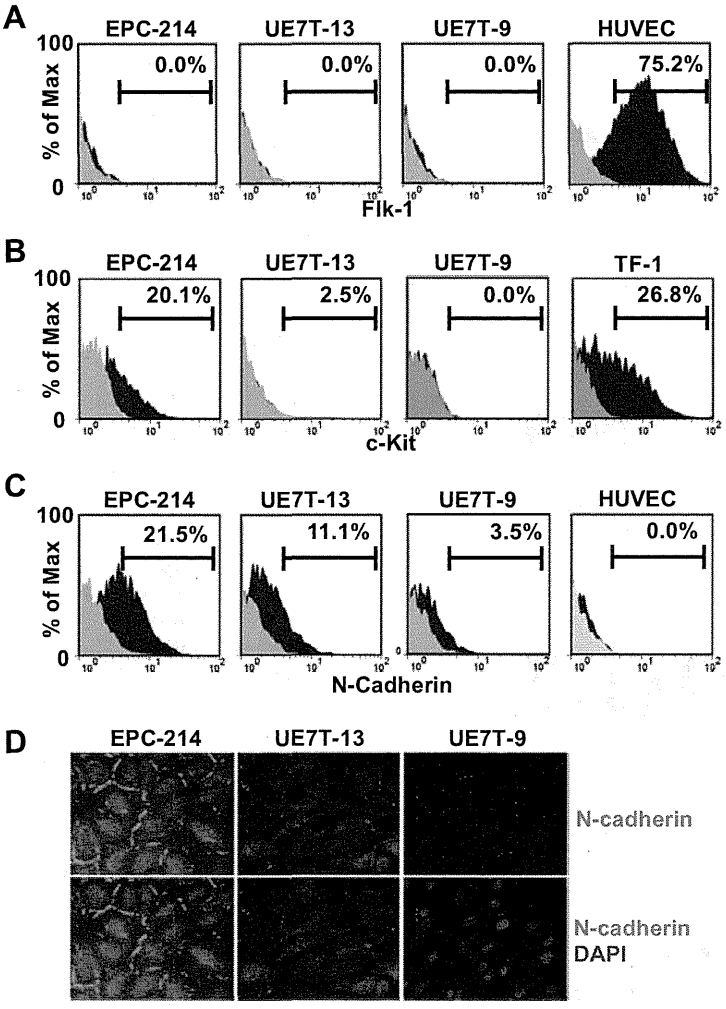


**Fig. 2.** Representative FACS plots of cell surface protein expression in human MSC cell lines. The live cells were immunostained with (A) Flk-1, (B) c-Kit, or (C) N-cadherin antibodies. Propidium iodide-positive cells were excluded from the analysis. (D) Localization of N-cadherin in human MSCs. The indicated human MSC lines were immunostained with N-cadherin antibody (green) and DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To characterize the cardiomyogenic N-cadherin-positive population, we analyzed the gene expression profiles of the MACS-sorted fractions with microarray. Gene ontology analysis of 3056 genes (among 44,000 genes) that exhibited more than a 1.5-fold difference identified molecular functions associated with zinc ion binding, transition metal ion binding, and nucleic acid binding (Fig. S4A) and biological functions involved in DNA binding, gene expression, transcription, and metabolic processes of nucleic acid (Fig. S4B). These results suggested that N-cadherin-positive cells showed higher expression of specific DNA-binding proteins and elevated metabolic activity. On the other hand, the N-cadherin-negative population showed higher expression of genes involved in the MHC class I protein complex, vacuole organization, and GTPase activity (Fig. S4C and S4D).

When the expression of various lineage marker genes was compared, the N-cadherin-positive fraction showed up-regulated expression of genes involved in the differentiation of cardiomyocytes and skeletal myocytes, such as *Nkx2.5*, *Hand1*, *Tnni3* (*cTnI*), and *Myog* (Fig. 4C). In contrast, ectodermal and endodermal lineage markers were the same among the MACS-sorted fractions, with the exception of *Pax4*, a transcription factor involved in pancreatic development. Although MSCs efficiently differentiate into osteoblasts, chondrocytes, and adipocytes, these specific markers did not show a large difference. The expression of MSC-specific cell surface markers was not increased in the N-cadherin-positive fraction.

Quantitative PCR analysis revealed significant up-regulation of a cardiomyogenic precursor-specific gene, *Nkx2.5*, in the N-cadherin-positive fraction, for more than 200-times higher than that in the N-cadherin-negative fraction. Two other transcription factors, *Hand1* and *Gata4*, but not Tbx5, also showed significantly elevated expression in the N-cadherin-positive fraction (Fig. 4D). Interestingly, the expression of *Myog*, a transcription factor involved in skeletal muscle development, was also elevated in the N-cadherin-positive fraction. Although terminal markers for cardiomyocytes, such as *Anp* and *cTnI*, showed higher expression in the N-cadherin-positive fraction (Fig. 4E), the expression levels of these terminal markers were very low, suggesting that N-cadherin-positive cells may be ready for differentiation, but not terminally differentiated into cardiomyocytes.

Interestingly, the expression of some pluripotency-specific genes such as *Oct4* (*Pou5f1*), *Sall4*, and *Nanog* was significantly up-regulated in the N-cadherin-positive population (Fig. 4F and G). However, these expression in human MSCs was not as high as that in human ES cells (Fig. 4F), suggesting that these genes up-regulated in N-cadherin-positive MSCs may not exhibit pluripotency as observed in ES/iPS cells.

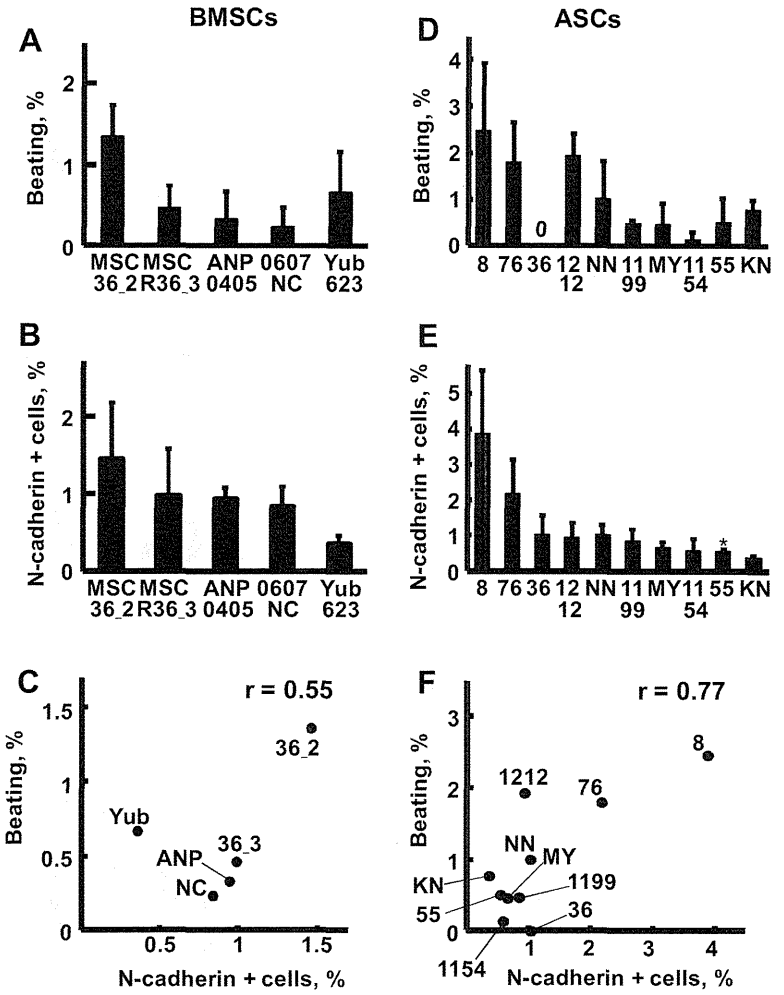


**Fig. 3.** Differentiation efficiency of primary human MSCs into cardiomyocytes and cell surface expression of N-cadherin. In this experiment, primary human MSCs derived from (A–C) the bone marrow (BMSCs) or (D–F) adipose-derived tissue (ASCs) were used. (A, D) Differentiation efficiency of primary human MSCs into cardiomyocytes. Autonomously beating cardiomyocytes differentiated from GFP-labeled human (A) BMSCs or (D) ASCs were counted using a microscope. (B, E) Flow cytometric analysis of cell surface expression of N-cadherin in primary human (B) BMSCs or (E) ASCs. $^*P < 0.05$. (C, F) Correlation between the differentiation efficiency into beating cardiomyocytes and the cell surface N-cadherin expression of primary human (C) BMSCs or (F) ASCs. The correlation coefficient ($r$) is shown on the graph.

## 4. Discussion

In this study, we have identified N-cadherin as a reliable cell surface marker for human MSCs with higher differentiation ability toward cardiomyocytes. N-cadherin is continuously expressed from cardiomyogenic progenitor cells to mature cardiomyocytes in the adult heart. N-cadherin maintains the functional gap junction complex at the plasma membrane in the adult heart, and conditional knockout of N-cadherin in mice resulted in arrhythmia in adult hearts with significant decreases in Cn43 and Cn40 [17].

We have previously shown that the cardiomyogenic progenitor cells differentiated from mouse ES cells expressed high levels of N-cadherin on the cell surface membrane, and an antibody against N-cadherin could be used to concentrate the progenitor cells from a heterogeneous cell population differentiated from mouse ES cells [18]. Although the possible differentiation pathway of cardiomyocytes from pluripotent ES cells and multipotent MSCs may not be the same, N-cadherin could be a common progenitor marker of the cardiomyogenic cells derived from these stem cells.

In addition to cardiomyogenic genes, we observed increased expression of pluripotency-specific transcription factors of ES cells, such as *Oct4*, *Sall4*, and *Nanog*, in the N-cadherin-positive fraction. Recently, *Oct4* has been suggested to be the gatekeeper into and out of the reprogramming expressway [19]. Therefore, the elevated expression of *Oct4* and related transcription factors could positively modulate the differentiation ability of MSCs. For example, overexpression of the *Oct4* gene enhanced the differentiation ability of MSCs [14], and knockdown of *Oct4* caused loss of multiple differentiation potential [15]. Nanog was also shown to possess similar activity in BMSCs [16]. Therefore, N-cadherin-positive cells with up-regulated expression of *Oct4* and other transcription factors responsible for cardiomyogenesis may increase the differentiation ability of MSCs into cardiomyocytes.

N-Cadherin is localized in the cell–cell contacts of cardiomyocytes and plays essential roles for formation of the cardiac intercalated disk structure that electromechanically couples adjacent cardiac myocytes. Addition of antibodies against N-cadherin to the cultured cardiomyocytes [20], mesodermal explants [21] or injected into embryos [22] caused a reduction in the number of myofibrils and destroy stress fibers [23]. In primary cardiomyocytes dissociated from adult rat heart, N-cadherin diffusely distributed around the cell periphery begins to co-localize with desmocollin, plakoglobin, and plakophilin-2 at the cell contact sites. The newly generated adhesive contacts sequentially recruit desmoplakin, intermediate filaments, connexin-43, and ankyrin-G. Subsequently, the voltage-gated sodium channel is incorporated into mature intercalated disks. This assembly process requires the clustering of transmembrane adhesive contacts with N-cadherin

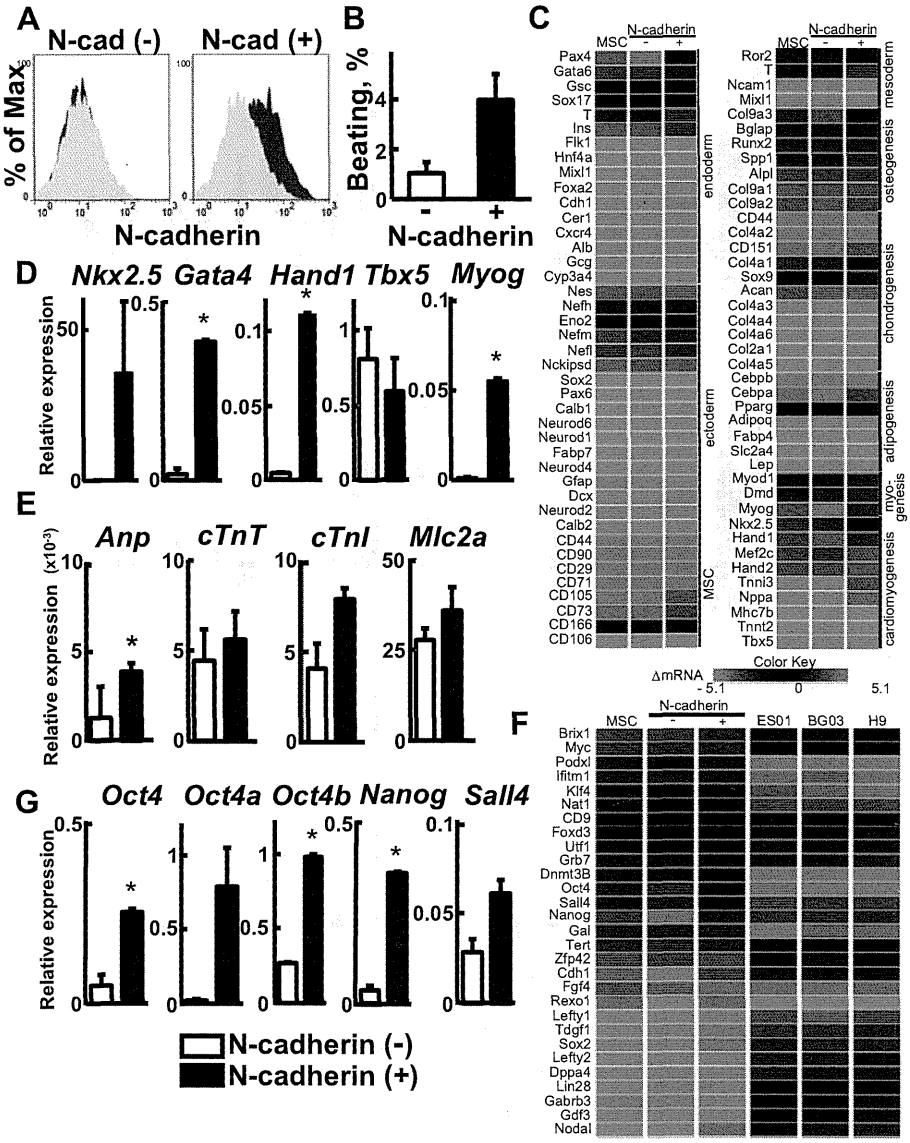


**Fig. 4.** Separation of N-cadherin-positive cells from the primary MSCs derived from adipose tissue (1212) with anti-N-cadherin antibody-conjugated magnetic beads. (A) The FACS histograms represent cell surface N-cadherin expression in the N-cadherin-negative fraction (left) and N-cadherin-positive fraction (right). (B) Differentiation efficiency of the purified primary ASCs into beating cardiomyocytes. Bar graphs represent the mean value of differentiation efficiency obtained from two independent experiments. (C) Heat map profile of lineage-specific differentiation marker expression in ASCs. The N-cadherin-positive fraction showed elevated expression of specific genes involved in cardiomyogenesis. (D, E) qPCR analysis of cardiomyogenic progenitor-specific transcription factors (D) and terminal differentiation markers for heart development (E). (F) Heat map profile of pluripotency-specific marker expression in human ASCs and human ES cells (ES01, BG03, and H9). (G) qPCR analysis of the expression of pluripotency-specific transcription factors in MACS-sorted fractions. $^{*}P < 0.05$. (For interpretation of the references to colour in (C) and (F), the reader is referred to the web version of this article.)

[24]. Therefore, MSCs with higher expression of N-cadherin may have preferable potential for the differentiation into cardiomyocytes. N-cadherin is expressed in pericytes and is involved in the interaction between pericytes and endothelial cells during vessel formation *in vivo* [25,26]. Pericytes in MSCs could be one of the possible cell sources that show higher differentiation ability toward cardiomyocytes.

## Disclosure statement

No competing financial interests exist.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.081.

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Regular Article

# Differentiation of Human Induced Pluripotent Stem Cells into Functional Enterocyte-like Cells Using a Simple Method

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

**Summary:** Human induced pluripotent stem (iPS) cells were differentiated into the endoderm using activin A and were then treated with fibroblast growth factor 2 (FGF2) for differentiation into intestinal stem cell-like cells. These immature cells were then differentiated into enterocyte-like cells using epidermal growth factor (EGF) in 2% fetal bovine serum (FBS). At the early stage of differentiation, mRNA expression of caudal type homeobox 2 (CDX2), a major transcription factor related to intestinal development and differentiation, and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), an intestinal stem cell marker, was markedly increased by treatment with FGF2. When cells were cultured in medium containing EGF and a low concentration of FBS, mRNAs of specific markers of intestinal epithelial cells, including sucrase–isomaltase, the intestinal oligopeptide transporter SLC15A1/peptide transporter 1 (PEPT1), and the major metabolizing enzyme CYP3A4, were expressed. In addition, sucrase–isomaltase protein expression and uptake of β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA), a fluorescence-labeled substrate of the oligopeptide transporter, were detected. These results demonstrate a simple and direct method for differentiating human iPS cells into functional enterocyte-like cells.

**Keywords: human iPS cells; intestinal differentiation; enterocytes; pharmacokinetics; drug metabolizing enzymes; drug transporters**

## Introduction

The small intestine and liver play important roles in all aspects of pharmacokinetics, including drug disposition, drug metabolism, drug transport, drug interactions, and bioavailability. Because drug-metabolizing enzymes such as cytochrome P450 (CYP) and UDP-glucuronyltransferase (UGT) and drug transporters such as ATP-binding cassette (ABC) and solute carrier (SLC) transporters are appreciably expressed in the small intestinal epithelia,[1,2] it is necessary to estimate intestinal metabolism and absorption during the early stages of drug development. To this end, various *in vivo* and *in vitro* systems have been employed to assess the intestinal first-pass effect. However, extrapolation of experimental animal data to humans is often hampered by species differences, and primary human intestinal cells are rarely available. Therefore, a system that accurately and easily estimates intestinal membrane permeability and metabolism is urgently required.

Human induced pluripotent stem (iPS) cells can be generated by transducing reprogramming factors (OCT3/4, SOX2, KLF4, c-MYC) into somatic cells[3] and these cells share many characteristics of embryonic stem (ES) cells.[4] Human iPS cells are expected to be useful not only in regenerative medicine but also in pharmacokinetic and toxicokinetic drug development studies because their use is not as ethically regulated as that of human ES cells.

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Therefore, human iPS cells have been differentiated into various cell types, including pancreatic cells,[5,6] neuron cells,[7] cardiomyocytes,[8] and hepatocytes.[9-13]

A few studies report the differentiation of iPS cells into enterocytes. In particular, mouse iPS cells were differentiated into a gut-like organ following the formation of embryoid bodies (EBs),[14] and human iPS cells were differentiated into intestinal tissue using a culture method for intestinal crypt stem cells.[15] However, functional characteristics of drug transporters and drug-metabolizing enzymes of differentiated cells are almost entirely unexplored in these reports. Thus, whether differentiated intestinal tissue or organoids can be used in drug development studies, particularly studies of the absorbability and metabolic capacity of drugs, remains unclear.

The small intestinal epithelium comprises absorptive cells, goblet cells, endocrine cells, and Paneth cells. Several signaling pathways such as Notch, Wnt, phosphoinositide 3-kinase, and bone morphogenic protein signaling are associated with intestinal development.[16] Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) has been identified as an intestinal stem cell marker.[17] Indeed, this was also observed in mouse LGR5-positive cells that formed a crypt–villus structure *in vitro*.[18] Improvements in this technique have enabled long-term culture of human epithelial cells isolated from the small intestine,[19] leading to advances in intestinal stem cell research. However, mechanisms of intestinal development are not sufficiently understood, and it is difficult to control differentiation into all four cell types.

In this study, we established a functional enterocyte-like cell line from human iPS cells for use in drug development studies. We propose a simple and direct differentiation method by two-dimensional culture. Our data may facilitate the development of an intestinal pharmacokinetic analysis system to identify safe drugs with favorable pharmacokinetic characteristics.

## Materials and Methods

**Materials:** FGF2, FGF4, activin A, and epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Wnt3a was purchased from R&D Systems, Inc. (Minneapolis, MN). BD Matrigel matrix Growth Factor Reduced (Matrigel) was purchased from BD Biosciences (Bedford, MA). Affinity-isolated rabbit polyclonal antihuman sucrase–isomaltase antibody and intestinal recombinant protein epitope signature tags were purchased from Sigma-Aldrich Co. (St. Louis, MO). The purified IgG fraction of polyclonal goat antiserum against rabbit IgG conjugated with Alexa Fluor 568 and KnockOut Serum Replacement (KSR) were purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys-AMCA) was purchased from BIOTREND Chemicals (Destin, FL), and (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Human adult small intestine total RNA from a 66-year-old male donor was purchased from BioChain Institute Inc. (Newark, CA). Murine embryonic fibroblasts (MEFs) were obtained from Oriental Yeast Co. (Tokyo, Japan). The RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). The PrimeScript RT Reagent Kit and TaKaRa SYBR Premix EX Taq II were purchased from Takara Bio Inc. (Otsu, Japan). All other reagents were of the highest quality available.

**Human iPS cell cultures:** A human iPS cell line (Windy) was provided by Dr. Akihiro Umezawa of the National Center for Child Health and Development. Human iPS cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) containing 20% KSR, 2 mM L-glutamine, 1% MEM nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/ml FGF2 at 37°C in humidified air with 5% $CO_2$. The human iPS cells were cultured on a feeder layer of mitomycin C-treated MEFs, and the medium was changed every day.

**Differentiation into enterocyte-like cells:** The human iPS cells were used for differentiation studies between passages 30 and 50. When the cells reached approximately 70% confluence, differentiation was initiated by replacing the medium with Rosewell Park Memorial Institute (RPMI) 1640 medium containing 2 mM GlutaMAX, 0.5% fetal bovine serum (FBS), 100 ng/ml activin A (a member of the transforming growth factor-β family that is known to efficiently induce differentiation into the definitive endoderm),[20,21] 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h, the medium was replaced with RPMI 1640 containing 2 mM GlutaMAX, 2% FBS, 100 ng/ml activin A, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the cells were cultured for 24 h. Subsequently, the culture medium was replaced with DMEM/F12 containing 2% FBS, 2 mM GlutaMAX, and 250 ng/ml FGF2 or FGF4 with or without 50 ng/ml Wnt3a for 96 h. The cells were then treated for 1 h with the selective Rho-associated kinase inhibitor Y-27632 at 10 μM.[22,23] The cells were then passaged on Matrigel-coated 24-well plates and cultured in DMEM/F12 containing 2% or 10% FBS, 2% B-27 supplement, 1% N2 supplement, 1% NEAA, 2 mM L-glutamine, antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and 20 ng/ml EGF for 1, 4, 13, or 19 days. Y-27632 was added at 10 μM during the initial 24 h of culture. The medium was changed every 3 days (**Fig. 1**).

**RNA extraction and reverse transcription reaction:** Total RNA was isolated from differentiated iPS cells using the RNeasy Mini Kit. First-strand cDNA was prepared from 500 ng of total RNA. The reverse transcription reaction was performed using the PrimeScript RT Reagent Kit according to the manufacturer's instructions.

**Real-time polymerase chain reaction (PCR) analysis:** Relative mRNA expression levels were determined using SYBR Green real-time quantitative reverse transcription-PCR (RT-PCR). Real-time PCR analysis was performed on the Applied Biosystems 7300 Real Time PCR System using 7300 System SDS software version 1.4 (Applied Biosystems, Carlsbad, CA). PCR was performed with the primer pairs listed in **Table 1** using SYBR Premix EX Taq II. mRNA expression levels were normalized relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

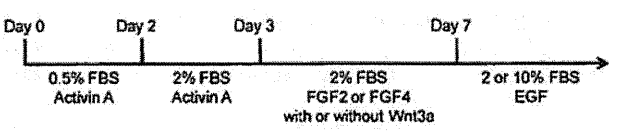


**Fig. 1. Schematic of the protocol for the differentiation of human iPS cells into enterocytes**
Human iPS cells were cultured in the presence of activin A (100 ng/ml) for 3 days. The cells were further cultured in medium containing FGF2 (250 ng/ml) or FGF4 (250 ng/ml) with or without Wnt3a (50 ng/ml) for 4 days. After 7 days of differentiation, the cells were treated with Y-27632 (10 μM), passaged, and subsequently cultured in the presence of 2% or 10% FBS and EGF (20 ng/ml) for 19 days.

**Table 1. Sequences of primers for real-time PCR analysis**

| Gene name | Sense (5′→3′) | Antisense (5′→3′) | Product length (bp) |
|---|---|---|---|
| CDX2 | ACCTGTGCGAGTGGATGC | TCCTTTGCTCTGCGGTTCT | 232 |
| LGR5 | TGCTCTTCACCAACTGCATC | CTCAGGCTCACCAGATCCTC | 193 |
| DPP4 | CAAATTGAAGCAGCCAGACA | GGAGTTGGGAGACCCATGTA | 212 |
| Sucrase–isomaltase | GGTAAGGAGAAACCGGGAAG | GCACGTCGACCTATGGAAAT | 195 |
| Villin 1 | AGCCAGATCACTGCTGAGGT | TGGACAGGTGTTCCTCCTTC | 169 |
| ISX | CAGGAAGGAAGGAAGAGCAA | TGGGTAGTGGGTAAAGTGGAA | 96 |
| CYP3A4 | CTGTGTGTTTCCAAGAGAAGTTAC | TGCATCAATTTCCTCCTGCAG | 298 |
| SLC15A1/PEPT1 | CACCTCCTTGAAGAAGATGGCA | GGGAAGACTGGAAGAGTTTTATCG | 105 |
| SLC46A1/PCFT | GGTCTTTGCCTTTGCCACTA | AGAGTTTAGCCCGGATGACA | 98 |
| GAPDH | GAGTCAACGGATTTGGTCGT | GACAAGCTTCCCGTTCTCAG | 185 |

**Immunofluorescence staining:** The cells differentiated with FGF2 and 2% FBS were washed three times with phosphate-buffered saline (PBS) without calcium or magnesium, fixed for 30 min at room temperature in 4% paraformaldehyde, and permeabilized in PBS containing 0.1% Triton X-100 for 5 min at room temperature. After being washed three times with PBS, the cells were blocked in PBS with 2% skim milk for 20 min at room temperature and were incubated with antisucrase–isomaltase antibody diluted at 1:200 for 60 min at room temperature. Rabbit serum was used as a negative control. The cells were washed three times with PBS and incubated with a 1:500 dilution of Alexa Fluor 568-labeled secondary antibody for 60 min at room temperature. After being washed three times with PBS, the cells were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and washed with PBS. The cells were mounted on a glass slide using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

**Uptake study of β-Ala-Lys-AMCA:** The cells differentiated with FGF2 and 2% FBS were rinsed several times with PBS and incubated with DMEM/F12 containing 25 μM β-Ala-Lys-AMCA for 4 h at 37°C. After incubation, uptake of β-Ala-Lys-AMCA was stopped by washing with ice-cold PBS. The cells were fixed for 30 min at room temperature in 4% paraformaldehyde, and immunofluorescence staining was performed using the primary and secondary antibodies as described above. The cells were then mounted using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope.

**Statistical analysis:** Levels of statistical significance were assessed using Student's *t*-test, and multiple comparisons were performed using analysis of variance (ANOVA) followed by Tukey's test.

## Results

**Early stages of differentiation into intestinal cells:** For efficient, selective, and direct differentiation, a protocol designed to mimic intestinal development is desirable. We attempted differentiation into enterocytes that mediate the formation of the definitive endoderm. Because the intestine is an endoderm-derived organ, the human iPS cells were initially differentiated into the endoderm using a high concentration of activin A (100 ng/ml). Subsequently, we investigated the effects of FGF2, FGF4, and Wnt3a, which promote the development of mid- and hindgut lineages,[24,25] during differentiation from the definitive endoderm to intestinal stem cells. In these experiments, mRNA expression of caudal type homeobox 2 (CDX2), a major transcription factor of intestinal development and cell differentiation,[26,27] was slightly higher in FGF4-treated cells than that in FGF2-treated cells (**Fig. 2A**). In contrast, mRNA expression of LGR5 in FGF4-treated cells was significantly lower than that in FGF2-treated cells (**Fig. 2B**). Under all conditions, these mRNA expression levels were higher than those in undifferentiated human iPS cells. No effects of Wnt3a were observed on mRNA expression of CDX2 or LGR5 during the early stages of differentiation.

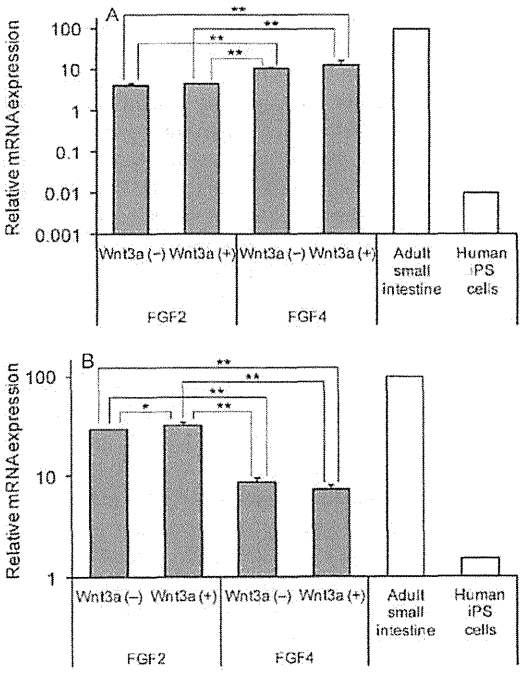


**Fig. 2. Relative mRNA expression levels of CDX2 (A) and LGR5 (B) in differentiated intestinal stem cell-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 or FGF4 with or without Wnt3a for 4 days and then in the presence of EGF for 1 day. After 8 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells. Levels of statistical significance were compared among all groups: $^{**}p < 0.01$, $^{*}p < 0.05$.

**Differentiation into enterocyte-like cells:** To effectively differentiate human iPS cells into enterocyte-like cells, we examined

the effects of FBS concentration in the differentiation medium. Expression of LGR5 in differentiated human iPS cells did not differ in the presence of 2% or 10% FBS (**Fig. 3A**). However, mRNA expression of sucrase–isomaltase was 3.5-fold higher in 2% FBS than that in 10% FBS (**Fig. 3B**). In addition, mRNA expression levels of SLC15A1/peptide transporter 1 (PEPT1) and CYP3A4 were higher in the presence of 2% FBS (**Figs. 3C** and **3D**). In differentiated enterocyte-like cells, sucrase–isomaltase and CYP3A4, which were not detected in undifferentiated human iPS cells, were expressed, and mRNA expression levels of LGR5 and SLC15A1/PEPT1 were 30–40-fold higher than those in undifferentiated human iPS cells. Morphological changes in differentiating human iPS cells are shown in **Figure 4**. Similar to ES cells, undifferentiated human iPS cells had little cytoplasm and were small in size (**Fig. 4A**). When human iPS cells were cultured in the presence of activin A and FGF2, the cells gradually exhibited morphological changes such as enlargement and acquisition of spiky shapes (**Fig. 4B**). At the final stage of differentiation with EGF and 2% FBS, a number of dome-like structures formed and were assumed to contain liquids and cells (**Figs. 4C** and **4D**). In cells differentiated with activin A, FGF2, EGF, and 2% FBS, villin 1[28,29] and intestine specific homeobox (ISX)[30] were expressed, whereas intestinal fatty acid-binding protein (IFABP) was not expressed. Interestingly, mRNA expression levels of CDX2, dipeptidyl peptidase 4 (DPP4), and SLC46A1/proton-coupled folate transporter (PCFT) were similar to those in the adult small intestine, which was used as a positive control (**Fig. 5**). However, gene expression levels of UGT1A1 and ABCB1/multidrug resistance 1 (MDR1) were similar to those in undifferentiated human iPS cells (data not shown).

To determine the optimal duration of differentiation, we examined time-dependent variations in expression levels of specific small intestine genes such as LGR5, sucrase–isomaltase, and SLC15A1/PEPT1. After short-term culture (11 days), mRNA expression levels of sucrase–isomaltase and SLC15A1/PEPT1 were very low but gradually increased with differentiation until day 26. Similarly, CYP3A4 mRNA was not expressed after 11 days of differentiation but was expressed after 20 days (**Fig. 6**). LGR5 mRNA did not change with the duration of differentiation.

**Immunofluorescence staining of sucrase–isomaltase in enterocyte-like cells:** Sucrase–isomaltase is an essential carbohydrate digestion enzyme that is specifically expressed in brush border membranes of mature enterocytes. Therefore, sucrase–isomaltase expression is thought to be an indicator of differentiation into enterocytes. Indeed, protein expression of sucrase–isomaltase was confirmed in differentiated cells using immunofluorescence staining, in particular, in dense clusters of cells (**Fig. 7**).

**Uptake of β-Ala-Lys-AMCA in enterocyte-like cells:** Oligopeptide transporters are expressed in the brush border membrane and participate in peptide absorption from the intestinal lumen.[2] As shown in **Figure 3C**, expression of SLC15A1/PEPT1 mRNA in differentiated enterocyte-like cells was more than 30-fold higher than that in undifferentiated human iPS cells. To determine whether this leads to active peptide transport in differentiated cells, we performed peptide uptake assays using β-Ala-Lys-AMCA, a fluorescence-labeled substrate of the oligopeptide transporter.[31] As shown in **Figure 8**, intracellular uptake of β-Ala-Lys-AMCA was observed in cells expressing the sucrase–isomaltase proteins. However, uptake of β-Ala-Lys-AMCA at 4°C was low compared with that at 37°C (data not shown).

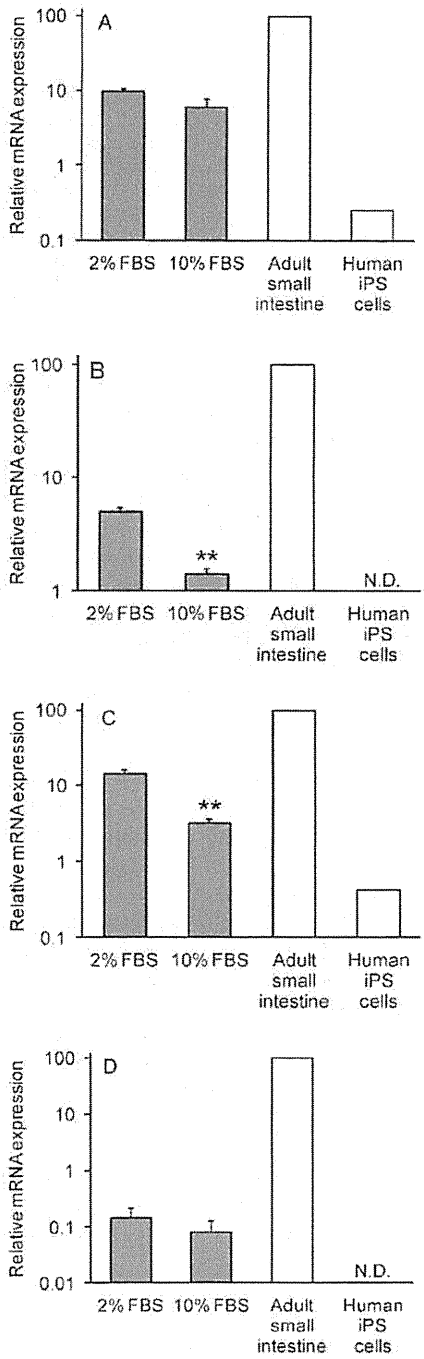


**Fig. 3. Relative mRNA expression levels of LGR5 (A), sucrase–isomaltase (B), SLC15A1/PEPT1 (C), and CYP3A4 (D) in differentiated enterocyte-like cells cultured in 2% or 10% FBS**

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% or 10% FBS and EGF for 17 days. After 24 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells. N.D., not detected. Levels of statistical significance were compared with the 2% FBS group: $^{**}p < 0.01$.

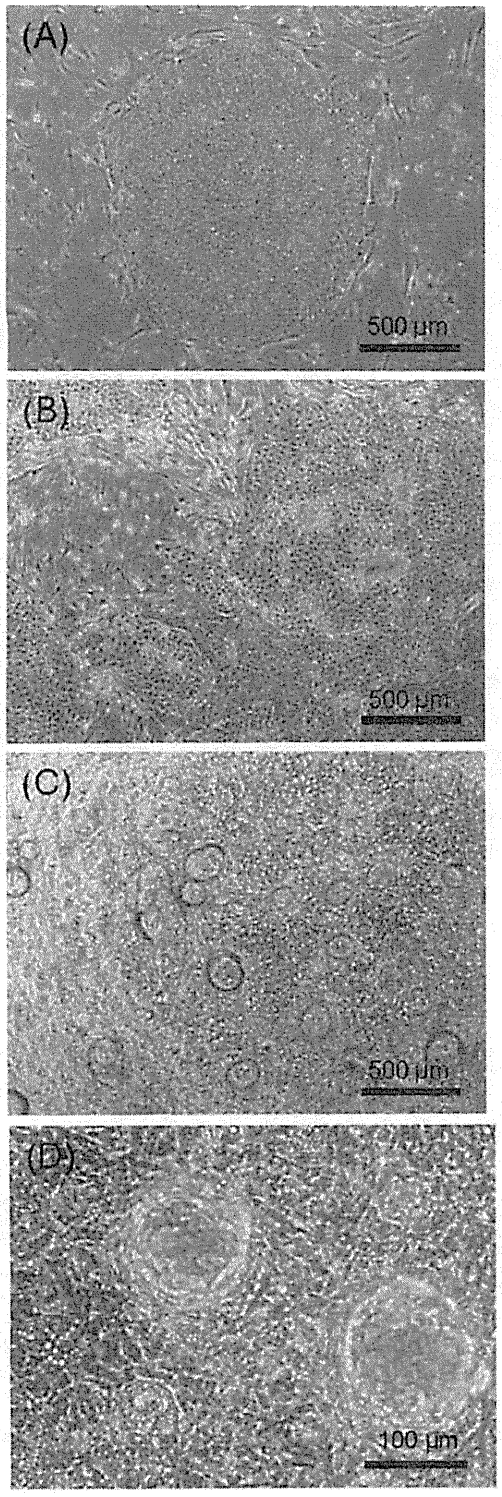


**Fig. 4. Morphological changes in human iPS cells during differentiation into enterocyte-like cells**
Human iPS cells were cultured in medium containing activin A for 3 days, FGF2 for 4 days, and 2% FBS and EGF for 17 days. (A) Undifferentiated human iPS cells; (B) midgut lineage cell-like cells after 7 days of differentiation; (C, D) enterocyte-like cells after 24 days of differentiation. Scale bar, 500 μm (A–C), 100 μm (D).

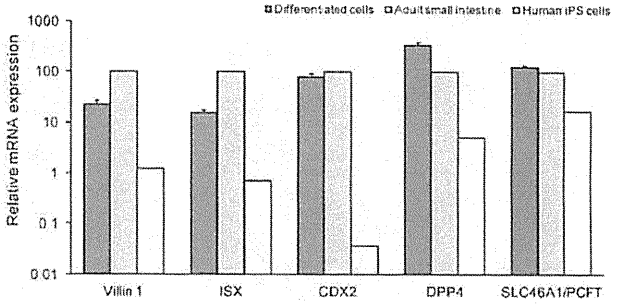


**Fig. 5. Relative mRNA expression levels of the intestinal markers villin 1, ISX, CDX2, DPP4, and SLC46A1/PCFT in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine and undifferentiated human iPS cells (shown as open columns) were used as positive and negative controls, respectively. Data are presented as the mean ± S.D. ($n = 3$), except for the adult small intestine and human iPS cells.

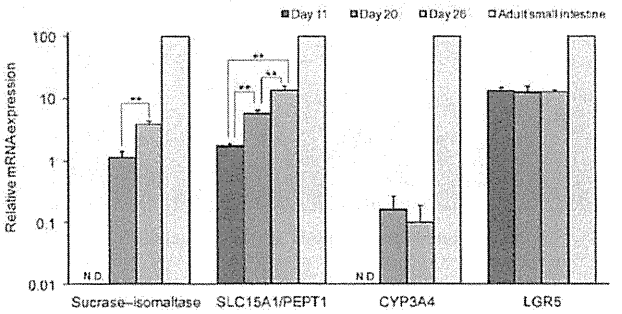


**Fig. 6. Time-dependent variation in mRNA expression levels of sucrase–isomaltase, SLC15A1/PEPT1, CYP3A4, and LGR5 in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 4, 13, or 19 days. After 11, 20, or 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time RT-PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which is set as 100. The adult small intestine was used as a positive control. Data are presented as the mean ± S.D. ($n = 4$) except for the adult small intestine. N.D., not detected. Levels of statistical significance were compared among all groups: $^{**}p < 0.01$.

## Discussion

Ueda *et al.*[14] reported the synthesis of gut-like organs from mouse iPS cells using the EB formulation technique with mouse ES cells. However, this hanging drop culture technique is hampered by its high requirement of skill, low EB formulation efficiency, unstable EB quality, and differing differentiation efficiencies between EBs. Spence *et al.*[15] reported the direct differentiation of human iPS cells into three-dimensional intestinal organoids. These organoids contained various cell types, including enterocytes, endocrine cells, goblet cells, and Paneth cells, although expression of drug-metabolizing enzymes and transporters, which are central to drug absorption and metabolism, was not examined. In their study, the intestinal crypt culture system, in which spheroids

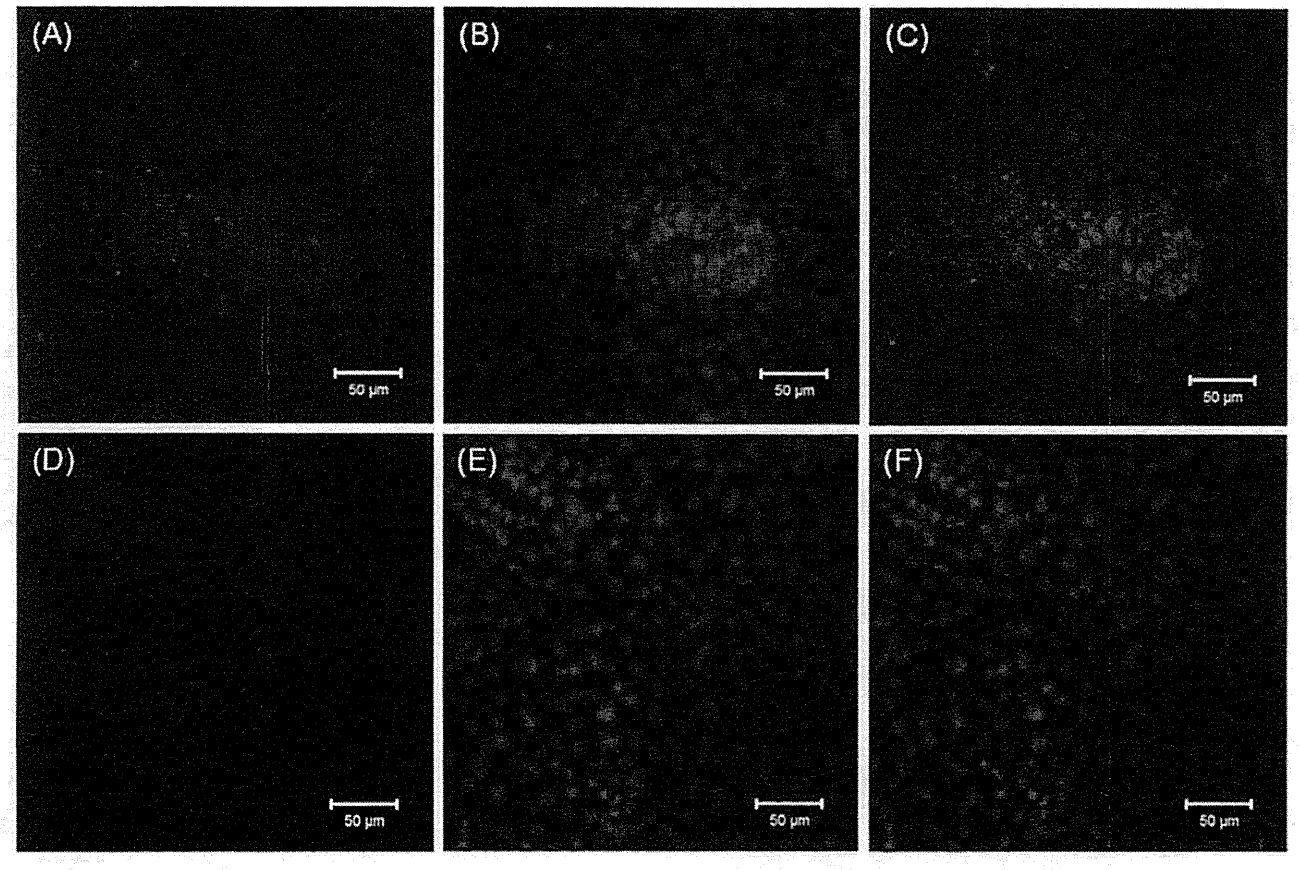


**Fig. 7. Immunofluorescence analysis of sucrase–isomaltase in differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, differentiated cells were stained with antisucrase–isomaltase antibody (A–C) or nonimmune rabbit serum as a negative control (D–F). Nuclei were counterstained with DAPI. (A) Immunofluorescence staining of sucrase–isomaltase (red); (D) immunofluorescence staining of rabbit serum as a negative control; (B, E) DAPI-stained DNA (blue); (C, F) overlay (Merge) image of sucrase–isomaltase and DAPI. Scale bar, 50 μm.

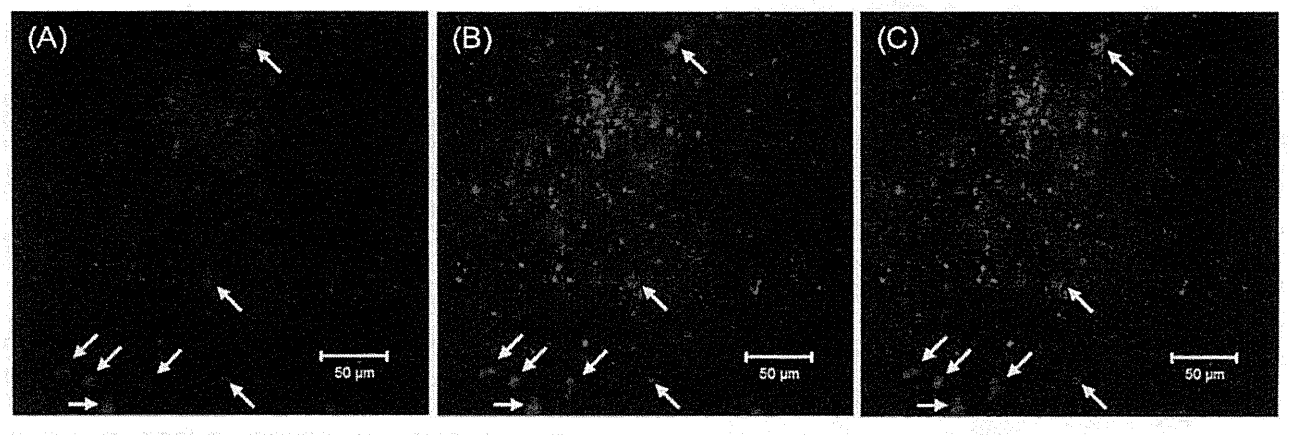


**Fig. 8. Uptake of β-Ala-Lys-AMCA into differentiated enterocyte-like cells**
Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of 2% FBS and EGF for 19 days. After 26 days of differentiation, the differentiated cells were incubated with β-Ala-Lys-AMCA (25 μM) for 4 h at 37°C. After uptake was stopped, the differentiated cells were fixed and stained with antisucrase–isomaltase antibody. Typical images from β-Ala-Lys-AMCA uptake experiments. Arrows indicate co-localization of β-Ala-Lys-AMCA and sucrase–isomaltase protein. (A) Intracellular uptake of β-Ala-Lys-AMCA (blue); (B) immunofluorescence staining of sucrase–isomaltase (red); (C) overlay (Merge) image of β-Ala-Lys-AMCA and sucrase–isomaltase. Scale bar, 50 μm.

are formed by three-dimensional culture, was applied during differentiation from the endoderm to intestinal organoids, and several factors were added in large quantities to induce differentiation. This method may be suitable for the culture of intestinal stem cells but not for selective differentiation into enterocytes, and it is complicated and costly. Therefore, we generated enterocyte-like cells using a simple two-dimensional culture method.

Spence *et al.*[15] also reported that the combination of FGF4 (500 ng/ml) and Wnt3a (500 ng/ml) effectively posteriorized the endoderm. Ameri *et al.*[32] reported the induction of CDX2 with FGF2 (256 ng/ml) in a concentration-dependent manner in the human ES cell-derived endoderm. In the present study, we examined the effects of FGF2, FGF4, and Wnt3a on the induction of the midgut endoderm lineage. Our results showed that the expression level of CDX2 was comparable in the FGF2 and FGF4 treatment groups, whereas no effect of Wnt3a was observed. These results were inconsistent with those of Spence *et al.*,[15] possibly because of the lower concentrations of Wnt3a used in our study (50 ng/ml). Sherwood *et al.*[33] demonstrated that β-catenin-dependent Wnt signaling, activated by the glycogen synthase kinase-3 (GSK3) inhibitor, induced the expression of the intestinal master regulator Cdx2 and induced intestinal differentiation of the ES cell-derived endoderm and large intestinal gene expression. They also indicated that because of poor bioactivity, the induction level of Cdx2 expression by Wnt3a was lower than that by the GSK3 inhibitor. Therefore, superior stability and bioactivity of small-molecule compounds may lead to more effective experimental regulation of these signaling pathways. However, the striking activation of Wnt signaling also induced large intestinal gene expression, and further investigations of the extent of activation of Wnt signaling during small intestinal differentiation may be required. The expression of LGR5[17] was higher in the FGF2 treatment group than that in the FGF4 treatment group. Therefore, subsequent differentiation experiments were performed using FGF2.

We have demonstrated that enterocyte-like cells, which express the specific intestinal markers sucrase–isomaltase,[34,35] villin 1, ISX, and pharmacokinetics-related genes, were differentiated from an intestinal stem cell-like cell population by two-dimensional culture with EGF and a low serum concentration. Sucrase–isomaltase and SLC15A1/PEPT1 mRNA gradually increased with the duration of differentiation, indicating that longer duration may be necessary to efficiently obtain mature enterocytes. In contrast, LGR5 mRNA expression remained unchanged, suggesting that enterocytes matured during intestinal stem cell proliferation. DPP4 (serine protease) and SLC46A1/PCFT (folate transporter) are known to be abundantly expressed in epithelial cells of the small intestine.[36,37] Expression of DPP4 and SLC46A1/PCFT in differentiated cells was higher than that in adult small intestine samples. Intestinal differentiation was promoted by low FBS concentrations in this study. Potentially, the growth of extra-enterocytic cells such as fibroblasts may be suppressed with decreasing FBS concentrations, and some differentiation and growth-inhibiting factors may be present in FBS, although the mechanisms underlying these effects remain unclear.

At present, human intestinal epithelial cells are difficult to obtain, and no appropriate model cell system exists. Instead, other tissue cell-derived cell lines, including Caco-2 cells (human colon carcinoma cell line) and Madin–Darby canine kidney (MDCK) cells, have been used as intestinal models in drug absorption studies.[2,38] However, drug transporter expression patterns in these cells considerably differ from those in enterocytes. In particular, CYP3A4 is expressed at very low levels in these cell lines. In the present study, cells expressing the sucrase–isomaltase protein showed uptake of β-Ala-Lys-AMCA, the substrate of the oligopeptide transporter (**Fig. 8**). Differentiated cells also expressed CYP3A4 mRNA, albeit at levels lower than those in the adult small intestine (**Fig. 3**), suggesting that enterocyte-like cells differentiated from human iPS cells have peptide-transporting activity and may be useful in the study of drug absorbability.

In this study, we used a human iPS cell line, which is easily differentiated into hepatocytes of an endodermal lineage, because the intestine is also an endoderm-derived tissue. Differentiation propensity is known to be markedly different among human ES and iPS cell lines.[39,40] Thus, there may be a difference in the degree of intestinal differentiation depending on the human iPS cell line being tested. Regarding this point, we believe it is necessary to perform studies comparing intestinal differentiation among human iPS cell lines in the future.

In conclusion, because the intestine is an endoderm-derived tissue, human iPS cells were directly differentiated into the endoderm using activin A. Subsequently, we devised a simple method for differentiation into enterocyte-like cells with functional peptide transport by two-dimensional culture and the addition of several growth factors. These data suggest that human iPS-derived enterocytes may facilitate future drug development studies. If enterocyte-like cells, which have functional features similar to those of enterocytes, can be generated from human iPS cells, it may be possible to construct systems for easy estimation of overall intestinal function, including absorption and metabolism.

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CHEMICAL REVIEWS

Review

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# Physical Cues of Biomaterials Guide Stem Cell Differentiation Fate

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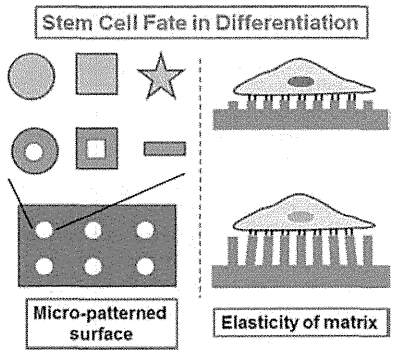


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## 1. INTRODUCTION

Millions of people lose or damage their organs or tissues due to disease, birth defects, or accidents each year. Stem cells, such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adult stem cells, and fetal stem cells are an attractive prospect for regenerative medicine and tissue engineering.[1−3] ESCs derived from preimplantation embryos have the potential to differentiate into any cell type derived from the three germ layers: the ectoderm (nerves and epidermal tissues), mesoderm (bone, muscle, and blood), and endoderm (lungs, liver, gastrointestinal tract, and pancreas).[2−7] iPSCs are known to have similar properties to ESCs, including the expression of certain pluripotent stem cell genes and proteins and differentiability into many types of cells derived from the three germ layers.[8−12]

The pluripotent nature of ESCs and iPSCs opens many avenues for potential stem cell-based regenerative therapies and the development of drug discovery platforms.[2,3,5,13] The nearest-term therapeutic use of ESCs and iPSCs may be in the treatment of disorders of single cell types, such as the transplantation of differentiated nerve cells ($TH^+$ cells, dopamine-secreting cells) for the treatment of Parkinson's disease or $\beta$ cells (insulin-secreting cells) for the treatment of diabetes.[2,3,13] However, it is difficult to guide iPSCs and ESCs

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to be differentiated into specific lineages of cells due to their pluripotent ability of differentiation.[14−24]

Adult and fetal stem cells can be isolated from a variety of somatic tissues, that is, bone marrow, umbilical cord blood, umbilical cord tissue, amniotic fluid, dental pulp, and other tissues such as fat.[16−22] There have been no reports to date of mesenchymal stem cells (MSCs) or fetal stem cells differentiating into tumors, such as have been reported in ESCs and iPSCs. MSCs are currently the most widely available autologous source of stem cells for practical and clinical applications. However, adult and fetal stem cells have aging problems and limited passage numbers.[25−28]

Stem cell characteristics, such as proper differentiation and maintenance of pluripotency, are regulated not only by the stem cells themselves but also by the microenvironment.[2,3,29] Therefore, mimicking stem cell microenvironments and niches using biopolymers (biomaterials) could facilitate the production of large numbers of stem cells and specifically differentiated cells needed for in vitro regenerative medicine.

Biological cues, such as growth factors, hormones, small chemicals, and extracellular matrix, can decide the stem cell fates of differentiation and pluripotency.[30,31] Therefore, many research efforts have been chiefly devoted to identifying soluble differentiation factors to mimic the stem cell microenvironment. However, investigators have begun to evaluate the potential importance of physical cues influencing stem cells, such as cell shape, stiffness of cell culture substrates, mechanical forces (e.g., shear stress of culture medium), external forces (e.g., electrical force, magnetic force), and light signaling.[18,32−44] Some excellent reviews and original articles addressing biomaterials guiding stem cell differentiation are listed in Table 1.[2,3,19,20,22,32,36,40,41,45−55] However, few review articles have specifically addressed the physical cues of cell culture biomaterials for stem cell differentiation, and the review articles that have been written do not describe the methods and results in comprehensive detail for chemists and materials scientists.[33,36,37,40,47,52,56,57] Therefore, this review focuses on physical cues of biomaterials guiding the differentiation of MSCs, ESCs, and iPSCs into several lineages, such as adipocytes, chondrocytes, osteoblasts, muscle cells, endothelial cells, and neural cells. We do not focus on the differentiation of stem cells triggered by mechanical[33,36−38] and external forces[18,36,37,47] in this review because these effects are not directly related to the biomaterials used in stem cell culture. The physical cues of biomaterials in stem cell culture described in this review are classified as biomaterial (a) elasticity and (b) topography. Recently, not only biological cues such as growth factors and bioactive molecules, but also physical cues of biomaterials such as elasticity and topography are considered to be important factors for stem cell differentiation into specific lineages. This is because small and large biomolecules that induce differentiation of stem cells have been highly investigated. Nowadays, it is difficult to find novel biomolecules for differentiation of stem cells and to find much higher efficiency of stem cell differentiation into desired lineages solely by combination of these biomolecules in culture medium. Biomaterials for stem cell culture are focused as a tool for fine-tuning of stem cell differentiation, because it is quite recent for researchers to realize biomaterials can guide stem cell fate of differentiation. For example, the morphology of stem cells can be regulated by elasticity and topography of cell culture biomaterials, which indicates that the elasticity and topography of the biomaterials can regulate signal transduction of stem cells and subsequently guide stem cell differentiation into specific lineages.

**Table 1. Some Key Review and Original Articles Addressing Biomaterials Guiding Stem Cell Differentiation**

| author | contents | ref (year) |
|---|---|---|
| Lee and Mooney | hydrogels for tissue engineering | 45 (2001) |
| Engler et al. | matrix elasticity directs stem cell lineage | 32 (2006) |
| Benoit et al. | small functional groups for controlled differentiation of hydrogel-encapsulated hMSCs | 20 (2008) |
| Liao et al. | stem cells and biomimetic materials strategies for tissue Engineering | 55 (2008) |
| Wescoe et al. | biochemical and biophysical environment in chondrogenic stem cell differentiation | 40 (2008) |
| Little et al. | biomaterials for neural stem cell microenvironments | 46 (2008) |
| Dellatore et al. | mimicking stem cell niches to increase stem cell expansion | 47 (2008) |
| Boskey and Roy | cell culture systems for studies of bone and tooth mineralization | 48 (2008) |
| Burdick and Vunjak-Novakovic | engineered microenvironments for controlled stem cell differentiation | 41 (2009) |
| Guilak et al. | control of stem cell fate by physical interactions with ECM | 36 (2009) |
| Mei et al. | combinatorial development of biomaterials for clonal growth of human pluripotent stem cells | 49 (2010) |
| Melkoumian et al. | synthetic peptide-acrylate surfaces for long-term self-renewal of hESCs | 50 (2010) |
| Gilbert et al. | substrate elasticity regulates skeletal muscle stem cell self-renewal | 51 (2010) |
| Huebsch et al. | harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate | 19 (2010) |
| Ghafar-Zadeh et al. | stem cell microenvironment for cardiac tissue regeneration | 52 (2011) |
| Balakrishnam and Benerjee | biopolymer-based hydrogels for cartilage tissue engineering | 53 (2011) |
| Higuchi et al. | biomaterials for the feeder-free culture of hESCs and human iPSCs | 2 (2011) |
| Kim et al. | design of artificial extracellular matrices for tissue engineering | 54 (2011) |
| Higuchi et al. | biomimetic cell culture proteins for stem cell differentiation | 3 (2012) |
| Trappmann et al. | ECM tethering regulates stem-cell fate | 22 (2012) |

These effects are described in detail in the following sections. The chemical schematics of the biomaterials described in this review are summarized in Figure 1. Some of the genes and proteins mentioned in this review that are typically utilized to verify the differentiation of stem cells into specific lineages and their descriptions are summarized in Table 2.[2,3,19,32,35,58−84] Various staining methods used to characterize specific lineages are summarized in Table 3.[2,3,18,19,22,32,51,64,85−87]

## 2. EFFECT OF ELASTICITY OF CELL CULTURE MATERIALS ON STEM CELL DIFFERENTIATION

Diverse microenvironmental factors contribute to overall stem cell fate (i.e., differentiation into specific lineages). In particular, physical interactions between cells and the elasticity (or rigidity and stiffness) of the extracellular matrix (ECM) where they are cultured can influence stem cell fate, although the control of stem cell fate has been classically attributed to genetic or molecular mediators.[36,88] Recently, many researchers have begun to realize that the elasticity of cell culture substrates


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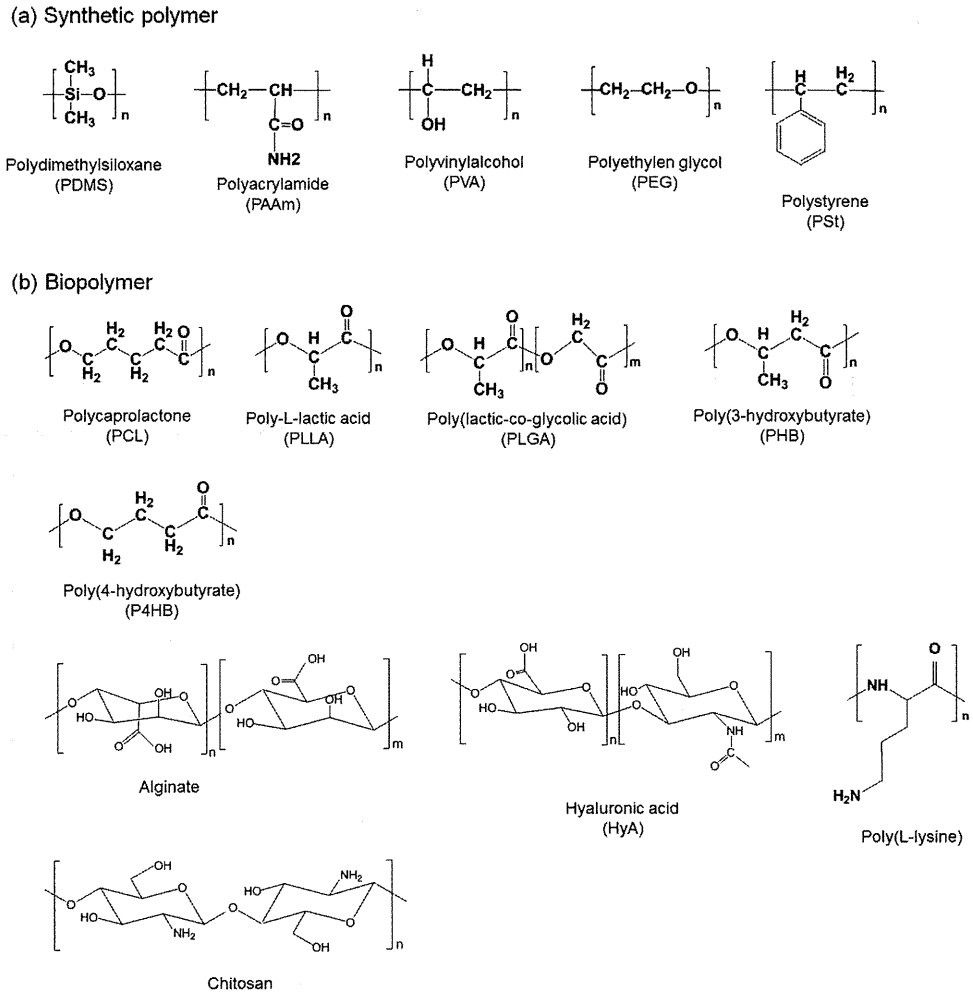


**Figure 1.** Chemical schematics of synthetic polymers (polydimethylsiloxane [PDMS], polyacrylamide [PAAm], poly(vinyl alcohol) [PVA], poly(ethylene glycol) [PEG], and polystyrene [PSt]) (a) and biopolymers (poly($\varepsilon$-caprolactone) [PCL], poly(L-lactic acid) [PLLA], poly(lactic-*co*-glycolic acid) [PLGA], poly(3-hydroxybutyrate) [PHB], poly(4-hydroxybutylate) [P4HB], alginate, hyaluronic acid [HyA], poly(L-lysine), and chitosan) (b) used as substrates, hydrogels, and scaffolds for the proliferation and differentiation of stem cells.

defines the lineage commitment of human MSCs (hMSCs). Stem cells tend to efficiently differentiate into specific tissue lineages when they are cultured on biomaterials with similar elasticity to those tissues. Figure 2 provides examples of the elasticity of various human tissues and synthetic and natural polymers derived from the literature.[32,49,77,87,89–92]

The elasticity of cell culture substrates can clearly influence cell morphology, cell phenotype, and focal adhesions, especially in two-dimensional (2-D) culture.[51,58,62,66,72–75,86,88,93,94] Mechanosensing of substrates by stem cells is considered to be generated by integrin-mediated focal adhesion signaling.[34] Integrins are receptors mediating the attachment between cells and ECM in cell culture substrates or tissues. They are composed of obligate heterodimers containing two distinct chains of $\alpha$ and $\beta$ subunits. Integrins contribute to cell-matrix signaling by activating intracellular tyrosine kinase and phosphatase signaling to elicit downstream biochemical signals important for the regulation of gene expression and stem cell fate.[89]

Previous studies have suggested that nonmuscle myosin IIA (NMMIIA)-dependent contractility of the actin cytoskeleton is an important mediator of the mechanosensing and mechanotransduction processes in different types of stem cells.[32,34,89,95–97] Furthermore, the elasticity of cell culture substrates affects intracellular signaling through mechanotransducers such as Rho kinase (ROCK) and focal adhesion kinase (FAK) and subsequently regulates the differentiation lineages of stem cells in 2-D culture.[74] Here, we review the effect of substrate elasticity (or rigidity) on the differentiation lineages of stem cells in 2-D and three-dimensional (3-D) culture. Tables 4 and 5 show examples from the literature of the effects of substrate elasticity on the differentiation of stem cells in 2-D and 3-D culture, respectively (refs 16, 19, 21, 22, 32, 58, 59, 62–66, 70, 73–75, 77, 85–87, 93, 94, 98–114).

### 2.1. Elasticity of Substrate Directs Stem Cell Differentiation Fate in 2-D Culture

Engler et al. cultured hMSCs on polyacrylamide (PAAm) substrates (hydrogels) of different stiffness coated with collagen type I in expansion medium (i.e., culture medium containing no differentiation-inducing biochemical factors).[32] Figure 3 shows the proteins and transcription profiles reported by Engler et al. of neuronal markers (P-NFH and $\beta$-III tubulin, Table 2), a muscle transcription factor (MyD1), and an osteoblast transcription factor (Runx2) expressed in hMSCs cultured on substrates with varying stiffness.[32] Several other proteins and transcription factors expressed in MSCs cultured on substrates with varying stiffness investigated by other research-

**Table 2. Genes and Proteins To Investigate the Differentiation of Stem Cells into Specific Lineages**

| differentiation lineage | gene or protein | specification | ref (example) | differentiation lineage | gene or protein | specification | ref (example) |
|---|---|---|---|---|---|---|---|
| osteoblasts | Runx2 (CBFα1) | early osteoblast marker | 19, 32, 66 | muscle | myocardin | smooth muscle cell marker | 67 |
| | osterix | early osteoblast marker | 3 | | smoothelin | smooth muscle cell marker | 67 |
| | osteocalcin (OCN) | late osteoblast marker | 32, 59, 61 | | collagen type 4 | myogenic marker | 69 |
| | osteopontin (OPN, Spp1) | late osteoblast marker | 58, 61 | | desmin | myogenic marker | 69 |
| | alkaline phosphatase (ALP) | early osteoblast marker | 61 | | Pax3 | myogenic marker | 32, 69 |
| | bone sialoprotein (BSP) | osteoblast marker | 78, 79 | | Pax7 | myogenic marker | 32, 69 |
| | collagen type I (Col I) | osteoblast marker | 74 | | myogenin (MYOG) | myogenic marker | 69 |
| chondrocytes | Sox 9 | chondrocyte marker | 35, 63, 67 | | MyoD, MyoD1 | myogenic marker | 69 |
| | Col2A1 | chondrocyte marker | 67 | neural cells | nestin | neural stem/progenitor cell marker | 71, 72, 77 |
| | aggrecan (ACAN) | chondrocyte marker | 80 | | ENO2 | neural cell | 59 |
| | collagen type II (Col II) | chondrocyte marker | 64 | | β-tubulin III or β-III tubulin (Tuj-1) | neuronal marker | 32, 66, 68 |
| | collagen type X (Col X) | chondrocyte marker | 3 | | tyrosine hydroxylase (TH) | neuronal marker (dopamin secreting cells) | 71 |
| | cartilage oligomeric protein (COMP) | chondrocyte marker | 80 | | neurofilament light chain (NEFL, NFL) | neuronal marker | 71−73 |
| adipocytes | adipocyte lipid-binding protein (ALBP) | adipocyte marker | 3 | | neurofilament heavy chain (NFH) | neuronal marker | 32, 73 |
| | PPARγ | adipocyte marker | 35 | | microtubule-associated protein 2 (MAP2) | mature neuronal marker | 72, 76 |
| | aP-2 | adipocyte marker | 81 | | glial fibrillary acidic protein (GFAP) | astrocyte marker | 62, 68 |
| | lipoprotein lipase (LPL) | adipocyte marker | 75 | | galactosylceramidase (GalC) | oligodendrocyte marker | 71 |
| cardiomyocytes | cardiac troponin T (cTnT) | cardiomyocyte marker | 3, 84 | | RIP | mature oligodendrocyte | 71 |
| | desmin | cardiomyocyte marker | 82, 83 | | O4 | oligodendrocyte marker | 68 |
| | myosin heavy chain (MHC) | cardiomyocyte marker | 84 | | CNPase | oligodendrocyte marker | 76 |
| | myoshin light chain (MLC) | cardiomyocyte marker | 84 | endothelial cells | Flk-1 | endothelial cell marker | 70 |
| | Nkx2.5 | cardiomyocyte marker | 84 | hepatocytes | α-fetoprotein (AFP) | early hepatocytes | 65 |
| | GATA-4 | cardiomyocyte marker | 84 | | albumin (ALB) | mature hepatocytes | 65 |
| muscle | smooth muscle α-actin (SMA) | smooth muscle cell marker | 70 | | Epithelial cell adhesion molecule (EpCAM) | hepatic stem cells, hepatoblasts | 65 |
| | α-actin | smooth muscle cell marker | 60 | | neural cell adhesion molecule (NCAM) | hepatic stem cells (and neural cells) | 65 |
| | calponin 1 | smooth muscle (contractile) marker | 59, 67 | | E-cadherin (CDH1) | hepatic stem cells (and ESCs, iPSCs) | 65 |
| | | | | ESCs, iPSCs | Oct3/4 | pluripotent marker | 2 |
| | | | | | Sox2 | pluripotent marker | 2 |
| | | | | | Nanog | pluripotent marker | 2 |

ers[62,63,66,70,75] are also included in Figure 3. Softer materials, with similar stiffness to the brain at approximately 0.3 kPa, tend to cause cells to express neuronal morphologies and neural markers (P-NFH, β-III tubulin), whereas stiffer materials of approximately 10 kPa mimicking muscle guide hMSCs and tend to cause cells to express myogenic markers (MyoD) in Engler's study.[32] Rigid materials similar to collagenous bone induced the expression of osteogenic marker Runx2 at a stiffness of approximately 35 kPa.[32] The myoblast cell line C2C12 also exhibited substrate stiffness-dependent MyoD expression, where the highest expression of MyoD was found in C2C12 cells cultured on substrate with a stiffness of approximately 10 kPa, although the expression intensity of MyoD in the myoblast cell line was twice as high as in hMSCs. Similarly, the highest expression of osteogenic marker Runx2 was found in the osteoblast cell line hFOB cultured on substrate with a stiffness of approximately 35 kPa, with an expression intensity 1.5 times greater than that in hMSCs.[32] Stiff substrates promote focal adhesion growth and elongation. Focal adhesions provide hMSCs with force transmission


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**Table 3. Staining Method To Investigate the Differentiation of Stem Cells into Specific Lineages**

| staining method | detection site | characterization | ref (example) |
|---|---|---|---|
| paxillin labeling | paxillin | focal adhesion | 32 |
| phalloidin F-actin | F-actin | focal adhesion | 22, 19, 85 |
| vinculin | focal adhesion protein | focal adhesion | 22, 86 |
| oil red O | oil droplet | adipocytes | 87 |
| nile red | oil droplet | adipocytes | 3 |
| alizarin red | calcium | osteoblasts | 85, 87 |
| von Kossa | calcium phosphate | osteoblasts | 18 |
| alkali phosphatase | alkali phosphatase activity | osteoblasts | 86 |
| safranin-O | proteoglycan | chondrocytes | 64 |
| alcian blue | proteoglycan | chondrocytes | 3 |
| toluidine blue | proteoglycan | chondrocytes | 86 |
| DAPI | DNA | nucleus | 19 |
| Hoechst | DNA | nucleus | 51 |
| Masson'S trichrome | tissue | connective tissue, nuclei, cytoplasm | 64 |
| hematoxylin and eosin (H&E) | tissue | connective tissue, nuclei, cytoplasm | 2 |

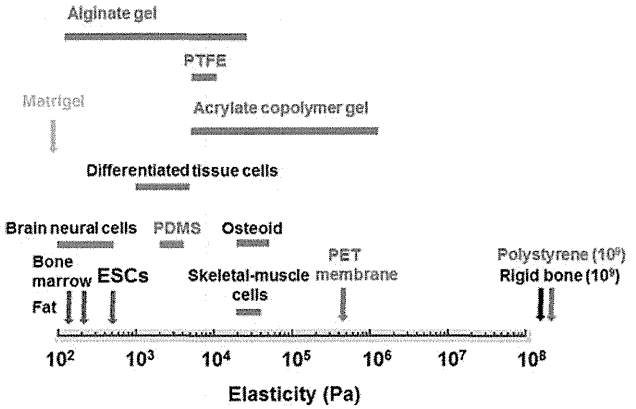


**Figure 2.** Examples of the elasticity of human tissues (blue bars and arrows), synthetic polymers (red bars and arrows), and natural polymers (green arrows).

pathways through which to influence their microenvironment via actin–myosin contractions. Therefore, stiffer culture substrates generate stiffer, more highly tensed cells. Cells alter their nonmuscle myosin expression to generate greater forces on the actin cytoskeleton, a necessary step to deform a stiffer matrix.[32] The forces generated on the actin cytoskeleton have been postulated to influence stem cell differentiation. Therefore, stem cells have different differentiation fates when they are cultured on different cell culture substrates.[3−88]

Several researchers have also reported that the stiffness of the cell culture substrate (matrix) is an important factor in the differentiation of stem cells in 2-D culture (refs 16, 34, 51, 58, 62, 66, 72−75, 85, 86, 88, 93, 94, 99, and 100).

The muscle microenvironment enables freshly isolated muscle stem cells to contribute to skeletal muscle regeneration when transplanted in animals. However, muscle stem cells cultured on conventional tissue culture plates lose their "stemness" easily, yielding progenitors with reduced regenerative potential.[51] Gilbert et al. investigated whether the elastic modulus of culture dishes plays a crucial role in muscle stem cell self-renewal and function in muscle regeneration.[51] They prepared cross-linked poly(ethylene glycol) (PEG) hydrogels with different stiffness values of 2, 12, and 42 kPa on plastic dishes (1 $\mu$m thick). Because laminin is a component of the native muscle stem cell niche, it was grafted onto the PEG hydrogels. Under time-lapse observation, the shortening velocity of muscle stem cells was found to decrease on soft PEG hydrogels (99 $\mu$m/h) compared with those cultured on stiff plastic culture dishes (120 $\mu$m/h).[51] The total number of muscle stem cells cultured on stiff plastic culture dishes did not change during 1 week of culture because cell division was offset by cell death. However, the number of muscle stem cells cultured on soft PEG hydrogels doubled compared with the cells cultured on rigid plastic culture dishes.[51] This result indicates that muscle stem cell culture on soft PEG hydrogels can augment cell survival. Muscle stem cells cultured on soft PEG hydrogels expressed only 1/3 as much of the myogenic transcription factor myogenin, which indicates differentiation of muscle stem cells, as those cultured on stiff plastic culture dishes after 1 week of culture.[51] It has been demonstrated that soft substrates seem to increase cell numbers by enhancing cell viability and by preventing the differentiation of muscle stem cells in vitro. The function of muscle stem cells cultured on stiff and soft culture substrates was also assessed in vivo to verify that muscle stem cells cultured on soft PEG hydrogels retain their stemness.[51] In vivo functional assays indicated that culturing muscle stem cells on PEG hydrogels matching the physiological modulus (12 kPa) of muscle tissue best preserved their stemness (pluripotency). Muscle stem cells cultured on PEG hydrogels with an elasticity of 12 kPa were retained in mice after 30 days of transplantation, whereas markedly reduced engraftment was observed for muscle stem cells cultured on stiff plastic culture dishes.[51] Mice transplanted with muscle stem cells cultured on soft PEG hydrogels developed new myofibers resulting from regeneration. A transplantation assay of muscle stem cells cultured on various substrates in mice demonstrated that soft PEG hydrogel, but not stiff plastic culture dishes, guided the self-renewal of muscle stem cells.[51]

Healy et al. developed an interfacial hydrogel prepared by creating an interpenetrating polymer network with an oligopeptide containing RGD (arginine-glycine-aspartic acid) sequences on the surface with stiffness values ranging from 10 to 10 000 Pa (Figure 4).[62] Rat neural stem cells proliferated when cultured in serum-free media on the RGD peptide-modified interpenetrating network hydrogels with elastic moduli greater than 100 Pa. The highest expression of neural marker $\beta$-III tubulin (Table 2) in rat neural stem cells was observed on the RGD peptide-modified interpenetrating network hydrogels with elastic moduli of 500 Pa, near the physiological stiffness of brain tissue.[62] It was found that neuronal differentiation was favored on softer RGD peptide-modified interpenetrating network hydrogels under mixed glial and neuronal differentiation medium, whereas glial differentiation was favored on stiffer RGD peptide-modified interpenetrating network hydrogels in the same medium. Furthermore, cell spreading, self-renewal, and differentiation were inhibited on the RGD peptide-modified interpenetrating network hydrogels with elastic moduli of approximately 10 Pa.[62] This study demonstrates that physical (elasticity of the cell culture biomaterials) and biochemical (RGD peptide and soluble biochemical factors) factors can regulate the self-renewal and specific differentiation lineages of rat neural stem cells.

**Table 4. Some Research Studies for Differentiation of Stem Cells Cultured on Biomaterials Having Different Elasticity in 2-D Culture[a]**

| stem cell source | materials for stem cell culture having different stiffness | differentiation | medium | ref (year) |
|---|---|---|---|---|
| hMSCs | HyA–gelatin–PEG hydrogels | proliferation and secretion of cytokines | expansion medium | 103 (2009) |
| hMSCs | PAAm gel coated with collagen type I | proliferation and cell morphology | expansion medium | 21 (2010) |
| murine ESC (OGR1) | PAAm gel coated with collagen type I and rigid dishes coated with collagen type I | proliferation with pluripotency | expansion medium | 94 (2010) |
| hMSCs | patterned cross-linked methacrylated HyA gel containing RGDS | proliferation and cell morphology | expansion medium | 111 (2010) |
| murine ESCs (CGR8) | polyion complex nanofilm composed of PLL and HyA | proliferation and cell morphology | expansion medium | 104 (2010) |
| hESCs (H1, H9) | PDMS micropost treated by oxygen plasma | proliferation with pluripotency | expansion medium | 105 (2012) |
| hMSCs | thiol-modified HyA gels and PAAm coated with collagen type I | proliferation and cell morphology | expansion medium | 98 (2012) |
| mESCs (TG2$\alpha$E14) | PDMS coated with collagen type I | proliferation, osteoblast, and mesendoderm differentiation | expansion and differentiation medium | 58 (2009) |
| rat MSCs | PDMS grafted with poly(acrylic acid) | osteoblast | differntiation medium | 99 (2009) |
| hMSCs | PAAm gel coated with collagen type I | osteoblast | differentiation medium | 74 (2011) |
| hMSCs | gelatin–hydroxyphenylpropionic acid–tyramine gels cross-linked with HRP and $H_2O_2$ | osteoblast | unknown | 112 (2012) |
| umbilical cord MSCs (Wharton's jelly) | PAAm gel grafted with Collagen type I | osteoblast | differntiation medium | 16 (2012) |
| rat MSCs | PDMS coated with fibronectin and gelatin | osteoblast | differentiation medium | 85 (2012) |
| hMSCs (Stro-1 enriched cells) | polyalkyl acrylate coated with fibronectin | osteoblast | expansion medium | 113 (2012) |
| hMSCs | PAAm gel coated with collagen type I and fibronectin | osteoblast, adipocyte | differentiation medium | 87 (2009) |
| human epidermal stem cells, hMSCs | PDMS and PAAm gel grafted with collagen type I | osteoblast, adipocyte | mixed differentiation medium of osteoblast and adipocyte | 22 (2012) |
| hMSCs | PEG gel immobilized fibronectin | osteoblast, adipocyte, neural cell | differentiation medium | 114 (2011) |
| human placenta-derived MSCs, hADSCs | layer-by-layer polyion complex of cationic PLL and anionic HyA | osteoblast, adipocyte, chondrocyte | differentiation medium | 86 (2009) |
| murine embryonic mesenchymal progenitor cells (C3H10T1/2) | PCL nanofibers and PCL-PES nanofiber by electrospinning method | osteoblast, chondrocyte | differentiation medium | 106 (2011) |
| MSCs | PAAm gel coated with collagen type I | osteoblast, myocyte, and neuron | expansion medium | 32 (2006) |
| hMSCs | PAAm gel grafted with polytrimethlphosphate, polyallyamine, poly(acrylic acid) , or collagen type I | osteoblast, myocyte, neuron | expansion and differentiation medium | 66 (2011) |
| murine cardiac progenitor cells, MSCs | PLLA, PCL, PLGA film | cardiomyocyte | differentiation medium | 107 (2008) |
| hMSCs | PAAm gel grafted with collagen I | adipocyte, chondrocyte, smooth muscle cell, schwann cell | expansion and differentiation medium | 75 (2011) |
| hMSCs | PAAm gel grafted with collagen I | myocyte, neural cell | expansion medium | 93 (2011) |
| hMSCs | gelatin–hydroxyphenylpropionic acid gel cross-linked with HRP and $H_2O_2$ | myocyte, neuron | expansion medium | 73 (2010) |
| rat NSCs | RGD conjugated PEG-PAAm interpenetrating network gel | neuron and astrocytes | differentiation medium | 62 (2008) |
| embryonic cortices | xyloglucan gel grafted with poly(D-lysine) | neuron | differentiation medium | 110 (2009) |
| rat NSCs | polymethacrylamide–chitosan gel coated with laminin | neuron, oligodendrocyte, astrocyte | differentiation medium | 108 (2009) |
| bovine limbal stem cells | collagen type I gel coated with laminin | limbal epithelial cell | expansion medium | 100 (2012) |
| murine ESCs (ESD3) | fibrin gel | endoderm cell | differentiation medium | 109 (2012) |

[a]ESC, embryonic stem cells; MSC, mesenchymal stem cells; hMSCs, human MSCs; hADSCs, human adipose-derived stem cells; NSCs, neural stem cells; HyA, hyaluronic acid; PAAm, polyacrylamide; PDMS, polydimethylsiloxane; PES, polyethersulfone; PEG, poly(ethylene glycol); PLL, poly(L-lysine); PLLA, poly(L-lactic acid); PCL, poly($\varepsilon$-caprolactone); HRP, horse radish peroxidase.

Cell culture substrates with elastic modulus gradients (or storage modulus) are sophisticated materials used to systematically study stem cell differentiation guided by substrate stiffness. Several methods for the preparation of substrates with elastic modulus gradients have been developed and are shown in Figure 5. A monomer solution including a cross-linker is placed under a temperature gradient[85] (Figure 5a) or UV light[93] (Figure 5b), creating a gradient of cross-linking degrees and elastic moduli in the resulting substrate.

Tse and Engler created cross-linked PAAm hydrogels with radial elastic modulus gradients (1 kPa/mm) with a range of 1 to 14 kPa using photopolymerization under a gradient-patterned photomask.[93] It was found that hMSCs in the expansion medium migrated to the stiffer matrix and then differentiated into a more contractile myogenic phenotype on the cross-linked hydrogels grafted with collagen type I, whereas hMSCs expressing the neuronal marker $\beta$-III tubulin remained on soft regions of the gradient hydrogels.[93] Several studies suggest that soft cell culture materials guide MSCs into


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**Table 5. Some Research Studies for Differentiation of Stem Cells Cultured on Biomaterials Having Different Elasticity in 3-D Culture[a]**

| stem cell source | materials for stem cell culture having different stiffness | differentiation | medium | ref (year) |
|---|---|---|---|---|
| hMSCs | PAAm gel coated with collagen type I | proliferation and cell morphology | expansion medium | 21 (2010) |
| hMSCs | thiol-modified HyA gels and PAAm coated with collagen type I | proliferation and cell morphology | expansion medium | 98 (2012) |
| rat MSCs | gelatin-$\beta$ tricalcium phosphate sponge | osteoblast | differentiation medium | 101 (2005) |
| hMSCs | PAAm gel coated with collagen type I and fibronectin | osteoblast, adipocyte | differentiation medium | 87 (2009) |
| hMSCs | RGD-modified alginate gel | osteoblast, adipocyte | mixed differentiation medium of osteoblast and adipocyte | 19 (2010) |
| rat MSCs | collagen–glycosaminoglycan scaffold | osteoblasts, chondrocytes | expansion medium | 63 (2012) |
| hMSCs | thiotrophic gel composed of PEG–silica and RGD–alginate gel | osteoblast, myocyte, and neural cell, | expansion medium | 59 (2010) |
| goat MSCs | tyramine–HyA gel cross-linked with HRP and $H_2O_2$ | chondrocytes | differentiation medium | 64 (2012) |
| murine cardiac progenitor cells, MSCs | PLLA, PCL, PLGA having hexagonal or square grid geometry | cardiomyocyte | differentiation medium | 107 (2008) |
| rat MSCs | PEG nanofiber coated with collagen type I | smooth muscle cell, endothelial cell | expansion medium | 70 (2012) |
| embryonic cortices | xyloglucan gel grafted with poly(D-lysine) | neuron | differentiation medium | 110 (2009) |
| rat NSCs | alginate hydrogels | neuron | expansion medium | 77 (2009) |
| human fetal liver stem cells | thiol–HyA–PEG gels | endoderm stem/progenitor cells | expansion medium | 65 (2011) |
| hESCs | PLLA, PLGA, PCL coated with matrigel | mesoderm, endoderm, and ectoderm cell | differentiation medium | 102 (2011) |
| murine ESCs (ESD3) | fibrin gel | endoderm cells | differentiation medium | 109 (2012) |

[a]ESCs, embryonic stem cells; hESCs, human ESCs; MSCs, mesenchymal stem cells; hMSCs, human MSCs; NSCs, neural stem cells; PAAm, polyacrylamide; HyA, hyaluronic acid; PEG, poly(ethylene glycol), PLLA, poly(L-lactic acid), PCL, poly($\varepsilon$-caprolactone); PLGA; poly(lactic acid-*co*-glyconic acid).

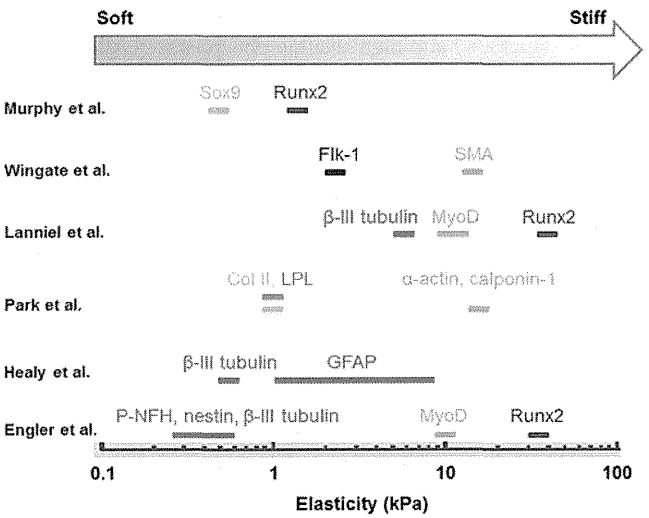


**Figure 3.** Proteins and transcription profiles of neural markers (red bar; nestin, P-NFH, and $\beta$-III tubulin), chondrocyte markers (yellow bar; collagen type II and Sox9), an adipocyte marker (orange bar; LPL), muscle transcription factors (green bar; MyD, $\alpha$-actin, calponin-1, and SMA), an osteoblast transcription factor (blue bar; Runx2), and an endothelial marker (dark blue bar; Flk-1) expressed in MSCs cultured on substrates of varied stiffness, as reported by Murphy et al.,[63] Wingate et al.,[70] Lanniel et al.,[66] Park et al.,[75] Healy et al.,[62] and Engler et al.[32]

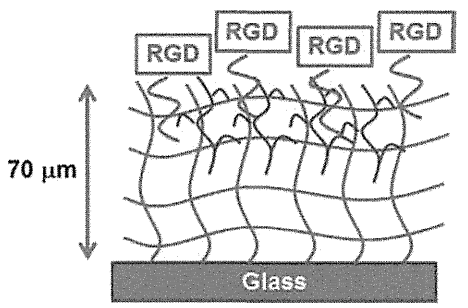


**Figure 4.** Schematic model developed by Healy et al. of an interpenetrating polymer network with oligopeptides containing surface RGD sequences with different stiffnesses. Modified with permission from ref 62. Copyright 2008 Elsevier Inc.

neuronal differentiation when MSCs are cultured in either expansion medium containing no induction factors or differentiation (induction) medium.[32,59,62,66,72,73,77]

Transforming growth factor $\beta$ (TGF-$\beta$) is known to promote MSC differentiation into either smooth muscle cells or chondrogenic cells. Therefore, Park et al. investigated whether the elasticity of the cell culture substrate affected the differentiation of hMSCs by culturing them on collagen type I gel (soft substrate), collagen-coated culture dishes (stiff substrate), and cross-linked PAAm hydrogels with varying stiffness (1, 3, and 15 kPa) grafted with collagen type I.[75] Cells cultured on soft substrates had less spreading, fewer stress fibers, and lower proliferation rates than hMSCs cultured on

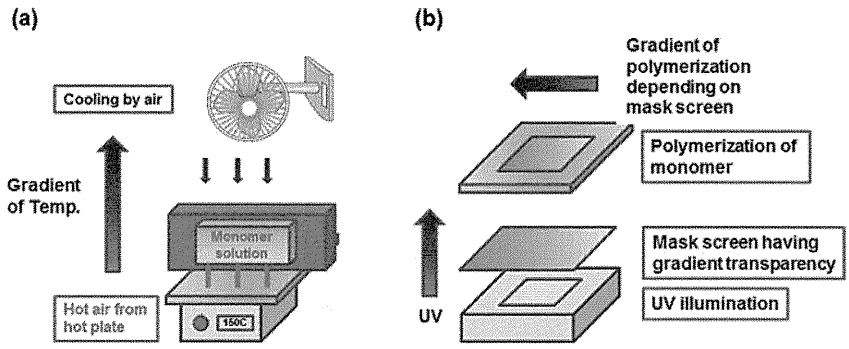


**Figure 5.** Preparation methods of substrates with elastic modulus gradients by monomer polymerization, shown by temperature gradient (a) or intensity of UV light (b). Modified with permission from ref 85 with Copyright 2012 Elsevier Inc. (a) and from ref 93 under a Creative Commons Attribution License (b).

stiff culture substrates. Additionally, hMSCs on stiff substrates displayed higher expression of smooth muscle cell markers ($\alpha$-actin and calponin-1) in expansion medium, whereas hMSCs on soft substrates displayed increases in expression of the chondrogenic marker collagen type II and an adipogenic marker (lipoprotein lipase, LPL) (Figure 3).[75] The addition of TGF-$\beta$ in the culture medium promoted the expression of smooth muscle cell markers and suppressed the expression of adipogenic markers on soft culture substrates. However, hMSCs were capable of differentiating into adipocytes on soft culture substrate when they were cultured in adipogenic differentiation medium.[75]

Lanniel et al. investigated differentiation lineages of hMSCs cultured on cross-linked PAAm hydrogels with varying elasticity (stiffness) and functional groups (trimethylphosphate, allylamine, and acrylic acid). Vinyl monomer was polymerized on the surface of the hydrogels using the plasma polymerization method. Cells were cultured on the hydrogels to evaluate the effect of different combinations of physical (substrate elasticity) and chemical cues (several functional groups) on hMSC differentiation.[66] The expression of the osteogenic marker Runx2 was highest in hMSCs cultured in expansion medium without added differentiation (induction) factors on PAAm hydrogels coated with phosphate polymer and with a stiffness of 41 kPa. The myogenic phenotype marker MyoD1 was most highly expressed in hMSCs cultured on PAAm hydrogels coated with poly(acrylic acid) with intermediate stiffness (10–17 kPa) (Figure 3).[66] Neurogenic differentiation as measured by $\beta$-III tubulin expression was highest on the softest hydrogels (6.5 kPa) coated with poly(acrylic acid) (Figure 3). Bone nodule formation and matrix calcification were observed on PAAm hydrogels stiffer than 10 kPa that had been coated with polyallylamine in osteogenic induction medium but not on hydrogels coated with collagen type I.[66] These results indicate that hMSC differentiation lineage can be regulated not only by the elasticity (stiffness) of cell culture substrates but also by surface chemistry (different types of functional groups) and differentiation induction factors.

### 2.2. Pluripotent Maintenance of ESCs, iPSCs, and MSCs on Soft Culture Substrate

It is important to maintain ESCs and iPSCs in an undifferentiated state in culture. However, several studies have suggested that undifferentiated ESC cultures actually contain heterogeneous populations identified by the fluctuating expression of various transcripts and cell-surface markers.[94,115−117] One of the major challenges in the field is to develop optimal culture conditions and culture substrates to maintain the self-renewal and pluripotency of ESCs and iPSCs.

Chowdhury et al. reported that mouse ESCs (mESCs) could maintain pluripotency (as measured by the expression of high levels of pluripotent genes and proteins (Oct3/4, Nanog) and by the generation of homogeneous undifferentiated colonies) when they were cultured in the absence of exogenous leukemia inhibitory factor (LIF) on soft substrates (0.6 kPa) matching the intrinsic stiffness of mESCs, while mESCs could not maintain their self-renewal and pluripotency on conventional stiff culture polystyrene dishes (>4 MPa) coated with collagen type I or on hydrogels with much stiffer moduli.[94] In general, it is necessary to add LIF to the culture medium during the culture and expansion of mESCs to maintain their self-renewal and pluripotency.[2]

However, in this study, several mESC cell lines were able to be cultured on soft substrates without the addition of LIF to the culture medium, maintaining the generation of homogeneous undifferentiated colonies with high expression of pluripotent markers (Oct3/4) and high alkaline phosphatase (ALP) activity (index of pluripotency, see Table 2) up to 15 passages, suggesting that these soft hydrogels could be used for long-term culture of mESCs.[94] It should be noted that laminin and vitronectin are more suitable proteins than collagen, which was used for their study, for ECM immobilized in the cell culture substrate for the maintenance of self-renewal and pluripotency of ESCs and iPSCs.[2] Therefore, it is interesting that mESCs can be cultured on soft hydrogels coated with collagen type I while keeping their self-renewal and pluripotency for 15 passages in the absence of LIF in the culture medium.[94] mESC colonies on soft cell substrates in culture medium without LIF generated low cell-matrix traction and had low stiffness. Both traction and stiffness of the colonies increased with increasing cell culture substrate stiffness, which was also accompanied by down-regulated expression of the pluripotent protein Oct3/4. This suggests that the self-renewal and pluripotency of mESCs can be maintained on soft cell culture substrates via the biophysical mechanism of facilitating the generation of low cell-matrix traction.[94]

However, there is a contradictory report that stiff (rigid) substrates can support the maintenance of hESC pluripotency.[105] Sun et al. macrofabricated elastomeric PDMS micropost arrays in which the height of the PDMS microposts controls substrate stiffness (elasticity) (Figure 6).[105] It is known that PDMS micropost arrays affect cell morphology, focal adhesions, cytoskeleton contractility, and stem cell differentiation.[105,118,119] Human ESCs were cultured on oxygen