CHEMICAL REVIEWS

Review

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Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation

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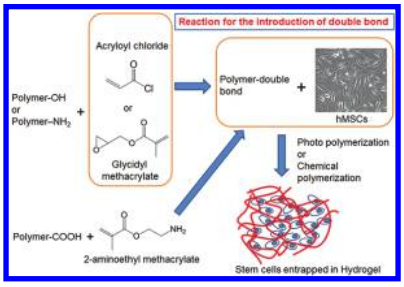

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1. INTRODUCTION

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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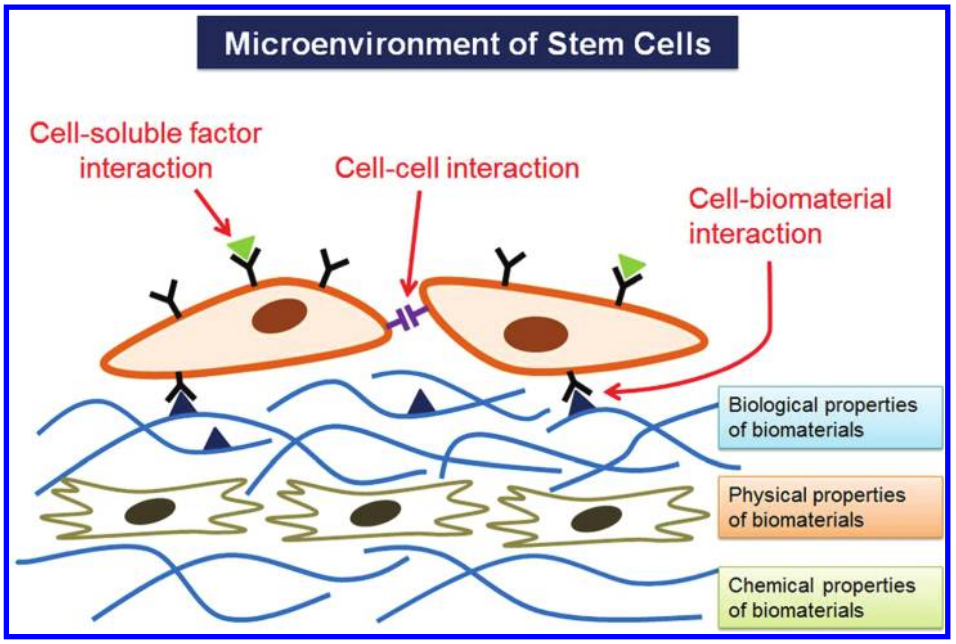

Figure 1. Schematic representation of the microenvironment and niches of stem cells and their regulation by the following factors: (a) soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (b) cell−cell interactions; (c) cell−biomaterial interactions. Biological, physical,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

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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 (GlcAC5E)	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 aminopeptidase (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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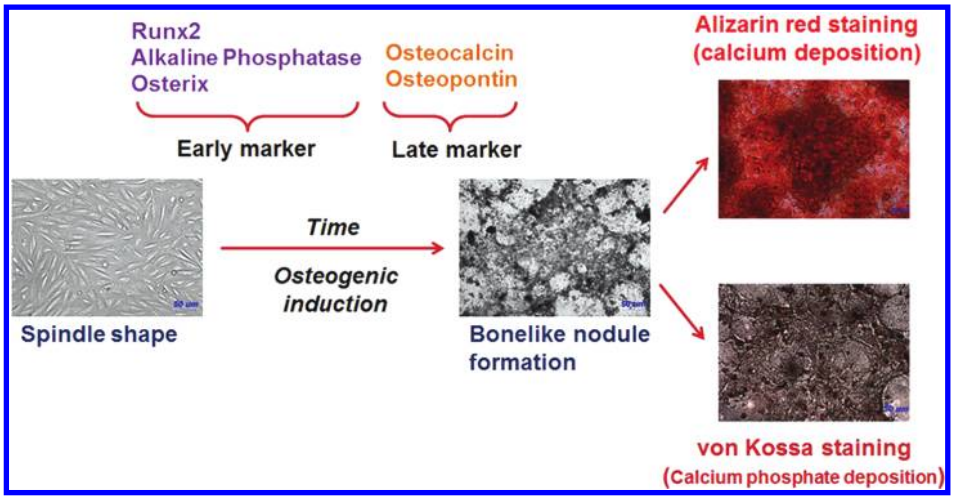

Figure 2. Osteogenic differentiation of MSCs, gene expression, and mineral deposition at early and late stages.

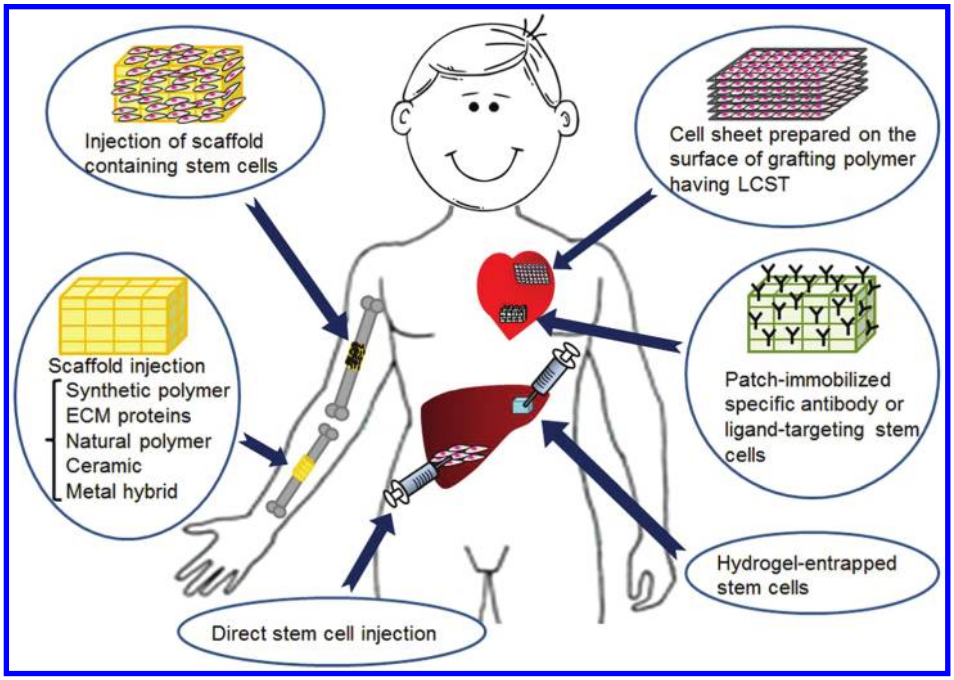

Figure 3. Some examples of biomaterial designs with and without stem cells for the injection of biomaterials in clinical applications: (a) injection of scaffold containing stem cells, (b) injection of scaffold without cells, (c) direct stem cell injection, (d) injection of cell sheets, (e) injection 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

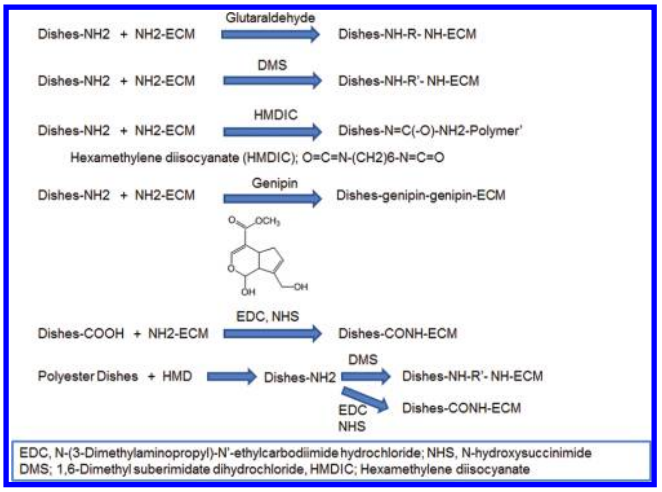

Figure 4. Surface reactions of covalent immobilization of ECM proteins and ECM-mimicking peptides on dishes.

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.

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
G**RGD**SPK	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
KGGPQVT**RGD**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

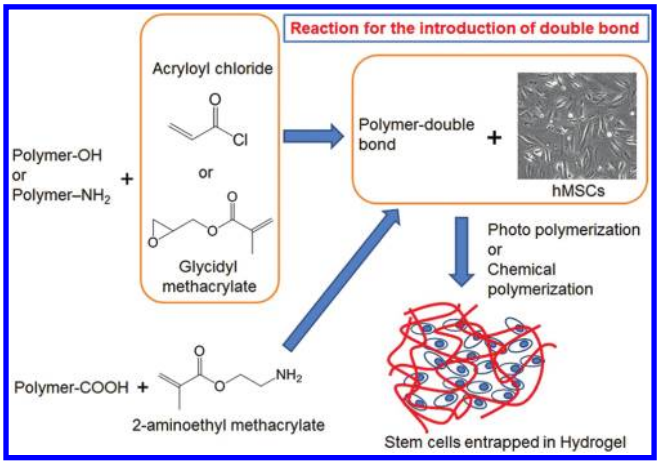

Figure 5. Schematic of the preparation method of hydrogels with entrapped stem cells by photopolymerization.

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.

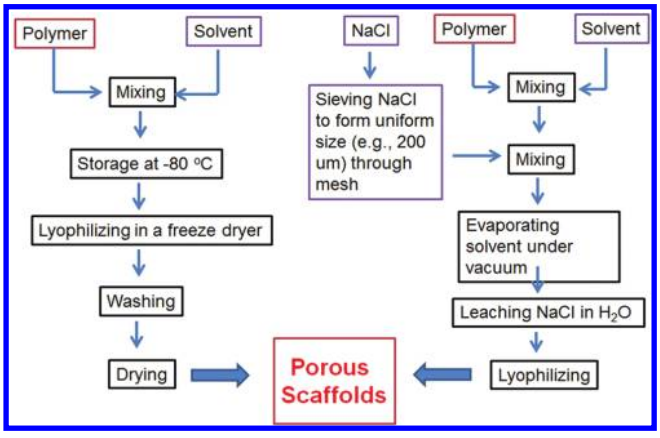

Figure 6. Typical preparation method of porous scaffolds by freeze-drying (a) and salt leaching (b).

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]

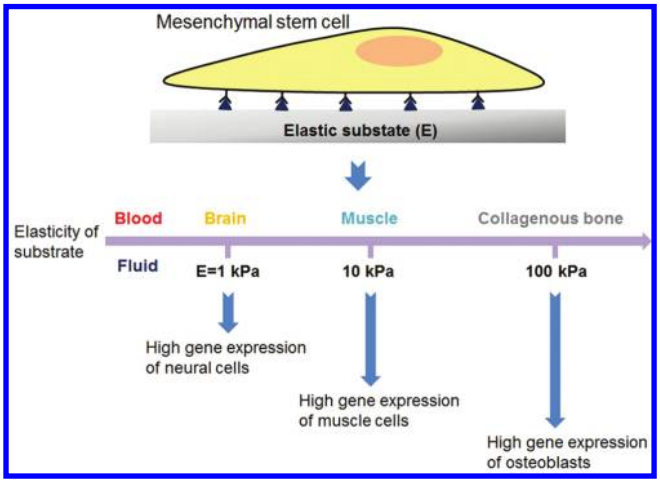

Figure 7. Physical properties decide the fate of stem cell cultured on biomaterials with different elasticity. Modified with permission from ref 19. Copyright 2006 Elsevier Inc.

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]

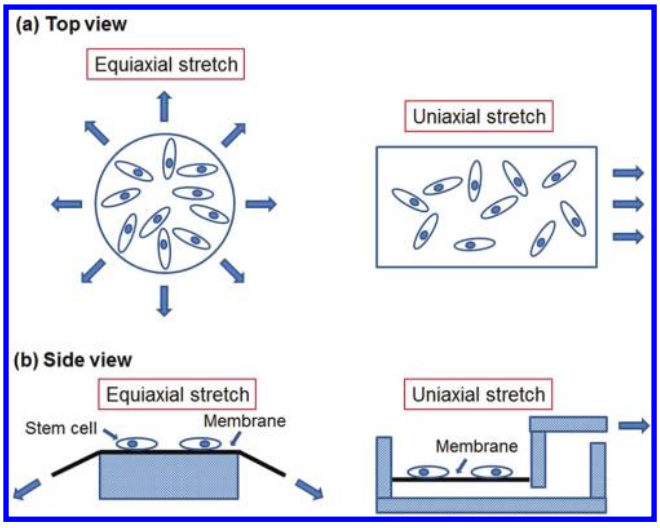

Figure 8. Schematic model of the apparatus that can apply equiaxial (a) and uniaxial (b) strain to MSCs. Modified with permission from ref 141. Copyright 2004 Wiley Periodicals.

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

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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, 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.

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

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 α5β1, α8β1, αIIbβ3, αVβ3, αVβ5, αVβ6, and αVβ8, 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 α2β1.[103] Collagen type I is also bound by integrins α1β1, α3β1, and αVβ3.[58,97] RGD in collagen type I is reported to associate with integrin αVβ3.[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%

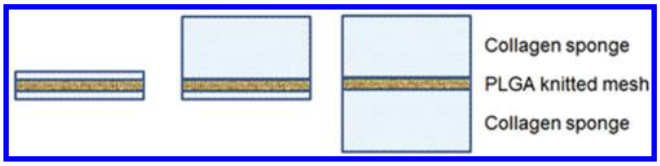

Figure 9. Schematic illustration of three structural designs of PLGA/collagen hybrid scaffolds. Black, PLGA knitted mesh; gray, collagen type I sponge. Modified with permission from ref 232. Copyright 2010 Elsevier Ltd.

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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

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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

scaffold was transplanted into mice as a cardiac patch to repair a surgical heart defect, more cells and capillaries infiltrated implants with immobilized anti-Sca-1 antibody.[40] Twelve weeks after surgery, the regeneration of cardiomyocytes was reported in antibody-conjugated cardiac patches, whereas collagen remodeling and tissue regeneration were retarded in control cardiac patches. Collagen scaffolds embedded with antibodies or ligands targeting specific stem cells represent another effective strategy for recruiting and maintaining stem cells at injury sites.

5.2.6. Differentiation into Ectoderm and Endoderm Lineages Using Collagen Scaffolds. Scaffolds and gels composed of collagen are mainly used in tissue engineering for osteogenic and chondrogenic differentiation of MSCs. However, collagen scaffolds have also been used for ectodermal and endodermal differentiation of MSCs.[189,216,217]

Poly(L-lactic acid-*co*-3-caprolactone) (PLCL), which is a synthetic and biodegradable polymer and a nontoxic copolymer of poly(L-lactic acid) (PLLA) and PCL, has been investigated as a biomaterial for surgery and drug-delivery systems.[45,255] Collagen, on the other hand, is a natural ECM protein with high cell-adhesion properties but weak mechanical strength. Prabhakaran et al. prepared electrospun nanofibers by blending collagen with PLCL, which improved its biocompatibility while preserving mechanical strength and providing a hydrophilic mesh with high porosity and small fiber diameters that are desirable for nerve tissue engineering.[45] MSCs differentiated on PLCL/collagen type I nanofibrous scaffolds showed neuronal morphology with multipolar elongations and expressed neurofilament (NF200) and nestin protein, as shown by immunofluorescent labeling.[45]

The mammalian central system (CNS) has little capacity for self-repair after injury, and neurons do not proliferate. Therefore, neural tissue engineering using hydrogels seeded with neural stem cells may expand the options for treatment of damaged CNS tissues. Ma et al. prepared collagen type I gels seeded with neural stem cells isolated from embryonic rat cortical or subcortical neuroepithelium and cultured them in serum-free medium.[189] The collagen-entrapped stem cells expanded and efficiently generated neurons, which developed neuronal polarity, neurotransmitters, ion channels/receptors, and excitability.[189] The differentiation from $BrdU^+/Tuj1^-$ to $BrdU^-/Tuj1^+$ cells was accompanied by a shift in the expression of functional receptors for neurotransmitters from cholinergic and purinergic to GABAergic and glutamatergic.[189] Spontaneous postsynaptic currents were recorded by patch-clamping from stem cell-derived neurons. These results suggest that neural stem cells cultured in collagen gels recapitulate CNS stem cell development.

5.3. Gelatin

Gelatin is heat-denatured collagen, which is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the boiled bones, connective tissues, organs, and intestines of animals.[256] Gelatin exists as a heterogeneous mixture of single- or multistranded polypeptides containing between 300 and 4000 amino acids. There are two general types of gelatin, type A and type B.[256] Gelatin type A is extracted and processed by acidic pretreatment of collagen, whereas gelatin type B is obtained by alkaline pretreatment.[256] The alkaline pretreatment converts glutamine and asparagine residues into glutamic and aspartic acids, respectively, which leads to a higher carboxylic acid content for gelatin type B than for gelatin type A. Gelatin has several potential advantages over other natural proteins, such as its biological origin, biodegradability, commercial availability, and low cost.[256] Gelatin melts to a liquid when heated and solidifies when cooled. Therefore, it is easy to prepare hydrogels and to entrap stem cells in gelatin. The chemical composition of gelatin is, in many respects, similar to that of its parent collagen. Table 9 summarizes several types of gelatin scaffolds or materials for MSC differentiation reported in the literature.[99,192,256−263]

Table 9. Some Research Studies for Stem Cell Differentiation on Gelatin Materials in 2D and 3D Culture

stem cell source[a]	material for stem cell culture	differentiation	ref
hBMSCs	gelatin (2D culture, coating on dishes)	osteoblasts	217
hBMSCs	gelatin/HA (2D culture, hydrogel particles)	osteoblasts	99
hBMSCs	gelatin (2D culture, coating on dishes)	pancreatic cells, neural cells, osteoblasts, adipocytes	257
rat BMSCs	gelatin (3D culture, scaffold)	osteoblasts	258
rat BMSCs	gelatin (3D culture, microparticles)	osteoblasts	259
hADSCs	gelatin (3D culture, scaffold)	chondrocytes	262
rBMSCs	gelatin/esterified HA (3D culture, scaffold)	chondrocytes	260
hBMSCs	gelatin (3D culture, scaffold)	cartilage	261
hADSCs	gelatin/PCL (3D culture, electrospinning mat), gelatin/collagen I/PCL (3D culture, electrospinning mat)		256

[a]ADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; hADSCs, human ADSCs; hBMSCs, human BMSCs; rBMSCs, rabbit BMSCs. [b]PCL, poly(ε-caprolactone); HA, hyaluronic acid.

5.3.1. Gelatin Scaffolds and Hydrogels. Ponticiello et al. used a porous gelatin sponge, Gelfoam (used as hemostatic agent), as a delivery vehicle for hMSCs in cartilage-regeneration therapy. hMSC in Gelfoam produced a cartilage-like ECM containing sulfated glycosaminoglycans and collagen type II after 21 days of cultivation in vitro.[261] Gelfoam cylinders containing hMSCs were observed to be biocompatible, with no evidence of immune response or lymphocytic infiltration at the site of implantation in an osteochondral defect in the rabbit femoral condyle. Gelfoam resorbable gelatin sponges may be a promising candidate as a carrier matrix for hMSC-based cartilage-regenerative therapies.[261]

Chondrogenic differentiation of human ADSCs (hADSCs) in gelatin scaffolds (Surgifoam) and in alginate and agarose hydrogels was investigated by Awad et al.[262] hADSCs in gelatin scaffolds showed more polygonal shapes, whereas cells encapsulated in alginate and agarose exhibited a spherical morphology. Significant cell-mediated contraction of the gelatin scaffolds (discs) was observed, with a reduction of up to 70% and 87% of their initial diameters under chondrogenic and control culture conditions, respectively, while alginate and agarose disks containing cells did not exhibit any contraction.[262] Protein and proteoglycan biosynthesis rates in the gelatin scaffolds were significantly higher than in agarose (31%) and alginate (68%) on day 1.[262] The number of cells in gelatin scaffolds was 37−51% greater than in agarose and alginate scaffolds on days 14 and 28. Sulfated glycosaminoglycan and

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hydroxyproline content increased significantly (by 2.5- to 9-fold) between days 1 and 28 for all scaffolds containing cells grown in chondrogenic conditions.[262] Gel contraction was generated in the regions enriched in chondroitin sulfate and collagen type II, which indicate cartilage generation. The gelatin scaffolds and agarose hydrogels had shear moduli three times greater than alginate hydrogels. However, it should be noted that the compressive and shear moduli of these scaffolds and hydrogels were on the order of 5% or less than those of native cartilage.[262,264,265] The increase in shear modulus was found to be significantly correlated with increases in sulfated glycosaminoglycan content and hydroxyproline content. Gelatin is an attractive biomaterial for scaffold of hMSCs or hADSCs. However, it is necessary to design gelatin-based scaffolds containing hMSCs or hADSCs that have similar compressive and shear moduli to native cartilage in future.[262]

Payne et al. investigated an injectable, in situ cross-linkable, degradable gelatin carrier for MSCs. MSCs were encapsulated in un-cross-linked gelatin microparticles with an average diameter of 630 μm, each containing ~53 cells.[259] Gelatin microparticles were cross-linked to a shell thickness of 75 μm via exposure to dithiobis(succinimidylpropionate) (DSP) solution. MSCs survived in un-cross-linked and cross-linked gelatin microparticles and retained their proliferative potential and osteoblastic phenotype over 28 days.[259] The encapsulation of cells in microparticles cross-linked with DSP holds promise for temporarily protecting cells from toxic local environments.[259]

MSCs are generated by plating cells from bone marrow (BM) or other sources in tissue culture polystyrene (TCPS) flasks and selecting plastic-adherent cells with fibroblastoid morphology. Battula et al. selected MSCs from BM and nonamniotic placenta (PL) by culturing Ficoll-selected cells in gelatin-coated flasks in serum-free medium containing bFGF, which was used for hESC expansion.[257] MSCs generated in gelatin-coated flasks in hESC medium showed a 4- to 5-fold higher proliferation rate than conventionally prepared MSCs, which were grown in TCPS in serum-containing medium. In contrast, the colony-forming unit-fibroblast (CFU-F) number was only 1.5- to 2-fold increased in PL-MSCs and was not affected in BM-MSCs. PL-MSCs and BM-MSCs grown on gelatin-coated dishes in hESC medium showed increased expression of the pluripotent stem and progenitor cell markers SSEA-4, Oct-4, nanog-3, and nestin, as well as frizzled-9 (FZD-9). PL-MSCs expressed Oct-4, SSEA-4, and FZD-9 at higher levels than BM-MSCs.[257] However, PL-MSCs and BM-MSCs cultured on TCPS expressed significantly lower levels of SSEA-4, Oct-4, and nestin than those cultured on gelatin-coated dishes. No expression of FZD-9 and nanog-3 was seen in BM-MSCs and PL-MSCs cultured on gelatin-coated dishes. The MSCs cultured on gelatin-coated dishes exhibited multilineage differentiation capacity, as demonstrated by their potential to give rise to cells of ectodermal (neuron-like) and endodermal (pancreatic-like) differentiation lineages, as well as mesodermal lineages (osteoblast, adipocytes).[257] Notably, the CFU-F capacity of BM-MSC and PL-MSC was not significantly altered by the different culture conditions, suggesting that the stem cell pool of MSCs was not affected. Battula et al. proposed that FZD-9 might represent a marker of primitive MSCs, which could distinguish them from mature MSCs, and can be explained by the fact that Wnt-FZD9 signaling is important for stem cell renewal.[257]

The optimal ECM for selecting primitive MSCs by culturing bone marrow, amniotic fluid, and adipose tissue on ECM-coated or ECM-grafted substrates has not yet been determined and should be a key research topic for biomaterial researchers in future. Specific ECM-coated or ECM-grafted dishes might select cells with higher pluripotency and greater quantities of primitive MSCs compared with gelatin-coated dishes or TCPS.

Photoinitiated cross-linking of gelatin hydrogels incorporated with chondrocytes has also been reported.[266] The gelatin molecule was modified with methacrylic acid (MA) to obtain cross-linkable gelatin, which formed a chemically cross-linked hydrogel by photoinitiated polymerization. The gelation time could be easily tuned and showed an inverse relationship with gelatin concentration. No detectable double carbon bonds were reported to be observed in the hydrogels from analysis of the hydrogen spectrum of high-resolution magic-angle spinning nuclear magnetic resonance spectroscopy.[266] The storage modulus and loss modulus of the hydrogels were found to increase with increasing gelatin concentrations, whereas the swelling ratio and mesh size were reported to decrease.[266] TGF-β1 was also incorporated into the gelatin hydrogel to improve its bioactivity.[266] In vitro chondrocyte culture showed that the gelatin hydrogel had excellent performance in supporting chondrocyte growth and maintaining the chondrocytic phenotype. Incorporation of TGF-β1 was found to further improve the biological activity in terms of both ECM secretion and cell proliferation.[266]

5.3.2. Gelatin Hybrid Scaffolds. Gelatin is reported to be an excellent substrate for cell attachment, proliferation, and differentiation.[260,267−269] However, the disadvantages of using gelatin as a scaffold in tissue engineering are its low biomechanical stiffness and rapid biodegradation.[260,268] Esterified hyaluronic acids are longer-lived biomaterial matrices, and scaffolds prepared from esterified hyaluronic acids persist long enough to be a useful in vivo substrate for differentiation of MSCs and matrix formation. However, esterified hyaluronic acid-based surfaces can impede cell attachment,[260,267] and MSCs on the surface are reported to (re)differentiate in vitro.[260] Hyaff11, a pure hyaluronic acid benzyl ester, was reported to undergo degradation by spontaneous hydrolysis of the ester bonds in two months in vitro[260,270] and in 3−5 months in vivo.[260,271] Cell-loaded gelatin sponges were reported to dissolve after 10 days in culture because of collagenolytic activity of infiltrating cells[260,271] and after 7−14 days in vivo.[260,272]

Angele et al. investigated the ability of a composite scaffold made of esterified hyaluronic acid (Jaloskin, 70%) and gelatin (30%) to facilitate the differentiation of rabbit BMSCs to engineer cartilage and bone. The composite scaffolds were prepared by a salt-leaching method.[260] The composite scaffolds had pores with two different size ranges, 50−150 μm and 250−500 μm in diameter, and contained mainly interconnected and a few blind-ended pores. Empty and cell-loaded composite scaffolds were cultivated for up to 28 days in the medium with and without TGF-β1. A collagen type II-rich ECM was produced by cells loaded in the composite scaffolds and cultured in the presence of TGF-β1.[260] The composite scaffolds supported osteochondrogenic cell differentiation of rabbit BMSCs when they were implanted subcutaneously into immunodeficient mice, whereas no osteochondral differentiation was found in implanted composite scaffolds without cells.[260] In vitro preculturing in a chondrogenic medium increased the percentage of osteochondral tissue in the

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composite scaffolds after 3 weeks in vivo. These results indicate that these composite scaffolds might be useful for tissue engineering.[260]

Takahashi et al. fabricated biodegradable gelatin sponges incorporating various amounts of β-tricalcium phosphate (βTCP) (gelatin−βTCP)[263] and investigated the in vitro osteogenic differentiation of MSCs isolated from rat bone marrow. The gelatin sponges incorporating βTCP had an interconnected pore structure with the average size of 180−200 μm, irrespective of the amount of βTCP.[263] The stiffness of the sponges became higher with increasing amounts of βTCP. When seeded by agitation, MSCs were homogeneously distributed throughout the sponge. The morphology of cells attached to the gelatin−βTCP became more spread with the greater amounts of βTCP.[263] The rate of MSC proliferation depended on the amount of βTCP and the culture method: the more βTCP in the stirring culture, the higher was the proliferation rate. The extent of deformation of the gelatin−βTCP sponges was reduced with increasing amounts of βTCP. ALP activity and osteocalcin content, as markers of osteogenic differentiation, were greatest for the sponge with a βTCP amount of 50% (wt).[263] ALP activity and osteocalcin content were found to be significantly higher in stirring cultures compared with those in static cultures. Thus, the attachment, proliferation, and osteogenic differentiation of MSCs are influenced by the composition of gelatin and βTCP sponges.

Electrospinning using natural ECM proteins is a promising technique for the fabrication of fibrous scaffolds for various tissue-engineering applications. One limitation of scaffolds electrospun from natural ECM proteins is the need to use a cross-linking agent for stability, which has been postulated to lead to many complications in vivo, including graft failure. Currently, glutaraldehyde has mainly been investigated as a cross-linking agent for electrospun collagen-based nanofibers.[273−276] Glutaraldehyde was required for intermolecular cross-linking of the fibers in the scaffolds for cell culture to prevent dissolution in culture medium. The cross-linked scaffolds showed markedly thickened fibers that frequently merged into one another, and the porosity decreased dramatically, making them unsuitable scaffolds for 3D culture of stem cells. Furthermore, residual glutaraldehyde is significantly toxic to tissue and stem cells.[256]

Heydarkhan-Hagvall et al. prepared hybrid nanofiber scaffolds of gelatin and poly(ε-caprolactone) (PCL), as well as hybrid nanofiber scaffolds of collagen, elastin, and PCL, using electrospinning without a toxic cross-linking agent.[256] Electrospun gelatin/PCL scaffolds showed a higher tensile strength compared to collagen/elastin/PCL constructs. PCL doping of the ECM protein solution as the electrospinning solution generated self-standing scaffolds in aqueous environment. It was necessary to increase the PCL concentration to at least 5% in the scaffolds to maintain their three-dimensional and porous structures without the use of glutaraldehyde.[256] Both hybrid scaffolds were seeded with ADSCs to determine the effects of pore size on cell attachment and migration. Complete cell attachment was reported on the surfaces of both hybrid scaffolds. It was found that cell migration into the scaffold was predominantly observed in the gelatin/PCL hybrid.[256] The combination of 10% PCL with 10% gelatin resulted in significantly higher tensile strength compared to gelatin or collagen and elastin alone, and this resulted in a uniform and pliant fiber mat.[256] We can conclude that electrospinning of hybrid scaffolds with natural proteins and synthetic polymers can be used to produce tissue-engineered scaffolds that better recapitulate key features of the native ECM, including its mechanical and biochemical properties. The combination of natural proteins and synthetic polymers to create electrospun fibrous structures results in scaffolds with favorable mechanical and biological properties.[256]

5.4. Laminin

Laminins are one of the major glycoproteins found in the basal lamina, which is critical for mediating a variety of cellular activities, including adhesion, proliferation, migration, and differentiation. Laminins are trimeric proteins that contain an α-chain, a β-chain, and a γ-chain, which have five, four, and three genetic variants, respectively.[100] Laminin molecules are named according to their chain composition., e.g., laminin-111 contains α1, β1, and γ1 chains (Laminin-1) and laminin-332 contains α3, β3, and γ2 chains (Laminin-5).[277] Laminin is frequently used as coating for cell culture materials, and it promotes differentiation into osteoblasts,[278] cardiocytes,[83,279] and neural cells.[76,79,101,215,280−282] Laminin is known to make direct contact with adult neural stem cells (hNSC's) via basal lamina-like extensions from blood vessels in the subventricular zone.[283] Therefore, laminin is frequently used as a coating material on the dishes for the culturing of neural cells.[22] Table 10 summarizes several types of laminin-coated scaffolds and dishes for MSC differentiation reported in the literature.[37,43,53,76,79,83,97,101,102,149,192,198,200,201,278,279,284−286]

Yu et al. developed an efficient method to induce the generation of proliferative dopaminergic neurons from rat NSCs in the presence of bFGF, heparin, and laminin in vitro and in vivo.[285] In their research, neurospheres of rat NSCs were cultured on dishes coated with 0.01% poly-D-lysine (PDL) and 1 μg/cm^2 laminin in culture medium supplemented with bFGF and heparin. The majority of cells remained nestin positive, which indicates neural stem cells, for one day of differentiation. Neurons were derived from neurospheres, of which some were TH positive (TH$^+$, dopaminergic) and a few cells were GFAP (glial fibrillary acidic protein) positive.[285] After differentiation for 7 days, more neurons were found to have become dopaminergic positive cells. Cells primed by bFGF and heparin and cultured on dishes coated with PDL and laminin for 7 days in vitro were injected into ventral tegmental area (VTA) and medial forebrain bundle (MFB) region of lesioned rats to evaluate whether the NSCs could become dopaminergic neurons in vivo.[285] TH$^+$ cells were found mainly near the injection sites after grafting of 5×10^4 primed NSCs. It was suggested that combination of bFGF and heparin could induce the generation of dopaminergic neurons from rat NSCs cultured on dishes coated with PDL and laminin in vivo and in vitro.[285]

Oligodendrocytes are glial cells responsible for myelin formation and maintenance in the central nervous system (CNS), and they are depleted in many acute and chronic diseases [e.g., Pelizaeus-Merzbacher disease and multiple sclerosis (MS)]. NSCs derived from human cord blood cells were reported to undergo oligogliogenesis when cultured on dishes coated with laminin, but not with poly-L-lysine, collagen type I, or fibronectin.[37] The adhesion of NSCs to laminin promoted a 2.4-fold increase in the oligodendrocyte number (11.8% on laminin versus 4.9% in controls).[37] Matrix metalloproteinase (MMP) expression was also reported to increase 3.6-fold on dishes coated with laminin (3.6% on

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Table 10. Some Research Studies for Stem Cell Differentiation on 2D and 3D Laminin Materials

stem cell source[a]	material for stem cell culture[b]	differentiation	ref
hBMSCs	laminin (2D culture, coating on dishes)	osteoblasts	149, 192
hBMSCs	laminin-1 (2D culture, coating on dishes)	osteoblasts	97
hBMSCs	laminin-5 (2D culture, coating on dishes)	osteoblasts	278, 284
hBMSCs	laminin-5 (2D culture, coating on dishes)	osteoblasts, chondrocytes	102
hADSCs	laminin (2D culture, coating on dishes)	adipocytes	53
hBMSCs	laminin (2D culture, coating on dishes)	smooth muscle cells	83
hADSCs	laminin (2D culture, coating on dishes)	cardiomyocytes	279
hESCs (TE03, TE06)	laminin/PDL (2D culture, coating)	neural cells	79
hBMSCs	laminin-1 (2D culture, coating on dishes)	neural cells	101
hBMSCs	laminin-10/11 (2D culture, coating on dishes)	neural cells	101
mESCs	laminin (2D culture, coating on dishes)	neural cells	198
rat neural stem cells	laminin (2D culture, coating on dishes)	dopaminergic neurons	285
human neural stem cells	laminin (2D culture, coating on dishes)	oligogliocytes	37
mESCs	laminin-332 (2D culture, coating on dishes)	lung epithelium	286
mouse hepatitic stem cells	laminin (2D culture, coating on dishes)	hepatocytes	200
mESCs	laminin-332 (3D culture, coating on PDDLA, sheet)	lung epithelium	286
rat neural stem cells	laminin (3D culture, coating on PES fiber mesh)	neural cells	43
hBMSCs	laminin (3D culture, coating on PLGA microcarrer)	dopamin-secreting neurons	76
hBMSCs	laminin (3D culture, coating on PLLA sheet)	smooth muscle cells	83

[a]ADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hBMSCs, human BMSCs; hESCs, human ESCs. [b]PDL, poly-D-lysine; PDDLA, poly-DL-lactic acid; PES, polyethersulfone; PLGA, poly(lactic-*co*-glycolic acid); PLLA, poly-L-lactic acid.

laminin, 3.0% on fibronectin, 2.0% on poly-L-lysine and collagen type I, and 1% in controls), which suggested a link between ECM, especially laminin, and the activity of metalloproteinases in the cells.[37]

Tate et al. investigated the transplantation of laminin- or fibronectin-based scaffolds containing neural stem cells into traumatically injured mouse brain.[215] Survival of neural stem cells was enhanced in the laminin-based scaffold compared to the fibronectin-based scaffold. The mice that received neural stem cells in the laminin-based scaffold performed significantly better than untreated mice on a spatial learning task. These findings support the idea that selecting the appropriate ECM for the scaffold loading neural stem cells can improve cell-transplantation therapy.[215]

Ma et al. reported the effect of ECM proteins on neural differentiation of hESCs.[79] Embryoid bodies derived from hESCs were plated on dishes coated with PDL, PDL/fibronectin, PDL/laminin, collagen type I, and Matrigel and cultured in neural differentiation medium. Neural progenitors and neuronal differentiation were observed to different degrees depending on the substrate on which the embryonic bodies were cultured. Neural progenitor generation, neuronal generation, and neural outgrowth were found to be significantly greater on dishes coated with laminin and laminin-rich Matrigel substrates than on other ECM protein-coated dishes.[79] Laminin stimulated hESC-derived neural progenitor expansion and neural outgrowth in a dose-dependent manner. The cells from embryoid bodies of hESCs interacted with laminin through $\alpha6\beta1$ integrin receptors, implicating the role of laminin/$\alpha6\beta1$ integrin signaling in directing neural differentiation of hESCs.[79]

Mruthyunjaya et al. investigated the neurite outgrowth induction potential of hBMSCs cultured on dishes coated with fibronectin, collagen type I, collagen type IV, laminin-1, and laminin-10/11 in the absence of growth factors and induction agents.[101] All of ECM proteins evaluated were found to support adhesion of hBMSCs to different degrees, but only direct interaction with laminin-1 triggered sprouting of neurite-like processes. hBMSCs plated on dishes coated with laminin-1 exhibited neurites with contracted cell bodies and neuronal morphology and neurite outgrowth by 24 h.[101] The interaction of hBMSCs with laminin-1 was mediated through $\alpha6\beta1$ integrin receptors and the MEK/ERK signaling pathway, as neurite outgrowth was suppressed by inhibiting these signals.[101]

Laminin-5 is known to be present in bone and is also expressed by hBMSCs.[278] hBMSCs synthesize laminin-5 and adhere to exogenous laminin-5 through $\alpha3\beta1$ integrin. Laminin-5 contributes to the development of bone tissues by promoting proliferation and by suppressing the chondrogenic differentiation of hBMSCs.[102]

Klees et al. reported that the adhesion of hBMSCs to laminin-5 activated ERK within 30 min and led to phosphorylation of the osteogenic transcription factor Runx2/CBFA-1 within 8 days.[278,284] hBMSCs cultured on dishes coated with laminin-5 for 16 days expressed increased levels of osteogenic marker genes including ALP, osteocalcin, and osteopontin. Cells cultured for 21 days deposited a mineralized matrix, which indicated osteogenic differentiation.[278] Addition of the ERK inhibitor PD98059 to the culture medium inhibited osteogenic differentiation of hBMSCs cultured on dishes coated with laminin-5 as well as of cells cultured on tissue culture plates in osteogenic induction medium. It was suggested that the contact of hBMSCs with laminin-5, but not with fibronectin, is sufficient to activate ERK and to stimulate osteogenic differentiation in hBMSCs in the absence of induction reagents (e.g., dexamethasone) in the culture medium.[278]

Salasznyk et al. also reported that contact of hBMSCs with laminin-5 was sufficient to induce osteogenic differentiation of hBMSCs through an ERK-dependent pathway.[284] They further reported that FAK-mediated signaling pathways link integrin $\alpha3\beta1$/laminin-5 binding and activation of ERK1/2 and that laminin-5 promoted osteogenic differentiation through this pathway.[284]

Cardiomyocyte differentiation of ADSCs cultured on laminin-coated, fibronectin-coated, and uncoated culture plates was reported by van Dijk et al.[279] Expression of an early cardiomyocyte marker, myosin light chain-2a (MLC-2a), increased significantly in cells on all dishes after 1 week of cardiomyocyte induction, whereas the late cardiomyocyte marker SERCA2a was only significantly increased in ADSCs cultured on laminin-coated dishes after 5 weeks. The number of

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desmin-positive cells (a late cardiomyocyte marker, a 52 kD protein that is a subunit of intermediate filaments in cardiac muscle tissue) was only significantly increased in ADSCs cultured on laminin-coated dishes. Thus, human ADSCs cultured on laminin-coated dishes can be effectively differentiated into cardiomyocytes, especially during the late differentiation period.[279]

ECM proteins also play a pivotal role in the phenotypic modulation of smooth muscle cells (SMCs). ECM proteins may contribute to the differentiation of MSCs into SMC lineages. Therefore, Suzuki et al. investigated whether hBMSCs could differentiate into smooth muscle cell (SMC) lineages for cardiovascular tissue engineering by culturing them on dishes coated with laminin, fibronectin, and collagen type IV, as well as noncoated dishes, in expansion medium lacking differentiation factors (such as TGF-β1) for 7 days, and the expression of SMC-specific genes and proteins was evaluated.[83] The expression of SMC-specific genes and proteins (α-smooth muscle actin [ASMA] and h1-calponin [CALP]) in hBMSCs was significantly upregulated in cells plated on laminin but not on fibronectin and collagen type IV, whereas the number of hBMSCs was increased on dishes coated with collagen type IV, fibronectin, and laminin compared to noncoated dishes.[83] Laminin-coated biodegradable PLLA sheets seeded with hBMSCs were also subcutaneously implanted in rats. These cells showed significantly increased expression of ASMA and CALP proteins in vivo. The full differentiation marker of SMCs (smooth muscle myosin heavy chain, SM2) was expressed in hBMSCs on the laminin-coated sheets by 2 weeks after implantation.[83]

Lung epithelial differentiation of mESCs cultured on TCPS and poly-DL-lactic acid (PDDLA) coated with collagen type I, laminin 332 (laminin 5), fibronectin, and Matrigel was investigated by Lin et al.[286] Laminin-332- or Matrigel-coated surfaces induced enhanced surfactant protein C gene expression in differentiating mESCs, which indicates a direct indication of lung epithelial differentiation. The choice of the ECM protein coating on culture dishes can greatly affect the differentiation of ESCs as well as MSCs. In particular, laminin-332-coated PDLLA provides an ECM-degradable scaffold in combination with defined materials, which will be suitable for tissue engineering of lung tissue constructs.

5.5. Fibronectin

Fibronectin is a high-molecular-weight glycoprotein (~440 kDa) that binds to integrins[287] and to extracellular matrix components of collagen, fibrin, and heparan sulfate proteoglycans (e.g., syndecans).[288] Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds,[287] and is reported to play a major role in cell adhesion, growth, migration, and differentiation. Its RGD sequence (Arg-Gly-Asp) is the site of cell attachment via α5β1 and αVβ3 integrins. Fibronectin also contains a cell-adhesion domain of the connecting segment-1 (CS1, EILDVPST), which is mostly recognized by hematopoietic stem and progenitor cells. Table 11 summarizes several types of fibronectin scaffolds or fibronectin-coated dishes used for MSC differentiation reported in the literature.[37,48,53,79,83,97,101,149,192,195,196,200,201,279,289,290]

The adhesion of hADSCs to fibronectin is reported to be mediated by β1 integrin and heparin-binding domain based on inhibition experiments using an antibody against β1 integrin and heparin-binding peptide (HBP), whereas the adhesion of collagens and laminin seem to be solely mediated by β1 integrin.[53] β1 integrins are a common receptors on MSCs that mediate cell adhesion to collagen type I and type IV, fibronectin, and laminin.

Heparan sulfate proteoglycans are involved in cell adhesion of MSCs via the heparin-binding region of fibronectin, and they modulate the osteogenic differentiation of MSCs via bone morphogenetic protein pathways.[291,292] hADSCs cultured on fibronectin-coated dishes differentiated into adipocytes to a greater extent than cells cultured on TCPS.[54] However, hADSCs cultured on fibronectin-coated dishes differentiated into adipocytes less than those on heparin-binding domain substrates[54] because the cells maintained a much rounder morphology when cultured on a heparin-binding domain substrate than on fibronectin-coated dishes and TCPS. Moreover, it has been reported that hMSCs differentiate into osteoblasts under culture conditions that maintain spread shapes, whereas rounded cells differentiate into adipocytes.[54]

Chang et al. reported that a pellet suspension culture of hMSCs with the addition of fibronectin promoted differentiation of MSCs to pancreatic, insulin-producing cells, with increased insulin and Glut2 gene expression.[293] A four-stage protocol that containes neuronal differentiation factor and insulin-producing cell (IPC)-conversion reagent (nicotina-

Table 11. Some Research Studies for Stem Cell Differentiation on 2D and 3D Fibronectin Materials

stem cell source[a]	material for stem cell culture[b]	differentiation	ref
hBMSCs	fibronectin/CP/HAP (2D culture, coating on HAP)	osteoblasts	289
hBMSCs	fibronectin (2D culture, coating on dishes)	osteoblasts	97
hBMSCs	fibronectin (2D culture, coating on dishes)	osteoblasts	97, 149, 192
hBMSCs	fibronectin (2D culture, coating on dishes)	osteoblasts adipocytes	196
mBMSCs	fibronectin (2D culture, coating on dishes)	osteoblasts, adipocytes	195
hADSCs	fibronectin (2D culture, coating on dishes)	adipocytes	53
hADSCs	fibronectin (2D culture, coating on dishes)	cardiomyocytes	279
hBMSCs	fibronectin (2D culture, coating on dishes)	smooth muscle cells	83
hBMSCs	fibronectin (2D culture, coating on dishes)	neural cells	101
hESCs (TE03, TE06)	fibronectin/PDL (2D culture, coating on dishes)	neural cells	79
human neural stem cells	fibronectin (2D culture, coating on dishes)	oligogliocytes	37
mESCs	fibronectin (2D culture, coating on dishes)	lung epithelium	286
BMSCs	fibronectin (2D culture, coating on dishes)	hepatocytes	48
mouse hepatitic stem cells	fibronectin (2D culture, coating on dishes)	hepatocytes	200
hESCs (H9) Fibronectin	fibronectin/PLGA+PLLA (3D culture, scaffold)	endoderm cells, ectoderm cells, chondrocytes	290

[a]ADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hBMSCs, human BMSCs; mBMSCs, murine BMSCs; hESCs, human ESCs; and mESCs, murine ESCs. [b]CP, calcium phosphate; HAP, hydroxyapatite; PDL, poly-D-lysine; PLGA, poly(lactic-*co*-glycolic acid); PLLA, poly-L-lactic acid.

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Table 12. Some Research Studies for Stem Cell Differentiation on Decellularized ECM Materials

stem cell source	material for stem cell culture	differentiation	ref
mBMSCs	decellularized ECM from mBMSCs (2D culture)	pluripotency, osteoblasts, adipocytes	195
mESCs (E14 TG2a)	ECM from decellularized osteoblasts and nonosteogenic cells (2D culture)	osteoblasts	308
rat BMSCs	decellularized ECM on electrospinning fibers of poly(ε-caprolactone) from osteoblasts differentiated from rat BMSCs	osteoblasts	318
rat BMSCs	decellularized ECM from osteoblasts differentiated from rat BMSCs on titanium fiber mesh (3D culture)	osteoblasts	307
hBMSCs	decellularized bovine endosteum-derived particles (3D culture)	osteoblasts, chondrocytes, adipocytes	62
rBMSCs	decellularized ECM scaffold from porcine cartilage (3D culture)	chondrocytes	72
hBMSCs	decellularizaed ECM from chondrocyte-encapsulated collagen microspheres (3D culture)	chondrocytes	306
hADSCs	porous scaffold derived from decellularized articular cartilage (3D culture)	chondrocytes	310
hBMSCs	decellulzarized scaffolds on PLGA, which are derived from hBMSCs and chondrocytes	chondrocytes	311, 322
embrionic rat brain cortical cels	decellularized ECM from hBMSCs (2D culture)	neural cells	309
human urine-derived stem cells	decellularized small intestinal submucosa scaffold (3D culture)	urethral tissue composed of urothelial and smooth muscle cells	28

mide) is generally used for derivation of IPCs from embryonic stem cells but was reported to be insufficient to induce MSCs to undergo IPC differentiation in monolayer cultures.[293] However, pellet suspension culture of hMSCs with the addition of fibronectin enhanced pancreatic differentiation. The differentiated cells secreted insulin in response to elevated glucose concentrations, and this was regulated by reagents that increased cyclic AMP production and modified calcium influx.[293] It was also reported that laminin-1 promoted the differentiation of fetal mouse pancreatic β-cells.[293,294] Further investigation of the mechanisms by which ECM proteins mediate the promotion of IPC differentiation is needed.

Sogo et al. prepared hydroxyapatite (HYA) ceramic composites immobilized with fibronectin or collagen type I.[289] The ECM proteins and the calcium phosphate precipitate formed a composite surface layer, and ECM proteins were not released completely for 3 days into a physiological salt solution.[289] hMSCs cultured on the HYA ceramic composites with immobilized fibronectin showed higher ALP activity in osteogenic differentiation medium than those on the HYA ceramic composites immobilized with collagen type I, which indicates that hMSCs differentiated into osteogenic lineages on the HYA ceramic composites immobilized fibronectin only.[289] No synergetic effect of hMSC differentiation into osteoblasts was observed on the HYA ceramic composites with both fibronectin and collagen type I. Thus, the fibronectin−HYA composite, but not the collagen type I−HYA composite, seems to be useful for the enhancement of osteogenic differentiation of hMSCs in vitro.

5.6. Vitronectin

Vitronectin is an ECM glycoprotein and is involved in the differentiation of diverse cell types in embryonic and adult tissues.[295,296] Vitronectin is not commonly used for coating or scaffold materials, although it is abundant in serum. Only a few reports have described positive effects of vitronectin on differentiation of MSCs in 2D culture, hydrogels, and scaffolds.[97,149,196,295]

Vitronectin was shown to promote the generation of spinal motor neurons by synergistically interacting with sonic hedgehog (Shh) both in explants and neuroepithelial cell cultures of chick embryo spinal cord.[295,297] Oligodendrocytes and motor neurons were derived from a common pool of spinal cord progenitors.[298,299] Vitronectin is therefore a possible candidate to promote the differentiation of spinal cord oligodendrocytes as well as motor neurons.

Gil et al. found that the oligodendrocytic differentiation of hESCs was efficiently promoted by vitronectin.[295] Salasznyk investigated osteogenic differentiation of hMSCs cultured on dishes coated with fibronectin, collagen type I, collagen type IV, vitronectin, and laminin-1.[97] hMSCs were found to adhere to ECM proteins in this order: fibronectin > collagen type I ≥ collagen type IV ≥ vitronectin ≥ laminin-1. However, cells cultured on dishes coated with vitronectin and collagen type I differentiated into osteoblasts to a greater extent than cells on dishes coated with fibronectin or laminin-1, as shown by an evaluation of ALP activity, osteopontin expression, and mineral deposition.[97] The contact of hMSCs with vitronectin as well as with collagen type I seems to promote the osteogenic differentiation of hMSCs.

5.7. Decellularized ECM

The biological niche of cells in vivo dictates stem cell fate and guides MSCs to differentiate into specific lineages. It is rather difficult to reproduce biological niches using only pure ECM proteins, glycosaminoglycans, and other components in vitro. One idea to reproduce a biological niche in vitro is to use decellularized ECM.[300−303] Decellularization is a technique for removing cellular components from native tissues and is usually achieved by a combination of physical, chemical, or enzymatic methods.[304,305] This technique removes the allogenic or xenogenic cellular antigens, as well as cellular components, from the tissues, but preserves the ECM components.[306] Several studies have focused on the decellularization of tissues and organs such as heart valve, heart, liver, lung, blood vessel, skin, and nerves.[300−303] Decellularization is typically performed by freeze−thaw cycling or surfactant methods.[28,62,195,306−310] The freeze−thaw cycling method is as follows. The scaffolds were thawed in a water bath at 37 °C for 10 min, rinsed with phosphate-buffered saline (PBS) to remove cellular debris, and frozen in liquid N_2 for 10 min. Subsequently, the scaffolds were left at room temperature for 1 h to melt. The scaffolds then underwent three freeze/thaw cycles under sterile conditions to ensure complete removal of the cellular components. After treating in NH_4OH aqueous solution and rinsing with PBS, scaffolds were allowed to air-dry before being seeded with cells.[307,311] The typical surfactant method is as follows. Cells were treated with 0.1% Triton X-100 in water at room

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temperature for 30 min. Cell lysates were carefully aspirated, and a solution of concentrated ammonium hydroxide diluted 1:100 in water was slowly added to the wells for 5−7 min. The wells were carefully washed twice with PBS and used immediately or stored in PBS at 4 °C.[309,312] Acellular ECMs processed from allogenic or xenogenic tissues most closely approximate natural tissues and have been used as scaffolds for the tissue engineering of heart valves,[313,314] vessels,[315] nerves,[316] tendons, and ligaments.[306,317] Some landmark examples of MSC propagation and differentiation that are promoted by culture on decellularized ECM are summarized in Table 12.[28,62,72,195,306−310,318]

Several studies have shown that ECM modulates neuritogenesis and glial growth.[309,319,320] However, little is known about effects of MSC-derived ECM on neural cells. Aizman et al. demonstrated that the ECM produced by MSCs could support neural cell attachment and growth in vitro. They compared the neurosupportive properties of MSCs to MSC derivative SB623 cells, which were being developed as a cell therapy for stroke.[309] Embryonic rat brain cortical cells cultured for 3 weeks on hMSC- and SB623 cell-derived ECM exhibited about 1.5- and 3-fold higher metabolic activities, respectively, compared with cultures grown on PDL-coated dishes.[309] The MSC- and SB623-derived ECMs protected neural cells from nutrient and growth factor deprivation, and supported the growth of neurons, astrocytes, and oligodendrocytes.[309] Morphologically, neurons on cell-derived ECM formed more complex and extended neurite networks than those cultured on PDL-coated dishes. It was suggested that the cell-derived ECM could be a mediator of the neuroregenerative properties of the MSCs and SB623 cells observed in vivo.[309]

Cheng et al. investigated whether a scaffold derived from articular cartilage could induce chondrogenesis of hADSCs.[310] hADSCs were seeded on porous scaffolds derived from adult porcine articular cartilage and cultured in standard medium without exogenous growth factors. Chondrogenesis of hADSCs seeded within the scaffold was shown by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of cartilage-specific ECM genes (collagen type II and aggrecan).[310] Histological and immunohistochemical examination showed abundant production of cartilage-specific ECM components (collagen type II) after 4 or 6 weeks of culture. The morphology of cells in the hADSC-seeded constructs resembled that of native articular cartilage tissue, with rounded cells residing in the glycosaminoglycan-rich regions of the scaffolds after 6 weeks of culture.[310] Biphasic mechanical testing showed that the aggregate modulus of the hADSC-seeded constructs increased over time, reaching 150 kPa by day 42, more than 3-fold higher than that of the unseeded controls.[310] These results suggest that a porous scaffold derived from articular cartilage has the ability to induce chondrogenic differentiation of hADSCs without exogenous growth factors, leading to synthesis and accumulation of ECM macromolecules and the development of mechanical properties approaching those of native cartilage.[310] These findings support the potential for a processed cartilage ECM as a biomaterial scaffold for cartilage tissue engineering.[310]

Evans et al. investigated whether tissue-specific ECM influenced the differentiation of ESCs.[308] They induced murine ESCs to differentiate by embryoid body formation, followed by dissociation and culture on ECMs prepared by decellularization of either osteogenic cell (MC3T3-E1) or nonosteogenic cell (A549) cultures, or on defined collagen type I matrix.[308] The osteogenic differentiation was evaluated by formation of mineralized tissue and osteogenic gene expression and was significantly greater on ECM matrices derived from osteogenic cells (MC3T3-E1) than on any other ECM matrix. The osteogenic effect of the MC3T3-E1 matrix was reduced by heat treatment and abolished by trypsin, suggesting that bioactive proteinaceous components secreted by MC3T3-E1 cells were the key factors that promoted differentiation of ESCs into the osteogenic lineage.[308] These results demonstrate that decellularized, bone-specific ECM can promote the osteogenic differentiation of ESCs, incorporating tissue-specific ECM signals and stimulating stem cell differentiation.

Datta et al. investigated the effect of ECM laid down by osteoblastic cells on the osteoblastic differentiation of rat BMSCs.[307] Primary rat BMSCs seeded in titanium (Ti) fiber scaffolds were differentiated into osteoblasts in static culture, and then the scaffolds were decellularized by rapid freeze−thaw cycling. Decellularized scaffolds were reseeded with rat BMSCs, and osteogenicity was determined by DNA, ALP, calcium, and osteopontin analysis. Calcium was deposited at a greater rate by cells grown on decellularized scaffolds than on control scaffolds by 16 days.[307] The Ti/BMSC constructs showed negligible calcium content at 16 days, compared with 213 mg/construct for the Ti/ECM/MSC constructs cultured without any osteogenic supplements.[307] These results indicate that bonelike ECM synthesized in vitro can enhance the osteoblastic differentiation of MSCs.

Wu et al. developed engineered urethral tissue from urothelial cells (UCs) derived from the differentiated urine-derived stem cells (USCs), which were seeded on a 3D porous scaffold prepared by decellularization of pig small intestinal submucosa (SIS).[28] Differentiated UCs and smooth muscle cells (SMCs) were seeded onto SIS scaffolds in a layered coculture process and cultured for 1 week. The seeded cells formed multiple uniform layers on the SIS and penetrated deeper into the porous matrix.[28] USCs were induced to differentiate expressed UC markers (Uroplakin-III and AE1/AE3) or SMC markers (α-SM actin, desmin, and myosin) after implantation into athymic mice for 1 month.[28] Thus, UCs and SMCs derived from USCs could be maintained on 3D porous SIS scaffold. The dynamic culture system further promoted 3D cell-matrix ingrowth and development of a multilayer mucosal structure similar to native urinary tract tissue.[28] USCs may serve as an alternative cell source for cell-based tissue engineering for urethral reconstruction or other urological tissue repair.

Depending on the cells from which decellularized ECMs are isolated, the ECM can not only promote specific differentiation lineages of MSCs but also prevent MSC differentiation. Chen et al. reported that ECM produced by murine BMSCs facilitated the expansion of MSCs and prevented their differentiation into osteoblasts.[195] The differentiation ability of MSCs was progressively lost with extensive passaging when MSCs were cultured on TCPS.[321] This is because bone marrow microenvironment that facilitates retention of stem cell properties is missing in TCPS dish culture.[195] Therefore, the ability of BMSC-derived ECM to support the maintenance of the stemness of MSCs in vitro was evaluated. The BMSC-derived ECM was found to be made of collagen types I, III, and V, syndecan-1, perlecan, fibronectin, laminin, biglycan, and decorin, similar to the composition of the marrow ECM.[195] This ECM preparation promoted mesenchymal colony-forming unit (MCFU) replication, restrained their "spontaneous"

Table 13. Some Research Studies for Stem Cell Differentiation on ECM-Peptide Materials

stem cell source[a]	material for stem cell culture containing ECM peptide[b]	differentiation	ref
hBMSCs	ECM-mimicking peptide (RGDS, DGEA, KRSR) amphiphile nanofiber (2D culture, coating on dishes)	osteoblasts	130
rat BMSCs	RGD peptides (2D culture, grafting on PEG gel)	osteoblasts, adipocytes	153
hADSCs	RGD, YIGSR, and IKVAV grafted PCL (2D culture, disk)	ADSC culture	329
rat neural stem cells	outer membrane protein A having ECM-peptide motif [RGDS, GTPGPQGIAGQRGVV (collagen I), PHSRN (fibronectin), MNYYSNS (collagen IV), YIGSR (laminin)] (2D culture, coating on dishes)	neural cells	109
neural stem cells	bacterial peptide (2D culture, coating on dishes)	neural cells	331
gBMSCs	PEODA (polyethylene glycol diacrylate) incorporated with YRGDS (3D culture, gel)	osteoblasts	125
gBMSCs	PEG hydrogel containing ECM-peptide motif (collagen mimetic peptide ([Pro-Hyp-Gly]$_7$-Tyr) (3D culture, gel)	chondrocytes	65
hBMSCs	PEG hydrogel-containing ECM-peptide motif (CRGDSG, CPENFFGGRGDSC) (3D culture, gel)	chondrocytes	128
mBMSCs	PEG hydrogel-containing matrix metalloproteinase-sensitive peptide (QPQGLAK) and chondroitin sulfate A (3D culture, gel)	chondrocytes	129
hBMSCs	PEG hydrogel-containing RGDS (3D culture, gel)	chondrocytes	126
hESC-derived MSCs	PEG hydrogel-containing ECM-peptide motif (YRGDS) (3D culture, gel)	chondrocytes	127
hBMSCs	elastin-like polypeptide [ELP, pentaoeotide repeat (Val-Pro-Gly-Xaa-Gly)] hydrogel[c] (3D culture, gel)	chondrocytes	155
hBMSCs	silk scaffold bound GRGDS covalently (3D culture, scaffold)	osteoblasts	328
hBMSCs	collagen mimetic peptide (DGEA, P15 (GTPGPQIAGQAGVV), QAGVV, GFOGER) and GPenGRGDSPCA (3D culture, coating on HYA)	osteoblasts	103
no cell loading	collagen mimetic peptide (GGYGGGPC[GPP]$_5$ GFOGER[GPP]$_5$GPC) where O is hydroxyproline (3D culture, coating on PCL)	bone formation	330
murine neural stem cells	nanofiber scaffold of self-assembled peptide containing motif of laminin (YIGSR, IKVAV, PDSGR), collagen (DGEA, FPGERGVEGPGP, PRGDSGYRGDS), fibronectin (RGDS), and bone marrow homing peptides (SKPPGTSS, PFSSTKT) (3D culture, scaffold)	neural cells	116

[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. [c]Xaa is any naturally occurring amino acid with the exception of proline.

differentiation toward the osteoblast lineage, and preserved their ability to differentiate into osteoblasts or adipocytes, where MCFUs comprised MSCs and their transit-amplifying progeny.[195] The transplantation of MCFUs expanded on the BMSC-derived ECM into immunocompromised mice generated 5 times more bone and 8 times more hematopoietic marrow than MCFUs expanded in TCPS dishes.[195] On the basis of this study, ECM in BMSCs can be considered to play an important role in the maintenance of MSC stemness.

Lu, Chen, and co-workers prepared ECM scaffolds derived from MSCs and chondrocytes on PLGA mesh.[311] Cell−ECM−PLGA constructs were decellularized by freeze−thaw techniques and subsequently immersed into aqueous Na_3PO_4 solution to remove the PLGA mesh template. The decellularized ECM scaffolds were reported to have a stronger stimulatory effect on chondrogenesis of MSCs compared with conventional pellet culture.[311] In particular, decellularized ECM scaffolds prepared from MSCs showed higher promotion of MSCs into chondrogenesis than did those prepared from chondrocytes.[311] This preparation method opens an avenue for efficiently creating autologous ECM (aECM) scaffolds by culturing autologous cells and decellularizing the resulting cell−ECM constructs.[311,322] The use of ECM scaffolds and patient BMSCs are expected to elicit the desired responses for clinical application.[311,322−325]

5.8. Biomaterials with ECM-Mimicking Oligopeptides

We have observed that MSCs on hydrogels or scaffolds with immobilized ECM proteins or dishes coated with ECM proteins can effectively promote the differentiation of MSCs into specific lineages. However, some technical challenges remain. We cannot store the hydrogels, scaffolds, and dishes containing ECM proteins at room temperature, and we should store those containing ECM proteins in a refrigerator under sterile conditions. Furthermore, it is difficult to sterilize hydrogels, scaffolds, and dishes with immobilized ECM proteins because denaturation of ECM proteins should be avoided when immobilized ECM proteins are to be used in clinical applications. Including cell-adhesion peptides from ECM proteins, which are highly stable and have lower molecular weights than ECM proteins, in the design of hydrogels, scaffolds, and coating materials on dishes is a potentially useful strategy. ECM protein-derived peptides (ECM peptides) can be directly coated or grafted onto cell culture dishes for 2D culture of MSCs,[85,326,327] and ECM peptides may be covalently or noncovalently incorporated into scaffolds or hydrogel networks for 3D culture.[40,65,68,103,121,125−129,155,328−330] Furthermore, ECM peptides can generate nanofiber configurations by self-assembly.[109,116,130]

Table 4 shows several cell-binding sites of ECM proteins, together with original ECM proteins from which they are derived and the binding sites of integrins, if they are known. Oligopeptides of RGD (binding to $\alpha5\beta1$ integrin or VLA-5), DGEA (binding to $\alpha2\beta1$ integrin), YIGSR, and IKVAV are frequently used for this purpose. The surface reaction of the grafting of the ECM-binding peptides was described in section 3.1, and the synthesis method of copolymerization with ECM peptides and acryloyl monomers was described in section 3.2. Table 13 summarizes some examples of research on MSC culture and differentiation in hydrogels or scaffolds with immobilized ECM peptides or on dishes coated (or grafted) with ECM peptides.[65,103,109,116,125−130,153,155,328−331]

Santiago et al. prepared the poly(ε-caprolactone) (PCL) surfaces covalently attached with RGD, YIGSR, and IKVAV

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peptide sequences derived from laminin and evaluated the attachment and proliferation of ADSCs.[329] IKVAV-treated surfaces were found to have a significantly greater number of bound ADSCs at 2 and 3 days after cell seeding compared to other peptide sequences.[329] Their results indicated that IKVAV is a suitable peptide sequence for use in surface-modification techniques aimed at improving the attachment of ADSCs to a tissue-engineered scaffold.[329] However, several other groups have reported that other ECM peptides were as or more effective for stem cell attachment on dishes and scaffolds, depending on the base materials of dishes and scaffolds.[65,103,125−128,130] The effect of ECM peptides in the hydrogels, scaffolds, or dishes with immobilized ECM peptides on differentiation ability of MSCs into specific lineages is discussed in the next sections.

5.8.1. MSC Differentiation on Self-Assembled ECM-Peptide Nanofibers. Self-assembled nanostructures in scaffolds are especially interesting because they mimic the hierarchical structure and self-assembled formation of native tissues. Peptide amphiphile (PA) is known to spontaneously generate self-assembled nanofibers above critical micelle concentrations.[109,116,130] Anderson et al. prepared peptide amphiphile nanofibers inscribed with specific cellular adhesive ligands (i.e., RGDS, DGEA, and KRSR) and investigated whether they could direct osteogenic differentiation of hMSCs without osteogenic supplements.[130] The peptide amphiphile nanofibers existed as self-assembled 2D coatings on the dishes. hBMSCs cultured on the RGDS-containing peptide amphiphile nanofibers, but neither DGEA nor KRSR nanofibers, showed significantly greater ALP activity, indicating the early promotion of osteogenic differentiation, and showed a progressive shift toward osteogenic morphology and positive staining for mineral deposition.[130] The peptide amphiphile nanofibers, which mimic the native ECM in bone, were found to direct the osteogenic differentiation of hBMSCs without the aid of supplements to some extent and provided an adaptable environment that allowed different adhesive ligands to control cellular behaviors.[130]

5.8.2. Osteogenic Differentiation on ECM-Peptide Immobilized Scaffolds and Dishes. Hennessy et al. evaluated the interaction between hBMSCs and hydroxyapatite (HYA) disks coated with the collagen-mimetic peptides DGEA, P15 (GTPGPQGIAGQRGVV), and GFOGER.[103] hBMSCs adhered equally well to disks coated with DGEA, P15, or collagen type I, and all three substrates, but not GFOGER, supported greater cell adhesion than uncoated HYA disks.[103] However, another study revealed that polycaprolactone scaffolds coated with GFOGER could promote bone formation in critically sized segmental defects in rats.[330] The combination of specific ECM peptides and scaffold materials might also be important for controlling MSC differentiation.

When peptide-coated HYA disks were overlaid with proteins from serum or the tibial microenvironment, collagen mimetic-coated HYA disks did not inhibit hBMSC adhesion, whereas RGD peptide-coated HYA disks did.[103] However, they did not enhance adhesion either. Osteocalcin secretion and ALP activity from hBMSCs adhering to DGEA or P15-coated disks were promoted by activation of collagen-selective integrins, which stimulated osteogenic differentiation.[103] Both of these osteogenic markers were upregulated by DGEA and P15 in the presence or absence of differentiation-inducing media. Bone formation on HYA tibial implants was enhanced by the collagen mimetic peptides. Therefore, collagen-mimetic peptides improve osteointegration of HYA disks, probably by stimulating osteoblastic differentiation, rather than adhesion, of MSCs.[103]

Although RGD-peptide-coated HYA scaffolds did not promote osteogenic differentiation,[103] poly(ethylene glycol) diacrylate hydrogel-incorporated RGD peptides were reported to promote osteogenic differentiation of goat BMSCs.[125] RGD peptides helped BMSCs maintain cbfa-1 expression in the hydrogel. Soluble RGD was found to completely block the mineralization of BMSCs, as shown by quantitative calcium assay, phosphorus elemental analysis, and von Kossa staining.[125] This research demonstrated that RGD-conjugated hydrogels promoted the osteogenesis of BMSCs in a dosage-dependent manner, with 2.5 mM being the optimal concentration in their preparation of hydrogels.[125] The combination of ECM peptides and scaffold materials seems to affect MSC differentiation in the scaffolds and hydrogels.

Porous biodegradable silk scaffolds and hBMSCs were used to engineer bonelike tissue in vitro.[328] Two different scaffolds with the same microstructure were studied: collagen (to assess the effects of fast degradation) and silk with covalently bound RGD sequences (to assess the effects of enhanced cell attachment and slow degradation).[328] hMSCs were isolated, expanded in culture, and characterized with respect to the expression of surface markers and the potential for chondrogenic and osteogenic differentiation. Cells were then seeded on scaffolds and cultured for up to 4 weeks. Histological analysis and microcomputer tomography showed the development of up to 1.2 mm long, interconnected, and organized bonelike trabeculae with cuboid cells on the silk−RGD scaffolds, features that were present to a lesser extent on silk scaffolds and absent on the collagen scaffolds.[328] The X-ray diffraction pattern of the deposited bone corresponded to hydroxyapatite in the native bone. Biochemical analysis showed increased mineralization on silk−RGD scaffolds compared with either silk or collagen scaffolds after 4 weeks.[328] Expression of bone sialoprotein, osteopontin, and bone morphogenetic protein 2 was significantly higher in hMSCs cultured in osteogenic than control medium after 2 and 4 weeks in culture.[328] These results suggest that RGD−silk scaffolds are particularly suitable for autologous bone-tissue engineering, presumably because of their stable macroporous structure, tunable mechanical properties matching those of native bone, and slow degradation.[328]

5.8.3. Chondrogenic Differentiation on ECM-Peptide-Immobilized Scaffolds and Dishes. Poly(ethylene oxide) diacrylate (PEODA) hydrogel provides 3D structural support for in vitro and in vivo chondrogenic differentiation of stem cells. However, PEODA gels are bioinert, as are most synthetic scaffolds, and nonadhesive to stem cells and proteins.[158,332] Therefore, several researchers have designed PEODA scaffolds conjugated with ECM peptides, such as collagen mimetic peptides (CMPs)[65] and RGD peptide[126−128] or chondroitin sulfate[129] for chondrogenic differentiation of BMSCs.

The collagen mimetic peptides (CMPs) are sequences of −(Pro-Hyp-Gly)$_7$−, where Hyp is hydroxyproline, and they have a unique collagen-like triple helical conformation that has been shown to associate with collagen fibers via a strand-invasion process.[333,334] Lee et al. showed that the CMP-mediated microenvironment enhanced the chondrogenic differentiation of goat BMSCs. BMSCs were photoencapsulated in the CMP-conjugated PEODA hydrogels.[65] Histological and biochemical analysis of the CMP-conjugated PEODA hydrogels revealed twice as much glycosaminoglycan and collagen

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contents as in control PEODA hydrogels after 3 weeks.[65] BMSCs cultured in CMP-conjugated PEODA hydrogels exhibited a lower level of the hypertrophic markers cbfa-1 and collagen type X than BMSCs in PEODA hydrogels by evaluation by gene expression and immunohistochemisty.[65] These results indicate that CMP-conjugated PEODA hydrogels provide a favorable microenvironment for encapsulated BMSCs and regulate their chondrogenic differentiation.[65]

Hwang et al. investigated the chondrogenic capacity of hESC-derived MSCs in pellet culture and after encapsulation in PEODA hydrogels with exogenous extracellular biomolecules (hyaluronic acid and collagen type I) or conjugated with RGD peptides.[127] The hESC-derived MSCs exhibited growth factor-dependent matrix production in pellet culture but did not produce tissues with characteristic cartilage morphology. No significant cell growth or matrix production was observed in PEODA hydrogels containing exogenous hyaluronic acid or collagen type I.[127] In contrast, neocartilage with basophilic ECM deposition, cartilage-specific gene upregulation, and ECM production was observed within 3 weeks of culture for hESC-derived MSCs encapsulated in PEODA hydrogels conjugated with RGD peptide.[127] These findings suggest that precursor cells characteristic of a MSC population from differentiating hESCs through embryoid bodies can generate cartilage tissues using hydrogels conjugated with RGD peptide.[127]

Betre et al. examined the potential of a genetically engineered elastin-like polypeptide (ELP) to promote chondrocytic differentiation of hADSCs without exogenous chondrogenic supplements.[155] ELPs have a repeated oligomeric pentapeptide motif composed of valine-proline-glycine-Xaa-glycine (Val-Pro-Gly-Xaa-Gly), where Xaa is termed the guest residue and can be any of the naturally occurring amino acids with the exception of proline.[335] ELPs form aggregates in aqueous solution at a specific transition temperature, termed an inverse temperature phase transition (T_t). Below T_t, ELPs are structurally disordered, highly solvated, and, therefore, soluble in aqueous solutions. When the temperature is above T_t, ELPs undergo desolvation and form a gelatinous aggregate termed a coacervate.[155,336] Encapsulation of hADSCs in ELP hydrogels can be easily prepared by ELP coacervate formation.

hADSCs were reported to be cultured in ELP hydrogels in either chondrogenic or standard medium at 5% O_2 for up to 2 weeks.[155] The ELP hydrogel containing hADSCs cultured in either medium exhibited significantly increased sulfated glycosaminoglycan and collagen production, where the matrix produced by hADSCs consisted mainly of collagen type II but not collagen type I.[155] The composition of the ELP hydrogels containing hADSCs cultured in either medium did not differ significantly.[155] The ELP hydrogels containing hADSCs were cultured in standard medium at either 5% or 20% O_2 for 7 days to evaluate the effect of oxygen tension on the differentiation of hADSCs in ELP hydrogels. These hADSCs showed upregulated SOX9 and collagen type II gene expression at both oxygen concentrations, and the gene expression of collagen type I was downregulated.[155] However, the ELP hydrogels containing hADSCs cultured in 20% O_2 had highly upregulated gene expression of collagen type X, indicating hypertrophic conditions, which was not detected in the 5% O_2 cultures.[155] The study suggests that ELP hydrogels can promote chondrogenesis of hADSCs in the absence of exogenous TGF-β1 and dexamethasone, especially under low oxygen tension.

Hydrophobic polyhydroxyalkanoate (PHA) scaffolds were made of a copolymer of 3-hydroxybutyrate-*co*-hydroxyhexanoate (PHBHHx). Several amphiphilic proteins can be coupled to the surface of PHA granules in vivo, such as PHA synthase PhaC and PHA granule-associated proteins, PhaP.[337] You et al. prepared PhaP−RGD fusion proteins by recombinant gene techniques.[68] hBMSCs on the PHA scaffolds coated with PhaP−RGD fusion proteins were cultured to evaluate the formation of articular cartilage derived from chondrogenic differentiation.[68] The scaffolds coated with PhaP−RGD fusion proteins induced more homogeneous spreading of cells, better cell adhesion, proliferation, and chondrogenic differentiation compared with those coated with PhaP or uncoated scaffolds in serum-containing medium.[68] In addition, more ECM protein was produced by the differentiated cells over 14 days on scaffolds coated with PhaP−RGD fusion proteins, which was evidenced by enhanced expression of chondrocyte-specific genes including SOX9, aggrecan, and collagen type II. This result indicated a positive effect of RGD on ECM production.[68] Furthermore, sulphated glycosaminoglycans (sGAG's) and total collagen content, which are cartilage-specific, were produced significantly more on the scaffolds coated with PhaP−RGD fusion proteins than on uncoated scaffolds or those coated with PhaP proteins.[68] Homogeneously distributed chondrocyte-like cells forming cartilage-like matrices were observed on the scaffolds coated with PhaP−RGD fusion proteins after 3 weeks.[68] These results can support engineered cartilage tissue.

It is challenging to generate a hierarchical tissue structure that mimics the highly organized zonal architecture of articular cartilage. The articular cartilage consists of four spatially distinct zones: the superficial, transitional (middle), deep, and calcified zones.[129] Each zone is characterized by unique ECM compositions, mechanical properties, and cellular organization. The cartilage−ECM is primarily composed of collagen type II and glycosaminoglycans (GAGs) whose relative concentrations vary spatially from the superficial to the deep zone, leading to varying mechanical properties.[129,338] The superficial zone contains high levels of collagen type II and low levels of GAG.[129,339] The transitional zone has lower collagen type II content and a higher GAG concentration.[129] The deep zone contains the highest concentration of GAGs and the lowest level of collagen type II fibers.[129,340] The calcified cartilage zone contains high levels of collagen type X and integrates the cartilage to the subchondral bone.[129,339,340]

Nguyen et al. showed that different combinations of synthetic and natural biopolymers created unique niches that could direct BMSCs to differentiate into the superficial, transitional, and deep zones of articular cartilage.[129] PEG hydrogels incorporated with chondroitin sulfate (CS) and matrix metalloproteinase-sensitive peptides (MMP-pep), PEG:CS:MMP-pep, induced high levels of collagen type II and low levels of proteoglycan expression, resulting in a low compressive modulus similar to the superficial zone.[129] PEG hydrogels incorporated with CS (PEG:CS) produced intermediate levels of both collagen type II and proteoglycans as in the transitional zone, whereas PEG hydrogels incorporated with hyaluronic acid (HA), PEG:HA, induced high proteoglycan and low collagen type II levels with a high compressive modulus, similar to the deep zone.[129] The compressive moduli of these zone-specific matrices following cartilage generation showed a similar trend to the corresponding zones of articular cartilage, with PEG:CS:MMP-pep having the lowest compressive modulus, followed by PEG:CS, and PEG:HA having the highest modulus.[129] These results illustrate the potential for composite scaffold structures incorporating biomaterial compo-

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sitions and BMSCs to generate zonally organized and functional articular cartilage-like tissue.

5.8.4. Neural Differentiation on ECM-Peptide-Immobilized Scaffolds and Dishes. Cellular adhesive motifs can be engineered into the extracellular loops of outer membrane protein A (OmpA). Cooke et al. engineered outer-membrane proteins to form self-assembled monolayers (SAMs) on gold surface where the proteins were correctly oriented on a gold surface, enabling the presentation of the peptide in a highly controlled manner.[109] The cellular adhesive motifs used in their study were RGDS and PHSRN from fibronectin, P15 (GTPGPQGIAGQRGVV) from collagen type I, MNYYSNS from collagen type IV, and YIGSR from laminin.[109,121] Adult neural stem progenitor cells cultured on monolayers of OmpA inscribed with collagen type I (P15, GTPGPQGIAGQRGVV) and fibronectin (PHSRN) motifs differentiated into beta-III tubulin-positive cells, whereas the cells on OmpA inscribed with collagen type IV did not.[109] This study demonstrates how biomimetic protein surfaces presenting the active peptide domains of ECM proteins can regulate the neural differentiation of stem cells in vitro.

N-cadherin is a cell−cell-adhesion molecule and plays important roles in neural development. Yue et al. developed an artificial ECM to mimic *N*-cadherin-mediated cell adhesion.[341] They constructed a chimeric protein that contained extracellular domain of *N*-cadherin and Fc domain of immunoglobulin G (IgG), N-cad-Fc protein.[341] N-cad-Fc protein could stably adsorb to hydrophobic surfaces. Both P19 (embryonal carcinoma) and MEB5 (neural stem) cells cultured on N-cad-Fc protein-coated surfaces showed scattering morphologies without colony formation and higher proliferating capacity than conventional culture systems, with maintenance of their undifferentiated state.[341] Both cell lines cultured on an N-cad-Fc protein-coated surface also differentiated into neural cells at the single cell level when induced with proper conditions.[341] It was proposed that the N-cad-Fc protein may be used as an artificial ECM for stem cell culture.[341] A recombinant E-cadherin fusion protein with IgG Fc region, E-cad-Fc protein, was also prepared in the similar recombinant gene expression method by Haque et al.[85] ESCs cultured on dishes coated with E-cad-Fc protein could effectively differentiate into hepatocytes with characteristic single-cell morphologies. These recombinant ECMs could be effectively used as in vitro models for studying the mechanisms of early stages of liver development of ESCs at the single-cell level.[85]

6. CONCLUSION

ECM proteins not only serve as supporting materials for stem cells but also act to regulate cellular functions, especially determination of stem cell fate.[311,342] Furthermore, ECM proteins can modulate signal transduction activated by various bioactive molecules, including growth factors.[311,343] The morphology of MSCs is regulated by controlling the adhesion of cells to ECM proteins, and cell morphology can, in turn, regulate cell differentiation. ECMs engineered in culture dishes or scaffolds can control MSC morphology and differentiation with high efficiency, which provides many possibilities for the application of stem cells in regenerative medicine.[53]

The interaction between specific ECM proteins and MSCs can guide differentiation of MSCs into specific lineages. The most widely used ECM proteins that promote differentiation of MSCs into specific lineages are summarized in Figure 10.

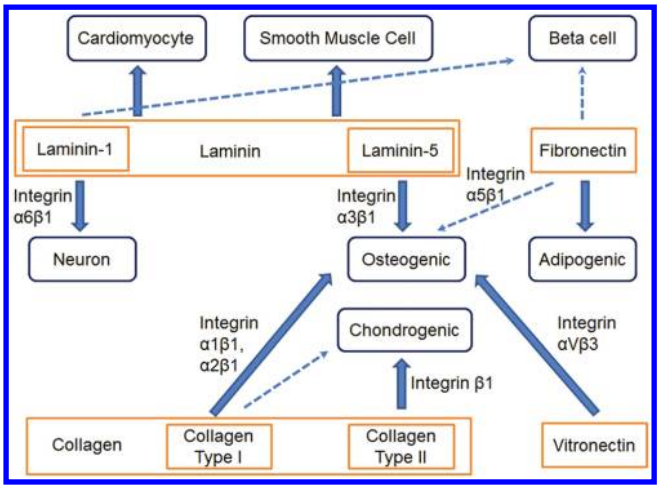

Figure 10. ECM proteins guide stem cell fate through integrin and nonintegrin binding.

Collagen type I, vitronectin, and laminin-5 promote MSCs into osteogenic differentiation.[97,102,196] The binding of integrin receptors of MSCs differs depending on the ECM protein. Integrin $\alpha 3\beta 1$ mediates the adhesion of BMSCs to laminin-5,[102] whereas integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ mainly bind collagen type I.[97,196] Integrin $\alpha V\beta 3$ mediates binding between BMSCs and vitronectin.[97] Laminin promotes differentiation of BMSCs into cardiomyocytes and smooth muscle cells,[83,279] whereas laminin-1 leads BMSCs into neural differentiation via integrin $\alpha 6\beta 1$.[101] The differentiation of BMSCs into β-cells may be promoted by interactions between MSCs and fibronectin and/or laminin-1.[76,290] Fibronectin seems to promote the differentiation of MSCs into adipocytes.[53]

Decellularized ECM scaffolds are attractive biomaterials, as these scaffolds can potentially retain the architecture of the original tissue and reproduce biological niches more precisely than scaffolds prepared from single ECM proteins. Decellularized ECM scaffolds might be effective tools for the differentiation of MSCs into some difficult lineages, such as β-cells, dopamin-secreting cells, and hepatocytes.

Synthetic or natural polymers containing ECM peptides are promising biomaterials for hydrogels or scaffolds containing MSCs. A variety of material designs for hydrogels and scaffolds containing MSCs are possible using polymers that have ECM peptides, which allow cell adhesion, proliferation, and differentiation into specific lineages. However, it is currently difficult to summarize the direction of specific differentiation lineages from the interaction of specific ECM peptides and MSCs. The combination of base polymers and ECM peptides on scaffolds, as well as the chemical and physical characteristics of scaffolds, determines the differentiation of MSCs into specific lineages.

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Notes

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