Table 8. Sequences of Self-Assembled Peptide Amphiphile for Stem Cell Immobilization

name (model ECM)	chemical sequence	ref (year)
PA-RGDS (collagen I, fibronectin, osteopontin)	$CH_3(CH_2)_{14}$CONH-GTAGLIGQ-**RGDS**	180 (2009)
PA-DGEA (collagen I)	$CH_3(CH_2)_{14}$CONH-GTAGLIGQ-**DGEA**	180 (2009)
PA-KRSR	$CH_3(CH_2)_{14}$CONH-GTAGLIGQ-**KRSR**	180 (2009)
PA-RGES (dummy of RGDS)	$CH_3(CH_2)_{14}$CONH-GTAGLIGQ-RGES	180 (2009)
PA-S (control)	$CH_3(CH_2)_{14}$CONH-GTAGLIGQ-S	180 (2009)
IKVAV-PA (laminin)	**IKVAV**-Glu(E)-$A_4G_3(CH_2)_{15}CH_3$	128 (2004)
EQS-PA (control)	EQS-Glu(E)-$A_4G_3(CH_2)_{15}CH_3$	128 (2004)
RGD-PA (collagen I, fibronectin, osteopontin)	**RGD**-Glu(E)-$A_4G_3(CH_2)_{15}CH_3$	172 (2006)
RGD-PA (collagen I, fibronectin, osteopontin)	**RGD**-Glu(E)-$A_4G_3(CH_2)_{15}CH_3$	176 (2006)
RADA16-PDSGR (laminin)	Ac-$(RADA)_4$-GG**PDSGR**-$CONH_2$	175 (2006)
RADA16-SDPGYIGSR (laminin)	Ac-$(RADA)_4$-GG**SDPGYIGSR**-$CONH_2$	175 (2006)
RADA16-IKVAV (laminin)	Ac-$(RADA)_4$-GG**IKVAV**-$CONH_2$	175 (2006)
RADA16-SKPPGTSS (bone marrow homing)	Ac-$(RADA)_4$-GG**SKPPGTSS**-$CONH_2$	175 (2006)
RADA16-PFSSTKT (bone marrow homing)	Ac-$(RADA)_4$-GG**PFSSTKT**-$CONH_2$	175 (2006)
RADA16-FLGFPT (bone marrow homing)	Ac-$(RADA)_4$-GG**FLGFPT**-$CONH_2$	175 (2006)
RADA16-DGEA (collagen I)	Ac-$(RADA)_4$-GG**DGEA**-$CONH_2$	175 (2006)
RADA16-RGDS (collagen I, fibronectin, osteopontin)	Ac-$(RADA)_4$-GG**RGDS**-$CONH_2$	175 (2006)
RADA16-FPGERGVEGPGP (collagen I)	Ac-$(RADA)_4$-GG**FPGERGVEGPGP**-$CONH_2$	175 (2006)
RADA16-PRGDSGYRGDSG (collagen VI)	Ac-$(RADA)_4$-GG**PRGDSGYRGDSG**-$CONH_2$	175 (2006)
RADA16 (control)	Ac-$(RADA)_4$-$CONH_2$	175 (2006)
MMP2-RGDS (collagen I, fibronectin, osteopontin)	Ac-GTAGLIGQ**ERGDS**	177 (2008)
MDP-RGDS (collagen I, fibronectin, osteopontin)	Ac-EESLSLSLSLSLSLEEG**RGDS**-CO-NH_2	183 (2011)
RADA16-RGDSP (collagen I, fibronectin, osteopontin)	Ac-$(RADA)_4$-**RGDS**P	178 (2010)

the self-assembly of peptide amphiphile molecules.[128,172,174,176,178,180,184]

Anderson et al. prepared peptide amphiphile nanofibers inscribed with specific cellular adhesive ligands (i.e., RGDS, DGEA, and KRSR) and investigated whether they could direct osteogenic differentiation of hMSCs without osteogenic supplements.[3,180] The peptide amphiphile nanofibers were used to create self-assembled 2-D coatings on cell culture dishes. Human MSCs cultured on RGDS-containing peptide amphiphile nanofibers, but not DGEA- or KRSR-containing nanofibers, exhibited significantly greater alkaline phosphatase activity, indicating early promotion of osteogenic differentiation, and showed a progressive shift toward osteogenic morphology and positive staining for mineral deposition.[3,180] The peptide amphiphile nanofibers, which mimic the native ECM in bone, were found to direct the osteogenic differentiation of hMSCs to a certain degree without the aid of supplements and provided an adaptable environment that allowed various adhesive ligands to control cellular behaviors.[3,180]

Silva et al. prepared a 3-D network of nanofibers formed by the self-assembly of peptide amphiphile molecules (IKVAV-PA in Table 8) in which neural progenitor cells were encapsulated *in vitro*.[128] The neurite-promoting laminin epitope IKVAV (isoleucine-lysine-valine-alanine-valine) was included in the peptide amphiphile molecules. Self-assembly was triggered by mixing cell suspensions in media and peptide amphiphile molecules. The resulting self-assembled nanofibers placed the bioactive epitopes (IKVAV) on their surfaces at van der Waals packing distances and produced a gel-like solid containing 99.5 wt % water.[128] The nanofibers had high aspect ratios and large surface areas; they were 5−8 nm in diameter and ranged from hundreds of nanometers to a few micrometers in length. Thus, these nanofibers were able to present the IKVAV epitopes to neural progenitor cells at an extremely high density relative to natural laminin ECM.[128]

Neurite length and cell-body area within the nanofiber networks were found to be noticeably larger than in neurons cultured on 2-D dishes.[128] Neural progenitor cells were found to differentiate into neurons on the self-assembled nanofiber scaffolds, in contrast to cells cultured on laminin-coated or poly(D-lysine)-coated dishes, which suppressed astrocyte differentiation.[128] It was found that the physical entrapment of IKVAV in the self-assembled nanofibers, not solely its presence in the scaffold, was important to the neuronal differentiation of neural progenitor cells because the addition of IKVAV-soluble peptide into gels containing neural progenitor cells where the IKVAV sequence had been changed into the nonbioactive sequence of EQS (glutamic acid−glutamine−serine) did not promote selective neuron differentiation.[128]

Gelain et al. also prepared 3-D networks of nanofibers, formed by the self-assembly of peptide amphiphile molecules with several functional motifs, including cell adhesion (SDPGYIGSR and IKVAV as laminin models, RGDS as a fibronectin model, and FPGERGVEGPGP as a collagen type I model), differentiation, and bone marrow homing (SKPPGTSS, PFSSTKT, and FLGFPT) motifs, in which neural progenitor cells were encapsulated in vitro.[175] The peptide amphiphile nanofiber gels with bone marrow homing motifs (SKPPGTSS and PFSSTKT) enhanced neural cell survival without added soluble growth factors or neurotrophic factors in the culture medium.[175] The populations of β-III tubulin$^+$ (neuron) (superscript of "+" indicates the expression of this protein or gene), GFAP$^+$ (astrocyte), and Nestin$^+$ (neural progenitor) cells cultured on peptide amphiphile nanofiber gels with the appropriate motifs were significantly larger than those in conventional 2-D culture (i.e., TCPS) and were similar to those cultured on Matrigel.[175] Matrigel is composed of isolated components from the sarcomas of Engelbreth−Holm−Swarm mice,[2,185,186] including laminin, collagen type IV, heparan sulfate proteoglycans, enactin, and growth factors (e.g., TGF-β, EGF, and FGF). Matrigel contains unknown ingredients and is extracted from mice, whereas synthetic peptide amphiphiles are chemically defined. These are important considerations for the clinical application of stem cell scaffolds, although Matrigel is an attractive biomaterial for the maintenance of stem cell pluripotency[2] or the specific differentiation of stem cells.[187,188] Self-assembling peptide nanofiber gels prepared by Gelain et al. can mimic the characteristics of Matrigel to guide stem cell differentiation into specific lineages.[175] They succeeded in guiding neural stem cells

Table 9. Some Research Studies for Stem Cell Differentiation on Nanofiber Materials Prepared by Self-Assembled Peptide Amphiphile[a]

stem cell source	self-assembled peptide amphiphile for nanofiber preparation	medium	differentiation	ref (year)
hMSCs	oligopeptides containing RGDS, DGEA, or GRES	differentiation medium	osteoblasts	180 (2009)
rat MSCs	oligopeptides containing RGD	differentiation medium	osteoblasts	172 (2006)
rat MSCs	oligopeptide containing RGDS	differentiation medium	osteoblasts	176 (2006)
human dental pulp stem cells, stem cells from human exfoliated deciduous supplements	oligopeptide containing RGDS (GTAGLIGQERGDS)	differentiation medium	osteoblasts	177 (2008)
rat marrow-derived cardiac stem cells	oligopeptides containing RGDSP	expansion medium	cardiomyocytes	178 (2010)
rat cardiac progenitor cells	oligopeptides containing insulin-like growth factor-1	expansion medium	cardiomyocytes	174 (2009)
murine neural progenitor cells	oligopeptides containing IKVAV	expansion medium	neuronal cells	128 (2004)
murine NSCs	oligopeptides containing IKVAV, YIGSR, DGEA, RGDS, PDSGR	differentiation medium	neural cells	175 (2006)

[a]MSCs, mesenchymal stem cells; hMSCs, human MSCs; NSCs, neural stem cells.

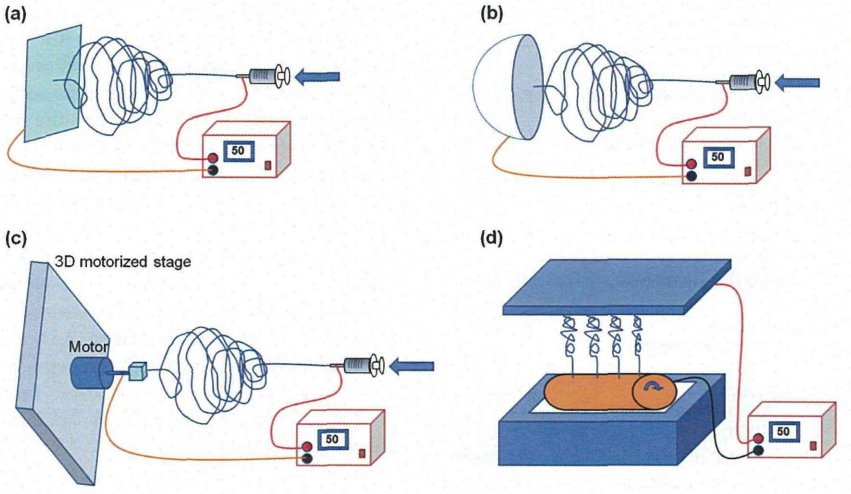

Figure 15. Typical examples of electrospinning methods. Traditional electrospinning method to prepare nonwoven fabric scaffolds (a), the electrospinning method to prepare cotton ball-like scaffolds (b), the electrospinning method to prepare double-layered 2-D architectures (crosshatch pattern) using a 3-D stage (c), and the Nanospider electrospinning method (d).

into neural and glial differentiation without the addition of extra growth factors by entrapping the neural stem cells in self-assembled peptide amphiphile nanofiber gels.

Hosseinkhani et al. prepared a 3-D network of nanofibers formed by the self-assembly of peptide amphiphile molecules containing RGD sequences (RGD-PA in Table 8) in which rat MSCs were encapsulated in vitro.[176] A 3-D nanofiber network was also formed in a hydrogel by mixing MSC suspensions in media with dilute aqueous solutions of the peptide amphiphile. The attachment, proliferation, and osteogenic differentiation of MSCs were successfully facilitated in the peptide amphiphile nanofiber gels with and without RGD sequences in comparison to MSCs in conventional 2-D culture on cell culture dishes.[176] However, the presence of the RGD sequence in the self-assembled amphiphile nanofibers enabled MSCs to promote greater attachment, proliferation, and osteogenic differentiation in comparison to those without the RGD sequence. This result can be explained by the possibility that binding of MSC integrin receptors with the RGD of the peptide amphiphile enhanced cell attachment, along with proliferation and osteogenic differentiation, on the nanofibers.[176]

The 3-D scaffolds used in conventional tissue engineering require surgery for their implantation, which is undesirable for clinical applications. Gel scaffolds consist of nanofiber networks formed by the aggregation of the peptide amphiphiles, and the process is physically triggered by the addition of a cell suspension to the aqueous peptide amphiphile solution. The gels formed by this process could be delivered to injured tissue by injecting the combined cell suspension and peptide amphiphile solution, allowing the injected solution to form a gel at the injection site.[176,177]

Self-assembling peptide nanofiber gels will be useful for the 3-D culture of stem cells in tissue engineering and in general molecular and cell biology.

3.5.2. Stem Cell Differentiation on Nanofibers Prepared by Electrospinning. Electrospun nanofibers can be generated from a spinning nozzle when high voltage is applied between the spinning nozzle and a flat metal collector. Several different nanofiber morphologies can be prepared with

Table 10. Some Research Studies for Stem Cell Differentiation on Nanofibers Prepared by Electrospinning[a]

stem cell source	materials for stem cell culture	medium	differentiation	ref (year)
hMSCs	PCL nanofibers	differentiation medium	osteoblasts, chondrocytes, adipocytes	201 (2005)
hMSCs	PLLA nanofibers	differentiation medium	osteoblasts	203 (2005)
hMSCs	nonwoven collagen type I nanofibers (diameter (d) = 50−200, 200−500, and 500−1000 nm)	differentiation medium	osteoblasts	191 (2006)
hMSCs	nanofibers composed of nanosized demineralized bone powders with PLLA composite material	differentiation medium	osteoblasts	192 (2008)
hMSCs	BMP-2-incorporated PLLA nanofibers	differentiation medium	osteoblasts	204 (2008)
hADSCs	collagen type I nanofibers	differentiation medium	osteoblasts	193 (2008)
hMSCs	PLLA-collagen I blend nanofibers	differentiation medium	osteoblasts	194 (2009)
hUSSC	plasma-treated PLLA nanofibers coated with nanohydroxtapatite	differentiation medium	osteoblasts	195 (2010)
hMSCs, hAFSCs	PCL nanofibers	differentiation medium	osteoblasts	202 (2010)
rabbit MSCs	nanofibers composed of nanosized hydroxyapatite and PCL having 340 nm diameter	differentiation medium	osteoblasts	196 (2011)
hUSSC	plasma-treated or collagen-grafted PES nanofibers	differentiation medium	osteoblasts	197 (2011)
hMSCs	nonwoven PLGA nanofibers with an diameter of 760 nm	differentiation medium	osteoblasts, chondrocytes	198 (2007)
MSCs	PLLA nanofibers	differentiation medium	chondrocytes	205 (2008)
calf MSCs	PCL nanofibers	differentiation medium	fibrocartilagious cells	199 (2011)
hMSCs	aligned and randomly oriented nanofibers prepared from thermally responsive hydroxybutyl chitosan	expansion medium	myocytes	69 (2007)
hATSPCs	aligned and randomly oriented PLLA nanofibers	expansion and differentiation medium	tendon	200 (2010)
murine ESC (CE3, RW4)	alighned and randomly oriented PCL nanofibers	differentiation medium	neural cells	68 (2009)
hMSCs	PLCL/collagen nanofibers	differentiation medium	neural cells	206 (2009)
rat ANSCs	PLO and laminin coated PCL nanofibers by electrospinning (d = 260, 480, 930 nm)	differentiation medium	neural cells	182 (2010)
mouse NSCs (C17.2)	collagen nanofiber cross-linked with rose bengal as photoinitiator by laser irradiation	expansion medium	neuronal cells	207 (2010)
rat NSCs	aligned, single- and double layer polystyrene nanofiber meshes coated with PLO and laminin (d = 800 nm)	expansion medium	neuronal lineages	76 (2011)
hESCs	PLO/laminin-coated PCL	expansion medium	neural cells	208 (2011)
hMSCs	PCL-gelatin nanofibers immobilized retinoric acid (d = 240−280 nm)	expansion medium	neural cells	71 (2012)
hESCs	tusan silk fibroin nanofibers coated with poly-D-lysine (PDL)/laminin (d = 400 and 800 nm)	differentiation medium	neuronal cells	209 (2012)
UCBPCs	aminated PES nanofibers	differentiation medium	endothelial and smooth muscle cells	210 (2009)
rat MSCs	photopolymerized PEG nanofibers coated with collagen type I	expansion medium	endothelial and smooth muscle cells	70 (2012)
rat MSCs	PLGA and collagen nanofibers immobilized CD29 antibody	expansion medium	epidermal cells	211 (2011)
hUSSCs	oxygen-plasma treated nanofibers of poly(ε-caprolactone)	differentiation medium	hepatocytes	212 (2009)
hMSCs	collagen-grafted PLLA nanofibers	differentiation medium	hepatocytes	213 (2012)
murine limbal stem cells, MSCs	polyamide 6/12 nanofibers by electrospinning (d = 290−539 nm)	expansion medium	proliferation and transplantation into damaged ocular surface	214 (2012)

[a]MSCs, mesenchymal stem cells; hMSCs, human MSCs; hADSCs, human adipose-derived stem cells; hUSSC, human unrestricted somatic stem cells; hAFSCs, human amniotic fluid stem cells; hATSPCs, human fetal achilles tendon stem/progenitor cells; NSCs, neural stem cells; ESCs, embryonic stem cells; hESCs, human ESCs; ANSCs, adult neural stem cells; UCBPCs, UCB-derived progenitor cells ($CD133^+$ cells); PES, polyethersulfone; PLC, poly(ε-caprolactone); PLCL, poly(L-lactic acid-*co*-3-caprolactone); PLGA, poly(lactic acid-*co*-glyconic acid); PLLA, poly(L-lactic acid), PLO, poly(L-ornithine).

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the electrospinning method, such as nonwoven fabric-like sheets, oriented fabric-like sheets, and structures resembling cotton balls. The typical electrospinning method is schematically shown in Figure 15a. Electrospun products are flat and highly interconnected scaffolds with a nonwoven fabric sheet-like morphology in most cases.[3] These characteristics hinder cell infiltration and growth throughout the scaffold. Blakeney et al. developed a three-dimensional cotton ball-like electrospun scaffold consisting of low-density, uncompressed nanofibers.[189] A grounded spherical dish and an array of needle-like probes were used instead of a traditional flat-plate collector to create a cotton ball-like scaffold (Figure 15b).[3] Scanning electron microscopy revealed that the cotton ball-like scaffold consisted of electrospun nanofibers with similar diameters but larger pores and less-dense structures than traditional electrospun scaffolds.[189] These cotton ball-like structures will be interesting for use as scaffolds for guiding specific stem cell differentiation lineages. Aligned nanofibers prepared using the electrospinning method have also been reported (Figure 15c). The rotating fiber collector enables nanofibers to align with one another. Double-layer 2-D architecture (crosshatch pattern) can be achieved by orthogonal substrate orientation and repeated nanofiber deposition.

One of the disadvantages of nanofiber fabrication with the electrospinning method is the extremely low production speed. To solve this problem, a rotating metallic drum dipping into polymer solution was used as a spinning nozzle to fabricate multiple nanofibers from the drum instead of a single-nozzle spinning needle (Nanospider, Figure 15d).[190] In the future, this new technique may contribute to the production of nanofiber scaffolds on an industrial scale.

Table 10 summarizes nanofibers fabricated with the electrospinning method for stem cell differentiation that have been reported in the literature.[68−71,76,182,191−214]

In general, human MSCs are difficult to differentiate into chondrocytes in 2-D monolayer culture. Pellet and hanging drop culture of hMSCs are the gold standards for chondrogenic differentiation.[215] This is likely because high seeding density leads to greater chondrogenic differentiation. Cell−cell contact and autocrine growth factors are important in chondrogenesis. Condensation of hMSCs initiates chondrogenesis during skeletal development,[216] suggesting the rationale for chondrogenic high-density pellet cultures.[217,218] Furthermore, the cell morphology in pellet and hanging drop culture is round as opposed to spread, as it is in monolayer culture. Morphological regulation is also an important parameter promoting hMSC chondrogenesis.[3]

Nanofibers fabricated with the electrospinning method have high surface area-to-volume ratios that maximize cell−material contact. Several researchers have reported that hMSCs on electrospun nanofibers can differentiate into chondrocytes, osteoblasts, and adipocytes.[191,195,198,201,205] Xin reported that hMSCs could differentiate into both chondrocytes and osteoblasts, depending on the induction media, when they were cultured on PLGA nanofibers. These results are important for tissue engineering applications for osteoarthritis because of the continuous differentiation of hMSCs into osteoblasts and chondrocytes.[198]

3.5.2.1. Effect of Nanofiber Size on Stem Cell Differentiation. Shih et al. prepared collagen type I nanofibers of varying diameters (50−200, 200−500, and 500−1000 nm) using the electrospinning method upon which hMSCs were seeded and examined for morphology, growth, adhesion, cell motility, and osteogenic differentiation.[191] Cells on all nanofiber sizes had more polygonal and flattened cell morphologies than those on TCPS. Moreover, hMSCs grown on 500−1000 nm nanofibers had significantly higher cell viabilities than TCPS controls.[191]

Christopherson et al. investigated the impact of nanofiber diameter on the differentiation of adult rat hippocampal-derived NSCs.[76,219] They found that NSCs cultured on smaller diameter (i.e., 283 nm) fibers differentiated preferentially into oligodendrocyte precursors in the presence of retinoic acid in medium containing serum, while NSCs preferentially differentiated into neuronal precursors on larger diameter fibers (i.e., 749 nm).

3.5.2.2. Effect of Nanofiber Alignment on Stem Cell Differentiation. Bakhru et al. prepared highly aligned, single-layer (uniaxially aligned) and double-layer (crosshatch pattern) polystyrene nanofiber meshes and investigated NSC fate as influenced by the physical microenvironment of the cells. Aligned nanofibers coated with poly(L-ornithine) (PLO) and laminin induced polarized NSC morphology and cellular elongation in the direction of fiber alignment, important for NSC neuronal differentiation.[76] The aligned fiber substrates promoted NSC neuronal lineage differentiation with an efficiency of 82.3%, whereas NSCs on conventional flat TCPS preferentially differentiated into glia (astrocytes) and not into neuronal lineages (efficiency of only 7%).[76] This research shows that microenvironmental physical cues determine stem cell differentiation fate.

Mahairaki reported that hESC-derived neural precursors (NPs) cultured on aligned fibrous substrates exhibited a higher rate of neuronal differentiation than those on other matrices; 62% and 86% of NPs become TUJ-1$^+$ cells (early neurons) on aligned microfibers and nanofibers, respectively, whereas only 32% and 27% of NPs acquired the same fate on random microfibers and nanofibers, respectively.[208]

Xie et al. induced mouse ESCs to differentiate into neural progenitor cells (NPCs) by adding retinoic acid to embryoid bodies (EBs). They examined biodegradable PCL nanofiber scaffolds seeded with neural progenitor cells and found culturing EBs on uniaxially aligned PCL nanofibers enhanced differentiation into neural lineages and promoted neurite outgrowth in comparison to EBs cultured on randomly oriented PCL nanofibers.[68] Neurites differentiated from EBs on aligned nanofibers extended along the direction of nanofiber alignment, while neurites cultured on randomly oriented nanofibers extended in all directions. More astrocytes were present on randomly oriented nanofibers than on aligned nanofibers.[68] The maximum length of neurite projections from EBs cultured on aligned nanofibers was significantly higher (500 μm longer) than that of neurites on randomly oriented nanofibers.[68] Aligned nanofibers seem to be able to enhance both the rate of EB neurite extension and neurite outgrowth direction.

Lim et al. also reported that higher fractions of adult rat NSCs on aligned PCL nanofibers coated with PLO and laminin exhibited neuronal differentiation compared with cells on randomly aligned PCL nanofibers or unpatterned surfaces.[182] Aligned nanofiber meshes 480 nm in diameter yielded the highest fraction of neural progenitors among nanofibers with 260, 480, and 930 nm diameters. This effect was in part due to neuron substrate selectivity, whereby aligned fiber substrates were less receptive to the attachment and survival of

oligodendrocytes than were randomly oriented fibers or unpatterned substrates.[182]

Aligned PCL–gelatin nanofibers (average diameter (d) = 270 nm) encapsulated with up to 0.3 wt % retinoic acid (RA) were prepared by Xu et al as scaffolds for hMSCs differentiated into neuronal lineages.[71] These nanofibers released RA for at least 14 days. Human MSCs cultured on aligned PCL–gelatin nanofibers with and without RA encapsulation upregulated expression of neural markers Tuj-1 (neuronal marker), MAP2 (mature neuronal marker), GalC (oligodendrocyte marker), and RIP (mature oligodendrocyte) (Table 2) at the mRNA and protein levels in comparison to hMSCs cultured on conventional TCPS or on randomly orientated PCL–gelatin nanofibers.[71] Human MSCs cultured on aligned PCL–gelatin nanofibers with encapsulated RA showed significantly enhanced neural marker expression in comparison to hMSCs on aligned PCL–gelatin nanofibers without RA encapsulation or randomly oriented PCL–gelatin nanofibers with encapsulated RA.[71] In particular, hMSCs cultured on aligned PCL–gelatin nanofibers with encapsulated RA, which allowed the controlled release of RA with lower loading amounts (>8 times lower), enhanced MAP2 and RIP expression compared with hMSCs cultured on nanofibers without RA encapsulation in culture medium containing high amounts of RA.[71] Higher expression of the mature neuronal marker MAP2 in hMSCs cultured on aligned PCL–gelatin nanofibers with encapsulated RA compared with the expression of glial markers at the mRNA and protein levels suggested that these nanofibers enhanced hMSC neuronal differentiation. Furthermore, positive staining for synaptophysin was detected only in cells cultured on aligned PCL–gelatin nanofibers with encapsulated RA.[71] These results illustrate the advantage of the nanofiber-based approach in enhancing the neuronal differentiation potential of hMSCs and demonstrate the importance of the drug delivery approach in directing stem cell fate. Such biomimicking drug-encapsulating nanofibers (used as scaffolds) may permit subsequent direct cell transplantation and may provide guidance cues to control the fate of endogenously recruited stem cells.

Dang and Leong prepared aligned nanofibrous scaffolds composed of a thermally responsive hydroxybutyl chitosan (HBC) blended with and without collagen type I.[69] Cell sheets could be generated by cooling hMSCs cultured on these scaffolds to 4 °C, allowing the cells in the polymer-free cell sheets to retain their elongated cell morphology and cytoskeletal alignment. The expression profiles of genes representative of three separate hMSC differentiation lineages were evaluated in the aligned hMSC cell sheets, where hMSCs were cultured on aligned HBC fiber scaffolds with and without collagen type I in proliferation medium and not in differentiation induction medium. These lineages included osteogenic, chondrogenic, and myogenic differentiation.

Expression of genes from all three differentiation lineages was detected in hMSCs cultured on both aligned HBC and HBC/collagen nanofibrous scaffolds. Interestingly, a definitive upregulation of myogenic genes was apparent for hMSCs on the aligned nanofibrous scaffolds when the genes expressed by hMSCs cultured on HBC films and TCPS were compared. Although MyoD expression was not detected in hMSCs on aligned nanofibrous scaffolds, elevated levels of myogenin, a gene involved in muscle differentiation and downstream of MyoD expression, suggested myogenic commitment.[69] The aligned nanofibrous topography induced an elongated nuclear shape, and this elongated nuclear shape was considered to be a major factor in the hMSC myogenic induction. The aligned nanofibers provide topographical cues to induce cell alignment, potentially guiding gene expression and influencing stem cell differentiation fate.

Tendons are specific connective tissues composed of parallel collagen fibers. It is known that human tendon stem/progenitor cells (hTSPCs) reside within a niche composed primarily of parallel collagen fibers and that this niche plays an important role in regulating their function and differentiation.[200,220−222] ECM or polymer electrospinning may be a suitable method to directly replicate the natural tendon ECM. Therefore, Yin et al. fabricated aligned and randomly oriented PLLA fibrous scaffolds, cultured hTSPCs on them, and evaluated the regulation of hTSPC orientation and differentiation into tendon by the aligned electrospun nanofibers.

Human TSPCs displayed spindle-shaped morphologies and were well-oriented on the aligned nanofibrous scaffolds. The expression of tendon-specific genes (Eya 2 and scleraxis) was significantly higher in hTSPCs cultured on aligned nanofibers compared with those on randomly oriented nanofibrous scaffolds in proliferation media and even in osteogenic media (due to tenogenesis and osteogenesis sharing a common signaling pathway).[223] In addition, alkaline phosphatase activity and alizarin red staining showed that hTSPCs on randomly oriented nanofibrous scaffolds experienced induced osteogenesis, while those on aligned nanofibrous scaffolds displayed hindered osteogenic differentiation.

In in vivo experiments, hTSPCs on aligned nanofibrous scaffolds were transplanted subcutaneously into immunocompromised mice. The efficacy of seeding hTSPCs on aligned nanofibrous scaffolds in inducing tendon tissue regeneration in vivo was investigated. From the observation of hematoxylin and eosin (H & E) and Masson's trichrome staining, it was determined that aligned nanofibers induced the formation of spindle-shaped cells and tendon-like tissue. These results suggest that aligned electrospun nanofibrous scaffolds provide an instructive microenvironment for hTSPC differentiation into tendon-like tissue and may lead to the development of desirable, intelligently engineered tendons.

3.5.2.3. Stem Cell Differentiation on Hybrid Nanofibers. Bone structure is composed of highly organized nanofibrillar proteins (mainly consisting of collagen type I), which serve as a pattern for the deposition of crystalline calcium phosphate minerals in the form of hydroxyapatite (HA).[195,224] A combination of nanofibrous organic and inorganic composite scaffolds, such as (a) calcium phosphates with nanofibrous scaffolds[195,196] and (b) composite nanofiber scaffolds with nanosized demineralized bone powders and biodegradable polymer,[192] may have promising potential for bone tissue engineering applications. Seyedjafari et al. prepared electrospun PLLA nanofibers coated with nanohydroxyapatite (n-HA) and investigated the capacity of these fabricated scaffolds for bone formation in vitro using human cord blood-derived unrestricted somatic stem cells (USSCs) under osteogenic induction.[195] Nanofibers coated with n-HA (n-HA/PLLA) supported attachment, spreading, and proliferation of USSCs. Higher ALP activity (an early marker of osteogenesis), biomineralization, and bone-related gene (Runx2, osteonectin, osteocalcin) expression were observed on nanofibers coated with n-HA compared with PLLA scaffolds without n-HA coating.[195] Furthermore, the expression levels of these markers were higher in USSCs on PLLA nanofibers than in those on TCPS. In addition, nanofiber scaffolds coated with n-HA demonstrated

the capacity for ectopic bone formation in the absence of exogenous cells in vivo after subcutaneous implantation of the nanofiber scaffolds into mice.[195]

Chen et al. also prepared nanocomposite scaffolds of n-HA dispersed in PCL using the electrospinning method.[196] Osteogenic differentiation of MSCs was enhanced on the composite nanofibers with an increase in n-HA content of up to 50%.[196] The extent of mineralization was significantly greater in nanocomposite scaffolds with 50% n-HA, which have Ca/P ratios similar to bone.

Nanofibrous organic and inorganic composite scaffolds containing demineralized bone powders (DBP) and PLLA were developed using the electrospinning method by Ko et al.[192] PLLA/DBP and PLLA scaffolds were transplanted into a full-thickness bony defect created in the central part of the rat cranial bone (8 mm diameter). Their results revealed that a larger amount of newly formed bone extended across the defect area 12 weeks after implantation with PLLA/DBP scaffold transplantation than in rats without implants and in PLLA scaffolds and that the defect size was almost 90% smaller.[197] Therefore, PLLA/DBP composite nanofiber scaffolds may serve as favorable matrices for the regeneration of bone tissue. The defect size decreased to 10% of its original size when PLLA/DBP composite nanofiber scaffolds were transplanted.[197]

Nanofibrous organic and inorganic composite scaffolds seem to be suitable for guiding MSCs into osteoblasts and for generating the mineralization of MSCs intended for bone tissue engineering.

Fibrocartilaginous tissues such as the meniscus serve critical load-bearing roles and rely on arrays of collagen fibers to resist tensile loads encountered during normal activity. The tissues of these structures are frequently injured and possess limited healing capacity; therefore, there exists demand for tissue-engineered replacements.[199] Baker et al. investigated scaffolds composed of aligned nanofibers that directed bovine MSC orientation and the formation of organized ECM under mechanical stimulation with the goal of recreating the structural features of these anisotropic tissues in vitro.[199] They examined the effect of cyclic tensile loading on MSC-laden nanofibrous PCL scaffolds made using the electrospinning method.[199] MSC fibrous gene expression (collagen type I, fibronectin, lysyl oxidase) and collagen deposition increased with mechanical stimulation, and the tensile modulus also increased by 16% relative to controls.[199] These results show that dynamic tensile loading enhances the maturation of MSC-laden aligned nanofibrous constructs, suggesting that recapitulation of the structural and mechanical environment of load-bearing tissues results in increases in functional properties that can be exploited for tissue engineering applications.[199]

3.5.3. Stem Cell Differentiation on Nanofibers Prepared Using Phase Separation. The phase separation method is a typical way to prepare porous membranes with pore sizes ranging from 1 nm to 10 μm. However, the porosity of membranes with pore sizes on the order of nanometers is extremely low under typical conditions (e.g., less than 10%), making them inadequate for use as cell culture scaffolds. Several researchers have created nanofiber scaffolds rather than porous membranes using phase separation techniques. These scaffolds have promise for the culture and differentiation of stem cells, although there have been only a few reports describing stem cell culture and differentiation on nanofibers prepared using phase separation techniques compared with those created by self-assembly of peptide amphiphiles or using the electrospinning method. This is because nanofiber scaffolds prepared using the phase separation method are quite similar to the 2-D structure of nanofiber mats, and stem cells cannot migrate inside the nanofiber scaffolds (mats) and must remain on the surface.

Nanofiber matrices are typically prepared from synthetic polymers as follows: (1) synthetic polymer is dissolved in a "good" solvent; (2) the polymer solution is cast on plates or dishes and phase-separated by cooling; (3) the polymer solution (gel) is immersed in water, and the solvent is removed from the gel, generating nanofiber scaffolds (mats); and (4) the nanofiber matrices are washed and then freeze-dried.

Smith et al. prepared nanofiber matrices using PLLA with the phase separation method to mimic the morphology of natural ECM. Their goal was to examine the contribution of ECM morphology to the differentiation of murine ESCs because natural ECM, such as collagen type I, typically has a nanofiber morphology.[225] The resulting nanofiber matrices had an average fiber diameter of 150 nm and a porosity of 92.9%. ESCs cultured on the nanofiber matrices displayed more extended morphologies than those on films prepared from the same PLLA and those on gelatin-coated control dishes. Furthermore, ESCs cultured on nanofiber matrices exhibited higher Brachyury expression, indicating mesoderm differentiation, and had stronger expression of osteogenic genes (also mesoderm), such as collagen type I, Runx2, osteocalcin, and bone sialoprotein; however, they expressed less nestin (a neural marker, ectoderm) and TUJ-1 (a neuronal marker, ectoderm) than ESCs cultured on PLLA films or gelatin-coated dishes (control experiments). It was found that osteogenic differentiation was more highly promoted when ESCs were cultured on the nanofiber matrices than on film or gelatin-coated dishes. The mechanism of the enhanced osteogenic differentiation observed on the nanofiber matrices was partially explained by high adsorption of serum proteins and fibronectin on the nanofibers prepared from PLLA. Several integrin subunits associated with cellular adhesion to collagen type I ($\alpha 2\beta 1$) and fibronectin ($\alpha 5\beta 1$) were upregulated on the nanofiber matrices compared with the film.[225] The increase in $\beta 1$ integrin transcription in ESCs on nanofiber matrices compared with those on film and gelatin-coated dishes supports increased mesoderm differentiation because increased $\beta 1$ integrin on stem cells is directly related to increased mesoderm differentiation while inhibiting neural differentiation.[226] Increased fibronectin adsorption on the nanofiber matrices compared with the film and gelatin-coated dishes likely accelerates ESC differentiation to the mesoderm and osteogenic lineages, as supported by $\alpha 5$ blocking experiments.[225] The nanofiber matrices had larger surface areas than the film and conventional flat culture dishes, which is favorable for the high adsorption of serum or ECM proteins. High, somewhat specific adsorption of serum and ECM proteins on nanofiber matrices prepared from selected chemical structures will be useful in designing dishes suitable for stem cell differentiation into specific lineages.

Polyhydroxyalkanoates (PHA) such as poly(3-hydroxybutyrate) (PHB), 3-hydroxybutyrate and 4-hydroxybutyrate (P4HB) copolymer, 3-hydroxybutyrate and 4-hydroxyhexanoate (PHBHHx) copolymer, and 3-hydroxybutyrate and 4-hydroxybutyrate (PHB4HB) copolymer are reported to have good biodegradability and no cytotoxicity in vitro and in vivo.[227,228] Xu et al. prepared nanofiber matrices using PHA,

which is structurally similar to natural ECM.[227] Rat NSCs were cultured on PHA nanofiber matrices and films. The viability of NSCs on PHA nanofiber matrices was significantly higher than that of those on PHA films.[227] This result indicates that nanofiber matrices with a 3-D nanostructure may be favorable for NSCs to absorb nutrients, ECM proteins, and growth factors. NSCs grown on PHBHHx nanofiber matrices expressed higher levels of neuronal marker β-III tubulin than those on PHA nanofiber matrices, except for PHBHHx or PHA films.[227] NSCs on PHBHHx nanofiber matrices appeared to be more suitable for NSC attachment, synaptic outgrowth, and synaptogenesis than other PHA nanofibers and films.[227]

Collagen type I in native tissue consists of three collagen polypeptide chains that form a ropelike superhelix conformation and assemble into nanofibers ranging in size from 50 to 500 nm.[225,229] However, typical collagen 3-D scaffolds do not appear to be composed of nanofiber networks, but rather generated hydrogels or porous sponges. There are only a few reported collagen nanofiber matrices in the literature that are prepared with the phase separation method for stem cell culture and differentiation.[230]

Orza et al. prepared gold-coated collagen nanofiber matrices by a single-step reduction process using collagen solution, a reduction agent (sodium citrate or sodium borohydrate), and $HAuCl_4$.[230] These matrices were electrically conductive due to their gold coating and had fiber widths of 20−65 nm depending on the preparation conditions. Gold-coated collagen fibers seem to maintain their native ropelike superhelix conformation and their nanofiber assemblies. It was determined that placental-derived MSCs experienced accelerated neural differentiation and developed more characteristic neural lineage morphologic features when they were cultured on gold-coated nanofiber matrices in neural differentiation medium.[230] MSCs grown on the gold-coated nanofiber matrices responded within 1−2 days to neuronal induction medium by generating cells bearing neuronal-like extensions and more neuronal-like morphologies compared with the cells cultivated on conventional control culture dishes.[230] Twenty-four hours of electrical stimulation with a neuronal differentiation protocol further accelerated the acquisition of neural morphology, and after 2 days, MSCs were largely oriented in the same direction.[230] Transmitting electrical stimulation to the MSCs was effective due to the electrically conductive properties of the gold-coated nanofiber matrices.

The gold-coated nanofiber matrices were also able to induce MSCs to differentiate into myocardiocytes efficiently when cultured in myocardiocyte induction media.[230] MSCs on gold-coated nanofiber matrices were strongly positive for the cardiac marker atrial natriuretic peptide (ANP), a cardiac hormone, and early cardiac-specific homeobox protein (Nkx2.5), in contrast to MSCs on conventional control culture dishes.[230]

The phase inversion method allows for the simple preparation of nanofiber matrices compared with the methods of peptide amphiphile self-assembly and electrospinning. However, it is difficult for cells to migrate into the inside of the nanofiber matrices prepared by the phase separation method, causing cells to generally remain on the surface. Therefore, due to the difficulty of 3-D culture on nanofiber matrices prepared by the phase separation method, stem cell culture and differentiation on nanofiber scaffolds prepared by peptide amphiphile self-assembly or electrospinning seem to be more useful than those prepared by the phase separation method for clinical applications. However, scaffolds with micropores prepared by the phase separation method, such as microporous sponges, are frequently used in tissue engineering and regenerative medicine for the immobilization and entrapment of stem cells.

4. CONCLUSION

The regulation of stem cell differentiation into specific lineages remains unclear. Cell culture materials should be developed with physical, biochemical, and biomechanical cues for this purpose. The development of biomaterials requires a multidisciplinary approach, combining the selection of specific ECM proteins, appropriately ordered scaffold structures, adequate elasticity, appropriate biomaterial morphology, and appropriate biomechanical stimulation, and it will open the door to the guided differentiation of stem cells into specific lineages. It is challenging to regulate stem cell differentiation fate by regulating their microenvironment, such as by controlling only physical matrix or substrate parameters in the stem cell niche, because biological cues can effectively decide stem cell fate. However, this topic also has a deep meaning in terms of human society; our ability and performance can be improved by our (micro)environment, which is not decided solely by heredity. We believe that the role of the stem cell microenvironment in guiding and deciding stem cell differentiation fate is similar to the fate of humans in our society.

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Notes

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