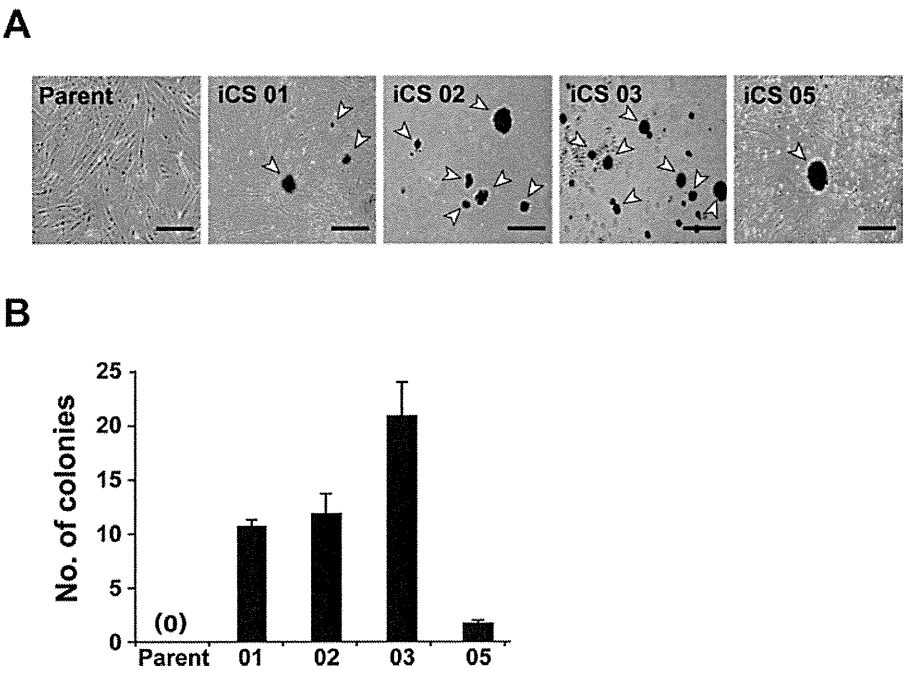

FIGURE 5: Colony formation of iCS cells. (A) Phase contrast micrograph of iCS colonies. iCS cells were cultured in methylcellulose medium on six-well plate dishes. Bars, 500 μm. (B) Number of iCS colonies. Error bars, SD ($n = 9$).

et al., 2005). In this study, *T* and *MITF* may act as inducers of chondrogenic fate determinant, and *BAF60C* may act as epigenetic modifier. *Klf4* was unnecessary for conversion from placental cells to chondrocytes, unlike the reprogramming described for iPS cells (Takahashi and Yamanaka, 2006) and chondrocytes (Hiramatsu *et al.*, 2011). *Baf60c* permits binding of *Gata4* to cardiac genes for reprogramming toward cardiomyocytes (Takeuchi and Bruneau, 2009). Likewise, *BAF60C* may initiate expression of chondrogenic gene sets in combination with *T* and *MITF* in iCS cells.

We showed that the transgene set was sufficient for chondrogenesis without exogenous *SOX9* gene. The *T* gene, one of the transcription factors used in this study, is a member of the T-box family of transcription factors (Papaioannou and Silver, 1998), all of which play key roles during early development, mostly in the formation and differentiation of normal mesoderm (Showell *et al.*, 2004). *T* is also transiently induced in vitro in rhesus monkey embryonic stem cells and mouse embryonal carcinoma cells undergoing mesodermal differentiation (Vidricaire *et al.*, 1994; Behr *et al.*, 2005). Both *T* and *Sox9* are downstream of mitogen-activated protein kinase signaling via fibroblast growth factor receptors in chondrogenesis (Hoffmann *et al.*, 2002). Chondrogenic conversion of the human extraembryonic cells was accompanied by induction of the endogenous *Sox9* gene; however, the induced *Sox9* did not complement reduction of chondrogenesis by siRNA to the *T* gene. *MITF* was also shown to be involved in chondrogenic conversion, although involvement of *MITF* in chondrogenesis has not been reported. *MITF* belongs to an evolutionarily ancient family of the bHLH/LZ proteins (Atchley and Fitch, 1997) and is known to regulate a number of genes of importance in differentiation and maintenance of the melanocytic lineage (Tachibana *et al.*, 1996); conversely, loss-of-function mutations in *MITF* produce depigmentation (Newton *et al.*, 2001). Disruption of *MITF* does not affect chondrocytic differentiation during development, and alternative factors may therefore be present in cells of chondrogenic lineage. The chondrogenesis by *T* and *MITF* may not reflect developmental or physiological pathway, but these two factors are requisite for in vitro chondrogenic conversion of nonchondrocytes.

Stability of the chondrogenic phenotype of iCS cells after long-term cultivation is probably due to lack of transgene silencing—in other words, continuity of transgene expression during cultivation and expression of endogenous chondrogenic genes in some cases. Chondrogenic induction is mediated at least in part by reprogramming, because expression of the endogenous *T* gene was initiated by the five factors. It remains unclear whether the process of the conversion is via dedifferentiation into multipotent, oligopotent stem cells or undifferentiated progenitors. Placenta is developmentally distinct from cartilage, and conversion from placenta to chondrocytes is considered lineage switching or transdifferentiation rather than differentiation. In β cells (Zhou *et al.*, 2008) and retinal cells (Osakada *et al.*, 2009), direct reprogramming is achieved with hepatocytes and iris cells, respectively, which are developmentally close to the generated cells. Successful reprogramming with other somatic cells for parental cells (Figure 6) indicates that the conversion is indeed reprogramming. Autoregulatory feedback and feedforward activation of downstream transcriptional regulators reinforces the expression of important cell fate–determining genes and helps to further stabilize the induced transcriptional program. Robust changes in transcriptional activity can be explained by genome-wide adjustments of repressive and active epigenetic features, such as DNA methylation, histone modifications, and changes of chromatin-remodeling complexes that further stabilize the new transcriptional network (Zhou *et al.*, 2008). It is possible that certain subpopulations of cells are "primed" to respond to these factors, depending on their preexisting transcriptional or epigenetic states (Yamanaka, 2009).

Our study opens an avenue to generate human chondrocytes. Even with the presence of retroviral integration, human iCS cells can possibly be used for tissue engineering experiments such as screening of suitable scaffold of cartilage and can be an alternative to murine ATDC5 teratocarcinoma cells, an ideal cell line for development of tissue engineering strategies aimed at cartilage generation. Once the safety issue, that is, cell transformation, is overcome, iCS cells should also be applicable for repair of defective cartilage in regenerative medicine. We should, however, exercise caution because human iCS cells are not identical to human chondrocytes from the viewpoint of global gene expression. Further studies are essential to determine whether a nontransformed counterpart of iCS can replace chondrocytes in medical applications.

MATERIALS AND METHODS

Preparation of tissue and procedure for cell culture

A human placenta was collected after delivery of a male neonate with the approval of the Ethics Committee of the National Research Institute for Child Health and Development, Tokyo, Japan. Signed informed consent was obtained from the donors, and the specimens were irreversibly deidentified. All experiments handling human cells and tissues were performed in line with the Tenets of the Declaration of Helsinki. This study was wholly carried out at the National Research Institute for Child Health and Development, Tokyo, Japan.

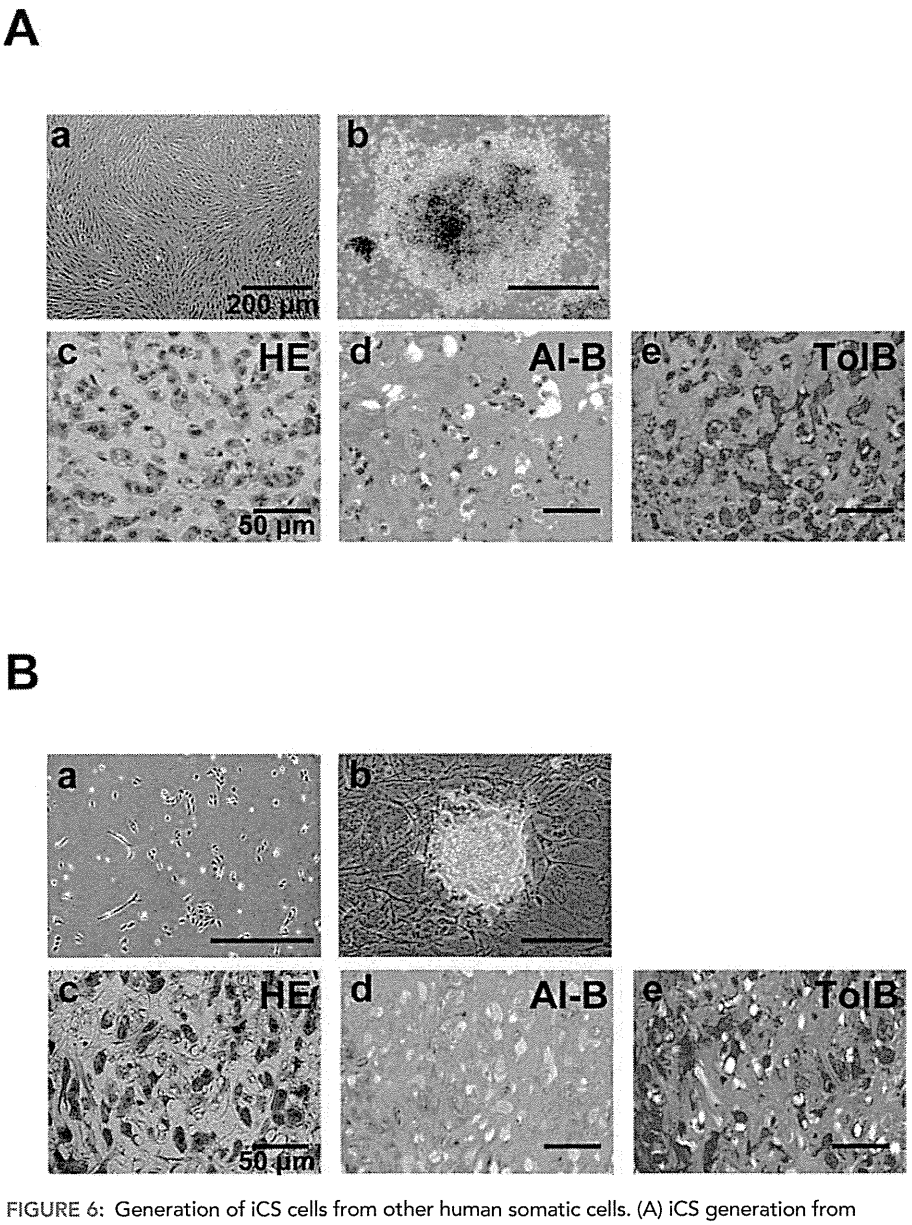

FIGURE 6: Generation of iCS cells from other human somatic cells. (A) iCS generation from human menstrual blood. (a) Phase contrast micrograph of menstrual blood–derived cells. (b) Phase contrast micrograph of iCS cells from menstrual blood. (c–e) Histological analysis of iCS cartilage generated from menstrual blood. (c) HE stain, (d) Alcian blue (Al-B), (e) toluidine blue (TolB). Bars, 200 (a, b) and 50 μm (c–e). (B) iCS generation from human placental artery (hPAE). (a) Phase contrast micrograph of hPAE cells. (b) Phase contrast micrograph of iCS cells from hPAE cells. (c–e) Histological analysis of iCS cartilage generated from hPAE cells. (c) HE stain, (d) Alcian Blue (Al-B), (e) toluidine blue (TolB). Bars, 200 μm (a, b) and 50 μm (c–e).

To isolate placenta-derived cells, we used the explant culture method, in which the cells were outgrown from pieces of chorion and decidual cells attached to dishes (Supplemental Figure S1). Briefly, the smooth chorion and decidua were cut into pieces ~2 mm^3 in size. The pieces were washed in DMEM (high glucose; Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin–streptomycin (Life Technologies, Carlsbad, CA), 1 μg/ml amphotericin B (Life Technologies), and 4 U/ml Novo-Heparin Injection 1000 (Mochida Seiyaku, Tokyo, Japan) until the supernatant was free of erythrocytes. Some pieces were attached to the substratum in a 10-cm dish. The cells migrated out from the cut ends after ~20 d of incubation at 37°C in 5% CO_2. The migrated cells were harvested with Dulbecco's phosphate-buffered saline with 0.1% trypsin and 0.25 mM EDTA for 5 min at 37°C and counted. The harvested cells were reseeded at a density of 3×10^5 cells in a 10-cm dish. Confluent monolayers of cells were subcultured at a 1:8 split ratio onto new 10-cm dishes. The culture medium was replaced with fresh culture medium every 3 or 4 d.

Plasmid construction

Full-length of transcription factors *BCL6*, *T*, *c-MYC*, *MITF*, and *BAF60C* were amplified from cDNAs prepared from total RNA of adult human heart cells (Clontech, Mountain View, CA) and embryonic body-formed iPS cells (day 3–4) by reverse transcription-PCR with the primers listed in Supplementary Table 1, and by digestion with *Hin*dIII sites of pMXs and T4 DNA polymerase accessory they were used for DNA synthesis. The cDNA plasmid was subcloned into pMXs vector using *Hin*dIII restriction sites. pMXs was a gift from T. Kitamura (Tokyo University, Tokyo, Japan).

Retroviral infection

293FT cells (5×10^6) were plated in a 10-cm dish and incubated overnight. The next day, the cells were cotransfected with pMXs-*BCL6*, pMXs-*T*, pMXs-*c-MYC*, pMXs-*MITF*, pMXs- *BAF60C*, pCL-GagPol, and pHCMV-VSV-G vectors with TransIT-293 reagent (Mirus, Madison, WI). The medium was replaced with fresh medium 24 h after transfection. The medium was collected after 48 h as the virus-containing supernatant. Placenta-derived cells in primary culture were seeded at 1×10^5 cells per six-well plate 1 d before infection. The virus-containing supernatants were filtered through a 0.45-μm pore-size filter, ultracentrifuged at 8000 × *g* for 24 h, and then resuspended in Knockout-DMEM (Invitrogen) supplemented with 2 mg/ml Polybrene (Nacalai Tesque, Kyoto, Japan). Cells in six-well plates were transfected with siRNA to the *p53* gene (si*TP53*) using RNAiMAX (Invitrogen, Carlsbad, CA) according to the protocols recommended by the manufacturer (Hong *et al.*, 2009). Cells were transfected overnight, washed, and resuspended in Opti-MEM (Life Technologies/Invitrogen) and were used for virus infection. Cells were trypsinized and plated in six-well plates at 1×10^5 cells per well for 24 h before transfection. Retrovirus cocktail was transferred to cells and incubated for 24 h. After infection, cells were cultured for 5 d and replaced on an irradiated MEF feeder layer in six-well plates. The medium was changed every 2 d with fresh Poweredby10 (GP Biosciences, Yokohama, Japan). Colonies were picked up and transferred into 2-ml Poweredby10 medium at ~10 d after infection. The colonies were mechanically dissociated to small clumps by pipetting up and down. The cell suspension was transferred on irradiated MEF feeder in 60-mm dish (Iwaki; Asahi Techno Glass, Tokyo, Japan). We defined this stage as passage 1.

RT-PCR

Total cellular RNA was isolated from cells using an Isogen extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (1.0 μg each) for RT-PCR was converted to cDNA with Superscript III RNase H– reverse transcriptase (Invitrogen), according to the manufacturer's manual. PCR conditions were optimized, and the linear amplification range was determined for each primer by varying annealing temperature and cycle number. PCR products were identified by positive control size. Primer sequences are provided in Supplemental Table S1 and Supplemental Figure S5. RT-PCR was performed by using the primers for the genes *BCL6*, *T*, *c-MYC*, *MITF*, *BAF60C*, *COL1A1*, *COL2A1*, *COL10A1*, *CRTL1*, *ACAN*, *SOX5*, *SOX6*, *SOX9*, *TERT*, and *GAPDH*. Adult chondrocyte RNA (Cell Applications (San Diego, CA), human heart RNA (Clontech), and human iPS cell RNA were used as positive controls for RT-PCR analysis. PCR was performed with KOD FX DNA polymerase and PCR buffer (Toyobo, Osaka, Japan), 35 cycles, with each cycle consisting of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s, with additional 5-min incubation at 72°C after completion of the final cycle. The PCR products were size fractionated by 2% agarose gel electrophoresis.

Quantitative RT-PCR

RNA was extracted from cells using an Isogen extraction kit according to the manufacturer's protocol. An aliquot of total RNA was reverse transcribed by using an oligo(dT) primer. For the thermal cycle reaction, the cDNA template was amplified (PRISM 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA) using the Platinum Quantitative PCR SuperMix-UDG with ROX (11743-100; Invitrogen) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s, and 60°C for 1 min) after an initial denaturation (95°C for 2 min). Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed using a melting curve analysis (using software provided by Applied Biosystems) and gel analysis. mRNA levels were normalized using *GAPDH* as a housekeeping gene.

Flow cytometric analysis

Cells were stained for 1 h at 4°C with primary antibodies and immunofluorescent secondary antibodies. The cells were then analyzed on a Cytomics FC 500 (Beckman Coulter, Brea, CA), and the data were analyzed with FlowJo, version 7 (Tree Star, Ashland, OR). Antibodies against human CD44, CD49c, CD151, and CD166 (all from BD Biosciences PharMingen, San Diego, CA) were adopted as primary antibodies.

Histological analysis

Infected cells were harvested by trypsin/EDTA treatment, collected into tubes, and centrifuged at 300 × *g* for 5 min, and the pellets were suspended in the DMEM medium. The same volume of Basement Membrane Matrix (BD Biosciences PharMingen) was added to the cell suspension. The cells were implanted subcutaneously to a BALB/c-nu/nu mouse (CREA, Tokyo, Japan) for 7 wk. Tumors were dissected and fixed with phosphate-buffered saline containing 4% paraformaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin.

Western blotting

Semiconfluent cells were lysed with CelLytic M cell Lysis Reagent (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates (20 μg each) were separated by electrophoresis on NuPAGE Novex Tris-Acetate gel (Invitrogen), and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA). The membrane was soaked in protein blocking solution (Blocking One solution; Nacalai Tesque) for 30 min at room temperature before an overnight incubation at 4°C with primary antibody for COL2A1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (1:1000; Cell Signaling, Beverly, MA) also diluted in blocking solution. The membrane was then washed three times with TBST (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20), incubated with a horseradish peroxidase–conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody for 45 min, and washed three times with TBST. The signal was detected by an enhanced ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ) and an LAS3000 imaging system (Fujifilm, Tokyo, Japan), following the manufacturers' recommendations.

siRNA transfection

The infected cells in six-well plates were transfected with siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the protocols recommended by the manufacturer (Cui *et al.*, 2011). The cells were harvested 48 h after transfection and analyzed by real-time PCR and RT-PCR.

Gene expression microarray

Total RNA was prepared from duplicate biological samples, and human adult chondrocyte RNA was purchased from Cell Applications. With the use of Low RNA Input Fluorescent Linear Amplification Kits (Agilent Technologies, Santa Clara, CA), cDNA was reverse transcribed from each RNA sample, as well as from a pooled reference control, and cRNA was then transcribed and fluorescently labeled with Cy3. cRNA was purified using an Agilent One Color Spike Mix Kit (Agilent Technologies). We hybridized 1650 ng of Cy3-labeled and amplified cRNA to Agilent 4 × 44 K whole human genome microarrays and processed it according to the manufacturer's instructions. The array was scanned using a G2505B DNA microarray scanner (Agilent Technologies). The image files were extracted using Feature Extraction software, version 10.7.3.1 (Agilent Technologies), with background subtraction and dye normalization. The data were analyzed using GeneSpring GX 10.0 (Agilent Technologies).

Hierarchical clustering analysis and principal component analysis

To analyze the microarray data, we used agglomerative hierarchical clustering and PCA. The hierarchical clustering techniques classify data by similarity using NIA Array Analysis (http://lgsun.grc.nia.nih.gov/ANOVA), and their results are represented by dendrograms. PCA is a multivariate analysis technique that finds major patterns in data variability using TIGR MeV (www.tm4.org/mev.html; Toyoda *et al.*, 2011).

Southern blot analysis

For Southern blot analysis, genomic DNA was isolated using the DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, digested with *Spe*I and *Bam*HI for *BCL6*, *Spe*I and *Nco*I for *BAF60C*, *Spe*I and *Bgl*II for *MITF* and *T*, and *Spe*I and *Mfe*I or *Bam*HI and *Mfe*I for *c-MYC*, and separated via 0.8% agarose gel electrophoresis. Transfer was to Hybond-N membranes (GE Healthcare). The membrane was fixed under UV irradiation. The probe was hybridized to the blot and detected using CDP-Star detection reagent (GE Healthcare). Signals from the labeled DNA were quantified using a Hyper film ECL (GE Healthcare).

Short-tandem repeat analysis

Genomic DNA was isolated from cultured cell samples using DNeasy columns (Qiagen). This was used as template for STR analysis using

the PowerPlex 16 System (Promega, Madison, WI) and PRISM instrumentation (Applied Biosystems). Numbers shown denote base pair lengths of the 15 autosomal fragments. The analysis was carried out at Nihon Gene Research Laboratories (Sendai, Japan).

Karyotypic analysis

The cells were fixed with methanol:glacial acetic acid (2:5) three times and dropped onto glass slides (Nihon Gene Research Laboratories). Chromosome spreads were Giemsa banded and photographed. A minimum of 10 metaphase spreads were analyzed for each sample, and they were karyotyped using a chromosome imaging analyzer system (Applied Spectral Imaging, Carlsbad, CA).

Colony formation assay

A total of 50,000 cells were resuspended in MethoCult H4034 medium and plated into a six-well plate. Colonies were counted 14 d after plating.

Gene Expression Omnibus accession numbers

National Center for Biotechnology Information Gene Expression Omnibus gene expression microarray data were submitted under accession number GSE29745.

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CHEMICAL REVIEWS

Review

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Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation

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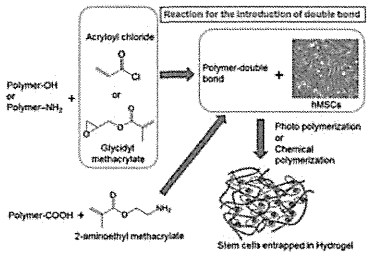

CONTENTS

1. INTRODUCTION

Each year, millions of people suffer loss or damage to organs and tissues due to accidents, birth defects, and disease. Stem cells are an attractive prospect for tissue engineering and regenerative medicine because of their unique biological properties. Embryonic stem cells (ESCs) derived from

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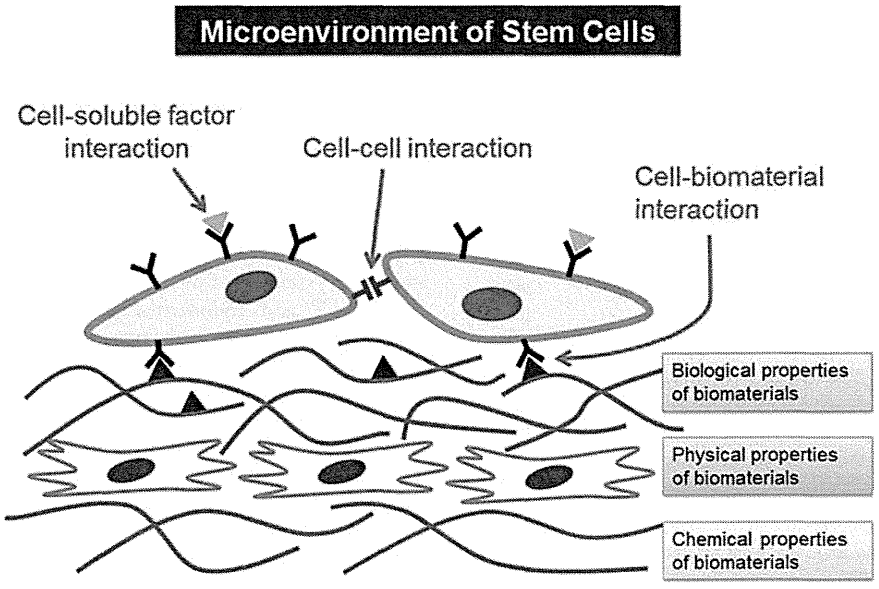

Figure 1. Schematic representation of the microenvironment and niches of stem cells and their regulation by the following factors: (a) soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (b) cell–cell interactions; (c) cell–biomaterial interactions. Biological, physical, and chemical properties of biomaterials also regulate stem cell fate.

preimplantation embryos have the potential to differentiate into any cell type derived from the three germ layers—the ectoderm (epidermal tissues and nerves), mesoderm (muscle, bone, and blood), and endoderm (liver, pancreas, gastrointestinal tract, and lungs).[1] The basis of pluripotency lies in conserved regulatory networks composed of numerous transcription factors and multiple signaling cascades. Together, these regulatory networks maintain human ESCs (hESCs) in a pluripotent and undifferentiated state, and alterations in the stoichiometry of these signals promote differentiation. hESCs have been shown to generate multipotent stem and progenitor cells in vitro and are capable of differentiating into a limited number of cell fates, and thus they have great potential for use in transplantation of cells and tissues into patients.[2]

Although hESCs are promising donor sources for cell transplantation therapies,[1] they face immune rejection after transplantation. Furthermore, ethical issues regarding human embryos hinder their widespread usage. These concerns can be circumvented if pluripotent stem cells can be derived directly from patients' own somatic cells.[3] Recently, pluripotent stem cells similar to ESCs, known as induced pluripotent stem cells (iPSC's), were derived from adult somatic cells by inducing a "forced" expression of certain pluripotent (stem cell) genes[4−6] such as Oct3/4, Sox2, (c-myc), and klf-4, or certain miRNAs[7] or proteins (piPS).[8] iPSC's are believed to be similar to ESCs in many respects, including the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, pluripotency, and differentiability.

The pluripotent nature of iPSC's opens many avenues for potential stem cell-based regenerative therapies and for development of drug-discovery platforms.[9,10] The nearest-term therapeutic uses of iPSC's may exist in the transplantation of differentiated nerve cells or β-cells for treatment of Parkinson's Disease and diabetes, respectively, which arise from disorders of single cell types. However, there are several barriers to the clinical application of iPSC's, such as the use of viral vectors, cultivation using xeno-derived materials [e.g., mouse embryonic fibroblasts (MEFs)], and the extremely low efficiency of iPSC generation.[11]

Stem cells have also been isolated from a variety of somatic tissues, including hematopoietic stem cells (HSCs) derived from umbilical cord blood and mesenchymal stem cells (MSCs) derived from bone marrow, umbilical cord blood, umbilical cord, dental pulp, and tissues such as fat. There have been no reports to date of MSCs or fetal stem cells differentiating into tumors, unlike ESCs and iPSC's. Consequently, HSCs, MSCs, and fetal stem cells are the most promising sources of cells for tissue engineering and cell therapies. Currently, MSCs are thought to be the most widely available autologous source of stem cells for practical and clinical applications. Fetal stem cells derived from amniotic fluid are pluripotent cells capable of differentiating into multiple lineages, including cell types of the three embryonic germ layers. Bone marrow MSCs, adipose-derived stem cells (ADSCs), and amniotic fluid stem cells may be more suitable sources of stem cells in regenerative medicine and tissue engineering than ESCs and iPSC's because of ethical concerns regarding their use and concerns about xenogenic contamination arising from the use of mouse embryonic fibroblasts (MEFs) as a feeder layer for ESC and iPSC culture.[11]

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. Therefore, mimicking stem cell microenvironments and niches using biopolymers will facilitate the production of large numbers of stem cells and specifically differentiated cells needed for in vitro regenerative medicine. Several factors in the microenvironment and niches of stem cells influence their fate: (i) soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (ii) cell–cell interactions; (iii) cell–biomacromolecule (or biomaterial) interactions; and (iv) physical factors, such as the rigidity of the environment (Figure 1). Some excellent review articles addressing the

engineering of stem cell microenvironments and niches using natural and synthetic biopolymers are listed in Table 1.[11−22]

Table 1. Key Review and Articles Dealing with Biopolymers for Culture and Differentiation of Stem and Progenitor Cells

author	contents	ref (year)
Lee and Mooney	hydrogels for tissue engineering	12 (2001)
Little et al.	biomaterials for neural stem cell microenvironments	13 (2008)
Higuchi et al.	polymeric materials for ex vivo expansion of HSCs	16 (2009)
Mei et al.	combinatorial development of biomaterials for clonal growth of human pluripotent stem cells	17 (2010)
Melkoumian et al.	synthetic peptide-acrylate surfaces for long-term self-renewal of hESCs	18 (2010)
G. J. Delcroix et al.	adult cell therapy for brain neuronal damages and the role of tissue engineering	22 (2010)
Higuchi et al.	biomaterials for the feeder-free culture of hESCs and human iPSC's	11 (2011)
Balakrishnam and Banerjee	biopolymer-based hydrogels for cartilage tissue engineering	14 (2011)
Kim et al.	design of artificial extracellular matrices for tissue engineering	15 (2011)
Engler et al.	matrix elasticity directs stem cell lineage	19 (2006)
Gilbert et al.	substrate elasticity regulates skeletal muscle stem cell self-renewal	20 (2010)
Huebsch et al.	harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate	21 (2010)

These articles focus on biopolymers employed for maintenance of pluripotency of hESCs, iPSC's, or hematopoietic stem cells (HSCs),[16−18] and for specific differentiation lineages such as chondrocytes (cartilage), muscle cells, and neural cells.[13,14,20] There have been no review articles specifically describing extracellular matrix (ECM) scaffolds (ECM in 3D) or ECM-immobilized dish coatings (ECM in 2D) that guide stem cell fates and differentiation. Therefore, this review focuses on the chemical, physical, and biological characteristics of natural biopolymers, especially ECM proteins, which are the major functional biopolymers, and deals with the ability of these biopolymers to guide differentiation of MSCs into osteogenic, chondrogenic, adipogenic, cardiomyogenic, and neural cell lineages.

2. CELL SOURCES AND ANALYSIS OF DIFFERENTIATION LINEAGES OF MSCS

2.1. Cell Sources

Human MSCs (hMSCs), including fetal stem cells, are one of the most widely available autologous sources of stem cells for clinical applications. hMSCs can be obtained from bone marrow,[23,24] adipose tissue,[25,26] dental pulp,[27] and urine,[28] among other sources. Fetal stem cells can be obtained from amniotic fluid,[29−31] umbilical cord,[32−34] menstrual blood,[35,36] umbilical cord blood,[25,34,37] and placenta.[38,39] hMSCs derived from bone marrow and fat are primarily used for biomaterials research on stem cell culture and differentiation because bone marrow MSCs and ADSCs are easily accessible and can be obtained in large quantities. Bone marrow MSCs (BMSCs) are now commercially available from several companies. Stem cell research is facilitated with these stem cell sources because it is not necessary to obtain permission from ethics committees of the Institutional Review Board (IRB) for use of commercially available MSCs. Otherwise, informed consent from donors and permission from the IRB must be obtained.

2.2. Analysis of Differentiation Lineages

MSCs are multipotent stem cells that can be differentiated into various mesodermal lineages, including osteoblasts, chondrocytes (cartilages), adipocytes, myocytes, and cardiomyocytes.[19,40,41] MSCs are also reported to be able to differentiate into ectodermal lineages (e.g., neuron, oligodendrocyte, astrocyte, neural stem cells, and dopamine-secreting cells)[22,42−45] and endodermal lineages (hepatocytes and β-cells),[31,46−52] although with lower probability than mesoderm lineages. Table 2 summarizes methods for characterizing specific differentiated cells from MSCs.[11,34,46,48,51−87]

MSCs differentiate into an osteogenic phenotype in vitro when supplements such as ascorbic acid, β-glycerophosphate, dexamethasone, and/or bone morphogenic protein 2 (BMP-2) are added to the culture medium. Figure 2 shows the expression of several genes and proteins, as well as mineral deposition, by MSCs upon osteogenic differentiation. Runt-related transcription factor 2 (Runx2, also known as Cbfa1, Pebp2αA, and AML3) is a master regulator of osteogenic gene expression and osteoblast differentiation, and it is an early marker of osteogenesis.[88−90] Runx2 activity is stimulated by mitogen-activated protein kinase (MAPK) signaling and is negatively regulated by thrombin-like enzyme 2 (TLE2). Alkaline phosphatase (ALP) activity is an early osteogenic marker, and osteopontin and osteocalcin are late osteogenic markers.[88] Mineral deposition is generated in the late stage of osteogenic differentiation and is detected by Alizarin Red staining (calcium deposition) and von Kossa staining (calcium phosphate deposition).[57,60,62]

MSCs commit to a chondrogenic phenotype when supplied with transforming growth factor-β1 (TGF-β1). Chondrogenic differentiation of MSCs is typically determined by immunostaining for specific proteins, such as collagen type II and Sox9, dye labeling of glycosamino glycans, and evaluation of expression of chondrogenic proteins or transcription factors (such as collagen type II and type X, cartilage oligomeric protein, aggrecan, and Sox9) (Table 2).[63,64,67,70,91] Sulfated glycosaminoglycans (sGAG's) are visualized by staining with Alcian blue.[91] Accumulation of sulfated proteoglycans are also visualized by Safranin O staining.[72]

Only a few groups have investigated adipogenic differentiation of MSCs cultured on natural and artificial biomaterials[53,62,70, 74,75,92] because adipose tissue is in less demand in clinical usage than osteoblasts and cartilage cells. Adipogenic differentiation is also analyzed by immunostaining for specific proteins (vimentin), dye staining of oil droplets, and measuring expression of transcription factors or other marker proteins, such as peroxisome proliferator-activated receptorγ [PPARγ] and adipocyte Protein 2 (aP-2).[53,61,62, 74,75,92] aP-2 is a carrier protein for fatty acids that is primarily expressed in adipocytes.[93] Preadipocytes and mature adipocytes contain multiple or single lipids in cell bodies, respectively. Therefore, Oil Red O or Nile red staining of preadipocytes and mature adipocytes is frequently used for the detection of lipids.

Neural differentiation of MSCs is primarily analyzed by observing characteristic morphologies of neurons, astrocytes, oligodendrocytes, and microglia. Neuronal progenitor cells and early-stage neurons are also identified by Sox1, Sox2, and CD133 gene expression and by nestin and β-tubulin-III

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Table 2. Characterization of Differentiation of MSCs into Specific Lineages [Osteoblasts and Chondrocyte (Cartilages)]

differentiation lineage	characterization	specification	ref (example)
1. Osteoblast	morphology	spread shape tends to differentiate into osteoblasts, bonelike nodule formation	53−55
	protein level (immunostaining)	**collagen I**, osteocalcin, osteonectin	56, 57
	surface marker analysis and immunostaining	osteopontin, bisphosphonate [2-(2-pyridinyl)ethylidene-BP] (PEBP), alkaline phosphatase (ALP)	34, 58
	enzyme activity	**alkaline phosphatase**	
	gene level	**runt-related transcription factor 2** [Runx2 or core binding protein A-1 (CBFA-1)], **osterix** (OSX), **osteocalcin** (OCN), **osteopontin** (OPN), **bone sialoprotein** (BSP), alkaline phosphatase, integrin-binding sialoprotein (IBSP), bone γ-carboxyglutamate protein (BGLAP)	34, 58−61
	dye staining	**Alizarin Red staining** (calcium)	62
	mineral deposition	**von Kossa staining** (calcium phosphate)	57, 60
2. Chondrocytes	protein level (immunostaining)	**collagen type II** (Col II), **collagen type X** (Col X), **aggrecan** (AGN), Sox-9, chondroitin-4-sulfate, chondroitin-6-sulfate, sulphated glycosaminoglycans	56, 57, 63−68
	glycosaminoglycan assay	glycosaminoglycan content	
	dimethylmethylene blue (DMMB) assay	proteoglycan (PG) content	69
	hydroxyproline assay	collagen content	65
	gene level	**collagen II**, collagen IX (Col IX), **collagen X**, collagen XI (Col XI), **aggrecan**, Sox 5, Sox 6, **Sox 9**, **cartilage oligomeric protein** (COMP), xylosyltransferase I (XT-1), α-4-*N*-acetylhexosaminyltransferase (EXTL2), β-1,4-*N*-acetylgalactosaminyltransferase (GalNAcT), glucuronyl C5 epimerase (GlcACSE)	63, 64, 67, 70−73
	dye staining	**Safanin O staining (proteoglycan)**, **Alcian blue staining (proteoglycan)**, EVG-staining, Masson's trichrome staining	34, 62, 64, 67, 70, 72
3. Adipocytes	morphology	round shape cells tends to differentiated into adipocytes	53, 54
	protein level	vimentin, adipocyte lipid-binding protein (ALBP)	53, 74
	enzyme activity	glycerol-3-phosphate dehydrogenase activity	75
	gene level	PPARγ, aP-2	61
	staining	*Oil red O and Nile red staining for lipid droplet*	62
4. Neural cells	morphology	neuronal-like cells having long neurites	76
	protein level	nestin, neuron-specific class III β-tubulin (TuJ1), galactosylceramidase (GalC), glial fibrillary acidic protein (GFAP), β-tubulin-III, microtubule-associated protein 2 (MAP2), O4, tyrosine hydroxylase (TH), neurofibromatosis (NFM), neurone-specific enolase (NSE)	76−81
	gene level	nestin, Musashi 1, neuron-specific class III β-tubulin (TuJ1), glial fibrillary acidic protein, microtubule-associated protein 2, Sox1, Sox2, CD133, tyrosine hydroxylase, neurofibromatosis, Nurr1, dopamine transporter (DAT), dihydropyrimidinase-related protein 2 (DRP-2), purine-sensitive aminopeptidase (PSA)	11, 61, 76, 81, 82
5. Cardiomyocytes	morphology	contractile cells	
	protein level	**cardiac troponin T (cTnT)**, **desmin**, myosin light chain (MLC), myosin heavy chain (MHC)	81
	gene level	Nkx2.5, GATA-4, MYH-6, TNNT2, TBX-5, myosin light chain (Mlc2a, MLC-2 V), tropomyosin, cTnI, ANP, **desmin**, **myosin heavy chain** (α-MHC, **β-MHC**), **cardiac troponin T**, Isl-1, and Mef2c	11
	electrocardiogram	electrocardiogram	
6. Smooth muscle cells	protein level	α-smooth muscle actin (ASMA), h1-calponin (CALP), SM2	83
	gene level	α-smooth muscle actin, h1-calponin, caldesmon, Smemb, SM22α, SM1, SM2	83
7. Epidermis	protein level	keratin 10 (early marker), filaggrin (intermediate marker), involucrin (late marker)	84
	gene level	keratin 10 (early marker), filaggrin (intermediate marker), involucrin (late marker)	84
8. Hepatocyte	morphology	oval cell morphology, small round cell morphology	46

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Table 2. continued

differentiation lineage	characterization	specification	ref (example)
	protein level	CXCR4 (endoderm), α-fetoprotein (AFP), albumin (ALB), asialoglycoprotein receptor (ASGPR), cytochrome P450 (CYP_1A_1), hepatocyte nuclear factor-1α (HNF-1α), hepatocyte nuclear factor-3β (HNF-3β), hepatocyte nuclear factor-4α (HNF-4α), CCAAT-enhancer binding protein α (C/EBPα), cytokeratin-18 (CK18), cytokeratin-19 (CK19), low-density lipoprotein (LDL), GATA4	46, 51, 52, 86, 87, 113
	gene level	Sox17 (endoderm), Foxa2 (endoderm), Gata6 (endoderm), α-fetoprotein, albumin, hepatocyte nuclear factor-1α, hepatocyte nuclear factor-3β, hepatocyte nuclear factor-4α, cytokeratin 18, cytokeratin-19, asialoglycoprotein receptor, tryptophan oxygenase (TO), cytochrome P450 (CYP1A1, CYP2B6), CCAAT-enhancer binding protein α, glucose 6-phosphate (G6P), GATA4	46, 51, 52, 86, 87, 113
	urea assay	urea production	46, 51, 113
	albumin assay	albumin production	52, 86, 113
	glycogen assay	glycogen production	46, 52, 113
	α-fetoprotein assay	α-fetoprotein production	52, 86
	pentoxyresorufin (PROD) assay	cytochrome P450 activity	113
	staining	periodic acid–Schiff (PAS) staining for glycogen storage	46, 113

immunostaining. Mature neurons express neuron-specific class III β-tubulin (Tuj1), microtubule-associated protein 2 (MAP2), neuron-specific enolase (NSE), and purine-sensitive amino-peptidase (PSA). Oligodendrocytes express galactosylceramidase (GalC) and O4. Dopaminergic neurons express tyrosine hydroxylase (TH), neurofibromatosis (NFM), and dopamine transporter (DAT). Nerve cells are electrically excitable cells that transmit information by electrical and chemical signaling. Therefore, electrical and action potentials in nerve cells can be monitored using electrodes.

3. PREPARATION OF CULTURE MATRIX

Biomimetic stem cell cultures can be categorized as two-dimensional (2D) or three-dimensional (3D). 2D culture is useful for basic research to investigate the fundamental interactions between cells and immobilized nanosegments on dishes, but 3D culture of stem cells in biomaterials is essential for clinical applications. Figure 3 shows some examples of biomaterial designs for carrying stem cells, as well as direct injection of biomaterials without cells. The injection of hydrogels or scaffolds containing stem cells is categorized as 3D cultures. Cell sheets prepared on a surface-grafting polymer having low critical solution temperature (LCST), such as poly(*N*-isopropylacrylamide) (poly(NIPAM)), can be prepared on 2D dishes.[94,95] Recently, patch sheets of immobilized antibodies or ligands targeting specific stem cells, which recruit the stem cells from the patient's body, are reported to be effective in gathering autologous stem cells at sites of injury.[40] The following sections describe methods for (a) surface immobilization of ECM proteins and ECM-mimicking peptides on 2D culture dishes and (b) preparing hydrogels or scaffolds containing ECM proteins and ECM-mimicking peptides for 3D culture of stem cells.

3.1. ECM Immobilization on 2D Dishes

Typically, 2D cell culture dishes are coated with ECM proteins or ECM-mimicking peptides. Tables 3 and 4 show examples of the ECM proteins and ECM-mimicking peptides used to coat culture dishes and their binding sites on stem cells.[16,18,53,58,71, 83,91,96−118] Collagen types I, II, and IV, gelatin, laminin, laminin-1, laminin-5, vitronectin, and fibronectin are typically used as coating materials.[58,71,83,91, 96−98,100−102] ECM-mimicking peptides (e.g., RGD, DGEA, YIGSR, IKVAV, KRSR, P15, and GFOGER) are commonly used as coating or grafting materials.[16,18,53,97,103−118] Covalent binding is preferable for long-term effects in culture, but noncovalent coating is the simplest method for the preparation of dishes with immobilized ECM proteins or ECM-mimicking peptides. Figure 4 summarizes typical surface reactions for the covalent immobilization of ECM proteins and peptides on dishes. Proteins and ECM-mimicking peptides should be used in aqueous solution, as they are unstable biomolecules. Reactions between amino groups and between amino groups and carboxylic acids can be used to bind ECM proteins and ECM-mimicking peptides to plastic dishes. These plastic surfaces should therefore have amino groups, carboxylic acid groups, or hydroxyl groups to bind and immobilize ECM proteins or peptides. For dishes made of polyesters, such as poly(ε-caprolactone) (PCL), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), or poly(lactic acid-*co*-glycolic acid) (PLGA), treatment with a diamine, such as hexamethylene diamine, generates amino groups on the surface by an aminolysis reaction. Then, ECM proteins and ECM-mimicking

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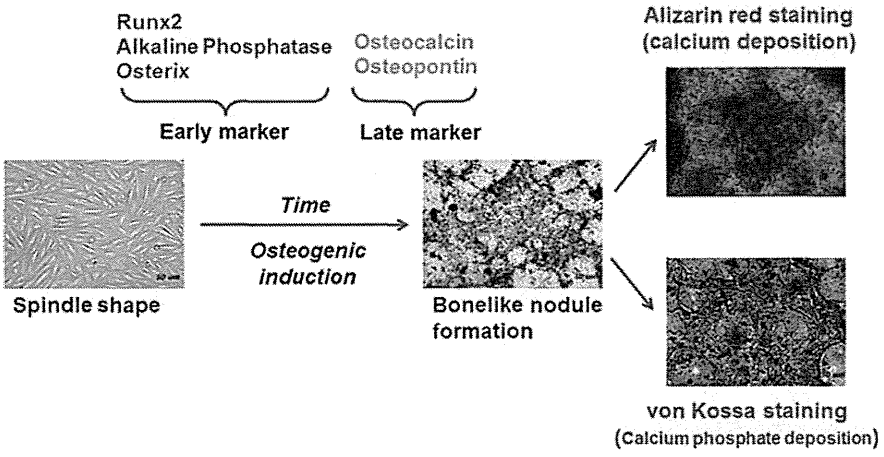

Figure 2. Osteogenic differentiation of MSCs, gene expression, and mineral deposition at early and late stages.

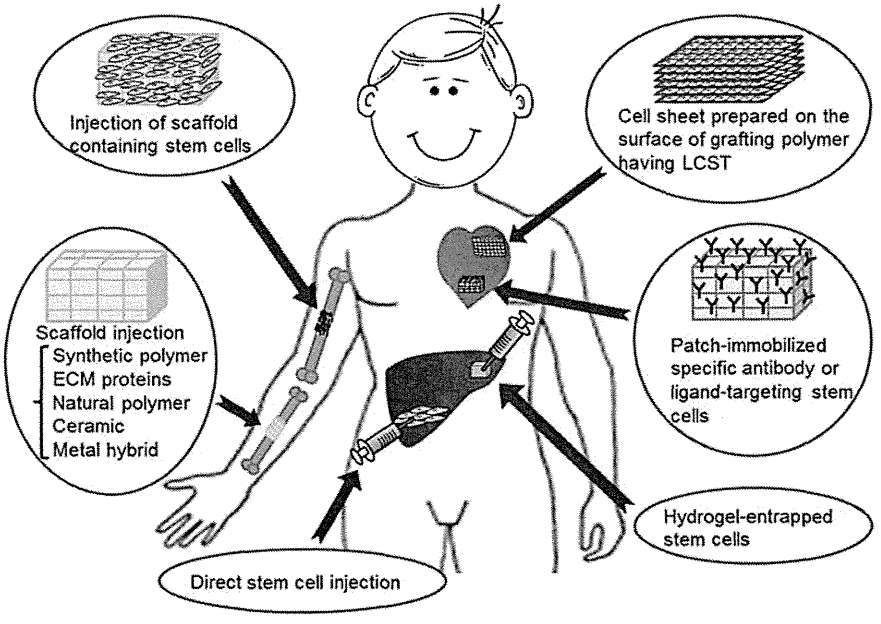

Figure 3. Some examples of biomaterial designs with and without stem cells for the injection of biomaterials in clinical applications: (a) injection of scaffold containing stem cells, (b) injection of scaffold without cells, (c) direct stem cell injection, (d) injection of cell sheets, (e) injection of patch-immobilized specific antibody or ligand-targeting stem cells, and (f) injection of hydrogel-entrapped stem cells.

peptides can be covalently immobilized using hexamethylene diisocyanate (HMDIC), 1,6-dimethyl suberimidate dihydrochloride (DMS),[119] or NHS/EDC reagent,[18] where NHS is *N*-hydroxysuccinimide and EDC is *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Figure 4). EDC is a water-soluble carbodiimide that is generally used in the 4.0–6.0 pH range. Therefore, it is possible to immobilize ECM proteins and ECM-mimicking peptides in aqueous solution using NHS/EDC reagents. The covalent bonding between amino groups can be reacted with aqueous DMS.[119]

Genipin is generally used to cross-link proteins, such as collagen and gelatin, and chitosan via amino groups.[120,121] Genipin can also be used for the immobilization of ECM proteins and peptides on the surface of culture dishes with amino groups (Figure 4). NHS/EDC, DMS, and genipin are the recommended reagents to covalently immobilize ECM proteins and ECM-mimicking peptides on culture dishes.

3.2. 3D Culture in Hydrogels

Hydrogels are physically or chemically cross-linked polymer networks that are able to absorb large amounts of water. Injectable hydrogels containing stem cells can be delivered to sites of damage in patients with minimal invasiveness, and the hydrogels ensure that stem cells remain localized to the damaged sites more effectively than injected cells alone. Physical cross-linking is performed on ECM proteins with thermosensitive properties of lower critical solution temperature (LCST) or upper critical solution temperature (UCST), such as collagen and gelatin. Collagen can be dissolved in aqueous solutions at low temperature and forms gels at ~37 °C because of its LCST characteristics, and gelatin can be dissolved in aqueous solution at high temperatures and forms gels at room temperature because of its UCST. Therefore, stem cells can be dissolved in ECM protein solutions and efficiently entrapped in ECM gels at 20–37 °C. However, most ECM

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Table 3. ECM Immobilized on Dishes for Adhesion, Differentiation, And Proliferation of Stem Cells and Some Examples of the Literature

ECM	binding site of cells	ref
collagen I	integrin ($\alpha V\beta 3$, $\alpha 2\beta 1$)	58, 96
collagen I	integrin ($\alpha 1\beta 1$)	97
collagen I	integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$)	71
collagen II	integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$)	71, 91
collagen IV	integrin ($\alpha 2\beta 1$, CD44)	98
gelatin		99
fibronectin	integrin ($\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha V\beta 6$, $\alpha V\beta 5$)	58, 96
laminin	integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)	100
laminin-1 (laminin 111)	integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 9\beta 1$), α-dystroglycan, suifade, and heparan sulfate proteoglycan	83, 101
laminin-5 (laminin 332)	integrin ($\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)	102
laminin-10/11	integrin ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)	100
vitronectin	integrin ($\alpha V\beta 3$, $\alpha V\beta 5$)	58, 96

proteins and ECM-derived oligopeptides (ECM peptides) need other forms of cross-linking to trap stem cells and generate hydrogels. Typically, photocross-linking and chemical cross-linking of ECM proteins and ECM peptides are used. There are several excellent reviews that discuss hydrogel preparation and reaction in detail.[12,14] Therefore, this section deals briefly with the preparation of ECM hydrogels using photocross-linking and chemical cross-linking with cross-linking agents. The application of ECM hydrogels containing stem cells is discussed in section 5 for specific ECM proteins and ECM peptides.

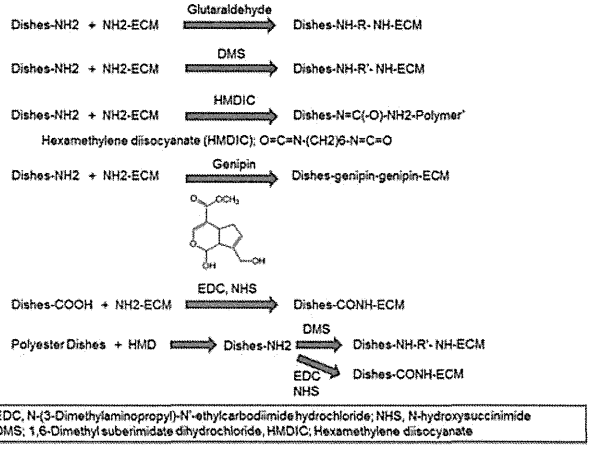

Figure 4. Surface reactions of covalent immobilization of ECM proteins and ECM-mimicking peptides on dishes.

3.2.1. Photocross-Linking of ECM Proteins and ECM Peptides. Hydrogels containing stem cells can be easily prepared by UV irradiation of ECM proteins and ECM–peptide solutions. These preparations can be used as injectable hydrogels via photocross-linking. However, it is first necessary to introduce double bonds into ECM proteins and ECM peptides for photocross-linking. ECM proteins and ECM peptides have $-OH$, $-NH_2$, and $-COOH$ functional groups. Double bonds can be introduced into ECM proteins and ECM

Table 4. ECM-Mimicking Peptides Immobilized on Dishes for Adhesion, Differentiation, And Proliferation of Stem Cells

ECM-mimicking peptide	ECM proteins for mimicking	binding site of cells	ref
DGEA	collagen I	integrin ($\alpha 2\beta 1$)	103–105
GTPGPQGIAGQRGVV (P15)	collagen I	integrin ($\alpha 2\beta 1$)	103, 106
$(RADA)_4$GG**DGEA**	collagen I	integrin ($\alpha 2\beta 1$)	116
$(RADA)_4$GGFPGERGVEGPGP	collagen I		116
GFOGER	collagen	integrin ($\alpha 2\beta 1$)	103, 107, 108
MNYYSNS	collagen IV		109
RGD	collagen I	integrin ($\alpha V\beta 3$)	97, 110
ELIDVPST (CS-1)	fibronectin	integrin ($\alpha 4\beta 1$); VLA-4	16, 111
FN-40	fibronectin	integrin ($\alpha 4\beta 1$, VLA-4)	16, 112
FN-120	fibronectin	integrin ($\alpha 5\beta 1$); VLA-5	16, 112
FN–CH296	fibronectin	integrin ($\alpha 4\beta 1$, $\alpha 5\beta 1$)	16, 112
KGGAVTG**RGD**SPASS	fibronectin	integrin ($\alpha 5\beta 1$); VLA-5	18, 113
GRGDSPK	fibronectin	integrin ($\alpha 5\beta 1$); VLA-5	18, 113
KNNQKSEPLIGRKKT	fibronectin	heparin-binding domain	53
RGDS	fibronectin		109
PHSRN	fibronectin		109
KYGAAS**IKVAV**SADR	laminin		18, 114
YIGSR	laminin		109
IKVAV	laminin		115
PPFLMLLKGSTR	laminin-5 (laminin332)	integrin ($\alpha 3\beta 1$)	
(RADA)4-GG**PDSGR**	laminin		116
(RADA)4-GGSDPG**YIGSR**	laminin		116
(RADA)4-GGIKVAV	laminin		116
KGGPQV**TRGD**VFTMP	vitronectin	integrin ($\alpha V\beta 5$)	18, 117
KGGNGEP**RGD**TYRAY	bone sialoprotein (BSP)		18, 118
PEO4-NGEP**RGD**TYRAY	BSP-linker		18, 118
RGD	osteopontin	integrin ($\alpha V\beta 3$)	97

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peptides by the reactions of acryloyl chloride,[122] glycidyl methacrylate,[12,123] and 2-aminoethylmethacrylate[12,124] (Figure 5). Figure 5 also shows a schematic for preparation method of

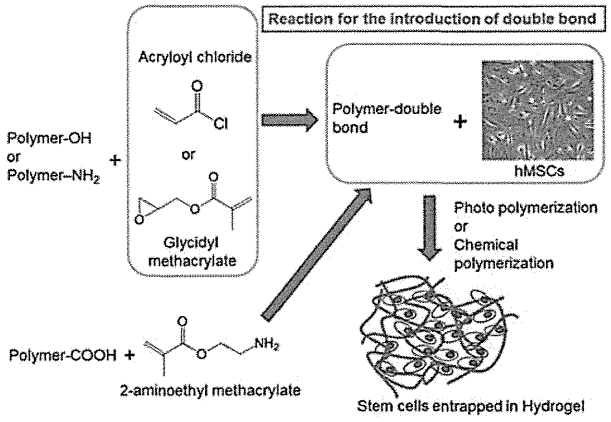

Figure 5. Schematic of the preparation method of hydrogels with entrapped stem cells by photopolymerization.

hydrogels with entrapped stem cells by photopolymerization. Aqueous solutions containing stem cells and macromers of ECM proteins and ECM peptides are irradiated with UV light to generate hydrogels with entrapped stem cells.

Poly(ethylene glycol)diacrylate (PEODA) is typically added to the reaction solution to generate optimal hydrogels.[65,125−129] Yang et al. prepared PEODA hydrogels incorporating RGD adhesive peptides and goat BMSCs by photopolymerization. They found that RGD-conjugated PEODA hydrogels promoted the osteogenic differentiation of BMSCs, and RGD enhanced differentiation in a dosage-dependent manner, with the highest concentration (2.5 mM) in the reaction solution being optimal in their study.[125]

3.2.2. Chemical Cross-Linking of Hydrogels. Hydrogels of ECM proteins can also be prepared by chemical cross-linking. Similar to ECM protein immobilization on 2D dishes, as discussed in section 3.1, NHS/EDC, DMS, HMDIC, and genipin are typically used as cross-linking agents. Glutaraldehyde is not commonly used for the preparation of hydrogels in tissue engineering because it is relatively toxic to stem cells. DMS, HMDIC, and genipin allow cross-linking between amino groups, whereas NHS/EDC leads to cross-linking between carboxylic acids and amino groups in ECM proteins.

Chang et al. compared gelatin hydrogels cross-linked with genipin and gelatin hydrogels cross-linked with glutaraldehyde.[120] They found that the degree of inflammatory reaction in wounds treated with the genipin-cross-linked gelatin was significantly less severe than those covered with the glutaraldehyde-cross-linked gelatin in vivo.[120] In addition, the healing rates of wounds treated with the genipin-cross-linked gelatin were notably faster than those with glutaraldehyde-cross-linked hydrogels.[120]

3.3. 3D Culture in Scaffolds

Scaffolds seeded with stem cells can support 3D tissue formation artificially. It is optimal for scaffolds (a) to allow cell attachment and migration, (b) to allow diffusion of nutrients, growth factors, and waste secreted by cells, and (c) to have mechanical properties similar to the natural tissue. Most of the scaffolds have high porosity (>80%) and large pore size (200−800 μm), which allow diffusion of nutrients, growth factors, and waste, but these properties also lead to weak mechanical properties. Biodegradability of scaffolds is often required because scaffolds should be absorbed by the surrounding tissues without the necessity of surgical removal. It is preferable that the degradation rate of scaffolds should be matched to the speed of tissue formation. The degradation speed of scaffolds can be regulated by the degree of cross-linking. Scaffolds prepared from ECM proteins and ECM peptides are desirable because of their biodegradable characteristics. ECM proteins used for the preparation of scaffolds are typically collagen type I, collagen type II, gelatin, fibronectin, laminin, and vitronectin. ECM proteins can be used as (a) coating materials, (b) blending materials, and (c) main materials of scaffolds.

3.3.1. Preparation of Scaffolds. There are several methods used to prepare scaffolds for tissue engineering and 3D culture of stem cells, including (a) freeze-drying, (b) salt leaching, (c) porogen leaching, (d) use of nonwoven fabric or mesh, (e) nanotopography, and (f) electrospinning. In the freeze-drying method, ECM proteins are dissolved in a buffer solution. The ECM solution is frozen at −20 or −80 °C and then lyophilized in a freeze-dryer before being washed and stored (Figure 6). If necessary, the scaffolds are also cross-linked.

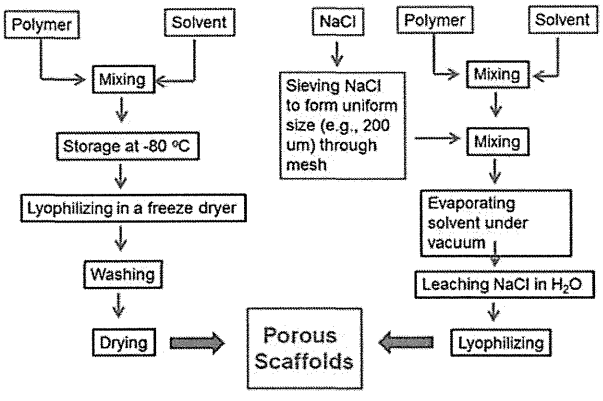

Figure 6. Typical preparation method of porous scaffolds by freeze-drying (a) and salt leaching (b).

The salt-leaching method is as follows. Biopolymers and/or ECM proteins are dissolved in a solvent. Salt, typically NaCl, is sieved to generate a uniform distribution of size using filtration through mesh and added into the solution. The solvent of the solution is vaporized under vacuum to generate dry scaffolds. Salt is then leached from the scaffolds by immersion in water after drying the scaffolds (Figure 6). The porogen-leaching method is a similar method to the salt-leaching method, but other uniformly sized particles, such as polymeric particles, are used instead of salt.

3.4. 3D Culture in Nanofibers

Peptide amphiphiles (PAs), which have a hydrophilic domain and a hydrophobic domain, are known to spontaneously generate self-assembled nanofibers above critical micelle concentrations.[109,116,130] MSC differentiation on self-assembled nanofibers using ECM peptides is discussed in section 5.8.1.

A typical method to create nanofibers is electrospinning. Electrospun scaffolds can support cell adhesion and growth and

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promote differentiation of stem cells.[131] Nanofibers can be generated from a spinning nozzle when high voltage is applied between the spinning nozzle and a flat metal collector. Typical electrospinning products are flat and highly interconnected scaffolds with a nonwoven fabric sheetlike morphology. These characteristics hinder cell infiltration and growth throughout the scaffolds. Blakeney et al. have developed a three-dimensional cotton ball-like electrospun scaffold that consists of low-density, uncompressed nanofibers.[131] A grounded spherical dish and an array of needle-like probes were used instead of a traditional flat-plate collector to create a cotton ball-like scaffold. Scanning electron microscopy showed that the cotton ball-like scaffold consisted of electrospun nanofibers with a similar diameter, but with larger pores and less dense structures than traditional electrospun scaffolds.[131] The cotton-ball like scaffolds prepared from ECM proteins by electrospinning will be interesting for use as scaffolds for guiding specific lineages of stem cell differentiation.

4. PHYSICAL PROPERTIES OF BIOPOLYMERS (BIOMATERIALS) GUIDE STEM CELL DIFFERENTIATION FATE (LINEAGE)

The interactions between MSCs and ECM proteins are classified as physical, chemical, and biological. It has recently been recognized that stem cell differentiation is directed by physical properties of culture materials as well as by biochemical responses to growth factors and ECM proteins.[19,20,132] Cells in bone, muscle, liver, and brain tissues reside in different environments that have diverse physical properties.[133] The matrix stiffness for differentiated cells is known to influence focal-adhesion structure and the cytoskeleton.[134−139] Engler et al. reported that soft materials, with similar stiffness to the brain, tend to differentiate MSCs into neurogenic cells, whereas stiffer materials that mimic muscle guide MSCs into myogenic cells and rigid materials similar to collagenous bone induce osteogenic differentiation (Figure 7).[19] However, this work was performed on a 2D surface of hydrogels coated with collagen. The effect of stiffness in 3D culture may produce different results than in 2D culture.

Gilbert et al. also reported that the elasticity of culture materials regulates self-renewal of skeletal muscle stem cells.[20]

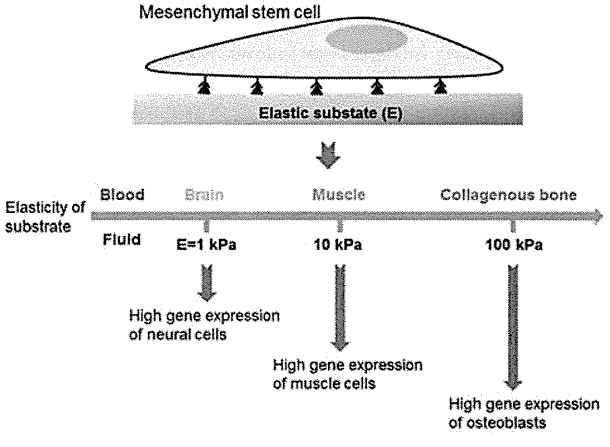

Figure 7. Physical properties decide the fate of stem cell cultured on biomaterials with different elasticity. Modified with permission from ref 19. Copyright 2006 Elsevier Inc.

Muscle stem cells (MuSC's) exhibit robust regenerative capacity in vivo, but this capacity is rapidly lost in culture. They showed that the elasticity of culture materials was a potent regulator of MuSC fate. MuSC's cultured on soft hydrogel substrates that mimicked the elasticity of muscle (12 kPa) self-renew in vitro and contributed extensively to muscle regeneration when transplanted into mice, unlike MuSC's grown on rigid plastic dishes (~106 kPa), as shown by histology and bioluminescence imaging. These studies provide evidence that propagation of adult muscle stem cells is possible by recapitulating physiological tissue rigidity.[20] This finding may contribute to future cell-based therapies for muscle-wasting diseases.

The effect of physical interactions between MSCs and culture materials on stem cell fate is discussed in several articles.[19,20,61,133,140−154] Some landmark findings are summarized in Table 5, and some examples of physical effects on differentiation of MSCs cultured on ECM proteins are reviewed here.

Table 5. Some Articles Discussing Physical Effect of Substrates on Differentiation of MSCs Cultured on the Substrates

authors	contents	ref (year)
J. R. Mauney et al.	**mechanical stimulation** promotes osteogenic differentiation of hBMSCs	140 (2004)
J. S. Park et al.	differential effects of **equiaxial and uniaxial strain** on MSCs	141 (2004)
V. E. Meyers et al.	**microgravity** disrupts collagen I/integrin signaling during osteogenic differentiation of hMSCs	142 (2004)
V. I. Sikavitsas et al.	**flow perfusion** enhances the calcified matrix deposition of marrow stromal cells in scaffolds	143 (2005)
H. Hosseinkhani et al.	**perfusion culture** enhances osteogenic differentiation of MSCs	144 (2005)
A. J. Engler et al.	**matrix elasticity** directs stem cell lineage specification	19 (2006)
R. D. Sumanasinghe et al.	osteogenic differentiation of hMSCs in collagen matrices: effect of **uniaxial cyclic tensile strain**	145 (2006)
D. F. Ward et al.	**mechanical strain** promotes osteogenic differentiation of hMSCs	61 (2007)
E. K. F. Yim et al.	**nanostructures** inducing differentiation of hMSCs into neurinal lihneage	154 (2007)
B. Lanfer et al.	growth and differentiation of MSCs on **aligned collagen** matrices	146 (2009)
Q. Li et al.	ECM with the **rigidity** of adipose tissue helps adipocytes maintain insulin responsiveness	147 (2009)
M. Zscharnack et al.	**low O_2** expansion improves subsequent chondrogenesis of BMSCs in hydrogel	148 (2009)
C. H. Huang et al.	interactive effects of **mechanical stretching** and ECM proteins on initiating osteogenic differentiation of hMSCs	149 (2009)
P. M. Gilbert et al.	substrate **elasticity** regulates skeletal muscle stem cell self-renewal in culture	20 (2010)
G. C. Reilly and A. J. Engler	intrinsic ECM properties regulate stem cell differentiation (**mechanobiology**)	150 (2010)
J. M. Kemppainen and S. J. Hollister	differential effects of **designed scaffold** permeability on chondrogenesis by BMSCs	151 (2010)
E. K. F. Yim et al.	**nanotopography**-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of hMSCs	152 (2010)
J. Tang et al.	regulation of stem cell differentiation by cell−cell contact on **micropatterned** material surfaces	153 (2010)
P. A. Janmey and R. T. Miller	mechanisms of **mechanical** signaling in development and disease	133 (2011)

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4.1. Mechanical Stretching Effect of Culture Surface-Coated with ECM Proteins

Mechanical strain and ECM proteins play important roles in the osteogenic differentiation of hMSCs.[61,140,145,149] Several studies have shown that mechanical strain can promote osteogenic or other lineage differentiation in cells cultured on ECM proteins even in the absence of osteogenic supplements in the culture medium.[61,145,149]

Park et al. reported that mechanical strain regulated the expression of vascular smooth muscle cell (SMC) markers in MSCs (Figure 8).[141] Cyclic equiaxial strain downregulated smooth muscle (SM) α-actin and SM-22α in MSCs on collagen- or elastin-coated membranes after one day and decreased the level of α-actin in stress fibers. In contrast, cyclic uniaxial strain transiently increased the expression of SM α-actin and SM-22α after one day, which subsequently returned to basal levels after the cells aligned in the direction perpendicular to the strain.[141] In addition, uniaxial but not equiaxial strain induced a transient increase in collagen type I expression. DNA microarray experiments showed that uniaxial strain increased SMC markers and regulated the expression of matrix molecules without significantly changing the expression of differentiation markers (e.g., ALP and collagen type II) in other cell types.[141] Their results suggest that uniaxial strain, which better mimics the type of mechanical strain experienced by SMCs, could promote MSC differentiation into SMCs if cell orientation is controlled.[141]

Ward et al. showed that application of a 3−5% tensile strain to a collagen type I substrate stimulated osteogenesis in attached hMSCs through gene focusing via a MAPK signaling pathway.[61] They found that mechanical strain led to an increase in the expression of osteogenic marker genes while simultaneously reducing expression of marker genes from three alternate lineages (chondrogenic, adipogenic, and neurogenic).[61] Mechanical strain also increased matrix mineralization (a hallmark of osteogenic differentiation) and activation of extracellular signal-related kinase 1/2 (ERK).[61] These results demonstrated that mechanical strain enhanced collagen type I-induced gene focusing and osteogenic differentiation in hMSCs through the ERK/MAPK signaling pathway.[61]

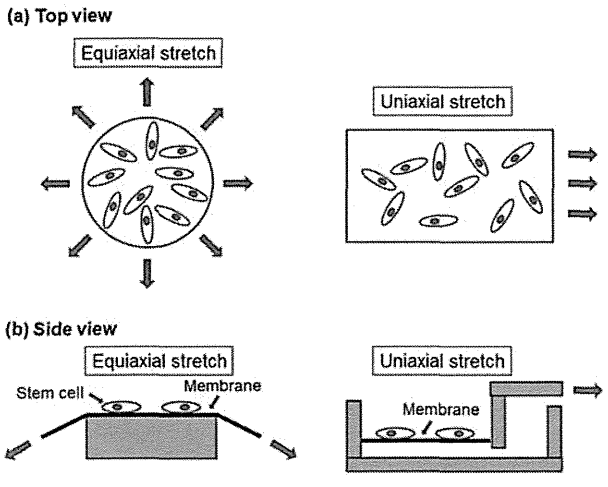

Figure 8. Schematic model of the apparatus that can apply equiaxial (a) and uniaxial (b) strain to MSCs. Modified with permission from ref 141. Copyright 2004 Wiley Periodicals.

Huang et al. investigated the combined effects of ECM proteins and mechanical factors (cyclic stretching) in driving hMSCs toward osteogenic differentiation.[149] hMSCs cultured in regular medium were grown on substrates coated with various ECM proteins (collagen type I, vitronectin, fibronectin, and laminin) and subjected to cyclic mechanical stretching.[149] All of the ECM proteins tested supported hMSC differentiation into osteogenic phenotypes in the absence of osteogenic supplements.[149] Cyclic mechanical stretching activated the phosphorylation of focal adhesion kinase (FAK), induced upregulation of the transcription and phosphorylation of Runx2, and subsequently increased ALP activity and mineralized matrix deposition.[149] Fibronectin and laminin exhibited greater effects of supporting stretching-induced osteogenic differentiation than did collagen type I and vitronectin.[149] It was suggested that the ability of ECM proteins and mechanical stretching to regulate osteogenesis in hMSCs may be exploited in bone tissue engineering by appropriate matrix design and by mechanical stimulation.[149]

4.2. Low Oxygen Expansion Promotes Differentiation of MSCs

Several groups have reported the effects of low oxygen tension on the differentiation of MSCs, especially in chondrogenic differentiation of MSCs cultured on ECM substrates.[148,155] Zscharnack et al. investigated the effect of low oxygen tension (5%) during the expansion of ovine MSCs on colony-forming unit-fibroblast (CFU-F) formation and chondrogenesis in pellet culture and in collagen type I hydrogels.[148] MSCs expanded in 5% O_2 showed a 2-fold higher CFU-F potential, and chondrogenic differentiation was enhanced in both pellet culture and collagen type I hydrogels. It was demonstrated that physiologically low oxygen tension during monolayer expansion of ovine MSCs was advantageous to improving cartilage tissue engineering in a sheep model.[148]

4.3. Other Physical Effect Affecting Differentiation of MSCs

There are several other physical effects that promote differentiation of MSCs on ECM protein surfaces. (i) Perfusion culture promotes osteogenic differentiation of MSCs cultured on ECM protein surface.[143,144] (ii) Microgravity disrupts collagen type I/integrin signaling during osteoblastic differentiation of hMSCs.[142] (iii) The mechanical properties of ECM proteins guide specific lineage differentiation of MSCs.[147,150,156,157] (iv) The topography of ECM proteins promotes differentiation of MSCs cultured on aligned or patterned substrates.[74,146,151−154,158]

5. MSC CULTURE ON ECM PROTEINS AND NATURAL BIOPOLYMERS

The ECM is the extracellular component of animal tissues that provides structural support for the cells, in addition to stimulating various important biological functions. ECM proteins are able to dictate whether cells will proliferate or undergo growth arrest, migrate or remain stationary, and thrive or undergo apoptotic death.[159] Therefore, the ECM proteins are an important factor in reproducing the biological niches of cells in vitro, which guides MSCs to differentiate into different lineages such as osteoblasts, chondrocytes, adipocytes, cardiomyocytes, neural cells, hepatocytes, and β-cells. The differentiation of MSCs in culture systems depends on the components, structure (morphology), origin, and quantity of ECM proteins that are used. Because ECM proteins are used as scaffolds for the organization of cells in tissues, ECM proteins

are the main cell culture materials used to control the proliferation and differentiation of MSCs in tissue engineering and regenerative medicine, both in vitro and in vivo. Therefore, this review focuses mainly on the differentiation of MSCs cultured on biomaterials made of specific ECM proteins and on the biological and chemical interactions between these cells and proteins.

5.1. Chemical and Biological Interactions of ECM Proteins and Stem Cells

ECM proteins have chemical functional groups of carboxylic acid, amine, phosphate, and/or sulfonic acid. They also have aspects of polyelectrolytes and characteristic isoelectric points (IEPs).[160−175] Table 6 shows the IEPs of some ECM proteins,

Table 6. Isoelectric Points of Some ECM Proteins, Growth Factors, And Polymers

materials	isoelectric point	ref
	ECM	
collagen type I	4.7, 6.4, 6.78, 7.02, and 8.26 depending on preparation conditions	172−174
gelatin sol	7.8, temp > 40, or increasing pH	344
gelatin gel	4.7, temp < 15, or decreasing pH	344
fibronectin	5.5−6.0	160
vitronectin	4.75−5.25	161
laminin	5.87, 4.89, and 5.08	162
heparin	3.4	163
hyaluronic acid	2.5	170
	growth factor	
FGF-1 (aFGF)	5.6	169
FGF-2 (bFGF)	9.6	169
rhBMP-2	9	171
insulin	5.3	168
PDGF	9.8	165
EGF	4.0−5.0	
TGF-β1	9.5	164
	polymer	
agarose	5.5	166
alginate	5.4	175
poly(lactic-*co*-glycolic acid) (PLGA)	2.75	163
poly(L-lysine)	9.5	163
chitosan	8.7	167
polyacrylamide	5.7	166

natural biopolymers, and growth factors.[160−172,174,175] IEPs are as follows: gelatin gel and collagen type I 4.7−8.3,[172,174] fibronectin 5.5−6.0,[160] laminin 4.9−5.9,[162] vitronectin 4.8−5.3,[161] heparin 4.7,[163] hyaluronic acid 2.5,[170] agarose 5.5,[166] and alginate 5.4.[175] Most ECM proteins and natural biopolymers are negatively charged under physiological conditions. The IEP of some growth factors is <7 (e.g., 5.6 for FGF-1[169] and 5.3 for insulin[168]), whereas for other growth factors, it is >7 (e.g., 9.6 for FGF-2,[169] 9.0 for BMP-2,[171] 9.8 for PDGF,[165] and 9.5 for TGF-β1[164]). Some binding between ECM proteins and growth factors (e.g., collagen type I and BMP-2) is mainly due to electrochemical interactions.

The binding of ECM proteins to cells is mainly mediated by integrin receptors. Integrins comprise a large family of cell-surface receptors that bind and mediate adhesion to ECM components, organize the cytoskeleton, and activate intracellular signaling pathways.[159] Each integrin consists of two type-1 transmembrane subunits: α and β. In mammals, 18 α- and 8 β-subunits associate in various combinations to form 24 integrin dimers that can bind to distinct subsets of ECM ligands.[176,177]

Most ECM proteins have molecular weights of 10−1000 kDa but only a few integrin-binding domains. These integrin-binding domains have specific sequences of a few amino acids (3−10), e.g., RGD, DGEA, YIGSR, IKVAV, and GFOGER. Table 4 summarizes the integrin receptors and amino acid sequences that mediate cell−ECM associations that are important for MSC proliferation and differentiation, as well as normal cell culture.

Many members of the integrin family, including $\alpha5\beta1$, $\alpha8\beta1$, αIIb$\beta3$, $\alpha V\beta3$, $\alpha V\beta5$, $\alpha V\beta6$, and $\alpha V\beta8$, recognize an Arg-Gly Asp (RGD) motif within fibronectin,[16,18,109] fibrinogen,[109] vitronectin,[18] von Willebrand factor, and other large glycoproteins. Collagen type I has a cell-binding domain of DGEA, which binds to integrin $\alpha2\beta1$.[103] Collagen type I is also bound by integrins $\alpha1\beta1$, $\alpha3\beta1$, and $\alpha V\beta3$.[58,97] RGD in collagen type I is reported to associate with integrin $\alpha V\beta3$.[97] The large size of ECM proteins, compared to the small integrin-binding motifs, provides not only structural support but also conformational regulation of the cell-binding domains. The differences in conformation of the cell-binding domains lead to different associations with specific integrin receptors.[178,179] MSC differentiation on culture materials composed of specific ECM and natural biopolymers is discussed in the following sections.

5.2. Collagen

Collagen is a typical ECM protein used in the culturing of MSCs, which is found in all animals, especially in the flesh and connective tissues of mammals.[180] Collagen is the main component of connective tissue and the most abundant protein in mammals,[181] making up ~25−35% of the whole-body protein content. Elongated collagen fibrils are found in fibrous tissues, including skin, ligaments, and tendons. Collagen is also abundant in the cornea, cartilage, bone, blood vessels, gut, and intervertebral discs. Because of its abundance, collagen, especially collagen type I, is relatively inexpensive compared to other ECM proteins such as laminin, vitronectin, and fibronectin, which allows us to use it in large quantities to make scaffolds and hydrogels for stem cell culture.[144,182−185]

To date, 29 types of collagen have been identified and described. The five most common types are (i) collagen type I (genes; COL1A1, COL1A2), which is the main component of bone and also found in skin and tendons; (ii) collagen type II (gene; COL2A), which is the main component of cartilage; (iii) collagen type III (gene; COL3A), which is the main component of reticular fibers; (iv) collagen type IV (genes; COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, and COL4A6), which is found in basement membranes;[186] and (v) collagen type V (COL5A1, COL5A2, and COL5A3), which is found in placenta and hair.[187]

Collagen undergoes many post-translational modifications, including extensive cross-linking. Defective cross-linking has been implicated in human syndromes (e.g., osteogenesis imperfecta and Ehlers-Danlos syndrome).[188] However, it was reported that the inhibition of cross-linking of collagen was not required for osteogenic differentiation of hMSCs, as shown by the expression of ALP and genome-wide gene-expression analysis, but it did enhance matrix mineralization.[188] Specific characteristics of collagen, such as stiffness, elasticity, degree of cross-linking, and origin (i.e., cow-, pig-, or fish-derived collagen

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from fetal or adult animals), might affect stem cell fate when it is used in the culture materials and scaffolds for MSC differentiation.

Collagen can form gels or scaffolds without elaboration. To prepare collagen gel, the protein is dissolved in acetic acid solution, and the solution is diluted with phosphate-buffered saline. After adjusting the pH of collagen solution to 7.4 by the addition of NaOH, the collagen solution is chilled in an ice bath to prevent gelation. Cells are then added into the collagen solution at the desired density, and the cell solution is incubated at 37 °C to allow gel formation. Once the gel has set, extra culture medium is added to the top of the gels and the cultures are returned to the incubator.[189] Tables 7 and 8 summarize several types of collagen materials and scaffolds for MSC differentiation that have been reported in the literature.[34,37,40,45,46,53,56,61,63,70,71,79,83,84,91,97,98,101,105,110,141,144,146,148,149,151,154,182−185,189−217]

5.2.1. Collagen Type I Scaffolds. Collagen sponges (scaffolds) can be fabricated by the conventional freeze-drying method followed by cross-linking.[144,202] Collagen type I is frequently used for scaffolds and culture materials to promote osteogenic[105,182,183,190,193,218] and chondrogenic[183] differentiation of MSCs.

Many reports have focused on the osteogenesis of MSCs cultured on collagen type I scaffolds,[105,183] because collagen type I is a major organic component of bones.[91] Activation of specific integrins ($\alpha1\beta1$ and/or $\alpha2\beta1$) by collagen type I was reported to mediate the osteogenic response of hBMSCs (human BMSCs).[70,97,105,188,194]

The proliferation and differentiation of MSCs into osteoblasts on collage type I-coated dishes and scaffolds are promising. It was reported that the tissue culture dishes coated with collagen type I, but not fibronectin, laminin, gelatin, or poly L-lysine, enhanced late cell proliferation and promoted osteogenesis by hBMSCs, as evidenced by an increase in Alizarin Red S staining, ALP activity, and mRNA levels of Runx2 and osteocalcin.[192] Tsai et al. found that collagen type I coating induced the activation of extracellular signal regulated kinase (ERK) and Akt, but not FAK.[192] Antibody blocking of $\alpha2\beta1$ integrin did not inhibit collagen type I-induced osteogenesis of hBMSCs.[192] This result indicates that cell signaling via $\alpha2\beta1$ integrin is not required for osteogenesis of hBMSCs cultured on collagen type I.

Donzelli et al. reported osteogenic differentiation of rat MSCs in a commercially available collagen scaffold, Gingistat. MSC commitment to osteogenic differentiation was demonstrated by the expression of osteopontin and osteocalcin, as well as increased ALP activity. Nodular aggregates and Alizarin Red-stained calcium deposits were observed in MSCs induced toward osteogenic differentiation cultured in the collagen scaffold.[183]

A honeycomb structure of collagen scaffold was reported to promote BMSC proliferation and differentiation.[110] BMSCs on honeycomb collagen scaffolds were able to differentiate into osteoblasts even without osteogenic induction medium to some extent, as shown by ALP activity and observation of mineral deposition by von Kossa staining.[110]

In another study, collagen type I nanofibers were prepared by electrospinning and seeded with hBMSCs. The morphology, growth, adhesion, cell motility, and osteogenic differentiation of hBMSCs on nanosized fibers of varying diameters (50−200, 200−500, and 500−1000 nm) were examined. The cells on all the nanofibers had a more polygonal and flattened cell morphology than those on tissue culture polystyrene dishes (TCPSs). Moreover, hBMSCs grown on 500−1000 nm nanofibers had significantly higher cell viability than the TCPS control.[182] Sefcik et al. also prepared collagen type I scaffolds by the electrospinning method.[184] Osteogenic genes (collagen type I, ALP, osteopontin, osteonectin, osteocalcin, and Runx2) were reported to be upregulated (>1-fold) in adipose-derived stem cells (ADSCs) cultured on nanofiber

Table 7. Some Research Studies for Stem Cell Differentiation on 2D Collagen Materials

stem cell source[a]	material for stem cell culture	differentiation	ref
hBMSCs	collagen I (2D culture, coating on dishes)	osteoblasts	97, 149, 190−192
rat BMSCs	collagen I (2D culture, coating on dishes)	osteoblasts	193
rBMSCs	collagen I (2D culture, gel)	osteoblasts	194
mBMSCs	collagen I (2D culture, coating on dishes)	osteoblasts, adipocytes	195
hBMSCs	collagen I (2D culture, coating on dishes)	osteoblasts, adipocytes	196
hBMSCs	collagen I (2D culture, aligned collagen on dishes)	osteoblasts, adipocytes	146
hBMSCs	collagen I (2D culture, aligned heparin on collagen I matrix)	osteoblasts, adipocytes	146
pBMSCs	collagen I (2D culture, coating on dishes)	osteoblasts, adipocytes	197
hBMSCs	collagen I (2D culture, coating on dishes)	osteoblasts, chondrocytes	61
hADSCs	collagen I (2D culture, coating on dishes)	adipocytes	53
hESCs (TE03, TE06)	collagen I (2D culture, coating on dishes)	neural cells	79
hBMSCs	collagen I (2D culture, coating on dishes)	neural cells	101, 154,
mESCs	collagen I (2D culture, coating on dishes)	neural cells	198
monkey ESCs	collagen I (2D culture, coating on dishes)	mesoderm cells, endoderm cells	199
mouse hepatitic stem cells	collagen I (2D culture, coating on dishes)	hepatocytes	200
hBMSCs, hAFSCs	collagen I (2D culture, coating on dishes)	hepatocytes	46
human neural stem cells	collagen I (2D culture, coating on dishes)	oligogliocytes	37
teratocarcinoma stem cells (F9)	collagen I (2D culture, coating on dishes)	visceral endoderm cells	98
hBMSCs	collagen I (2D culture, coating on dishes)	vascular smooth muscle cells	141
mESCs	collagen I (2D culture, coating on dishes)	lung epithelium	201
hBMSCs	collagen IV (2D culture, coating on dishes)	osteoblasts	97
hADSCs	collagen IV (2D culture, coating on dishes)	adipocytes	53
hBMSCs	collagen IV (2D culture, coating on dishes)	neural cells	101
mouse hepatitic stem cells	collagen IV (2D culture, coating on dishes)	hepatocytes	200
teratocarcinoma stem cells (F9)	collagen IV (2D culture, coating on dishes)	visceral endoderm cells	98
hBMSCs	collagen IV (2D culture, coating on dishes)	smooth muscle cells	200

[a]ADSC's, adipose-derived stem cells; BMSCs, bone marrow stem cells; ESCs, embryonic stem cells; hBMSCs, human BMSCs; rBMSCs, rabbit BMSCs; mBMSCs, mice BMSCs; pBMSCs, porcine BMSCs; hADSCs, human ADSCs; hESCs, human ESCs; mESCs, mice ESCs; hAFSCs, human amniotic fluid-derived stem cells.

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Table 8. Some Research Studies for Stem Cell Differentiation on 3D Collagen Materials

stem cell source[a]	material for stem cell culture[b]	differentiation	ref	stem cell source[a]	material for stem cell culture[b]	differentiation	ref
rBMSCs	collagen I (3D culture, gel)	osteoblasts	193	hBMSCs	collagen I (3D culture, sponge)	chondrocytes	208
rat BMSCs	collagen I (3D culture, gel)	osteoblasts	105	rBMSCs	collagen I (3D culture, microsphere)	osteochondrocytes	209
hBMSCs	collagen I (3D culture, scaffold)	osteoblasts	202	hBMSCs	collagen I (3D culture, microsphere)	chondrocytes	210
rBMSCs	collagen I (3D culture, scaffold)	osteoblasts	183	bBMSCs	collagen I/alginate (3D culture, gel)	chondrocytes	91
hBMSCs	collagen I (3D culture, cross-linked scaffold)	osteoblasts	185, 190	rBMSCs	collagen I/alginate (3D culture, gel)	chondrocytes	211
hADSCs	collagen I (3D culture, electrospinning nanofiber)	osteoblasts	184	hBMSCs	collagen I/HA/PCL (3D culture, scaffold)	chondrocytes	151
hBMSCs	collagen I (3D culture, electrospinning nanofiber)	osteoblasts	182, 203	rat cardiac stem cells	collagen I/PLGA (3D culture, scaffold)	cardiomyocytes	212
rBMSCs	collagen I/PGA fiber (3D culture, sponge)	osteoblasts	144	mESCs	collagen I/Matrigel (3D culture, scaffold)	cardiomyocytes	213
rat BMSCs	collagen I/bioglass/PSN (3D culture, scaffold)	osteoblasts	204	mBMSCs	collagen I immobilized Sca-1 antibody (3D culture, scaffold)	cardiomyocytes	40
rBMSCs	collagen I/PGA (3D culture sponge)	osteoblasts	205	hBMSCs	collagen type I/PLCL (3D, electrospinning nanofiber)	neural cells	45
hBMSCs	collagen I/HYA (3D culture, scaffold)	osteoblasts	191	neural stem cells	collagen I (3D culture, grafting on elctrospinning mat)	neural cells	214
rBMSCs	collagen I/chitosan (3D culture, sponge)	osteoblasts	206	neural stem cells	collagen I (3D culture, gel)	neural cells	189
hBMSCs, Wharton's Jelly of UCB	collagen I/collagen III (3D culture, scaffold)	osteoblasts	34	rat neural stem cells	collagen I (3D culture, gel)	neural cells	217
rBMSCs	collagen I/chondroitin 6-sulfate (3D culture, scaffold)	osteoblasts, chondrocytes	56	mice neural stem cells	collagen I (3D culture, gel)	neural cells	215
hBMSCs	collagen I/HYA (3D culture, scaffold)	osteoblasts, chondrocytes	70	mice neural stem cells	collagen I/laminin (3D culture, gel), collagen I/fibronectin (3D culture, gel)	neural cells	215
pBMSCs	collagen I/PCL/TCP (3D culture, scaffold)	osteoblasts, adipocytes	197	rat stem cells	collagen I (3D culture, gel)	neuronal circuits	216
hBMSCs, hUCB-BMSCs	collagen I/collagen III (3D culture, gel)	osteoblasts, adipocytes	34	hBMSCs	fibroblast-embedded collagen I (3D culture gel)	epidermis	84
bBMSCs	collagen I (3D culture, gel)	chondrocytes	148	hADSCs	collagen II (3D culture, gel)	chondrocytes	71
hBMSCs	collagen I (3D culture, gel)	chondrocytes	63	bBMSCs	collagen II/aliginate (3D culture, gel)	chondrocytes	91
hADSCs	collagen I (3D culture, gel)	chondrocytes	71	rat BMSCs	atelocollagen (3D culture, honeycomb structure)	osteoblasts	110
mESCs	collagen I (3D culture, gel)	chondrocytes	207				

[a]ADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; gBMSCs, goat BMSCs; hBMSCs, human BMSCs; mBMSCs, murine BMSCs; hESCs, human ESCs. [b]PCL, poly(ϵ-caprolactone); HYA, hydroxyapatite; PEG, polyethylene glycol.

scaffolds compared to 2D collagen coatings by day 21.[184] Extensive synthesis of mineralized extracellular matrix was observed on the nanofiber scaffolds assessed on day 21 with Alizarin Red staining. The results demonstrate that 3D nanoscale morphology plays a critical role in regulating cell fate determination and in vitro osteogenic differentiation of ADSCs under serum-free conditions.[184]

Chondrogenic differentiation of MSCs induced by collagen type I-based hydrogels has also been reported by several groups.[63,210,211,219] Chang et al. compared chondrogenesis of immortalized hBMSCs embedded in collagen type I gel to those grown in pellet culture.[219] The hBMSCs in collagen scaffolds expressed more glycosaminoglycan than those in pellet culture. Expression of the chondrogenic genes Sox9, aggrecan, collagen type II, and collagen type I (which indicates dedifferentiation) increased over time in pellet culture. However, only collagen type II and aggrecan expression in hBMSCs in the collagen gels increased over time, whereas Sox9 expression remained unchanged and collagen type I expression decreased, which indicated that there was no dedifferentiation from the chondrogenic lineage. These results indicate that chondrocytes differentiated from hBMSCs in collagen gel are superior to those generated in pellet culture because of their lower levels of dedifferentiation.

The regulation of ESCs in specific lineages of differentiation is a complex and technically challenging subject. Collagen type I microspheres encapsulated with mouse ESCs (mESCs) have been reported to be a suitable microenvironment for supporting mESCs and maintaining their undifferentiated state for a certain period.[207] However, Yeung et al. reported that the proportion of undifferentiated mESCs in the microspheres gradually decreased, and the proportion of MSCs was increased at later time points.[207] This result points to inductive properties of the collagen matrix for differentiating mESCs toward MSC lineages. It was reported that a lower initial collagen concentration facilitated the differentiation of mESCs into chondrogenic lineages, while mESCs differentiated into a more advanced stage of chondrocytes at a later time point using chondrogenic differentiation medium.[207] The cultivation of hESCs and human iPSC's in hydrogels or scaffolds of collagen type I or other ECM proteins and natural biopolymers could yield efficient differentiation into MSC

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lineages, including osteoblasts, chondrocytes, and cardiomyocytes. This strategy would provide a larger-scale source of MSC lineage cells, which at present is limited to autologous patients.

Bioengineering complex tissues, which consist of multiple tissue phases with different structures and functions, is extremely challenging. In particular, it is difficult to create biological interfaces between mechanically dissimilar tissues such as cartilage and bone. The formation of the osteochondral interface with proper zonal organization is quite difficult, although tremendous efforts have been devoted to the developing osteochondral plugs.[209,220,221] An osteochondral interface is essential for preventing mechanical failure and maintaining normal function of cartilage.[209]

Cheng et al. demonstrated in vitro formation of a stem cell-derived osteochondral interface, with a calcified cartilage interface separating a noncalcified cartilage layer and an underlying bone layer, using BMSCs adhered to collagen type I microspheres.[209] Rabbit BMSCs were entrapped in collagen microspheres composed of a self-assembled nonfibrous meshwork.[209] BMSCs in the collagen microspheres were separated into two groups; one group was immersed in chondrogenic differentiation medium to drive differentiation into a chondrogenic lineage, whereas the other group was immersed in osteogenic differentiation medium and differentiated into an osteogenic lineage. Hundreds of cartilage-like and bonelike microspheres were aggregated to form chondrogenic and osteogenic layers, respectively.[209] Layers of these functional subunits were brought into contact with a central undifferentiated BMSC–collagen layer in a trilayered configuration for 3D cocultures. By 5 weeks, a calcified cartilage interface was formed between the noncalcified cartilage layer and the underlying bone layer. The cells at the interface region were found to be hypertrophic chondrocytes, and the extracellular matrix in this region contained collagen type II and type X, as well as calcium deposition. The osteochondral interface was reported to successively resemble the native osteochondral interface, based on the presence of hypertrophic chondrocytes, calcium phosphate deposits, collagen type II and type X, GAGs, and vertically aligned collagen bundles.[209] Thus, an osteochondral construct with proper zonal organization can be engineered using rabbit BMSCs and collagen in vitro.

Collagen type I hydrogels and scaffolds have also been used to promote differentiation of stem cells into neural cells. Ma et al. reported differentiation of central nervous system (CNS) mammalian stem cells into neuronal circuits in collagen type I hydrogels.[189] The proliferative capacity and differentiating potential of neural progenitors in 3D collagen gels suggest their potential use to promote neuronal regeneration.

5.2.2. Organic Hybrid Scaffolds of Collagen Type I. The mechanical strength, swelling properties, and degradation behavior of scaffolds, as well as their biocompatibility, play crucial roles in the long-term performance of tissue-engineered stem cell/biomaterial constructs.[206,222–226] The shrinkage and weak mechanical strength of scaffolds present a serious problem for the use of purely collagen scaffolds in tissue engineering. Therefore, synthetic polymers or natural biopolymers are commonly blended into collagen scaffolds or hydrogels to enhance their mechanical strength (Table 8). No shrinking was observed in the scaffolds or hydrogels prepared from collagen blended with synthetic or natural biopolymers seeded with MSCs. Synthetic biopolymers, such as poly(L-lactic acid)-*co*-poly (3-caprolactone) (PLCL), poly(lactic-*co*-glycolic acid) (PLGA), and poly(glycolic acid) (PGA), and natural biopolymers of alginate, chitosan, and hyaluronic acid are blended with collagen for this purpose.

It should be noted that the contractile properties of skeletal cells are physiologically important, and the in vivo functions of contractility must be accounted for when developing tissue-formation strategies.[70,227,228] It was reported that a reduction in contraction induced by altering the cross-linking method of collagen–glycosaminoglycan scaffolds resulted in delayed collagen type II synthesis by articular chondrocytes.[229] Thus, malleable ECM proteins and synthetic biopolymers may provide environmental cues that direct cell differentiation, and these considerations should be included in scaffold design.

Fujita et al. prepared three kinds of scaffolds: a collagen type I sponge, a PGA–collagen type I sponge, and a PGA–collagen type I (UV) sponge seeded with rat BMSCs.[205] The PGA–collagen type I (UV) sponge was cross-linked by irradiation with UV light.[205] The collagen type I sponges with BMSCs shrank considerably, whereas PGA–collagen type I and PGA–collagen type I (UV) sponges maintained their original shapes. PGA–collagen type I sponges with and without cross-linking by UV induced high ALP activity (indicative of osteogenic differentiation) in medium containing the osteogenic supplement dexamethasone. The addition of bFGF together with dexamethasone promoted increased cell proliferation. However, extremely low osteogenic differentiation of BMSCs was found in collagen type I, PGA–collagen type I, and PGA–collagen type I (UV) sponges without osteogenic supplements in the culture medium.[205]

Osteoblasts were reported to maintain their phenotype and MSCs to undergo osteogenesis when cultured in ECMs containing collagen type I.[91,230,231] The interaction between collagen type I and $\alpha 2\beta 1$ integrin in MSCs, which was the major collagen type I receptor, was responsible for the osteoblastic differentiation of MSCs.[91,231]

Hybrid-type scaffolds made by a simple preparation method have also been reported. This collagen type I sponge can be formed in and on a mechanically strong PLGA knitted mesh. Dai et al. prepared three types of scaffolds (Figure 9): (i) collagen microsponges formed in the interstices of PLGA mesh; (ii) collagen microsponges formed on one side of PLGA mesh; (iii) collagen sponges formed on both sides of PLGA mesh.[232] All three groups of transplants showed homogeneous cell distribution, natural chondrocyte morphology, and abundant cartilaginous ECM deposition. Production of glycosaminoglycans and the expression of type II collagen and aggrecan mRNA were much higher in the collagen sponges formed on one or both sides of PLGA mesh than in the collagen sponges formed in interstices of the PLGA mesh. The engineered cartilage reached 54.8% (one side of PLGA mesh) and 49.3% (both sides of PLGA mesh) of the Young's modulus of native articular cartilage and 68.8% (one side) and 62.7%

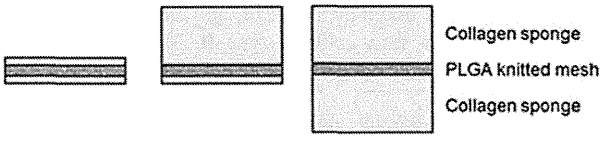

Figure 9. Schematic illustration of three structural designs of PLGA/collagen hybrid scaffolds. Black, PLGA knitted mesh; gray, collagen type I sponge. Modified with permission from ref 232. Copyright 2010 Elsevier Ltd.

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(both sides) of the stiffness of the native tissue.[232] These scaffolds, therefore, could be used for the tissue engineering of articular cartilage with adjustable thickness. The design of the hybrid structures provides a potential strategy for the preparation of 3D porous scaffolds.

Hybrid scaffolds composed of collagen type I and natural biopolymers have also been studied for regeneration of bone, cartilage, and other tissues. Scaffolds composed of collagen type I and glycosaminoglycan have been developed for tissue engineering using stem cells by several researchers.[56] Farrell et al. prepared scaffolds composed of collagen type I and chondroitin 6-sulfate. Rat BMSCs underwent osteogenesis when grown on these scaffolds and stimulated with osteogenic factors (dexamethasone, ascorbic acid, and β-glycerophosphate), as evaluated by expression of collagen type I and osteocalcin and mineral deposition analyzed by Alizarin Red and von Kossa staining.[56] The stimulation by osteogenic factors was linked to activation of ECM-regulated protein kinase (ERK), which plays an important role in osteogenesis of MSCs.[56]

Chitosan is a partially deacetylated derivative of chitin that is conducive to osteoblast growth.[206,233] To improve the mechanical and biological properties of collagen scaffolds, Arpornmaeklong et al. prepared hybrid sponges composed of chitosan–collagen type I for osteogenic differentiation of rat BMSCs.[206] The BMSCs attached successfully to the structure of the sponges. The expression of ALP and osteocalcin on collagen and chitosan–collagen type I composite sponges were greater than on chitosan sponges. A 1:1 chitosan–collagen sponge showed the highest compressive strength.[206] Thus, combined chitosan–collagen matrixes promoted osteoblastic differentiation of BMSCs and improved their mechanical and physical properties.

5.2.3. Scaffolds Using Collagen Type II and Type III. Whereas collagen type I is used for culture and scaffold materials that promote osteogenic differentiation of MSCs by mimicking the bone environment, collagen type II should be the ideal material for scaffolds that promote chondrogenic differentiation. However, only collagen type I has already been approved for clinical usage by the FDA, and collagen type I is much less expensive than collagen type II. Therefore, many investigators study chondrogenic differentiation of MSCs in collagen type I gels.

It is extremely difficult for MSCs to differentiate into chondrocytes in 2D monolayer cultures. Hanging-drop and pellet cultures of MSCs are the gold standards for chondrogenic differentiation of MSCs.[219] High seeding density promotes greater chondrogenic differentiation, indicating that cell–cell contact and autocrine growth factors are important in the chondrogenesis. The condensation of MSCs triggers the initiation of chondrogenesis during skeletal development,[234] providing the rationale for chondrogenic high-density pellet cultures.[70,235] The inhibition of *N*-cadherin, a cell–cell adhesion molecule transiently upregulated during chondrogenesis, was found to disrupt cell condensation and BMP-2/β-catenin-mediated chondrogenic gene expression in vitro.[70,236] In addition, cell morphology in hanging-drop and pellet cultures is round as opposed to spread, as it is in monolayer culture. Morphological regulation is another key factor that promotes chondrogenesis of MSCs.

Bosnakovski et al. investigated chondrogenic differentiation of bovine BMSCs in different hydrogels compared to tissue culture polystyrene plates (monolayer culture).[91] BMSCs were cultured in alginate, collagen type I, and collagen type II hydrogels. The chondrogenic differentiation marker genes Sox9, collagen type II, aggrecan, and cartilage oligomeric protein (COMP) were upregulated in collagen type I and collagen type II hydrogels. No significantly different expressions of these chondrogenic differentiation genes were found between the different collagen hydrogels, but the genes were expressed at extremely low levels by cells in monolayer cultures.[91] Chondrogenic differentiation of BMSCs in both collagen type I and type II was superior to that in alginate gels, based on the expression of chondrogenic genes; however, chondrogenic differentiation in alginate was higher than that of monolayer cultures. This finding indicates that both collagen type I and type II are suitable biopolymers for chondrogenic differentiation of BMSCs. Interestingly, the expression of chondrogenic differentiation genes in BMSCs in collagen type I and type II hydrogels in normal expansion medium was not very different from that of cells chondrogenic medium (supplemented with TGF-β1 and dexamethasone) in this study.[91] Cells adopted a round, plump shape and could not spread out in hydrogels. Therefore, both the physical space effects that induce the round morphology of BMSCs and the biological interactions between cells and collagen promote chondrogenic differentiation of BMSCs.[71] The expression of collagen type I gene is a marker of dedifferentiation of chondrocytes.[91] The expression of collagen type I gene, which was relatively high in expansion medium, could be suppressed in BMSCs in collagen hydrogels using chondrogenic differentiation medium supplemented with TGF-β1 and dexamethasone.[91]

In summary, BMSCs cultured solely in collagen type I scaffolds or hydrogels cannot be differentiated into osteoblasts without supplementation (dexamethasone, ascorbic acid, and/or BMP-2), whereas hydrogels composed of collagen type I and type II can induce chondrogenesis without supplements. Chondrogenic differentiation of BMSCs in collagen type II hydrogels seems to be better than in collagen type I. Collagen type II is the predominant component of hyaline cartilage. Chondrocytes bind to collagen type II through $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha10\beta1$ integrins, which promote the formation of signaling complexes for differentiation, matrix remodeling, cell survival, and response to mechanical stimulation.[91,237] Mitogen-activated protein kinase (MAPK) plays an important role in mediating the downstream signal from integrins, and it can regulate gene expression through activation of transcription factors such as NFκB and AP-1.

Lu et al. investigated whether collagen type II favors chondrogenic induction by affecting cell shape through $\beta1$ integrins and Rho A/Rock signaling using ADSCs entrapped into collagen type I and type II hydrogels.[71] The following points were observed. (a) ADSCs in collagen type II hydrogels showed more efficient chondrogenic induction and higher expression of chondrocyte marker genes (collagen type II, collagen type X, Sox6, Sox9, and aggrecan) than those in collagen type I hydrogels, when cells were cultured in expansion medium and chondrogenic induction medium. (b) ADSCs in collagen type II hydrogels showed lower Rock 2 expression and a more round shape than those in collagen type I hydrogels in expansion medium. (c) $\beta1$ integrin blocking not only reduced the differences in chondrogenic gene expression but also eliminated the differences in Rock 1 and Rock 2 gene expression and cell shape compared with ADSCs in collagen type I and type 2 hydrogels.[71] It can be concluded that collagen

type II provides the inductive signal for chondrogenic differentiation in ADSCs by promoting a round cell shape through β1 integrin-mediated Rho A/Rock signaling.[71]

A combination of collagen type I and type III, which are the most abundant proteins in the osteocyte environment, is osteoinductive, and hybrid scaffolds comprised of collagen type I and type III have been used for MSC culture materials.[34,238−240] Schneider et al. investigated the osteogenic differentiation of BMSCs and perinatal MSCs from Wharton's jelly of the umbilical cord (UC-MSC) in hybrid scaffolds of collagen type 1 (90%) and type III (10%).[34] Because of their primitive state, UC-MSCs were expected to possess a higher differentiation potential than BMSCs, which lack the expression of embryonic stem cell markers (Oct4 and Nanog). However, UC-MSCs had a poor ability to differentiate into adipocytes in monolayer culture and in 3D culture.[34,241,242] Furthermore, BMSCs exhibited the most robust osteogenic induction and extracellular mineralization when cultured under osteogenic conditions in a monolayer. However, UC-MSCs in hybrid scaffolds of collagen type I and type III exceeded BM-MSCs in ECM protein synthesis.[34] UC-MSCs and BMSCs displayed all the features needed for effective bone fracture healing in vivo. The expression of ECM proteins differed considerably in the two cell types, suggesting different mechanisms for bone formation.

5.2.4. Hybrid Collagen Scaffolds Using Inorganic Materials. The major components of human bone are inorganic hydroxyapatite (a natural ceramic) and organic collagen type I. In addition, there are small amounts of ground substances, such as glycoproteins, proteoglycans, and velum lipids, which have been demonstrated to play important roles in regulating bone regeneration and mineralization.[204,243]

Natural bone is composed of nanosized carbonate substituted hydroxyapatite (nano-HYA) crystals within a collagen network. The generation of scaffolds closely resembling the composition and microstructure of collagen and nano-HYA in bone should be useful for osteogenic differentiation of BMSCs.[70] Several researchers have suggested that hydroxyapatite (HYA) promotes differentiation of MSCs into osteoblasts.[191,244] Dawson et al. prepared collagen−HYA scaffolds as follows: HYA solution was added to a collagen solution, and the solution was frozen at −30 or −80 °C. Then, the frozen collagen−HYA solid was dehydrated. Critical point drying with liquid CO_2 resulted in dry porous scaffolds.[70] Primary hBMSCs were seeded onto collagen−HYA scaffolds and following 72 h of osteogenic induction were subcutaneously implanted into immunodeficient mice. After 4 weeks, the implanted cell−scaffold constructs were slightly compacted within the subcutaneous cavity and surrounded with host neovasculature.[70] The collagen−HYA scaffolds were fully integrated with the host tissue, and significant cell invasion into the scaffolds was observed. New osteoid matrix was evidenced by the characteristic appearance of cells embedded in lacunae within the matrix and the birefringence of organized collagen fibers under polarized light.[70] In addition, collagen−HYA scaffolds seeded with hBMSCs and cultured for 48 h in osteogenic conditions were implanted subcutaneously in immunodeficient mice on a devitalized mouse femur with a segmental "v"-shaped defect. Implanted cell−scaffold constructs demonstrated good integration with mouse femurs, as evidenced by large areas of deposited matrix surrounding the defect site and encapsulation of the femur edges. Thus, collagen−HYA scaffolds can support osteogenesis in vivo. Both collagen and HYA enhance the osteogenic response in collagen−HYA scaffolds embedded with MSCs. It is proposed that collagen−hydroxyapatite or collagen−nanocrystalline hydroxyapatite scaffolds have better osteoconductive properties than hydroxyapatite or collagen alone.[70,191,245,246]

Bioactive glasses (BGs) such as $CaO−P_2O_5−SiO_2$ are similar to natural inorganic components of bone and have been shown to stimulate the formation of calcium phosphates from physiological solutions, resulting in enhanced bone−matrix interface strength.[204,247] Composite materials composed of a bioactive glass and collagen type I have been reported as bone tissue engineering scaffolds.[204,248] Matrix vesicles, extracellular lipid bilayer-enclosed microstructures released by calcifying cells, have been reported to initiate mineral formation during bone formation.[249] In particular, phosphatidylserine (PPS) has a high affinity for calcium ions and should be an important component of newly forming bone.[250,251] Xu et al. prepared biomimetic composite scaffolds of bioglass−collagen−phosphatidylserine (BG−COL−PPS) using a freeze-drying technique.[204] The BG−COL−PPS composite scaffolds consisted of 65 wt % inorganic components and 35 wt % organic components, where the organic component was composed of 80% collagen type I and 20% PPS. BMSCs in BG−COL−PPS composite scaffolds exhibited a higher degree of cell attachment, growth, and osteogenic differentiation than those on BG−COL scaffolds in vitro, which was determined by dsDNA content, ALP activity, osteogenic gene expression (ALP, osteopontin, and osteocalcin), and Alizarin Red staining.[204]

BG−COL−PPS scaffolds seeded with and without rat BMSCs were implanted in rat femur defects to investigate their in vivo biocompatibility and osteogenesis.[204] BG−COL−PPS scaffolds exhibited good biocompatibility and extensive osteoconductivity with host bone. BG−COL−PPS with BMSCs dramatically enhanced the efficiency of new bone formation compared to BG−COL−PPS without BMSCs or BG−COL with BMSCs.[204] This study demonstrates the usefulness of PPS in collagen−bioglass hybrid scaffolds for inducing enhanced bone formation.

5.2.5. Collagen Scaffolds Immobilized Antibody-Targeting Stem Cells. Although some stem cells are known to circulate in the body, mobilized stem cells cannot be specifically recruited into the injury sites in the body.[40] In heart disease, tissue-engineered cardiac patches made of ECM proteins have been used to treat heart failure, but myocardial repair was limited due to the low capacity for stem cell infiltration.[40,252,253] A new approach, in which stem cells are recruited from circulation system using scaffolds with immobilized antibodies or ligands that bind specific stem cells, was reported by Shi et al.[40] (Figure 3e). They developed collagen scaffolds, and membranes covalently immobilized anti-Sca-1 monoclonal antibody using Traut's reagent and sulfosuccinimidyl-4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC).[40] Sca-1 is a member of the Ly 6 family and is a common marker for adult murine hematopoetic stem cells. Furthermore, Sca-1-positive cells derived from skeletal muscle and heart were reported to be multipotent.[254] Shi et al. attempted to enrich autologous stem cells at wound sites using a stem cell-capturing collagen scaffold conjugated with a Sca 1 monoclonal antibody in mice.[40] The antibody-conjugated scaffold was implanted in the hind leg muscles. Sca-1-positive cells were found to be enriched 3-fold in the scaffolds conjugated with anti-Sca-1 monoclonal antibody than in the scaffolds without antibody. When the functional collagen

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