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.

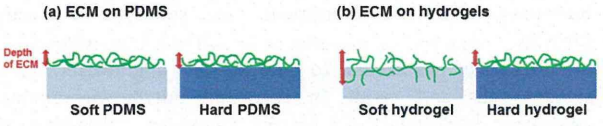

Figure 9. Morphology of ECM on PDMS (a) and hydrogel (b) with soft and hard characteristics.

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 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]

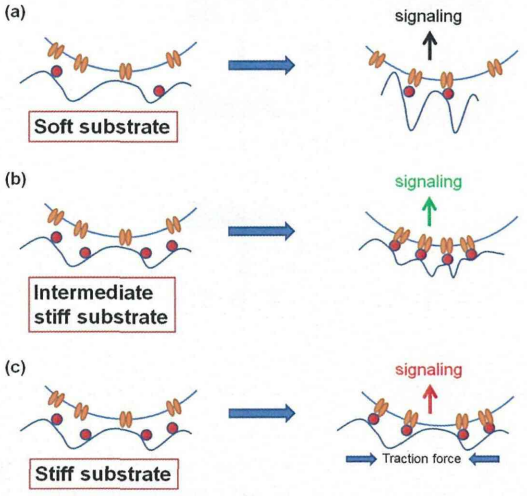

Figure 10. Matrix stiffness (soft substrate [a], intermediate substrate [b], and stiff substrate [c]) regulates integrin binding and reorganization of adhesion ligands, which are traction-dependent, at the nanometer level.

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

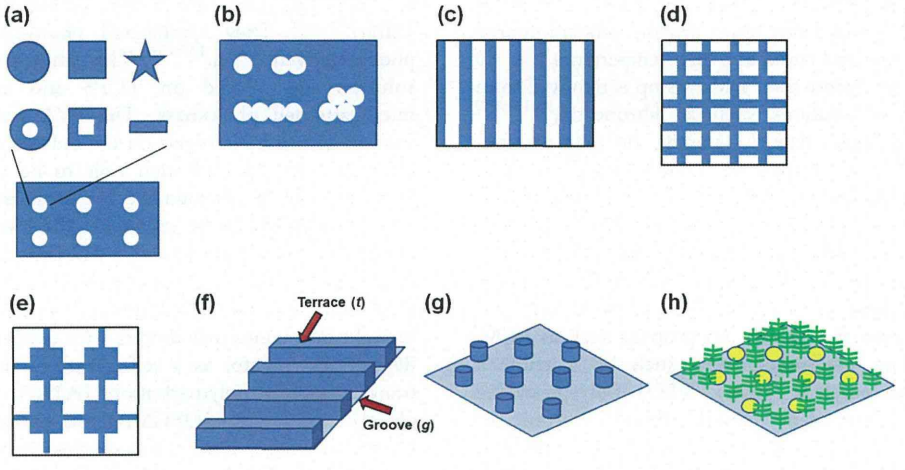

Figure 11. Examples of shapes and morphologies of micropatterned surfaces reported in the literature. Circles, squares, and stars (a), circle combinations (b), stripes (c), grids (d), grid and square combinations (e), microgroove morphologies (f), microposts (g), and circles surrounded by polymer brushes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

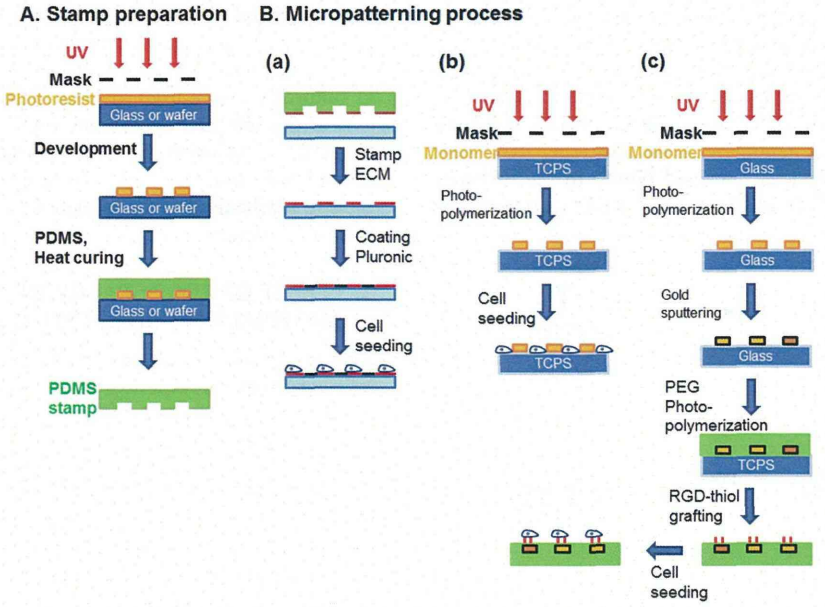

Figure 12. Typical microcontact printing preparation methods in two-dimensional (2-D) stem cell culture. Stamp preparation method (a) and several micropatterning processes (b).

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

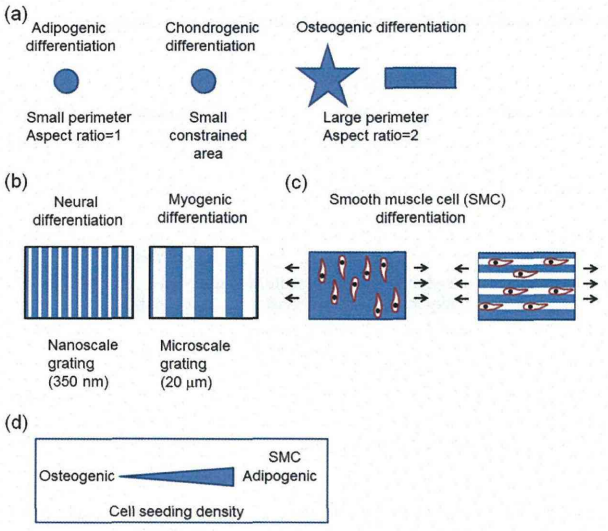

Figure 13. Stem cell differentiation on several different micropatterned surfaces. Adipogenic stem cell differentiation is promoted on surfaces with smaller perimeters and lower aspect ratios. Chondrogenic stem cell differentiation is promoted on surfaces of constrained area. Osteogenic stem cell differentiation 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

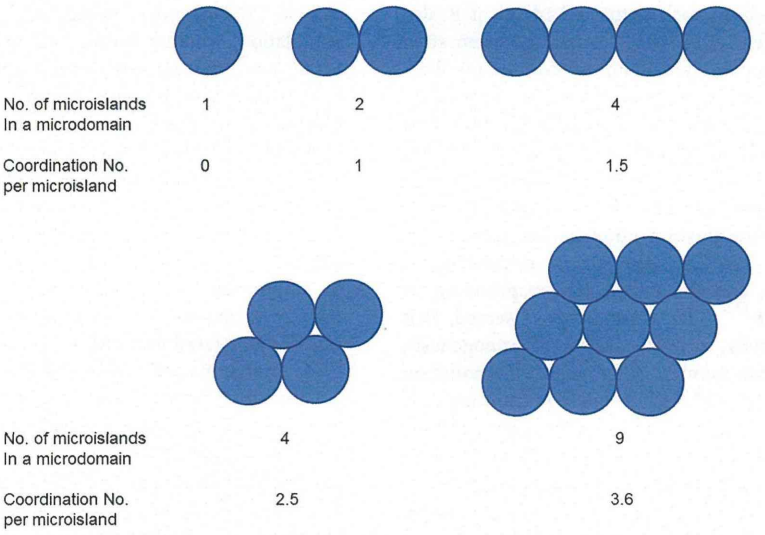

Figure 14. Micropatterned domains consisting of 1–9 microislands with 30 μm diameters. Coordination numbers per island are estimated to be 0, 1, 1.5, 2.5, and 3.6 in microdomains consisting of 1, 2, 4, 4, and 9 microislands, respectively.

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

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

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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

decrease in the mean number of neurites per neuron compared with the control flat PDMS plates.[135] The micropatterning of neural stem cells seems to influence the number of neurites per neuron. Neuronal cells predominantly exhibited a single neurite (83%) when the cells were cultured on micropatterned surfaces with narrow terraces and grooves (t = 5 μm and g = 5 μm), while only 27% of neural cells had single neurites on micropatterned surfaces with wider terraces and grooves (t = 10 μm and g = 60 μm).[135] The proportion of neurons developing two or three neurites increased with the microchannel width. Neurite length was also affected by the microchannel width. A significant decrease in neurite length was observed on micropatterned surfaces with narrow grooves (t = 5 μm and g = 5 μm) compared with those with wider grooves (e.g., t = 20 μm and g = 20 μm or t = 10 μm and g = 60 μm).[135] Small micropatterns appeared to hinder neurite development.

As for neurite direction and orientation, neural cells preferentially aligned their neurites along the axes of the line patterns. Therefore, alignment was stronger in the smaller microchannels. The proportion of neurites forming angles smaller than 10° to the microchannel direction was 95% on surfaces with narrow grooves (t = 5 μm and g = 5 μm), whereas that same proportion decreased to 44% on surfaces with wide grooves (t = 10 μm and g = 60 μm).[135] Micropatterned surfaces with narrow channels (t = 5 μm and g = 5 μm) generated sharp neurite alignments parallel to the microchannel direction, while the differentiation rate and neurite length were drastically decreased.

Nanotopography may influence stem cell differentiation into specific lineages, such as neural lineages (neurons, astrocytes, and oligodendrocytes), because ECM in vivo has nanoscale topography in stem cell niches. Yim et al. cultured hMSCs on micropatterned and nanopatterned PDMS substrates with striped groove morphologies with (a) 700 nm terrace widths, 350 nm groove widths, and 350 nm depths, (b) 20 μm terrace widths, 1 μm groove widths, and 350 nm depths, (c) 20 μm terrace widths, 2 μm groove widths, and 350 nm depths, or (d) 20 μm terrace widths, 10 μm groove widths, and 350 nm depths.[126] The micropatterned and nanopatterned PDMS substrates were coated with collagen type I.

When hMSCs were cultured on nanopatterns with 350 nm groove widths in expansion medium where the groove size was 1 order of magnitude smaller than the cell body, hMSC nuclei and cell bodies were significantly elongated.[126] F-actin fibers were stretched predominantly along the long axis of the cells. The alignment of cells on nanopatterned surfaces was 86.5%, while no alignment was observed on unpatterned surfaces.[126] Gene expression and microarray studies showed that neuronal (SOX2, neurofilament light peptide [NFL], and tyrosine hydroxylase [TH]) and muscular (myosin light chain and myf5) gene markers were significantly upregulated on nanopatterned surfaces even in expansion medium; the changes in expression were not significant on unpatterned or micropatterned surfaces.[126] Notably, the mature neuronal markers microtubule associated protein 2 (MAP2) and TuJ-1 were also detected on nanopatterned surfaces in expansion medium. A significant terrace width dependency of neuron differentiation was observed on patterned surfaces.[126] MAP2 expression increased with decreasing groove width when hMSCs were cultured in expansion medium. Synaptophysin expression was detected in hMSCs cultured on nanopatterned surfaces in expansion and differentiation media (i.e., with retinoic acid) but not on unpatterned surfaces, suggesting synapse formation in the cells cultured on nanopatterned surfaces.[126]

These studies show that nanotopography plays an important role in regulating stem cell differentiation. Nanotopography alone can induce significant upregulation of neuronal markers in hMSCs, suggesting induction into the neuronal lineage.

3.5. Stem Cell Differentiation on Nanofiber Surfaces

Stem cell culture on nanofibers can be considered a sophisticated 3-D example of stem cell culture on nanopatterned surfaces. Furthermore, structural protein fibers, such as native collagen and elastin in tissue, have diameters ranging from several dozen to several hundred nanometers.[172,173] Nanoscaled protein fibers are entangled with each other and generate nonwoven protein fibers, providing tensile strength and elasticity in native tissue.[172] Therefore, stem cell culture on nanofibers can be considered to mimic the environment of the stem cell niche in vivo.

There are four types of nanofibers: (1) nanofibers formed by the self-assembly of peptide amphiphile molecules,[128,172,174−181] (2) nanofibers prepared by electrospinning, (3) nanofibers prepared by micro(nano)phase separation, and (4) nanofibers formed by self-assembly of ECMs such as collagen. Nanofibers prepared by the self-assembly of peptide amphiphile molecules have small diameters in the lower end of the range of natural extracellular matrix collagens, whereas nanofibers prepared with the electrospinning method have large diameters on the upper end of that range.[176] Nanofibers prepared using the microphase separation method have similar diameters to natural extracellular matrix collagens and have macropore structures.[176]

3.5.1. Stem Cell Differentiation on Nanofibers Formed by Self-Assembly of Amphiphile Peptides. Self-assembling peptides form nanofibers that can be controlled at physiological pH by altering salt concentration.[175] A transparent gel-like solid is formed by mixing cell culture medium (e.g., DMEM supplemented with 15% fetal bovine serum [FBS]) with peptide amphiphile solution (e.g., 1 wt %). The transparent gel-like solid solution is composed of nanofibers, as verified by atomic force microscopy in solution[178] and scanning electron microscopy in dried samples.[128,172,175,176,182] When these nanofiber hydrogels formed by self-assembling peptides undergo shear thinning, they quickly recover nearly 100% of their elastic modulus when the shearing force is released. Therefore, there is great potential that nanofiber hydrogels can be used as an injectable delivery agent of stem cells to injured sites in vivo.[128,181]

Several types of self-assembling peptides have been designed, and some examples are shown in Table 8.[128,172,175−178,180,183] Self-assembling peptides have hydrophobic and hydrophilic regions to create amphiphilic characteristics for the generation of self-assembled nanofibers. In most self-assembling peptides, the hydrophobic regions are composed of alkyl chain (e.g., $[CH_2]_{15}CH_3$) and RADA16 (i.e., $[RADA]_4$) or hydrophobic oligopeptides (e.g., $(Ala)_4(Gly)_3$ $[A_4G_3]$),[128] whereas the hydrophilic parts are composed of cell receptor-binding sequences, such as RGDS, DGEA, KRSR, IKVAV, YIGSR, etc. Some self-assembling peptides are designed to have biodegradable characteristics. For this purpose, an enzyme-degradable site for matrix metalloproteinase-2 (MMP-2), GTAGLIGQ (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln), may also be included.[180] Table 9 summarizes various research studies on the differentiation of stem cells cultured on nanofibers formed by