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

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-$(RADA)_4$-**RGDSP**	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.

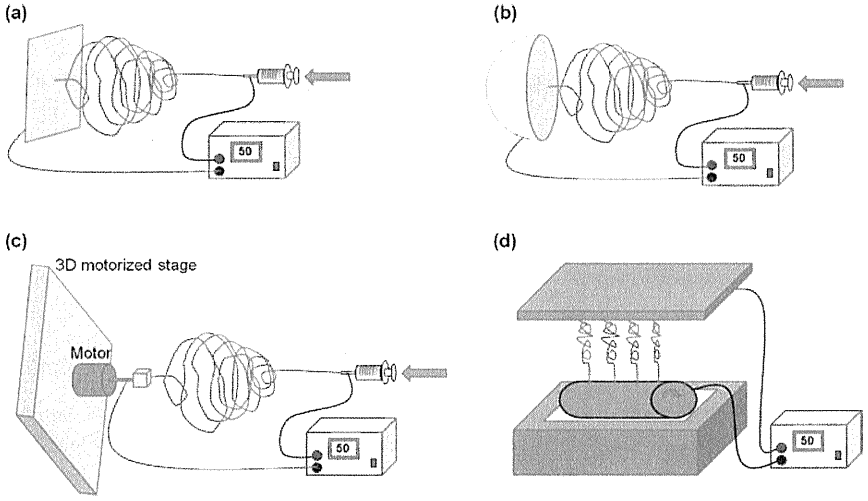

Figure 15. Typical examples of electrospinning methods. Traditional electrospinning method to prepare nonwoven fabric scaffolds (a), the electrospinning method to prepare cotton ball-like scaffolds (b), the electrospinning method to prepare double-layered 2-D architectures (crosshatch pattern) using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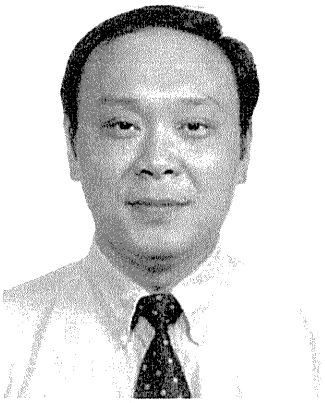

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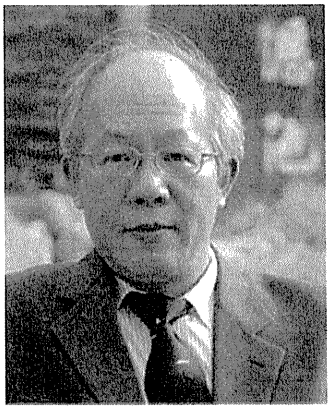

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シリーズ（医薬品評価をめぐる最近の話題）

再生医療製品の素材としての多能性幹細胞（ES/iPS 細胞）の品質

Quality of Pluripotent Stem Cells as Substrates Used for Production of Cell-Processed Therapeutic Products

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Abstract

Pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, have opened new avenues for regenerative medicine/cell therapy. PSCs are expected to be new materials used for production of cell-processed therapeutic products (CTPs), especially for the treatment of serious or life-threatening diseases/conditions, for which no adequate therapy is currently available. At present, many attempts are being made to develop various types of PSC-derived CTPs. The most ideal base camp in the stable manufacture of a CTP is a cell bank that has been well-characterized, is stable, possesses the ability to propagate, can be regenerated and has a stable supply, and finally can differentiate into the desired cells. In most cases of the manufacture of PSC-derived CTPs, PSC bank systems are developed to supply substrates for stable production of the final products with reproducible quality. To establish quality management systems for ensuring quality, safety and efficacy of CTPs, it is necessary to well understand the quality of the cell banks/substrates, which have significant impacts on the characteristics of the final products. From a view point of manufacturing biologics, based on the concept of ICH Harmonized Tripartite Guideline Q5D (ICH-Q5D), this minireview provides a perspective on the meaning and specification method of the quality of pluripotent stem cells as cell banks/substrates for production of CTPs.

抄　録

胚性幹細胞や人工多能性幹細胞などの，いわゆる多能性幹細胞は，再生医療／細胞治療に新たな展開をもたらしている．多能性幹細胞は，特に従来十分な治療法が存在しなかった重篤ないし生命を脅かす疾患を対象とした再生医療製品を製造するために用いる新しい素材として期待されている．現在，多能性幹細胞を分化させることによって様々な種類の再生医療製品の開発の取り組みが数多くなされている．再生医療製品の安定な製造における最も理想的な起点は，十分に解析され，安定で，増殖能力を持ち，再生可能かつ安定供給可能で，その上，目的細胞に分化する能力をもつセル・バンクである．ほとんどの多能性幹細胞由来再生医療製品の製造においては，再現性のある品質を持った最終製品を安定的に製造するための細胞基材の供給源として，多能性幹細胞のセル・バンクが樹立される．セル・バンク／細

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胞基材の品質は，最終製品の特性に大きな影響を与えるものであり，最終製品の品質・安全性・有効性を確保するための品質マネジメントシステムを構築するためには，セル・バンク／細胞基材の品質を十分に把握することが必須である．本稿では，バイオロジクスの製造という観点から，ICH-Q5D の考え方をもとに，再生医療製品のセル・バンク／細胞基材としての多能性幹細胞の品質の意味合いとその設定のありかたについて概説する．

Key words: pluripotent stem cell, ES cell, iPS cell, cell bank, quality

1．はじめに

胚性幹細胞（ES 細胞，embryonic stem cell）や人工多能性幹細胞（iPS 細胞，induced pluripotent stem cell）などの「多能性幹細胞」は，その幅広い多能性ゆえに，いままで入手が困難であった各種細胞を作製することのできる素材となることが期待され，またその無限の自己複製能ゆえに，ひとたび目的細胞への効率的分化誘導方法が確立すれば，再生医療・細胞治療に利用できる細胞，すなわち，いわゆる再生医療製品の成分となる細胞を大量かつ安定に供給することが可能となることが期待されている．既に2011年１月に米国では，ヒト ES 細胞を加工した医薬品の再生医療における活用例として，世界初の治験（脊髄損傷治療）が開始され，2011年７月には同じく米国で網膜疾患治療を目的としたヒト ES 細胞加工製品の治験が開始されている（ただし，前者の治験は2011年11月に経済的理由により中断）．また，2007年に世界初のヒト iPS 細胞が樹立されたことを契機に，細胞のプログラミングを人為的に操作，制御できる時代が到来し，新規細胞基材，新規製造関連資材，新規製造方法，新規適用法等，新たなイノベーションを推進し，再生医療・細胞治療へ応用しようとする研究展開が国内外できわめて活発化している．この中に実用化に有望と考えられるシーズも数多くあり，例えば，2013年夏にはわが国においてヒト iPS 細胞を加工して作製した網膜色素上皮細胞を加齢黄斑変性の患者らに対して臨床応用する研究が開始されるに至っている．

再生医療製品の品質管理において重要なこととして，最終製品としての再生医療製品の規格及び試験方法の設定，製造工程の妥当性の検証と一定性の維持管理などに加えて，素材（原材料・中間製品）の品質管理を適正に行うことが挙げられる．従って，多能性幹細胞由来再生医療製品のような，一昔前には実現が想定されていなかった再生医療製品の開発では，製品に特有の品質・安全性確保のための基盤技術（例えば最終製品に残存する多能性幹細胞の造腫瘍性に起因する安全性上のリスクの評価法など）の確立が必要となると同時に，最終製品を再現性の高い品質で継続的に生産する方策，特に多能性幹細胞のような新しい素材の品質・規格の設定のあり方を理解することが重要となる．

多能性幹細胞を，幹細胞生物学や発生学の対象（客体的存在，Vorhandensein）としてではなく，再生医療製品の素材（道具的存在，Zuhandensein）として見た場合，その位置づけは，「対象とする特定の治療目的に適う，品質・有効性・安全性を備えた最終製品」を製造するのに適したもの，ということになる．一般的に，バイオロジクス（生物薬品）の製造においては，その製造管理の出発点を「ICH-Q5D 的な意味でのセル・バンク」（後述）に設定すること，つまり，解析が十分で，形質が安定で，増殖性を有し，更新も，安定供給も可能で，最終目的製品を高い再現性で効率よく生産することが可能なセル・バンクを製造工程上の起点として設定することが基本とされる．再生医療製品はバイオロジクスの一種と考えられ，また，多能性幹細胞は通常，細胞株として樹立され，チューブないしアンプルに分注した状態で保

存（バンク化）される．そこで本稿では，再生医療製品の素材としての多能性幹細胞の品質のあり方について，バイオロジクス製造のためのセル・バンクの品質という観点から概説することにする．

2．セル・バンクの定義

「セル・バンク」（ないし「細胞バンク」）という言葉はアカデミアから産業界まで幅広く用いられているが，その定義は，立場や目的によって複数あり，それぞれ意味合いが異なる．例えば，①「研究目的または体の損傷部位の外科的再建を目的とした凍結組織標本を保管する貯蔵施設」のように定義されていることもあるし[1]，②「提供されたヒトの細胞（中略）等について，研究用資源として品質管理を実施して，不特定多数の研究者に分譲する非営利的事業」と定義されることもある[2]．しかしバイオロジクスの製造においては，日米EU医薬品規制調和国際会議（ICH）のガイドライン Q5D（ICH-Q5D）に従い，セル・バンクの定義は③「均一な組成の内容物をそれぞれに含む相当数の容器を集めた状態で，一定の条件下で保存しているもの．個々の容器には，単一の細胞プールから分注された細胞が含まれている．」とされ，チューブないしアンプルに入った凍結細胞という実体を指す[3]．また，ICH-Q5D で扱っているのは，特定のバイオロジクスを生産する目的で使用される細胞基材（後述）としてのセル・バンクである．

本稿では，ヒト多能性幹細胞加工製品等の再生医療製品をバイオロジクスの一種ととらえ，「セル・バンク」という言葉を，特に断らない限り，ICH-Q5D に従い，「細胞基材としてのセル・バンク」という意味で用いることとする．

3．セル・バンク・システム構築の目的

ICH-Q5D に基づくセル・バンク・システムは大抵の場合2段階のシステムから成り立っている．即ち，大本の細胞を一定の培養条件下で最低限の継代数を経て増殖させることにより調製したセル・バンクを「マスター・セル・バンク」と呼び，マスター・セル・バンクから一定の条件で培養して得られる均質な細胞懸濁液を分注して調製した，実際の製造に使用されるセル・バンクを「ワーキング・セル・バンク」と呼ぶ．なお，「微生物細胞あるいはヒト又は動物由来の細胞で，ヒトを対象に *in vivo* 又は *in vitro* で投与されるバイオロジクスを生産する上で必要な能力を有するもの」は「細胞基材」と呼ばれる．ヒト多能性幹細胞加工製品をはじめとする再生医療製品の素材となる細胞はすべて細胞基材である．従って，セル・バンクも細胞基材の一種である．また，マスター・セル・バンクを調製する元になる親細胞株や親細胞株を樹立するために使用される親細胞も細胞基材である（**図1**）．

バイオロジクスの製造におけるセル・バンク・システム構築の目的は，「一定の品質の特定の最終製品を安定的かつ継続的に製造する」ということにある．逆に言えば，一定の品質の最終目的製品を安定的かつ継続的に製造する上で重要かつ科学的に合理的な場合に，セル・バンク・システムの構築またはその他の細胞基材の調製が必要となる．従って，再生医療製品の製造において全ての種類の細胞基材が必須であるというわけでない．

4．セル・バンクの品質

ICH-Q5D における「セル・バンク」の意味合いの中では必ず細胞の具体的な臨床用途・最終製品が特定されているのに対し，先述の第1・第2の定義では細胞の具体的用途は特定されない[*1]．この違いによってセル・バンクの品質の意味合いも大きく異なる．

第1・第2の定義，すなわち「具体的臨床用途が未特定のセル・バンク」[*2]における品質上の注意点は4つある．その一つは，①感染因子混入などの汚染が無いことの保証である．これは作業従事者の安全性の確保（および臨床グレードの細胞の場合にはさらに患者の安全性の確保）の意味合いがある．もう一点は，②学問的定義（一般的定

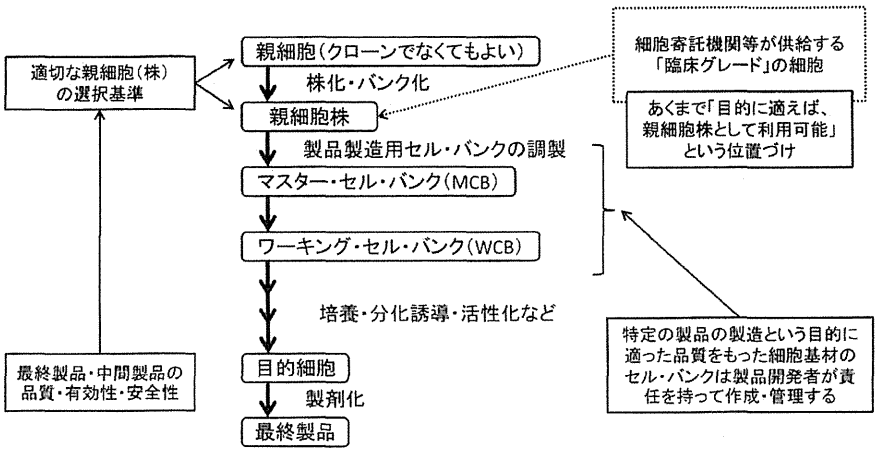

図1 再生医療製品の製造の概略

義）に基づく細胞種としての同一性・純度とその安定性を保証することである．例えば，リプログラミングされた「iPS細胞様細胞」を「iPS細胞」としてバンク化する場合には，三胚葉系への多分化能を確認することが必須である．

先の第3の定義（ICH-Q5D）に従った「特定の臨床用途・最終製品製造のためのセル・バンク」すなわち「細胞基材としてのセル・バンク」における品質上の注意点の1つは上の場合と同様に①感染因子混入などの汚染が無いことの保証である．ただし，臨床用であることから，患者の安全性の確保の意味合いがより強い．もう一つの注意点は具体的臨床用途が未特定のセル・バンクの場合とは異なり，②患者に投与される最終製品の品質・有効性・安全性の再現性を確保するための素材としての特性とその安定性である．例えば，リプログラミングされた「iPS細胞様細胞」を特定の分化細胞製造用の素材としてバンク化する場合には，目的とする細胞への分化効率の高さやその再現性の高さの方が多分化能よりも重要となる．

つまり，再生医療製品の製造における，素材としての細胞基材（セル・バンク等）の品質・規格については，製造プロセス全体として最終製品の有効性・安全性が確保できるように設定することが原則となる．

再生医療製品の製造においては，細胞という極めて複雑な構造と不確実性の高い特性を持つ要素が存在するために，素材の品質をもとに最終製品の品質を設計・デザインすることが不可能である．従って，再生医療製品の場合には，対象疾患，患者のQOL（Quality of Life），標的となる臓器・細胞・分子，製品の使用方法，製品の安全性・有効性（First-in-Humanの場合には，非臨床安全性試験や非臨床Proof-of-Concept試験（非臨床薬力学試験）等のデータ）などをもとに，最終製品の品質・規格が設定され，最終製品の品質・規格から目的細胞の品質・規格が決定される．同様に，目的細胞の品質・規格からセル・バンクの品質・規格が決定され，セル・バンクの品質・規格から親細胞の品質・規格が決定されることになる（図2）．

5．細胞基材としての多能性幹細胞の品質

ICH-Q5Dガイドラインの構成は，①細胞基材（細胞株）の起源．履歴・調製（すなわちドナー情報・培養歴及び株化の方法など），②細胞のバンク化の手法，および③セル・バンクの特性解析となっている．なお，セル・バンクの特性解析としては，特性解析試験，純度試験，細胞基材の安定性，核型分析・造腫瘍性試験が挙げられている．

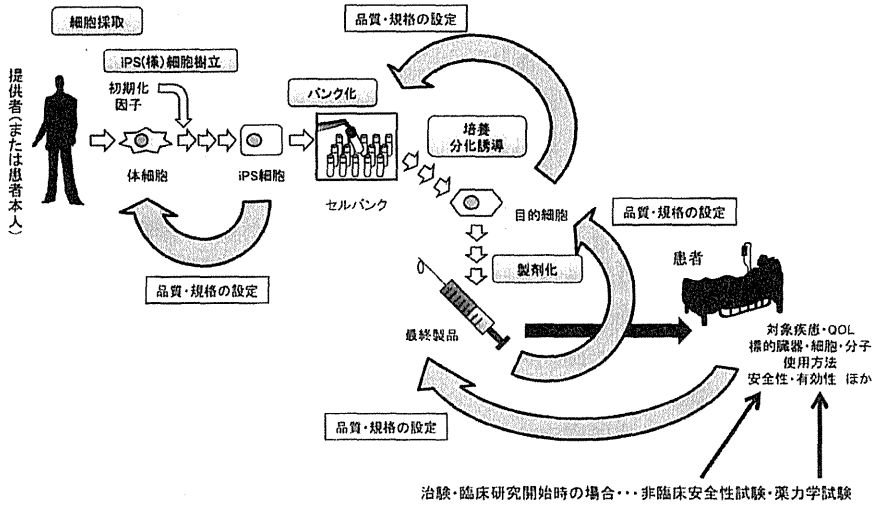

図2　再生医療製品とその素材の品質（iPS細胞由来製品の例）
最終製品としての再生医療製品の品質をもとに中間製品の品質，中間製品の品質から出発原料の品質，と逆行性に規定される．

これらをまとめると，バイオロジクス製造用細胞基材の主な留意事項が，「汚染がないことの保証」と，「同一性・均質性の確認・維持」であることが分かる．

例えば，米国WiCell研究所の保有する「臨床グレード」のヒトES細胞株（WA09株）の品質管理においては，①主要なウイルス・感染因子のチェックが実施されると同時に②フィーダー細胞フリー培養系（TeSR™，マトリゲル™）を使用し，感染因子の混入を防ぎ，また，③融解後生存率，④同一性試験（縦列型反復配列（short tandem repeat; STR）検査），⑤染色体異常検査（Gバンド，比較ゲノムハイブリダイゼーション（Comparative genomic hybridization, CGH）検査），および⑥ES細胞マーカーおよび分化マーカーの発現の検査等によって，同一性・均質性の確認・維持が行われている[4]．

ここで注意しなければならないのは，WA09株のような，臨床用途・最終製品が未特定な「臨床グレード」の多能性幹細胞における上記①～⑥のような留意事項と，特定の再生医療製品の製造という目的に適ったICH-Q5D的なセル・バンクの品質管理における留意事項とは同じとは限らないということである．

2011年にハーバード大学のBockらは，20株のES細胞と12株のiPS細胞について，様々な細胞系譜への分化傾向（プロペンシティ）を評価する目的で，各株の細胞を用いて形成させた胚葉体中の細胞種マーカー，胚葉マーカーの発現を検討したデータを報告している[5]．この報告では，各多能性細胞株は確かに多能性を保有するものの，株間で分化プロペンシティのプロファイルが様々であることが示されている．即ち，ヒトES/iPS細胞株のセル・バンクを多能性幹細胞の一般的／学問的定義に従い「未分化度」や「多能性」のみで品質管理した場合，目的とする細胞への分化効率に細胞株間で大きなバラツキが生じる恐れがある．従って，多能性幹細胞加工製品の細胞基材としてのセル・バンクにおいては，「目的に適った分化プロペンシティ」すなわち「目的細胞への高い分化効率とその高い再現性」を品質特性とする必要性がある．2011年12月，動物由来成分を全く使用せずに「臨床グレード」のヒトES細胞が樹立され，UK Stem Cell Bankに寄託されたとのニュースがNature Newsで報道されたが，同報道には「実際にヒトに投与されるまでには何年も

かかるかもしれない」「細胞株間で組織形成能力は様々であり，心筋を作りやすい株や軟骨を作りやすい株などが存在するので，臨床グレードの株の一連のセットが必要だ」というコメントも記されている[6]．ヒト多能性幹細胞株／バンクの分化プロペンシティの予測と管理は，今後のヒト多能性幹細胞加工製品の実用化の上で非常に重要な課題となると予想される．例えば既に米国では2013年3月に，ライフテクノロジー社がハーバード大学と契約を結び，目的細胞を作成するために必要な特性をもつ最も有望な細胞株を迅速に選抜するための分析ツールの開発を行うことを発表しており[7]，こうした研究の成果として今後出てくると想定される分析ツールや関連特許は，将来，iPS細胞樹立技術の基本特許が失効した後のわが国の多能性幹細胞由来再生医療製品のビジネス展開に大きな影響を及ぼす可能性がある．

6．多能性幹細胞のセル・バンクの樹立・管理

多能性幹細胞由来再生医療製品をはじめとする各種再生医療製品の製造においては，一定の品質の最終製品を安定的かつ継続的に製造する上で重要かつ科学的に合理的な場合に，セル・バンク・システムの構築またはその他の細胞基材の調製が必要となる．わが国の「ヒト幹細胞臨床研究」のように小規模かつ非継続的な医療ならば，多能性幹細胞の供給は細胞寄託機関等からの一時的なものでも済んでしまうかもしれない．しかしそれ以外の場合には，即ち産業・医療の一環として多能性幹細胞由来再生医療製品を安定的かつ継続的に供給するためには，抗体医薬や組換えタンパク質医薬品等の他のすべてのバイオロジクスと同様に，特定の製品の製造という目的に適った品質の細胞基材としてのセル・バンクを製品の開発者が自らの責任において樹立・管理するのが基本だと考えるべきである（**図1**）．その際には，最終製品または中間製品の品質・安全性・有効性を基に，適切な親細胞（株）を選択する必要がある．

細胞寄託機関等が供給する「臨床グレードの多能性幹細胞」は，最終製品の品質を安定的かつ継続的に確保するために重要かつ科学的に合理的である場合，つまり製品製造という目的に適う場合において利用可能であるが，細胞寄託機関等の「臨床グレードの多能性幹細胞」を利用することが製品製造の必須条件だというわけではない．むしろ，特定の製品を効率的かつ再現性良く製造するためのセル・バンクを，感染因子・免疫原性因子の混入を避けつつ，いかに効率的かつ安価に樹立・選別できるかどうかの方が再生医療製品の製造・実用化・継続的供給という目的のためには重要である．

7．おわりに

上で述べたように，細胞基材としての多能性幹細胞のセル・バンクの品質は，個々の最終製品の品質・態様・適用法・対象疾患等で決まる．細胞株／セル・バンク・システムの「標準化」はデータの相互参照性という意味において学問的には重要である．しかし，多能性幹細胞に由来する再生医療製品の製造においては「はじめにセル・バンクの品質（もしくは標準化）ありき」ということはありえず，特定の再生医療製品を一定の品質で再現性良く製造するという目的を達成するためにセル・バンクの品質・規格が決定される．標準化された部品・原材料から最終製品の品質が設計可能な多くの工業製品（建築，機械からコンピュータープログラムまで）の開発手法と同様な発想を，再生医療製品の開発に当てはめることはできない．むしろ再生医療製品の素材の品質についての考え方は，生きた素材であると言う意味で，醸造のそれに近い．ビールの製造の場合，酵母は例えば「芳醇な香り」という最終製品の品質を実現するために素材として厳選され，その上で酵母の品質・規格が決定されるのであり，標準化された酵母の品質をもとにビールの味や香りをデザインすることはありえない．また，選び抜かれた酵母はセル・バンクとして開発者が管理する，あるい

はビジネス戦略によっては囲い込んで門外不出とするものである．

一般的留意事項（必要条件）のみを満たした「臨床グレードの多能性幹細胞」から特定の再生医療製品を製造する場合には，それまで管理されていなかった幾つかの細胞の特性のバラツキにより，目的とする最終製品の品質が十分に確保できない恐れがある．従って，製品ごとに具体的目的に適った品質の多能性幹細胞のセル・バンクが必要となる．もちろん，細胞寄託機関等が供給する「臨床グレードの多能性幹細胞」のセル・バンクは，安価で簡単にアクセス可能な整理された細胞基材供給源（親細胞株）として非常に有用となる可能性はある．ただしその場合でも開発者はそこから改めて特定の製品製造に適う品質のセル・バンクを作成することが必要とされると考えるべきである．

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注

＊1 第1の定義によるセル・バンクは「研究目的または体の損傷部位の外科的再建を目的とした」ものではあるが，これはあくまで漠然とした目的であり，また，その目的に使用するものを貯蔵する「施設」である（例：理研セルバンク，American Type Culture Collection，Wisconsin International Stem Cell Bank，UK Stem Cell Bank）．予め「具体的な臨床用途・最終製品」を特定してから樹立されるものでなく，また細胞（を含むチューブ・アンプル）自体を指すものでもない点で，ICH-Q5Dの言う「細胞基材のセル・バンク」とは異なる．

＊2 具体的臨床用途・最終製品を予め特定することはせず，漠然と外科的再建・再生医療等での臨床利用を想定して樹立される，いわゆる「臨床グレード」と呼ばれる多能性幹細胞が国内外に存在する．これらは感染因子混入防止のための厳重な管理に加え，免疫原性を示す恐れのある動物由来成分等を含んだ試薬を細胞の樹立・維持に使用しないなど，より厳密な規格の下に製造された細胞であることを意味している．