of cells, indicating that adipocyte differentiation had been achieved (Fig. 3A). When the potentials of each cell type and the control to differentiate into adipocytes were compared, it was found that CD44 (+), CD54 (+), and CD271 (+) cells all have a higher differentiation potential into adipocytes compared to other marker-positive and -negative cells (Fig. 3B).

3.4. Differentiation into osteoblasts

We then investigated which cells have a differentiation potential into osteoblasts. After three weeks of inducing their differentiation into osteoblasts, calcium depositions were observed in all cells (Fig. 4A). When the differentiation

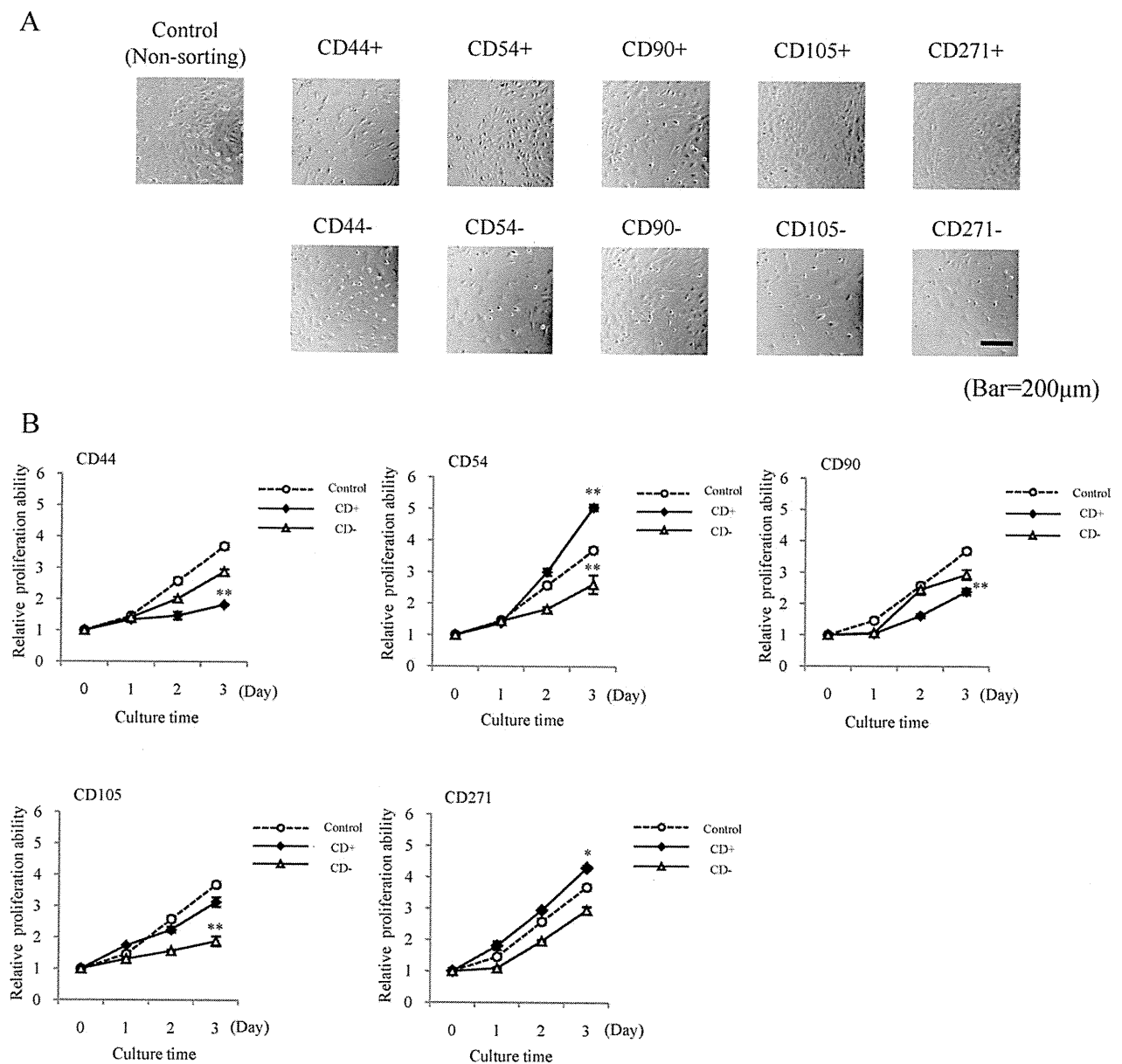


Fig. 2. The isolated cells attached to plastic dishes were harvested and seeded into 96-well plates. (A) Pictures showing the morphology of cells sorted according to their cell surface marker expression. The isolated cells were seeded into 96-well plates at a cell density of 2×10^3 cells/well and cultured for 3 days. (B) The proliferation potential of marker-positive and -negative cells type was compared to that of the non-sorted cells used as the control (dotted line). Data are presented as the mean \pm SD ($n = 4$, * $P < 0.05$, ** $P < 0.01$ compared to the control).

potentials into osteoblasts were compared, the calcium concentrations of the CD54 (+) and CD271 (+) cells were found to be higher than those of non-sorted cells, CD54 (-) and CD27 (-) cells, indicating that they have a high potential to differentiate into osteoblasts (Fig. 4B).

3.5. Differentiation into chondrocytes

We then investigated which cells have the potential to differentiate into chondrocytes. After two weeks of inducing the cells to differentiate into chondrocytes, the formation of chondro-

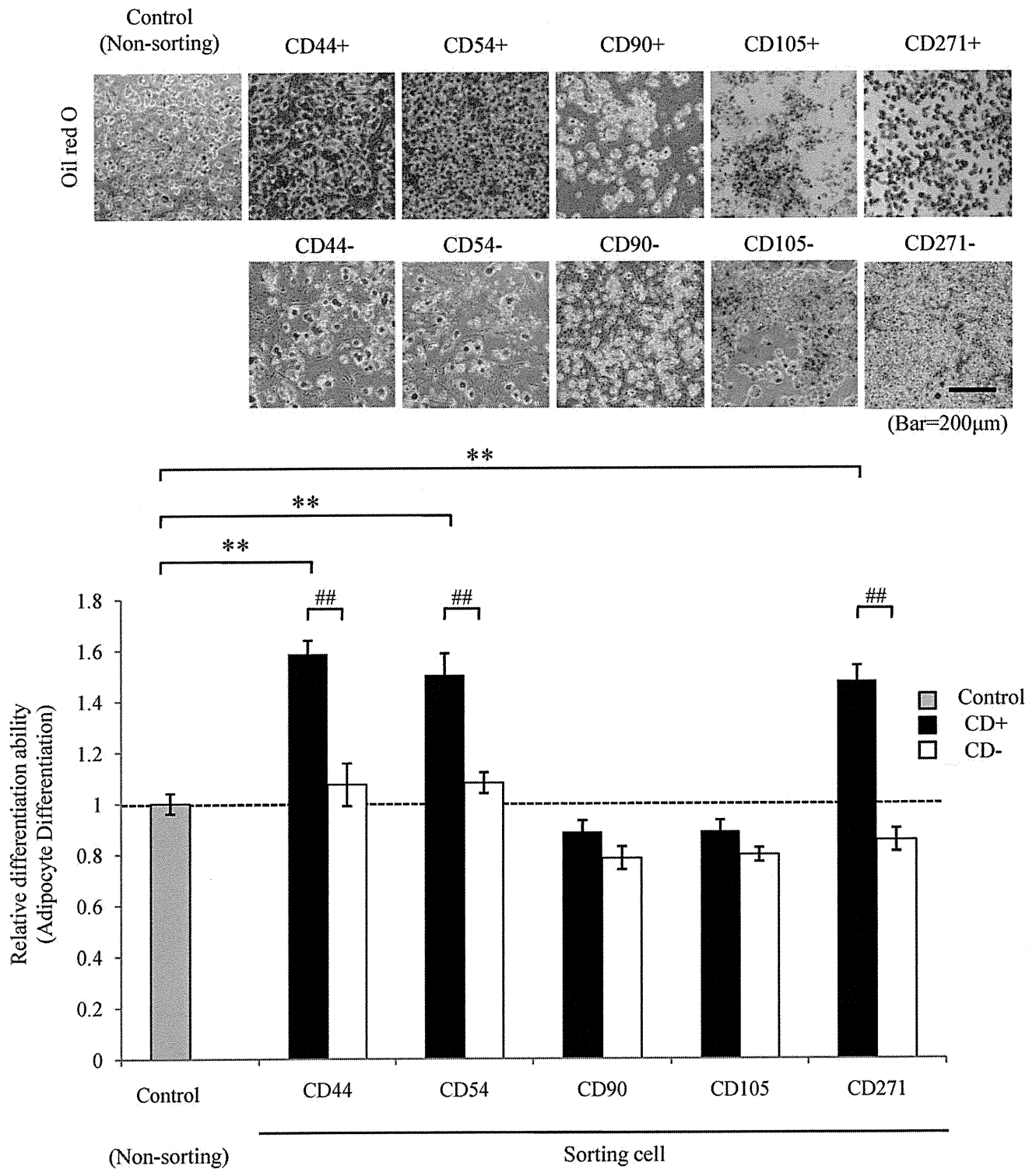


Fig. 3. Adipogenesis was assayed by measuring the amount of lipid accumulation in the differentiated cells with Oil Red O staining. (A) Oil Red O staining of the cells following the induction of adipogenic differentiation. (B) Oil Red O was extracted using 100% 2-propanol, and the optical density (OD) of the solution was measured at 520 nm. Gray bars indicate control, black bars indicate CD (+) cells, and outlined bars indicate CD (-) cells. Data are presented as the mean \pm SD ($n = 4$, ** $P < 0.01$ compared to the control, ## $P < 0.01$ compared to the cell surface marker-negative cells).

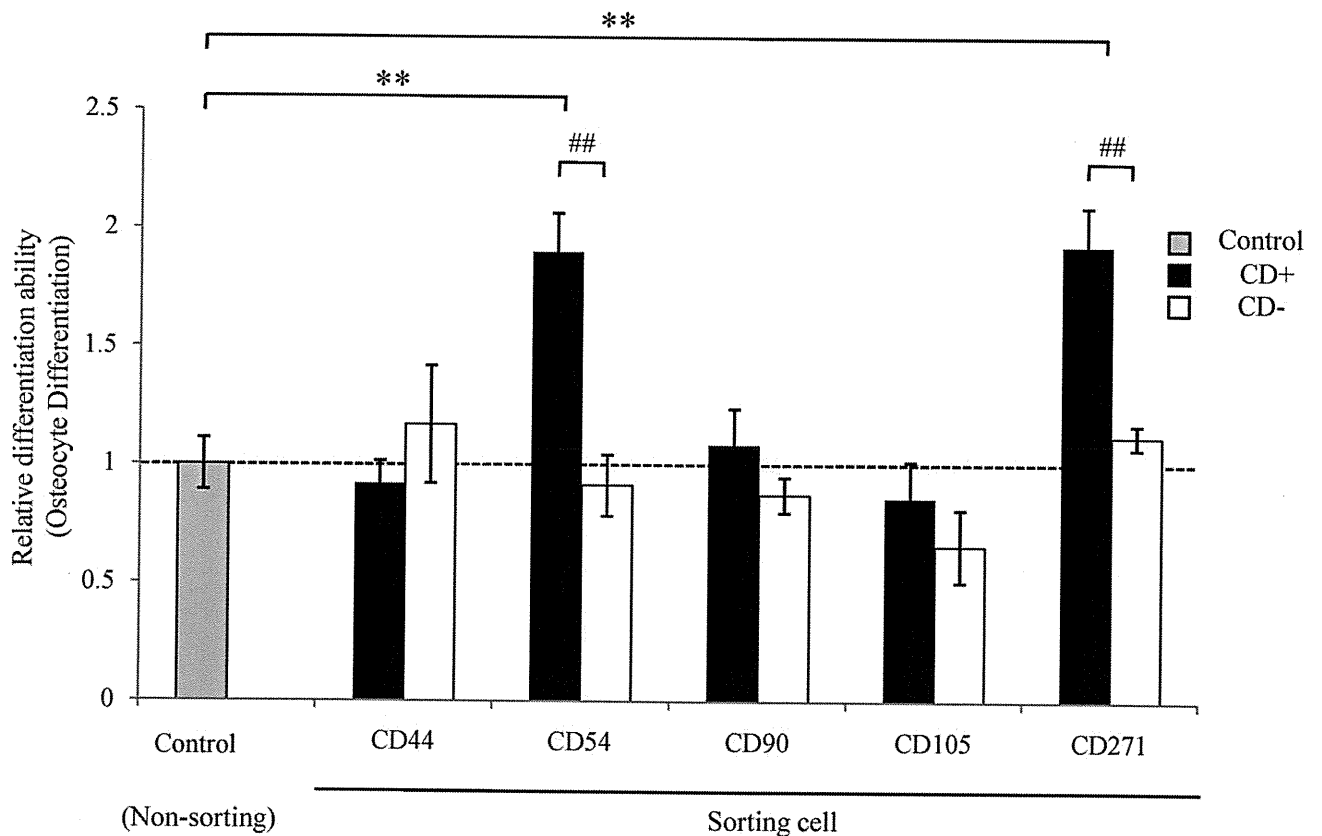
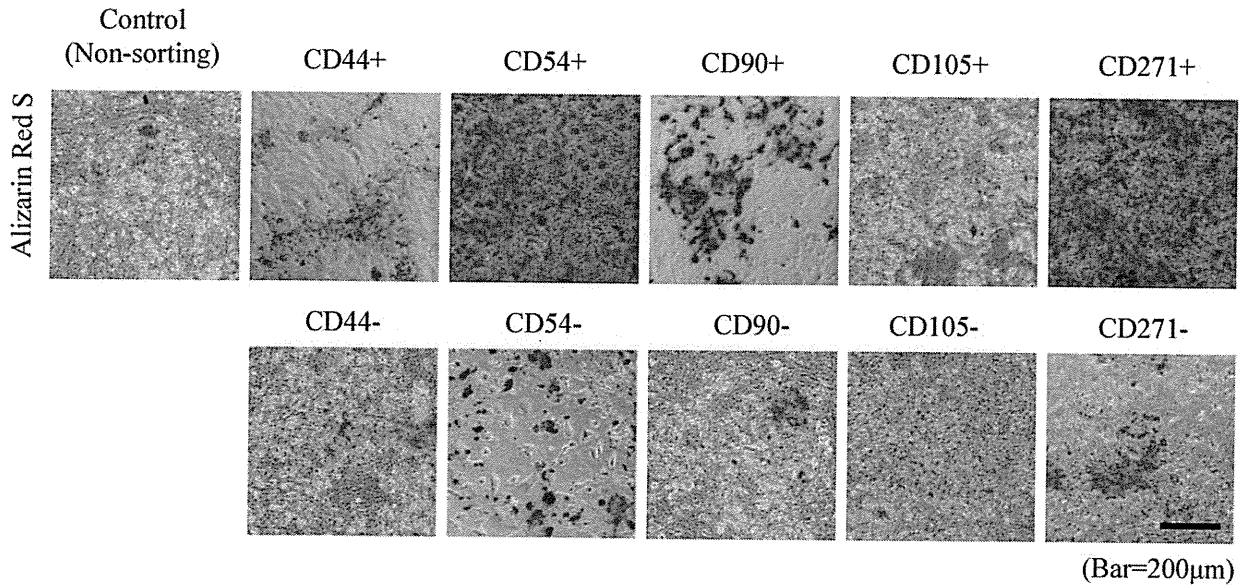


Fig. 4. Osteogenesis was assayed by matrix mineralization with Alizarin Red S staining, and the total calcium content was determined by a colorimetric assay. (A) Alizarin Red staining of the cells following the induction of osteogenic differentiation. (B) Quantification of calcium deposition in the cells following the induction of osteogenic differentiation. Data are presented as the mean \pm SD ($n = 4$, $**P < 0.01$ compared to the control, $##P < 0.01$ compared to the cell surface marker-negative cells).

cyte-like cell masses was observed in all cell types (Fig. 5A). Examination of the glycosaminoglycan concentration of the cells indicated that CD54 (+) and CD271 (+) cells have a significantly higher differentiation potential into chondrocytes compared to non-sorted cells, CD54 (-), CD271 (-) and other cells (Fig. 5B). The percentage expression of each cell surface marker in dermal tissues and the proliferation and differentiation potentials of the cells expressing them are summarized (Table 2). For evaluation of proliferation potential, cell populations with a significantly higher

proliferation potential than the non-sorted cells, a significantly lower proliferation potential than the non-sorted cells, and the remaining cells were classified as High, Low, and Middle, respectively. Differentiation potentials were indicated as values relative to that of the non-sorted cells and marker-negative cells. This table shows that CD54 (+) and CD271 (+) cells have a high proliferation potential and pluripotency. Furthermore, we have confirmed the presence of stem cells with various properties in dermal tissues.