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CHEMICAL REVIEWS

Review

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Physical Cues of Biomaterials Guide Stem Cell Differentiation Fate

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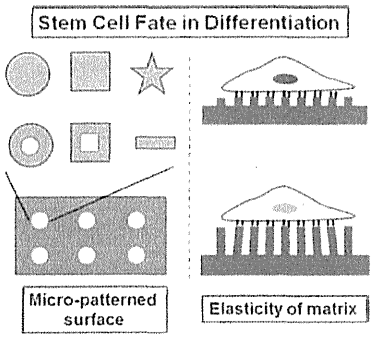

CONTENTS

1. INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

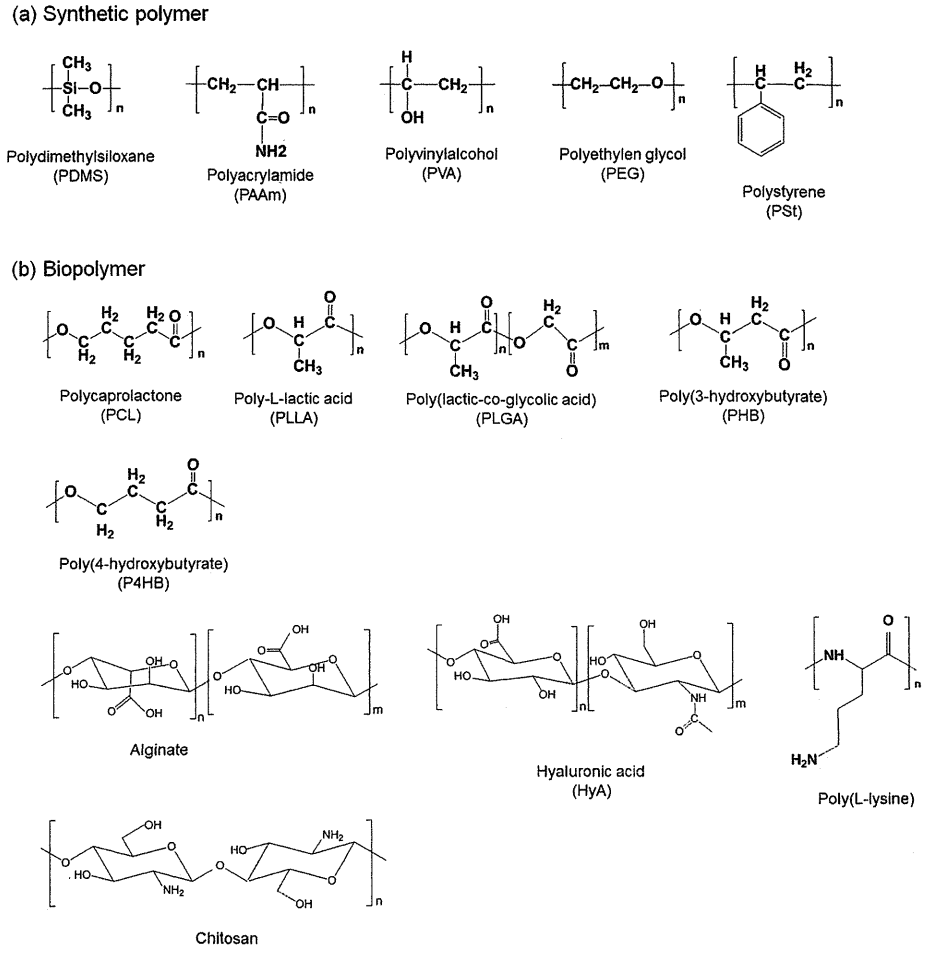

Figure 1. Chemical schematics of synthetic polymers (polydimethylsiloxane [PDMS], polyacrylamide [PAAm], poly(vinyl alcohol) [PVA], poly(ethylene glycol) [PEG], and polystyrene [PSt]) (a) and biopolymers (poly(ε-caprolactone) [PCL], poly(L-lactic acid) [PLLA], poly(lactic-*co*-glycolic acid) [PLGA],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

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

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

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

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

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

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

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

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

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

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

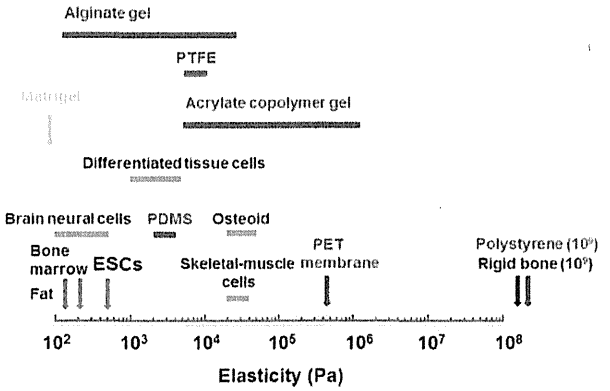

Figure 2. Examples of the elasticity of human tissues (blue bars and arrows), synthetic polymers (red bars and arrows), and natural polymers (green arrows).

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

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

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

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

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

stem cell source	materials for stem cell culture having different stiffness	differentiation	medium	ref (year)
hMSCs	HyA–gelatin–PEG hydrogels	proliferation and secretion of cytokines	expansion medium	103 (2009)
hMSCs	PAAm gel coated with collagen type I	proliferation and cell morphology	expansion medium	21 (2010)
murine ESC (OGR1)	PAAm gel coated with collagen type I and rigid dishes coated with collagen type I	proliferation with pluripotency	expansion medium	94 (2010)
hMSCs	patterned cross-linked methacrylated HyA gel containing RGDS	proliferation and cell morphology	expansion medium	111 (2010)
murine ESCs (CGR8)	polyion complex nanofilm composed of PLL and HyA	proliferation and cell morphology	expansion medium	104 (2010)
hESCs (H1, H9)	PDMS micropost treated by oxygen plasma	proliferation with pluripotency	expansion medium	105 (2012)
hMSCs	thiol-modified HyA gels and PAAm coated with collagen type I	proliferation and cell morphology	expansion medium	98 (2012)
mESCs (TG2αE14)	PDMS coated with collagen type I	proliferation, osteoblast, and mesendoderm differentiation	expansion and differentiation medium	58 (2009)
rat MSCs	PDMS grafted with poly(acrylic acid)	osteoblast	differntiation medium	99 (2009)
hMSCs	PAAm gel coated with collagen type I	osteoblast	differentiation medium	74 (2011)
hMSCs	gelatin–hydroxyphenylpropionic acid–tyramine gels cross-linked with HRP and H_2O_2	osteoblast	unknown	112 (2012)
umbilical cord MSCs (Wharton's jelly)	PAAm gel grafted with Collagen type I	osteoblast	differntiation medium	16 (2012)
rat MSCs	PDMS coated with fibronectin and gelatin	osteoblast	differentiation medium	85 (2012)
hMSCs (Stro-1 enriched cells)	polyalkyl acrylate coated with fibronectin	osteoblast	expansion medium	113 (2012)
hMSCs	PAAm gel coated with collagen type I and fibronectin	osteoblast, adipocyte	differentiation medium	87 (2009)
human epidermal stem cells, hMSCs	PDMS and PAAm gel grafted with collagen type I	osteoblast, adipocyte	mixed differentiation medium of osteoblast and adipocyte	22 (2012)
hMSCs	PEG gel immobilized fibronectin	osteoblast, adipocyte, neural cell	differentiation medium	114 (2011)
human placenta-derived MSCs, hADSCs	layer-by-layer polyion complex of cationic PLL and anionic HyA	osteoblast, adipocyte, chondrocyte	differentiation medium	86 (2009)
murine embryonic mesenchymal progenitor cells (C3H10T1/2)	PCL nanofibers and PCL-PES nanofiber by electrospinning method	osteoblast, chondrocyte	differentiation medium	106 (2011)
MSCs	PAAm gel coated with collagen type I	osteoblast, myocyte, and neuron	expansion medium	32 (2006)
hMSCs	PAAm gel grafted with polytrimethlphosphate, polyallyamine, poly(acrylic acid) , or collagen type I	osteoblast, myocyte, neuron	expansion and differentiation medium	66 (2011)
murine cardiac progenitor cells, MSCs	PLLA, PCL, PLGA film	cardiomyocyte	differentiation medium	107 (2008)
hMSCs	PAAm gel grafted with collagen I	adipocyte, chondrocyte, smooth muscle cell, schwann cell	expansion and differentiation medium	75 (2011)
hMSCs	PAAm gel grafted with collagen I	myocyte, neural cell	expansion medium	93 (2011)
hMSCs	gelatin–hydroxyphenylpropionic acid gel cross-linked with HRP and H_2O_2	myocyte, neuron	expansion medium	73 (2010)
rat NSCs	RGD conjugated PEG-PAAm interpenetrating network gel	neuron and astrocytes	differentiation medium	62 (2008)
embryonic cortices	xyloglucan gel grafted with poly(D-lysine)	neuron	differentiation medium	110 (2009)
rat NSCs	polymethacrylamide–chitosan gel coated with laminin	neuron, oligodendrocyte, astrocyte	differentiation medium	108 (2009)
bovine limbal stem cells	collagen type I gel coated with laminin	limbal epithelial cell	expansion medium	100 (2012)
murine ESCs (ESD3)	fibrin gel	endoderm cell	differentiation medium	109 (2012)

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

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

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

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

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

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

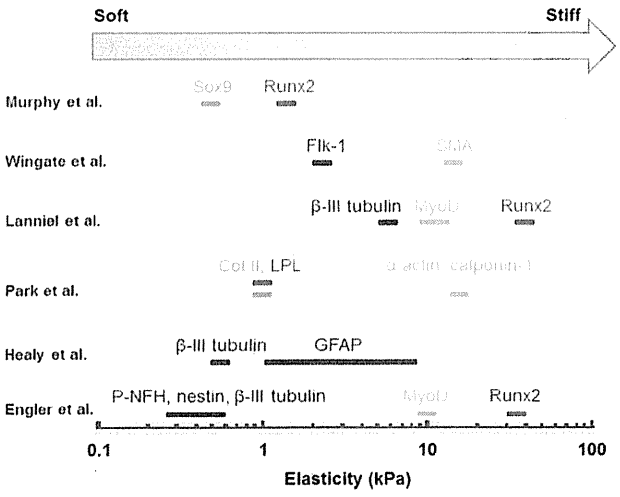

Figure 3. Proteins and transcription profiles of neural markers (red bar; nestin, P-NFH, and β-III tubulin), chondrocyte markers (yellow bar; collagen type II and Sox9), an adipocyte marker (orange bar; LPL), muscle transcription factors (green bar; MyD, α-actin, calponin-1, and SMA), an osteoblast transcription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

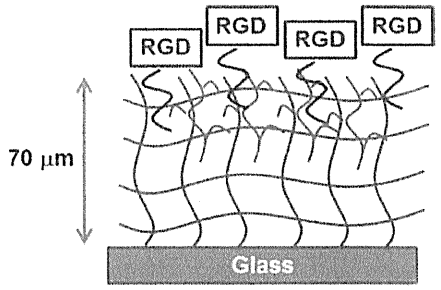

Figure 4. Schematic model developed by Healy et al. of an interpenetrating polymer network with oligopeptides containing surface RGD sequences with different stiffnesses. Modified with permission from ref 62. Copyright 2008 Elsevier Inc.

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

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

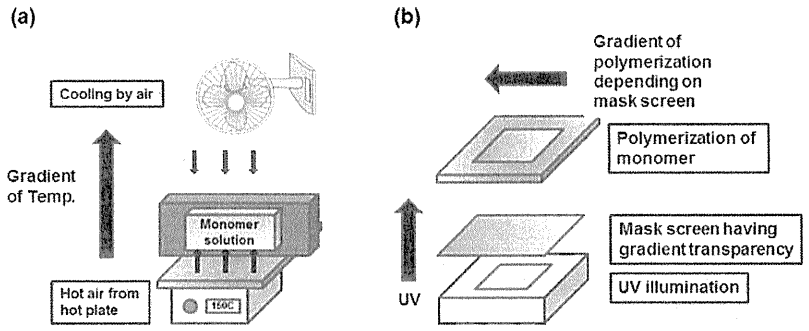

Figure 5. Preparation methods of substrates with elastic modulus gradients by monomer polymerization, shown by temperature gradient (a) or intensity of UV light (b). Modified with permission from ref 85 with Copyright 2012 Elsevier Inc. (a) and from ref 93 under a Creative Commons Attribution License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

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

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

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

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

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

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

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

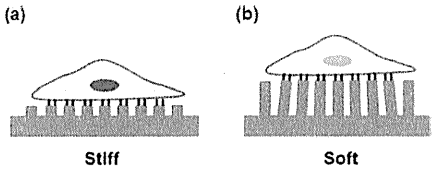

Figure 6. Elastomeric PDMS micropost arrays where the height of PDMS microposts control substrate stiffness (elasticity). Short and long microposts lead to stiff and soft surfaces, respectively, although both micropost arrays are prepared with the same components and cross-linking of PDMS. Modified with permission from ref 105 under a Creative Commons Attribution License.

plasma-treated micropost arrays, which were coated with vitronectin. These cells were mechanosensitive and increased their cytoskeleton contractility with matrix stiffness, and stiff substrates were supportive of the maintenance of hESC pluripotency.[105] Matrix mechanics-mediated cytoskeleton contractility seems to be functionally correlated with E-cadherin expression in cell–cell contacts and involved in hESC cell fate decisions. The microenvironment of hESC culture on micropost arrays should be different than that of conventional 2-D culture.[105] This difference may lead to different optimal elasticities (stiffness) for the maintenance of hESC pluripotency, where stiff surfaces are preferable in micropost array culture and soft surfaces are preferable in conventional 2-D culture.

The in vivo microenvironment of hMSCs regulates their self-renewal and differentiation. Human MSCs cultured ex vivo gradually lose their pluripotency after 10–20 passages, as characterized by a lack of proliferation and differentiation. Winer et al. cultured hMSCs on 250-Pa cross-linked PAAm hydrogels coated with the ECM proteins fibronectin and collagen I. These hydrogels mimic the elasticity of bone marrow (220 Pa of storage modulus for bovine bone marrow) and fat tissues (Figure 2).[87] Human MSCs cultured on a soft substrate halted cell cycle progression, despite the presence of serum; these nonproliferative hMSCs reproliferated when replated on a stiff substrate. Nonproliferative hMSCs on 250-Pa PAAm hydrogels also exhibited the capability to differentiate into adipocytes when cultured in adipogenic induction medium and differentiated into osteoblasts when incubated on a stiff substrate in osteoblast induction medium. These results demonstrate that hMSCs on soft (250-Pa) PAAm hydrogels are quiescent but competent to resume proliferation or initiate terminal differentiation when provided with appropriate cues. These observations suggest that the biomaterial physical cue of ECM elasticity is a factor enabling the bone marrow niche to maintain a reservoir of hMSCs for long periods.[87]

2.3. Mechanism of Regulation of Stem Cell Differentiation Fate by ECM and Substrate Elasticity in 2-D Culture

The mechanism by which the elasticity of ECM on culture substrates induces lineage specification of stem cell differentiation is currently difficult to explain. Figure 7 illustrates several mechanosensing models proposed by several researchers for detecting substrate elasticity and the direction of different MSC lineages.[60,72,74] The elasticity of the ECM on the substrate generates mechanical stimuli on the plated stem cells, thereby inducing changes in focal adhesion (FA) protein activity and remodeling.[72−75] The growth and elongation of focal adhesions vary depending on culture substrate stiffness, suggesting that ECM elasticity regulates focal adhesion assembly (Figure 7). Integrins are known as the most important mechanical sensors positioned at the starting point of the sensing pathway.[72] Du et al. reported that β1 integrin activation in MSCs was induced by soft culture substrate to a significantly greater degree than by stiff culture substrate.[72] However, the level of cell-surface integrins in MSCs on soft culture substrate was significantly lower than that on stiff culture substrate in 2-D culture (Figure 7). Soft substrate markedly enhanced the internalization of integrin; this internalization was mediated mainly through caveolae/raft-dependent endocytosis.[72] Enhanced integrin internalization in MSCs on soft culture substrate guides neural differentiation of MSCs by inhibiting the bone morphogenetic protein (BMP)–Smad pathway. The inhibition of integrin internalization by the caveolae/raft inhibitor methyl-β-cyclodextrin was found to block neural lineage differentiation of MSCs on soft substrate in 2-D culture. Soft substrate suppressed the BMP–Smad pathway partially via integrin-regulated BMP receptor endocytosis.[72]

Atomic force microscopy data suggested that integrin–receptor complexes are more easily ruptured on soft culture substrates than on stiff culture substrates. This phenomenon may contribute to the enhancement of integrin internalization on soft culture substrates. It has been suggested that ECM elasticity affects the integrin activity of MSCs and trafficking mechanisms modulating integrin–receptor internalization, thereby contributing to the specific lineage differentiation of

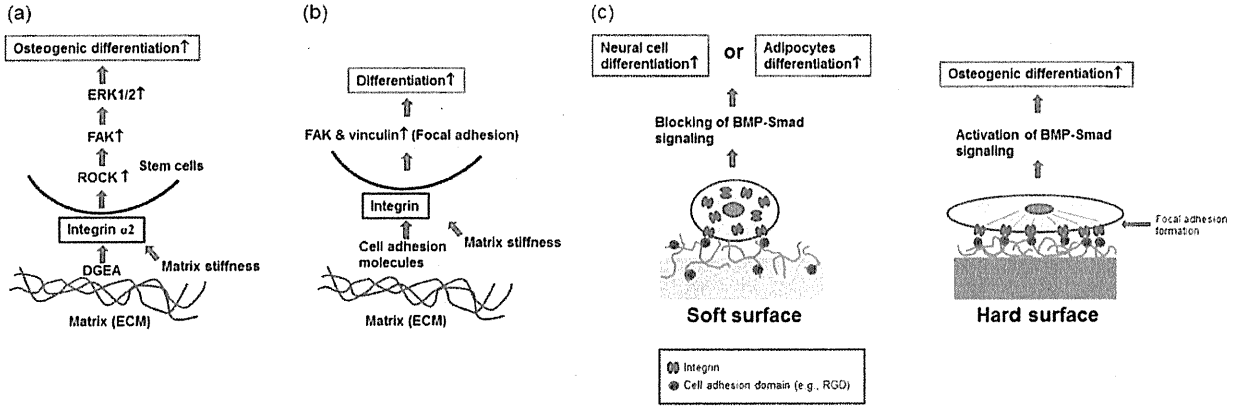

Figure 7. The mechanism of the effect of culture substrate stiffness on stem cell differentiation via the growth and elongation of focal adhesions, as suggested by Shih et al. (a),[74] Yim et al. (b),[60] and Du et al. (c).[72]

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stem cells cultured on ECM with specific elasticities in 2-D culture.[72]

2.4. Elasticity of Substrate Directs Stem Cell Differentiation Fate in 3-D Culture

Elasticity of scaffolds is another important factor for specific lineage differentiation of stem cells in three-dimensional (3-D) culture.[63] Several researchers have reported that the stiffness of cell culture scaffolds (matrix) affects specific stem cell differentiation lineages in 3-D culture.[59,63−65,70,77,98,102]

Murphy et al. prepared cross-linked collagen−glycosaminoglycan scaffolds as analogues of natural ECM. Two different glycosaminoglycans, chondroitin sulfate (CS) and hyaluronic acid (HyA), were used.[63] Cross-linking with dehydrothermal (DHT) treatment and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) produced three collagen−glycosaminoglycan scaffolds with stiffness values of 0.5, 1, and 1.5 kPa. In their study, the effect of scaffold stiffness and glycosaminoglycan composition on rat MSC differentiation was investigated in expansion medium in the absence of induction (differentiation) supplements.[63] The scaffolds prepared with HyA and with the lowest stiffness (0.5 kPa) facilitated a significant upregulation in Sox9 expression, indicating that MSCs were directed toward a chondrogenic lineage. In contrast, Runx2 expression was highest in the stiffest (1.5 kPa) scaffolds prepared with CS, indicating that MSCs were directed toward an osteogenic lineage in stiffer scaffolds.[63] This study demonstrated that scaffold stiffness can direct MSC fate in 3-D culture even in the absence of differentiation supplements; this capability is further enhanced by the selection of optimal scaffold components for specific differentiation lineages.

An injectable and biodegradable hydrogel composed of hyaluronic acid−tyramine (HyA-Tyr) conjugates was prepared by Toh et al.[64] HyA-Tyr was cross-linked in vivo by the addition of small amounts of peroxidase and hydrogen peroxide (H_2O_2) with independent tuning of the gelation rate and the degree of cross-linking, both of which affected hydrogel elasticity.[64] HyA-Tyr hydrogels of varying elasticities (5.4, 9.5, and 11.8 kPa) were explored as biomimetic matrices for caprine MSCs in cartilage tissue engineering where the compressive modulus, equilibrium swelling, and degradation rate were controlled by varying the concentration of H_2O_2 as the oxidant in the oxidative coupling reaction (Figure 8).[64,120] Cellular condensation, which was determined by measuring the increase in the effective number density of rounded cells in the lacunae, was found to enhance in softer hydrogel matrices with lower cross-linking that displayed enhanced scaffold contracture. Conversely, cells expressed a more elongated morphology with a reduced degree of cellular condensation in more highly cross-linked matrices.[64] The degree of hydrogel cross-linking also modulated matrix biosynthesis and cartilage tissue histogenesis. Matrices with less cross-linking enhanced chondrogenesis, demonstrating increases in the percentage of cells with chondrocyte morphology and increases in the biosynthetic rates of glycosaminoglycan and collagen type II where hyaline cartilage tissue formation was observed.[64] It may be that the tunable three-dimensional microenvironment of the HA-Tyr hydrogels modulates cellular condensation during chondrogenesis and has an impact on cell spatial organization and matrix biosynthesis.

Pek et al. created thixotropic poly(ethylene glycol)−silica (PEG−silica) nanocomposite hydrogels with and without the cell adhesive RGD (Arg-Gly-Asp) oligopeptide for 3-D cell

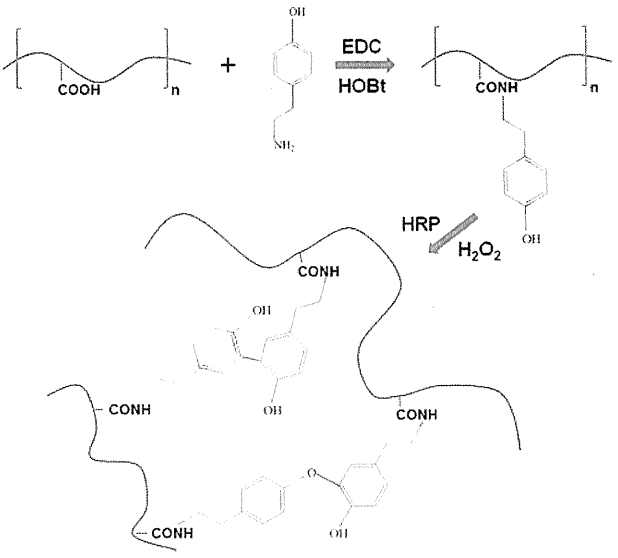

Figure 8. Preparation of hyaluronic acid−tyramine (HyA-Tyr) hydrogels in which their elasticity was controlled by varying the H_2O_2 concentration as the oxidant in the oxidative coupling reaction with HRP as an enzyme. Modified with permission from ref 120. Copyright 2005 Royal Society of Chemistry.

culture.[59] The thixotropic PEG−silica hydrogel can be liquefied simply by applying a shear stress. This allows stem cells in the thixotropic hydrogels to be easily introduced into defect parts in human body before the matrix material reverts to a gel state. The matrix stiffness of the hydrogel can be tuned by controlling the amount of fumed silica in the hydrogel. Hydrogels were created with 7, 25, 40, 75, and 100 Pa of liquefaction stress, where the liquefaction stress is defined as the minimum shear stress at which the hydrogels become liquid under the conditions of storage modulus (G') = loss modulus (G'') and $\tan^{-1}(G''/G') = 45°$.[59] When hMSCs were cultured in PEG−silica nanocomposite hydrogel in expansion medium for 1 week, the highest expression level of neural transcript factor ENO2 was observed in hMSCs cultured in hydrogels with the lowest liquefaction stress (7 Pa).[59] Furthermore, the highest expression levels of myogenic (MYOG) and osteogenic (Runx2 and osteocalcin) transcription factors were observed in hMSCs cultured in intermediate (25 Pa) and high liquefaction stress (75 and 100 Pa) hydrogels, respectively. All of the aforementioned transcription factors were expressed more highly in hMSCs cultured in the PEG−silica nanocomposite hydrogel than in hMSCs cultured on conventional 2-D tissue culture polystyrene plates (TCPS). This result indicates that hMSCs cultured in 3-D are in a more differentiated state compared with cells cultured in 2-D. Immobilization of the cell-adhesion peptide RGD promoted both proliferation and differentiation of hMSCs on the stiffer hydrogels (liquefaction stress >75 Pa).[59] It was concluded that matrix stiffness (elasticity) regulates hMSC differentiation fate when cultured in 3-D PEG−silica nanocomposite hydrogel in expansion medium. In their study, it was demonstrated that mechanical signals alone can control lineage specification of hMSCs in 3-D culture. It was proposed that the mechanism by which RGD promotes osteogenesis is via its role as a ligand to the integrin mechanoreceptor, allowing stem cells to detect high matrix stiffness for bone differentiation.

Banerjee et al. reported on the proliferation and differentiation of rat neural stem cells (NSCs) encapsulated within 3-D scaffolds of alginate hydrogels with elastic moduli ranging from 0.18 to 20 kPa created by modulating the concentration of alginate and calcium ions.[77] The rate of NSC proliferation decreased with increases in the hydrogel elastic modulus in expansion medium without added induction factors. Enhanced expression of the neuronal marker β-tubulin III was found within the softest hydrogels, which had elastic moduli comparable to that of brain tissue (approximately 180 Pa).[77] It was found that NSCs cultured on soft substrates were guided to differentiate into neuronal lineages in 3-D[77] and 2-D cultures.[62]

Engler et al. have shown that cytoskeletal motors may be involved in the matrix-elasticity sensing that drives lineage specification in MSCs grown on hydrogels.[32] It is interesting to probe for a possible role for cytoskeletal motors in influencing the function of NSCs in 3-D environments.

Few in vitro studies describe the effects of cell culture matrix elasticity on the differentiation of MSCs into vascular cell types.[37,70,121,122] Wingate et al. fabricated a 3-D PEG-based nanofiber hydrogel coated with collagen type I with tunable elasticity for use as a cellular substrate directing rat MSCs into vascular cell types. This hydrogel is prepared using electrospinning and photopolymerization techniques, and its elasticity is tuned by adjusting the photopolymerization time.[70] The elastic moduli of the hydrogels were determined by compression evaluation to be in the range of 2 to 15 kPa, similar to the in vivo elasticity of the intima basement membrane and media layer where endothelial cells are known to reside on top of the soft basement membrane and smooth muscle cells in the stiffer medial layer.[123,124] MSCs seeded on rigid matrices (8−15 kPa) exhibited an increase in cell area compared with those seeded on soft matrices (2−5 kPa).[70] It was found that the matrix elasticity guided the cells to express different vascular-specific phenotypes with high differentiation efficiency. Ninety-five percent of MSCs cultured on hydrogels with an elasticity of 3 kPa expressed Flk-1 (endothelial marker) protein within 24 h in expansion medium, whereas only 20% of MSCs seeded on matrices with elasticities >8 kPa expressed the Flk-1 marker.[70] In contrast, approximately 80% of MSCs cultured on hydrogels with elasticities >8 kPa expressed α-actin (smooth muscle marker) protein within 24 h in expansion medium, while fewer than 10% of MSCs seeded on hydrogels with elasticities <5 kPa expressed the α-actin marker.[70] In summary, the local elasticity of hydrogels encapsulating MSCs can guide MSC differentiation lineages into vascular cell types in expansion medium without the addition of induction factors, and the lineage commitment of MSCs toward specific vascular cell types can be controlled by the specific design of the substrate modulus.

Mechanical forces are also critical to embryogenesis in the lineage specification of the gastrulation phase, where the embryo is transformed from a spherical cell to a multilayered organism with properly organized endoderm, mesoderm, and ectoderm germ layers. Zoldan et al. investigated the germ layer formation process by culturing hESCs on 3-D scaffolds with stiffnesses corresponding to specific germ layers to understand the environmentally induced cell changes of the embryo in the gastrulation phase.[102] The materials of the scaffolds used in their study were poly(L-lactic acid) (PLLA), poly(lactic acid-*co*-glyconic acid) (PLGA), poly(ε-caprolactone) (PCL), and PEG. The scaffolds were prepared using the salt-leaching method.[3] Binary PLLA/PLGA and ternary PLLA/PLGA/PCL scaffolds were also prepared with selected weight ratios of these biodegradable polymers. The elastic moduli of these scaffolds ranged from 0.05 to 7 MPa. Human ESCs were mixed with Matrigel solution and seeded into the scaffolds to facilitate cell attachment.[102]

Human ESCs cultured on the stiffest scaffolds (>6 MPa) remained undifferentiated and exhibited reduced expression of the germ layer-specific genes evaluated in their study.[102] In contrast, scaffolds with medium-high elastic moduli promoted mesodermal differentiation, and endoderm- and ectoderm-associated gene expressions were not detected.[102] Scaffolds with intermediate elastic moduli (0.1−1 MPa) promoted endoderm differentiation and reduced expression levels of mesoderm-related genes (Brachyury and MIXL1). Scaffolds with low elastic moduli (<0.1 MPa) resulted in ectoderm differentiation, as evidenced by high expression levels of SOX1 and ZIC1 genes (ectodermal germ layer-associated genes).[102] In summary, the differentiation of hESCs into each germ layer was promoted by different scaffold stiffness thresholds, reminiscent of the forces exerted during the gastrulation process. It is possible that 3-D scaffolds could recapitulate the mechanical stimuli required for directing hESC differentiation, depending on the stiffness (elasticity) of the scaffolds.

The liver is one of the most complex organs in the body and is responsible for toxin removal, production of bile and hormones, regulation of nutrients, and synthesis of serum proteins.[65] Hepatic stem cells are partnered in vivo with mesenchymal precursors to endothelia (angioblasts) and stellate cells and reside in regulated microenvironments containing hyaluronic acid (HyA), laminin-5, collagen type III, and chondroitin sulfate proteoglycans. Lozoya et al. investigated the effects of 3-D-microenvironments on human hepatic stem cells by embedding them in HyA-based hydrogels prepared with a serum-free medium tailored for endodermal stem/progenitor cells by mimicking the liver's stem cell niche.[65] These HyA-based hydrogels matched the diffusivity of culture medium and had tunable stiffness (25−520 Pa), depending on their concentration of HyA and cross-linker (poly(ethylene glycol) diacrylate). The HyA-based hydrogels induced the transition of hepatic stem cell colonies toward stable heterogeneous populations of hepatic progenitors (hepatoblasts) depending on hydrogel stiffness, as shown by both their gene and protein expression profiles.[65] This study shows that the mechanical properties of the microenvironment can regulate differentiation in endodermal stem cell populations, such as human hepatic stem cells.

2.5. Results Contradictory to Engler's Research in 2-D Culture

Although Engler et al.[32] performed the landmark study, demonstrating that substrate matrix guides stem cell differentiation fate (1850 citation by Web of Science in September, 2012), the mechanism by which stem cells sense the mechanical and geometrical properties of the substrate has remained elusive.[125] Several researchers have reported conflicting results and different intriguing ideas on the effect of substrate elasticity on stem cell differentiation.[19,22]

Trappmann et al. investigated the differentiation of hMSCs and human epidermal stem cells on (a) polydimethylsiloxane (PDMS) with immobilized collagen type I and (b) PAAm hydrogels with immobilized collagen type I.[22] PDMS and PAAm substrates of varying stiffness in the range of 0.1 kPa to

2.3 MPa were prepared by varying the ratio of cross-linker to base monomers. They found that epidermal stem cells could spread fully and assemble a cortical F-actin cytoskeleton on all PDMS substrates, independent of stiffness. Furthermore, cells cultured on all PDMS substrates reached terminal epidermal stem cell differentiation (i.e., keratinocytes) and expressed the cornified envelope precursor involucrin at the same rate (i.e., approximately 25%). Thus, PDMS elasticity (stiffness) did not affect the differentiation or spreading of human epidermal stem cells.[22] Furthermore, osteoblast differentiation, as analyzed by alkaline phosphatase (Table 3), was observed to the same degree (approximately 35%) on PDMS substrates of varying stiffness in the range of 0.1−800 kPa. No effect of PDMS substrate stiffness on the differentiation of hMSCs into osteoblasts was observed. Adipogenic differentiation of hMSCs as analyzed by Oil Red O staining (Table 3) was also investigated on PDMS substrates of varying stiffness. As was found in osteogenic differentiation on PDMS substrates, no effect of PDMS substrate stiffness was noted on the differentiation of hMSCs into adipocytes. These results can be explained by the fact that the morphology and characteristics of the ECM (collagen I in this case) should be almost the same on PDMS substrates of different elasticities because ECM cannot penetrate the surface of PDMS substrates, whereas it can penetrate the surface of hydrogels, as depicted in Figure 9.

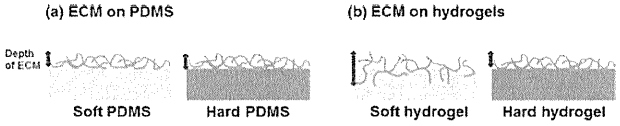

Figure 9. Morphology of ECM on PDMS (a) and hydrogel (b) with soft and hard characteristics.

Other researchers have also reported no effects of PDMS substrate stiffness on the differentiation of hMSCs.[85]

In contrast, adhesive cell area and actin polymerization of hMSCs cultured on PAAm hydrogels grafted with collagen type I increased with increasing elastic modulus of the PAAm hydrogels, the same tendency reported by several researchers in 2-D culture of hMSCs on PAAm hydrogels coated with collagen type I.[32] In summary, the differentiation of hMSCs is influenced by the stiffness of PAAm but not PDMS substrates.[22]

Stiffer PAAm hydrogels have higher cross-linking points. Therefore, differences in PAAm hydrogel network cross-linking will result in differences in collagen attachment, with the distance between covalent anchoring points being longer on softer gels, whereas anchoring point distance is shorter on stiffer gels, as described in Figure 9. ECM can anchor into stiffer hydrogels with higher anchoring points, whereas ECM can anchor into softer hydrogels with lower anchoring points. In our recent study, it was found via XPS ECM analysis that ECM concentration on the outer surface is higher on stiffer hydrogels and lower on softer hydrogels, whereas the total ECM amount on the hydrogels was found to be nearly unchanged using measurements of fluorescent probe binding to the ECM (unpublished data), as reported in several previous studies.[32,74] Therefore, the depth of ECM anchoring is expected to be deeper on softer hydrogels, as depicted in Figure 9. The ECM anchoring depth may be another factor regulating stem cell differentiation lineage fate.

When cells pull on covalently attached collagen, the mechanical feedback consists of the magnitude of the movement of the collagen segment coupled to the PAAm hydrogel. Therefore, the strength of the collagen elasticity feedback that cells sense upon integrin ligation decreases with increasing anchored collagen fiber length (i.e., on soft PAAm hydrogels).[22,125] In contrast, collagen stiffness on PDMS substrates of varying stiffness is suggested to be the same due to the same collagen cross-linking time leading to the same collagen cross-linking distance. This may be because collagen cannot penetrate into PDMS substrates of any stiffness.

Rowlands et al. demonstrated the interplay of stiffness and adhesive ligand presentation, exemplified by the observation that osteogenic differentiation of hMSCs occurred significantly only on collagen type I-coated substrates with the highest tested substrate stiffness.[88] The modulation of osteogenic and myogenic transcription factors by various ECM proteins demonstrated that substrate stiffness alone did not direct stem cell lineage specification, but the combination of substrate stiffness and specific ECMs, that is, the stiffness of specific ECMs, seemed to direct stem cell fate into specific differentiation lineages in 2-D culture.

It is concluded that Engler's landmark study demonstrating that substrate matrix guides stem cell differentiation fate is verified under the limited condition that the stem cells are cultured on hydrogels with immobilized collagen type I in a 2-D system where ECM (e.g., collagen type I) can penetrate the hydrogel surface to some extent, and not on solid substrates where ECM cannot penetrate the substrate surface, such as PDMS, glass, or metal. Hydrogels of varying stiffness lead to differences in ECM anchoring densities, thereby altering the mechanical feedback of ECM on stem cells. When the ECM is more loosely bound on soft hydrogels, we think that it cannot provide the mechanical feedback that the integrin complex requires to cluster in focal adhesions and signal through ERK/MAPK. It seems that mechanical feedback in stem cells leads them into specific differentiation lineages or causes them to remain undifferentiated.

2.6. Results Contradictory to Engler's Research in 3-D Culture

As discussed in previous sections, stem cells sense and respond to mechanical properties of the ECM. However, how ECM mechanics biophysically affect stem-cell fate in 3-D microenvironments is difficult to determine. Huebsch et al. demonstrated that the lineage commitment of murine MSCs changed in response to the rigidity of 3-D microenvironments. The highest osteogenesis of MSCs was predominantly generated in alginate gels grafted with RGD oligopeptide (RGD-modified alginate) at 11−30 kPa, whereas MSCs preferentially differentiated into the adipogenic lineage in softer alginate gels (2.5−5 kPa).[19] The tendency for substrate stiffness to affect MSC differentiation lineage was also observed in various types of hydrogels, i.e., RGD-modified agarose and RGD-modified PEG hydrogels, and RGD-modified alginate.[19] The relationship between stem cell morphology and differentiation direction in 3-D hydrogels was investigated because hydrogel matrix elasticity affects stem cell morphology in 2-D culture, which correlates with MSC differentiation fate. However, the elastic modulus had no significant influence on MSC morphology in the 3-D hydrogels.[19] In other words, in the 3-D hydrogels, stem cell fate was not correlated with cell morphology, in contrast to previous 2-D work.[32] Instead, matrix

stiffness regulated integrin binding and the nanoscale reorganization of adhesion ligands, both of which were traction dependent and correlated with MSC osteogenic commitment.[19] It seemed that the stem cells used traction forces to mechanically reorganize the RGD peptides presented by the hydrogel matrices on a nanometer scale, clustering RGD near integrins while the peptides remained bound to the hydrogel material (Figure 10).[19] It was found that RGD clustering was

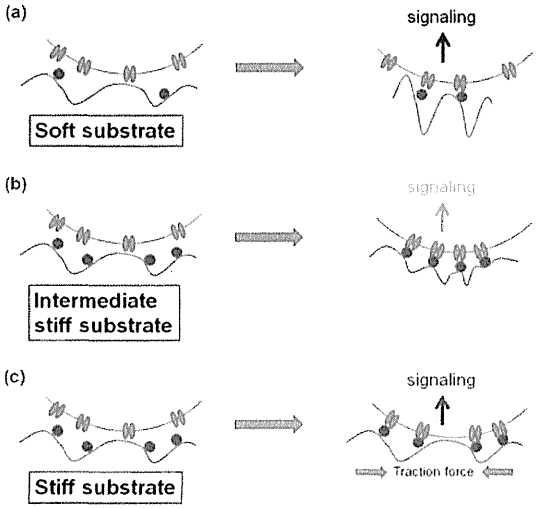

Figure 10. Matrix stiffness (soft substrate [a], intermediate substrate [b], and stiff substrate [c]) regulates integrin binding and reorganization of adhesion ligands, which are traction-dependent, at the nanometer level.

maximized in hydrogels of intermediate rigidity (22 kPa). This can be explained by the fact that stem cells in extremely soft hydrogels cannot assemble the cytoskeleton-associated adhesion complexes required to exert traction force, preventing them from deforming the hydrogel matrix to generate RGD clustering and maintain the RGD–integrin complex.[19] Blocking RGD–integrin binding to α_5 and α_V integrins by using anti-α_5- or anti-α_V-antibodies significantly decreased osteogenesis in RGD-modified alginate in 3-D culture and enhanced adipogenesis in an antibody dose-dependent fashion. In contrast, only α_5 integrins were expressed on the surfaces of MSCs in 2-D culture and were used to differentiate into osteoblasts.[19]

It is possible that MSCs interpret changes in the physical properties of adhesion substrates as changes in adhesion-ligand presentation and that MSCs themselves can be harnessed as tools to mechanically process materials into structures that feed back to manipulate their fate.

3. EFFECT OF TOPOGRAPHY OF CELL CULTURE MATERIALS ON STEM CELL DIFFERENTIATION

The topography of the extracellular microenvironment can influence stem cell responses from attachment and migration to differentiation and production of new tissues.[126−129] Cells in their natural environment interact with ECM components on a nanometer scale.[130] There is evidence of nano- or macro-topography-induced stem cell differentiation, suggesting that physical interactions between stem cells and the extracellular environment in the form of topography can modulate cell function and stem cell differentiation.[60] The regulation of cell spreading area and shape in 2-D culture is one of the physical factors affecting stem cell differentiation fate that can be guided by the topography of cell culture biomaterials (substrates). Micro- and nanopatterned surfaces with and without immobilized ECM are one of the most typical cell culture biomaterials used for topography regulation. In this section, we will discuss the effect of biomaterial topography on stem cell differentiation on micro- and nanopatterned surfaces.

3.1. Preparation of Micro- and Nanopatterned Surfaces

Micro- and nanopatterned surfaces are the most common biomaterials used to investigate the effect of cell culture topography on stem cell differentiation. Microcontact printing (soft lithography) and photolithography patterning (hard lithography) methods are typically used to create micro- and nanopatterned surfaces. A variety of shapes, morphologies, sizes, and microdomain stem cell attachment patterns can be designed using both of these methods.

Some examples of the shapes and morphologies of micro- and nanodomains of micropatterned surfaces are illustrated in

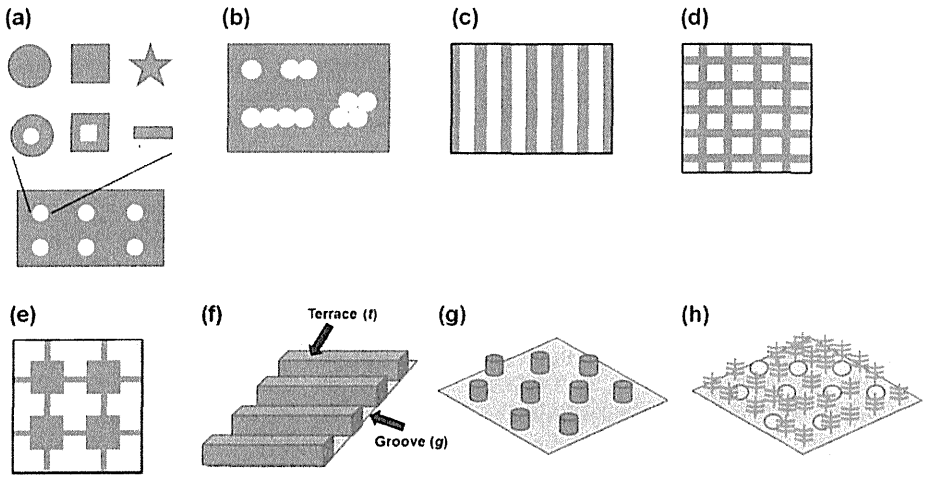

Figure 11. Examples of shapes and morphologies of micropatterned surfaces reported in the literature. Circles, squares, and stars (a), circle combinations (b), stripes (c), grids (d), grid and square combinations (e), microgroove morphologies (f), microposts (g), and circles surrounded by polymer brushes (h) are illustrated.

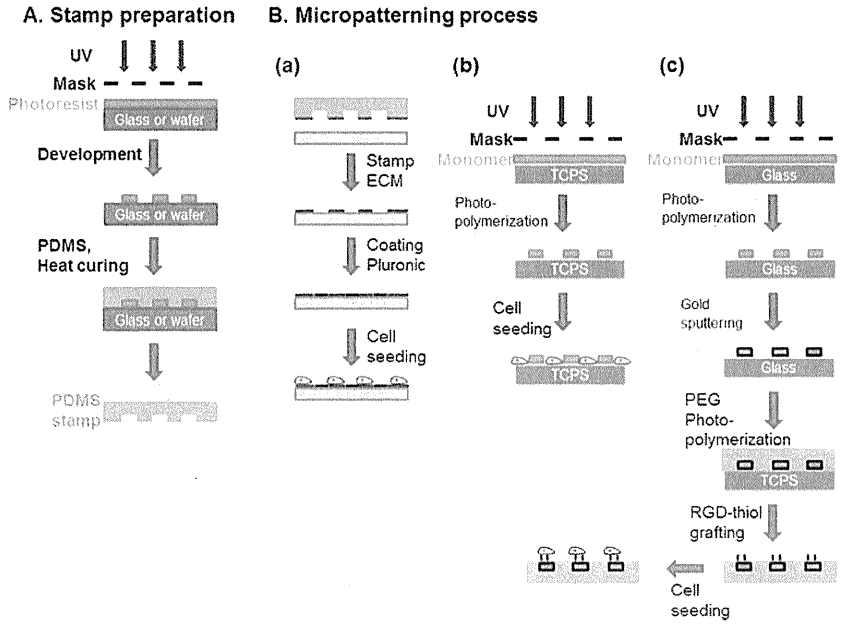

Figure 12. Typical microcontact printing preparation methods in two-dimensional (2-D) stem cell culture. Stamp preparation method (a) and several micropatterning processes (b).

Figure 11. Circles, squares, stars, striped lines, and grid lines are typically used as stem cell attachment macro-domains (Figure 11a,b,c,d).[131−133] Surface patterns of squares connected with narrow lines have also been investigated (Figure 11e).[134] Striped and microgrooved surface morphologies have been investigated for stem cell culture (Figure 11f).[135] Patterned cylinder (micropost) (Figure 11g),[136] and cave (Figure 11h)[137−139] structures have also been prepared.

A typical preparation method of microcontact printing for the 2-D culture of stem cells is illustrated in Figure 12. First, a polydimethylsiloxane (PDMS) elastomeric stamp is prepared using a mold. The mold can be fabricated by spin-coating a photoresist solution onto silicon wafers or glass cover plates and then exposing the photoresist to ultraviolet (UV) light through a chrome mask with the desired geometric features (Figure 12A).[131] Un-cross-linked polymer (photoresist) is removed by washing the silicon wafer or glass cover plates with solvent. PDMS and curing agent prepolymers are poured on the resulting mold and cured at a high temperature (i.e., 70 °C) for several hours before the PDMS stamp is removed from the mold.[131] ECM solutions such as fibronectin,[95,131,140] Matrigel,[141−143] collagen type I, laminin, or poly(L-lysine) (PLL) solution[134] are stamped on PDMS films,[95,140] Petri dishes,[134] TCPS dishes or slides,[141−143] and glass slides. Although stem cells specifically adhere to ECM or PLL domains, nonspecific adsorption outside the microcontact patterning domains on PDMS films, TCPS dishes or slides, or glass slides should be avoided. Because stem cells are adhered to the biomaterial surface via proteins such as ECM, low-protein-binding macromolecules, such as a triblock copolymer of poly(ethylene oxide) (PEO)−poly(propylene oxide) (PPO)−poly(ethylene oxide) (PEO), Pluronic or Poloxamer,[144−146] are typically coated on micropatterned dishes and slides after stamping with ECM or PLL. In other cases, ECM or PLL is stamped on Petri dishes coated with plasma-polymerized PEO using a PDMS microstamp in which PEO is one of the low-protein-binding polymers.[134] Microprinting is a simple way to prepare micropatterned surfaces; however, the drawback of microprinting ECMs or PLL is the low stability of the remaining micropatterned ECM or PLL on the surface due to their physical adsorption.

The self-assembly monolayer (SAM) method can be combined with microcontact printing. Wan et al. prepared micropatterned fibronectin using the SAM method.[131] An adhesive SAM octadecanethiol was transferred via PDMS microstamp onto gold-coated glass slides. Subsequently, the unstamped regions of the slides were coated with nonadhesive ethylene glycol-terminated SAM. Finally, the patterned surfaces were coated with fibronectin solution because fibronectin preferentially adsorbed on the hydrophobic domain of SAM octadecanethiol in the microcontacted region.[131]

Hydrogels such as poly(vinyl alcohol) (PVA) and PEO or PEG display low protein-binding activity. Chen et al. developed an interesting and simple micropatterning method (Figure 12B,b).[137,139] They synthesized photo-cross-linkable azidophenyl-derived PVA.[137,139] The photo-cross-linkable PVA solution was placed on TCPS and cross-linked via a micropatterned photomask. The PVA was cross-linked and used to generate hydrogels on the outside of stem cell culture domains. This allowed stem cells to be cultured on micropatterned TCPS surrounded by cross-linked hydrogels. If necessary, ECM can be coated on the micropatterned TCPS dishes. A similar strategy using a different method was reported by Connelly et al.[138] Micropatterned stamps inked with the thiol initiator ω-mercaptoundecyl bromoisobutyrate were brought into conformal contact with gold-coated coverslips to deposit the initiator as a self-assembled monolayer.[138] Atom transfer radical polymerization (ATRP) of oligo(ethylene glycol) methacrylate (OEGMA) was performed on the gold-coated coverslips. Finally, the micropatterned coverslips were coated with collagen type I, which could be loaded on the regions without micropatterned OEGMA polymer brush. Stem cells were cultured on bowls surrounded by hydrogel polymers on micropatterned dishes prepared from either PVA or

Table 6. Some Research Studies for Stem Cell Differentiation on Micropatterned Materials (Osteogenic and Adipogenic Differentiation)[a]

stem cell source	micropatterned materials for stem cell culture	pattern type	medium	differentiation	ref (year)
hMSCs	micropatterned amorphous diamond, titanium, tantalum, and chromium with square shape on silicon wafer	Figure 11a	differentiation medium	osteoblasts	147 (2010)
murine MSCs	micropatterned PDMS with grid (lattice) morphology coated with fibronectin	Figure 11d	differentiation medium	osteoblasts	151 (2011)
hADSCs	micropatterned fibronectin with ring shape or rectangles on gold-coated slides	Figure 11a	differentiation medium	osteoblasts, adipocytes	131 (2010)
hMSCs	micropatterned fibronectin with square, rectangular, flower, and star shape on octadecanethiol surface	Figure 11a	mixed differentiation media of adipocytes and osteoblasts	adipocytes, osteoblasts	79 (2010)
hMSCs	micropatterned fibronectin with square shape on PDMS surrounded by Pluronic F108	Figure 11a	mixed or single differentiation medium of adipocytes and osteoblasts	adipocytes, osteoblasts	95 (2004)
rat MSCs	micropatterned RGD with circle, square, triangle, and star shape on PEG hydrogel	Figure 11a	differentiation medium	adipocytes, osteoblasts	148 (2011)
hMSCs	micropatterned alkane thiol surface with circle, octagone, triangle, trapezoid, square, and pentagone shape surrounded by PEG-terminated alkanethiol on gold surface	Figure 11a	differentiation medium	adipocytes	137 (2008)
rat MSCs	micropatterned RGD with circle and aggregated circle shape on PEG hydrogel	Figure 11b	differentiation medium	adipocytes, osteoblasts	132 (2010)
hMSCs	TCPS surface of circle shape surrounded by micropatterned poly(vinyl alcohol)	Figure 11h	differentiation medium	adipocytes, osteoblasts	139 (2011)
hMSCs	TCPS surface of triangle, square, pentagon, hexagon, and circle shape surrounded by micropatterned poly(vinyl alcohol)	Figure 11h	differentiation medium	adipocytes	149 (2011)

[a]hADSCs, human adipose-derived stem cells; MSCs, mesenchymal stem cells; hMSCs, human MSCs; PDMS, polydimethylsiloxane; TCPS, tissue culture polystyrene.

OEGMA polymer brushes, making it possible to restrict the stem cells from leaving the bowl where they were intended to stay (Figure 11h).

Tang et al. developed a slightly different preparation method of the micropatterned surfaces described above (Figure 12B,c).[132] Clean glass slides were spin-coated with a positive photoresist, exposed to UV light through a chrome mask with the desired geometric features, and developed. Subsequently, the surface was sputtered with gold, and the unpolymerized photoresist was removed by washing with solvent. Allylmercaptan was grafted on the gold microislands in a vacuum. This process was necessary to transfer the gold onto the surface of the PEG hydrogel in the following step. PEG diacrylate with a photoinitiator was cast onto the micropatterned glass and photo-cross-linked under UV irradiation. Micropatterned PEG hydrogel was finally obtained by separating the hydrogel bound with the gold micropatterns from the glass slides. Cyclo-(RGDfk)-thiol (R, arginine; G, glycine; D, aspartic acid; f, D-phenylalanine; and k, lysine) was grafted onto the gold microislands on the PEG hydrogels. Stem cells were able to attach to the RGD microdomain on the PEG hydrogels via integrin receptors.

Microgrooved surfaces with striped terraces and grooves (Figure 11f) can be prepared using conventional photolithographic techniques. Beduer et al. prepared microgrooved surfaces on PDMS, and PLL and laminin were then coated on the microgrooved surface for neural stem cell culture.[135]

3.2. Adipogenic and Osteogenic Stem Cell Differentiation on Micropatterned Surfaces

Evidence that cell shape regulation by micropatterned surfaces leads to the commitment of stem cells into different lineages has been identified in 2-D cultures. Several researchers have investigated the effect of the spreading area and shape of stem cells cultured on micropatterned surfaces on differentiation lineage commitment.[67,79,95,131,133,135,139,147–150] Table 6 summarizes a number of studies of stem cell differentiation into adipocytes and osteoblasts on micropatterned surfaces.[79,95,131,132,137,139,147–149,151]

It is known that cell seeding density directly affects hMSC lineage commitment; hMSCs at high seeding density tend to differentiate into adipocytes, and those at low seeding density tend to differentiate into osteoblasts when cultured in mixed differentiation medium for osteoblasts and adipocytes (Figure 13d).[95] These phenomena can be explained by (a) a decrease in cell adhesion and spreading on the cell culture substrate or (b) an increase in cell–cell contact and paracrine signaling. However, conventional cell culture cannot distinguish between these two potential effects. Therefore, McBeath et al. investigated the effect of cell shape on stem cell differentiation commitment by controlling the degree of cell spreading in the absence of cell–cell communication.[95] They placed square shaped microcontact fibronectin prints onto PDMS substrates to generate "islands" of fibronectin surrounded by regions coated with Pluronic F108, which enables stem cells to adhere on fibronectin islands (Figure 11a).[95] Human MSCs were seeded on the micropatterned PDMS substrates by attaching as single cells per island and spread to varying degrees, depending on the size of the island (1000 or 10 000 μm^2) in mixed differentiation medium for adipocytes and osteoblasts. Adipogenesis occurred only on small islands, indicating that round hMSC morphology guided their differentiation commitment into adipogenesis, whereas osteogenesis was observed only on large islands, suggesting that hMSC spreading led to osteogenic differentiation (Figure 13a). In their results, it is suggested that the regulation of cell shape alone can mediate a switch in hMSC commitment between adipogenic and osteogenic fates in 2-D culture.[95] Shape-mediated commitment was facilitated by actin-cytoskeleton expression because disrupting the actin cytoskeleton by the addition of the actin-disrupting agent cytochalasin D or the Rho kinase inhibitor (ROCK) Y-27632 into the mixed differentiation medium increased adipogenesis and decreased osteogenesis of hMSCs in both cases of inhibitor addition where the Rho effector was involved in myosin

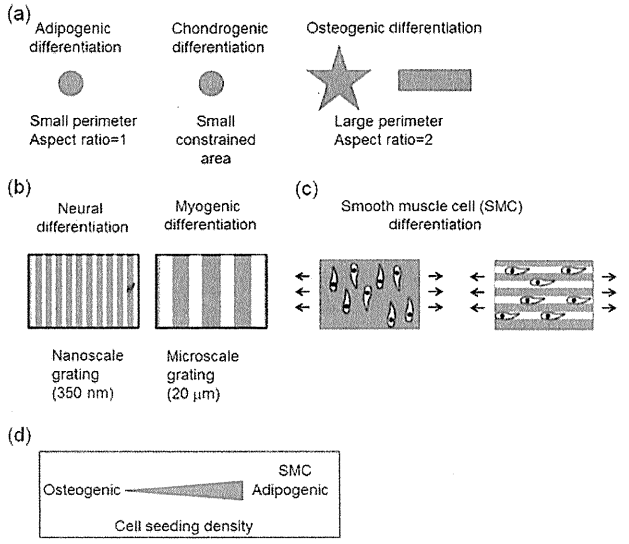

Figure 13. Stem cell differentiation on several different micropatterned surfaces. Adipogenic stem cell differentiation is promoted on surfaces with smaller perimeters and lower aspect ratios. Chondrogenic stem cell differentiation is promoted on surfaces of constrained area. Osteogenic stem cell differentiation is promoted on surfaces with larger perimeters and relatively high aspect ratios (a). Surfaces with nanoscale grating promote neural stem cell differentiation, whereas surfaces with microscale grating promote myogenic stem cell differentiation (b). Micropatterned surfaces with striped groove (grating) morphologies promote stem cell differentiation into smooth muscle under uniaxial strain (c). Lower cell seeding density promotes osteogenic stem cell differentiation, whereas higher cell seeding density promotes the differentiation of stem cells into smooth muscle cells or adipocytes (d).

activation. The commitment switch between adipocyte and osteoblast was suggested to be mediated through the RhoA–ROCK signaling pathway; cell shape regulation and RhoA activity were both necessary, but neither was sufficient, to drive the switch in hMSC commitment.[95]

Cell shape can be regulated by the shape of the adhesion domain on the micropatterned surface. Therefore, Kilian et al. precisely investigated the effect of adhesive area, aspect ratio, and subcellular curvature of micropatterned surfaces on adipocyte and osteoblast hMSC commitment. Human MSCs were cultured on micropatterned fibronectin islands with rectangular, star, and flower shapes on glass plates at varying aspect ratios and curvatures in mixed differentiation medium for adipocytes and osteoblasts. The aspect ratio was defined as the ratio of the width of the fibronectin shape at its long axis to its width at its short axis (Figure 11a).[79] When hMSCs were cultured on small islands (e.g., 1000 μm^2) of varying shapes, most of the cells differentiated into adipocytes in the mixed differentiation medium (Figure 13a). In contrast, hMSCs differentiated into osteoblasts when they were cultured on large islands (e.g., 5000 μm^2). These results indicate that the size of the cell adhesion area predominantly guides stem cell differentiation fate, as in McBeath's results.[95] Human MSCs cultured on several patterns of intermediate area (2500 μm^2) differentiated into a mixed population of adipocytes and osteoblasts. Therefore, the effect of shape, aspect ratio, and curvature of the cell adhesion domain on hMSC differentiation commitment was investigated at a constant cell adhesion area of 2500 μm^2 in mixed differentiation media.[79]

Human MSCs cultured on rectangular islands with aspect ratios of 1:1, 3:2, and 4:1 demonstrated that osteogenesis increased with aspect ratio (Figure 13a). Human MSCs cultured in rectangles with aspect ratios of 4:1 were 61% osteogenic, while hMSCs cultured in squares (aspect ratio 1:1) were only 46% osteogenic.[79] Human MSC cultivation on flower-shaped islands with large convex curves along each edge displayed 62% adipogenic differentiation, while hMSCs cultured on star-shaped islands with concave edges and sharp points at the vertices displayed 62% osteogenic differentiation. Human MSCs cultured on pentagon-shaped islands with straight lines for the edges were evenly differentiated into both adipocytes and osteoblasts.[79,137,148] These experiments are striking in that the subtle geometric differences of stem cell adhesion domains are significantly important in directing stem cell differentiation lineage commitment in 2-D culture. It has been suggested that the stem cell shape alone can influence the direction of their differentiation in 2-D culture.[79,137] Stem cell shape is organized by cytoskeleton components, such as stress fibers and focal adhesion complexes. Human MSCs cultured on the star-shaped islands exhibited larger focal adhesions and stress fibers than those cultured in flower shapes. Furthermore, a higher degree of actomyosin contractility along the edges was observed in immunofluorescent staining images of myosin IIa in hMSCs cultured in star shapes.[79] It may be that local curvatures of stem cell shapes that increase cytoskeletal tension and contractile stem cell cytoskeletons promote osteogenesis relative to adipogenesis. Microarray analysis and pathway inhibition studies suggest that stem cell contractility promotes osteogenesis by enhancing c-Jun N-terminal kinase (JNK) and extracellular-related kinase (ERK1/2) activation in conjunction with elevated wingless-type (Wnt) signaling (e.g., downstream effectors of RhoA and Rock signaling).[79] The geometric shapes of stem cells cultured on varying adherent shapes play roles in orchestrating mechanochemical signals and paracrine/autocrine factors that can direct hMSCs to appropriate differentiation lineage fates.

Chen et al. investigated whether differing geometries with small surface areas (e.g., 1100 μm^2) have an effect on the adipogenesis of hMSCs in an adipogenic induction medium.[149] In this investigation, micropatterned TCPS surrounded by photo-cross-linked PVA with cell adhesion geometries of triangles, squares, pentagons, hexagons, and circles of fixed surface area was used in a single hMSC culture. The cellular shapes of the hMSCs adopted nearly the same geometries as the micropatterns. Human MSCs cultured on islands with differing micropatterns predominantly assembled actin filaments along the peripheral edges of the micropatterns, indicating that the cells were sensing their peripheral microenvironment.[149] However, hMSCs cultured on nonpatterned TCPS dishes had much stronger actin filaments and stress fibers in their peripheral and central regions. Human MSCs cultured on islands of differing geometries with small surface areas showed similar adipogenic differentiation potentials.[149] However, hMSCs cultured on islands of micropatterned geometries differentiated into adipocytes at significantly higher rates than those cultured on nonpatterned TCPS dishes, consistent with the result reported by several researchers that smaller spreading areas favor adipogenic hMSC differentiation.[79,95]

Wan et al. investigated the expansion and differentiation potentials of human adipose-derived stem cells (hADSCs) on different shapes and sizes of micropatterned surfaces upon

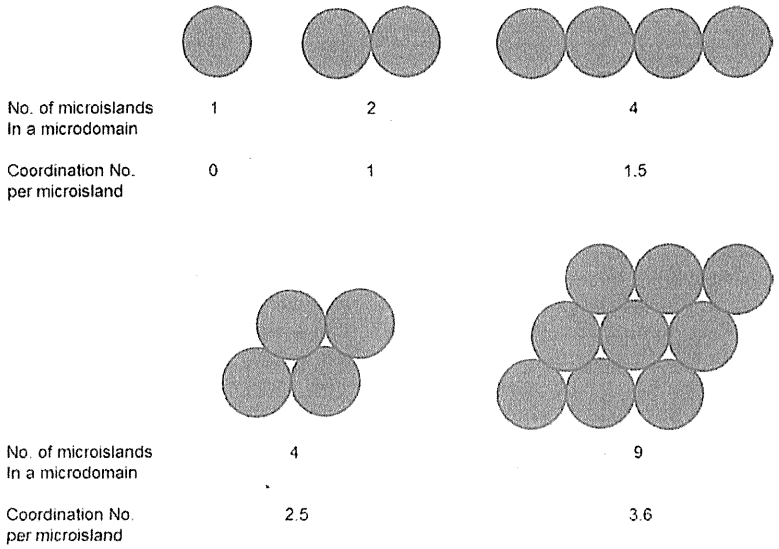

Figure 14. Micropatterned domains consisting of 1−9 microislands with 30 μm diameters. Coordination numbers per island are estimated to be 0, 1, 1.5, 2.5, and 3.6 in microdomains consisting of 1, 2, 4, 4, and 9 microislands, respectively.

Table 7. Some Research Studies for Stem Cell Differentiation on Micropatterned Materials (Cardiomyocyte, Chondrocyte, Smooth Muscle Cell, Neuronal Cell, Epidermal Cell, and Hepatocyte Differentiation)[a]

stem cell source	micropatterned materials for stem cell culture	pattern type	medium	differentiation	ref (year)
hESCs	micropatterned matrigel of circular shape surounded by Pluronic F-127 on TCPS	Figure 11a	differentiation medium	cardiomyocyte	143 (2009)
hMSCs	micropatterned fibronectin with square shape surroundedby Pluronic F127 on PDMS film	Figure 11a	expansion or differentiation medium	smooth muscle cell, chondrocyte	67 (2010)
hESCs	micropatterned matrigel of circular shape surounded by Pluronic F-127 on TCPS	Figure 11a	differentiation medium	mesoderm cells, cardiomyocyte	142 (2008)
hMSCs	microprinting fibronectin with stripe shape on PLGA	Figure 11c	expansion medium	cardiomyocyte	133 (2010)
hMSCs	micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 11f	expansion medium	smooth mucle cells	152 (2006)
iPSCs	micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 11f	differentiation medium	smooth muscle cells, neuronal cells	156 (2011)
mESCs	micropost array made of PDMS coated with fibronectin	Figure 11g	no description	cardiomyocyte	136 (2009)
NSCs	micropatterned PLL with square shape connected with and without line on PEO film	Figure 11a and 11e	differentiation medium	neuronal cells	134 (2008)
hNSCs	micropatterned PDMS with striped groove morphology coated with PLL and laminin	Figure 11f	differentiation medium	neuronal cells	135 (2012)
adult rat hippocampal progenitor cells	micropatterned polystyrene with striped groove morphology coated with PLL, laminin, or both	Figure 11f	differentiation medium	neuronal cells	153 (2006)
hMSCs	micropatterned amorphous carbon with striped groove morphology	Figure 11f	differentiation medium	neuronal cells	154 (2010)
hMSCs	micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 11f	expansion medium	neuronal cells	126 (2007)
human epidermal stem cells	micropatterned collagen-coated circle shape surrounded by grafted PEG on gold-coated coverslips	Figure 11h	differentiation medium	epidermal cells	150 (2011)
mESCs	micropatterned protein (collagen type I, fibronectin, and growth factors) microarray with circle shape on glass slide	Figure 11a	differentiation medium	hepatocytes	155 (2010)

[a]ESCs, embryonic stem cells; hESCs, human ESCs; hMSCs, human MSCs; NSCs, neural stem cells; hNSCs, human NSCs; iPSCs, induced pluripotent stem cells; TCPS, tissue culture polystyrene; PEO, poly(ethylene oxide).

which hADSCs were cultured.[131] The micropatterned surfaces had ring (rings 500 or 1000 μm in diameter and 100 or 200 μm in width) or rectangular structures (500 or 1000 μm long and 100 or 200 μm wide) coated with fibronectin on microprinted SAM octadecanethiol on a gold-coated glass surface.[131] The highest rates of cell proliferation were observed at the outer edge of the ring patterns and along the short axes of the rectangle patterns, where hADSC morphology was large and spreading.[131] Human ADSCs that exhibited high adipocyte and osteoblast differentiation were increased on the inner edges of the ring structures and on regions next to the narrow ends of rectangles, where hADSC morphology was small and elongated. These results can be attributed to the cytoskeletal tension associated with cell shape.[131]

Micropatterned surfaces make it possible to study the effects of stem cell spreading and shape on single-cell differentiation by

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the culture of a single cell on each patterned island; it is also possible to study the effect of cell–cell contact between stem cells on cell differentiation by controlling cell contact numbers via the design of specific micropatterned surfaces. Tang et al. designed several unique micropatterned domains grafted with RGD on PEG hydrogels as shown in Figure 14.[132] The micropatterned domain was composed of 1–9 microislands 30 μm in diameter, allowing single cells to bind on each microisland circle. The coordination numbers per island were estimated to be 0, 1, 1.5, 2.5, and 3.6 in microdomains consisting of 1, 2, 4, 4, and 9 microislands, respectively, as illustrated in Figure 14.[132] The researchers observed that alkaline phosphatase activity, an early marker of osteogenesis, increased with coordination number. Adipogenic differentiation of rat MSCs, as measured by Oil Red O staining (counting cells with lipid droplets), also increased with coordination number.[132] Inhibiting gap junctions between cells with 18α-glycyrrhetinic acid (AGA) treatment dramatically suppressed adipogenic and osteogenic differentiation of rat MSCs on all microdomains regardless of coordination number.[132] The micropatterned surface enables the study of the effects of cell–cell contacts and gap junctions on stem cell differentiation potential.

The effect of topographical variation on murine MSC osteogenic differentiation has also been reported. Seo et al. prepared micropatterned PDMS with an ordered lattice morphology consisting of a fixed pattern width of 2 μm, a fixed pattern height of 1 μm, and varied pattern intervals of 0, 1, 2, 3, 4, 6, and 8 μm.[151]

When murine MSCs were cultured on the micropatterned PDMS surface coated with fibronectin, gene expression of collagen type I, the major extracellular component of bone, osteocalcin, an osteogenesis marker, and alkaline phosphatase, an early marker of osteogenesis, was found to increase with increased pattern interval up until an interval of 3 μm.[151] However, with pattern intervals greater than 3 μm, gene expression of collagen type I, osteocalcin, and alkaline phosphatase decreased with increasing pattern interval. Therefore, gene expression indicating osteogenic differentiation of MSCs was highest on PDMS surfaces with lattice micropattern morphology pattern intervals of 3 μm.[151] These results demonstrated that the topography of micropatterned substrates is a significantly positive regulator of stem cell osteogenic differentiation in 2-D culture.

3.3. Chondrogenic, Myogenic, and Hepatic Stem Cell Differentiation on Micropatterned Surfaces

Table 7 summarizes a number of research studies regarding stem cell differentiation into chondrocytes, myocytes, hepatocytes, or neural cells on micropatterned surfaces (refs 67, 126, 133–136, 141–143, 150, and 152–156).

Chondrogenic differentiation of hMSCs can be induced in pellet culture mimicking cellular condensation during cartilage development with exposure to transforming growth factor β (TGF-β). TGF-β can also induce hMSC differentiation into smooth muscle cells (SMCs).[67] However, it is unclear which cell culture and environmental parameters can direct commitment between these two lineages. Gao et al. investigated differentiation switching between chondrogenic and SMC fates when hMSCs were cultured on micropatterned square surfaces (1000 μm^2 or 10 000 μm^2 islands) of fibronectin on PDMS substrates prepared with the microprinting method.[67] Human MSCs with well-spread morphologies on large micropatterned islands (10 000 μm^2) upregulated SMC genes with TGF-β stimulation, whereas hMSCs on small micropatterned islands (1000 μm^2) that were prevented from spreading and flattening upregulated chondrogenic genes.[67] hMSCs undergoing SMC differentiation exhibited little change in RhoA but had significantly higher Rac1 activity than chondrogenic differentiated cells. Rac1 activation inhibited chondrogenic hMSC differentiation and was necessary for SMC differentiation, whereas RhoA activity is known to mediate the shape-dependent control of hMSC lineage commitment to osteoblasts or adipocytes.[95] Rac1 signaling also upregulated N-cadherin, which was required for SMC differentiation.[67] In this study, it was demonstrated that hMSC commitment to chondrogenic or SMC lineage is mediated by cell shape, Rac1, and N-cadherin.[67]

Control of hESC differentiation into specific lineages with high efficiency is currently a difficult task. One reason for this is the heterogeneity of hESC colonies, while hESC culture on a micropatterned surface provides size-controlled aggregates of hESCs.[142,143] Niebruegge et al. prepared uniformly sized hESC aggregates by culturing hESCs on circular Matrigel islands 400 or 800 μm in diameter, which were microstamped on TCPS dishes and coated with (i.e., surrounded by) the non-protein-adsorbent Pluronic F127.[143] After 2–3 days, when uniformly sized hESC aggregates were formed, the aggregates were shifted into bioreactors and cultured in cardiac differentiation medium under hypoxia (4% oxygen tension). Under hypoxia, the uniformly sized hESC aggregates could differentiate toward cardiomyocytes with high efficiency.[143] Human ESC aggregates after differentiation were able to spontaneously beat, indicating that uniformly sized differentiated hESC aggregates generated some cardiomyocyte functions.[143]

Mechanical stimulation of blood vessel walls in vivo is considered to play an important role in the differentiation of MSCs into vascular SMCs. Several studies have suggested that mechanical strain enhances differentiation of MSCs and neural crest stem cells into vascular SMCs.[152,156–159] However, MSCs aligned perpendicularly to the axis of strain after long-term stimulation with cyclic uniaxial strain (Figure 13c), causing a decrease in SMC markers after an initial up-regulation. This cellular orientation differs from that observed in in vivo conditions, where vascular SMCs align in the circumferential direction.[152,160,161] To guide stem cells into parallel alignment with the axis of strain, Kurpinski et al. cultured hMSCs for two days on micropatterned PDMS sheets with a striped groove (grating) morphology of 10 μm terrace width, 10 μm groove spacing, and 3 μm depth and on which collagen type I had been coated under uniaxial strain (5%, 1 Hz) (Figure 13c).[152] Increases in contractile markers (e.g., calponin 1) and decreases in chondrogenic/osteogenic ECM markers were observed in hMSCs cultured on the micropatterned PDMS sheets under uniaxial strain.[152] Cartilage matrix proteins were observed to decrease significantly with uniaxial strain, suggesting that tensile stress suppressed the phenotype of compression-bearing tissue.[152] The surface of micropatterned biomaterials can regulate cell alignment direction even under uniaxial strain, thereby guiding the direction of stem cell fate, as in SMCs.[152,156] The micropatterned surface helps to mimic in vivo microenvironmental conditions of circumferential or helical SMC alignment within blood vessel walls.

Tay et al. investigated the differentiation of hMSCs toward the myogenic lineage when hMSCs were cultured on PLGA thin films with microstamped fibronectin in 20 μm stripes separated by nonadhesive gaps of 40 μm coated with Pluronic

F127.[133] Human MSCs cultured on micropatterned surfaces in expansion medium were found to be highly elongated with small adhesive areas of approximately 2000 μm^2, whereas hMSCs cultured on unpatterned surfaces had flat morphologies with large adhesive areas of approximately 10 000 μm^2.[133] Several hallmark neurogenesis (NeuroD1, nestin, GFAP, and MAP2) and myogenesis genes (GATA4, MyoD1, cTnT, and β-MHC) were upregulated in hMSCs on micropatterned surfaces in expansion medium, whereas osteogenic genes (alkaline phosphatase, RUNX2) were specifically downregulated or remained at normal levels. Myogenic lineage proteins, such as cardiac myosin heavy chain (MHC), predominantly existed in hMSCs cultured on micropatterned surfaces.[133] The enforced cell shape distortion resulted in the rearrangement of the cytoskeletal network and altered the shape of the nucleus, indicating the mechanical deformation of hMSCs translated into a biochemical response and ultimately contributed to specific differentiation toward a specific lineage, such as the myocardial lineage.

The differentiation of hESCs and hMSCs into endoderm lineages, such as hepatocytes and β cells, should be useful for clinical application but requires relatively difficult and complex procedures. Stimulation of stem cells with several growth factors and drugs is necessary at optimal concentrations with specific timing and duration. Tuleuova et al. developed a rather simple method of differentiating ESCs into the hepatic lineage by cultivating mESCs on micropatterned protein arrays with and without micropatterned coculture of human hepatic stellate cells.[155]

Protein microarrays were contact-printed on silane-modified glass slides using a microarray system with 500 μm-diameter circle-shaped protein spots in which the protein solution used for contact-printing was composed of hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and bone morphogenetic protein (BMP4) mixed with ECM composed of fibronectin and collagen type I.[155] Murine ESCs were cultured on the protein spots and exhibited hepatic differentiation. Coculture with nonparenchymal liver cells (hepatic stellate cells) on the protein spots enhanced hepatic differentiation of mESCs compared with mESC culture on the protein spots alone or with coculture of the hepatic stellate cells without micropatterning.[155] Microarrayed protein spots on dishes seem to guide or sort mESCs into hepatic lineages with high efficiency; hepatic differentiation of mESCs cultured on the printed protein spots was found to be enhanced compared with mESCs in conventional culture with media containing the same growth factors in soluble form.[155] Furthermore, a large amount of soluble growth factors were used in the conventional culture method, with daily media exchanges using media containing growth factors, while growth factors in the protein spots were printed once at the beginning of an experiment, with 60 times less total growth factor used than in the conventional culture medium.[155] This indicates that protein microarrays provide a more effective method of presenting functional growth factors to ESCs and allow more economical growth factor use. Growth factors bound to ECM and immobilized on the surface may have more stable and functional potential than soluble growth factors added to culture media.[155,162]

3.4. Neural Stem Cell Differentiation on Micropatterned Surfaces

Axonal regeneration in the central nervous system (CNS) is restricted by the inhibitory influences of the glial and extracellular environments after CNS injury.[153,163] Transplantation of NSCs and neural progenitor cells (NPCs) is a promising strategy for repairing the injured CNS. One instructive environment for axonal regeneration and restoration of function is the culture of NSCs, NPCs, or differentiated MSCs into neural cells on scaffolds with guidance channels.[128,134,153,154,164−166]

Ruiz et al. prepared PLL-micropatterned surfaces on Petri dishes coated with a plasma polymerized PEO using the microcontact printing method.[134] Various patterns of 1-nm-thick cell adhesive PLL were created on a cell-repellent PEO matrix. Neural stem cells cultured on the PLL patterns in differentiating medium over 20 days exhibited good confinement to the PLL domains.[134] Neural stem cells cultured on the PLL-micropatterned surface generated random axon-like projections outside of the patterns and expressed high amounts of neural markers in the differentiation medium. Migration and axon-like outgrowth were successfully guided by means of interconnected square patterns of PLL.[134]

Recknor et al. investigated directional growth and differentiation of adult rat hippocampal progenitor cells (AHPCs) on micropatterned PDMS substrates coated with PLL and laminin.[153] The micropatterned PDMS substrates had striped groove morphologies with 13 μm terrace widths, 16 μm groove widths, and 4 μm depths (Figure 11f). The micropatterned surface-directed AHPCs into over 75% alignment in the groove direction.[153] AHPCs were also cocultured with astrocytes, generating nearly double the percentage of cells (i.e., 35%) expressing class III β-tubulin (TuJ-1) on the micropatterned surface in comparison to those on nonpatterned surfaces or those growing in the absence of astrocytes.[153] This indicates that a physical biomaterial cue (micropatterned surface) in synergy with chemical (laminin) and biological (astrocytes) guidance cues facilitates the neuronal differentiation of AHPCs.[153] Integrating these cues seems to be important in understanding and controlling neural stem cell differentiation and in designing scaffolds for guided nerve generation in the future.

Microchannel surfaces patterned using photolithographic techniques have been reported to generate highly oriented neurites as described in the previous section.[167−169] However, most of these studies did not investigate the effects of the microstructure on stem cell differentiation into neural lineages. Beduer et al. investigated the effect of groove and terrace width of micropatterned surfaces with striped groove morphologies on the differentiation of human neural stem cells into neurons and intercommunication between neurons. In this study, hMSCs were cultured on the surfaces of micropatterned PDMS plates with varying dimensions of terrace (t) and groove (g) width: (a) t = 5 μm and g = 5 μm, (b) t = 10 μm and g = 10 μm, (c) t = 20 μm and g = 20 μm, and (d) t = 10 μm and g = 60 μm (Figure 11f).[135] The micropatterned PDMS plates were coated with PLL and laminin. A large majority of the adherent cells were located in the grooves and extended neurites inside the microgrooves along the walls.[170,171] Neuronal differentiation as evaluated by Tuj-1 immunostaining increased with increasing groove width, while adherent cell density did not depend on either groove or terrace width.[135] The differentiation of stem cells into neurons was especially affected and decreased on surfaces with micropatterned widths smaller than the cell soma diameter (12 μm). Furthermore, the size constraints imposed by the line microchannels of 5 μm−5 μm, 10 μm−10 μm, and 20 μm−20 μm caused a significant