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

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

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

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

5.3. Gelatin

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5.4. Laminin

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5.5. Fibronectin

Fibronectin is a high-molecular-weight glycoprotein (~440 kDa) that binds to integrins[287] and to extracellular matrix components of collagen, fibrin, and heparan sulfate proteoglycans (e.g., syndecans).[288] Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds,[287] and is reported to play a major role in cell adhesion, growth, migration, and differentiation. Its RGD sequence (Arg-Gly-Asp) is the site of cell attachment via $\alpha5\beta1$ and $\alpha V\beta3$ integrins. Fibronectin also contains a cell-adhesion domain of the connecting segment-1 (CS1, EILDVPST), which is mostly recognized by hematopoietic stem and progenitor cells. Table 11 summarizes several types of fibronectin scaffolds or fibronectin-coated dishes used for MSC differentiation reported in the literature.[37,48,53,79,83,97,101,149,192,195,196,200,201,279,289,290]

The adhesion of hADSCs to fibronectin is reported to be mediated by $\beta1$ integrin and heparin-binding domain based on inhibition experiments using an antibody against $\beta1$ integrin and heparin-binding peptide (HBP), whereas the adhesion of collagens and laminin seem to be solely mediated by $\beta1$ integrin.[53] $\beta1$ integrins are a common receptors on MSCs that mediate cell adhesion to collagen type I and type IV, fibronectin, and laminin.

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

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

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

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

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

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

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

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

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

5.6. Vitronectin

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

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

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

5.7. Decellularized ECM

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

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

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

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

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

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

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

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

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

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

[a]ADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; gBMSCs, goat BMSCs; hBMSCs, human BMSCs; mBMSCs, murine BMSCs; hESCs, human ESCs. [b]PCL, poly(ε-caprolactone); HYA, hydroxyapatite; PEG, polyethylene glycol. [c]Xaa is any naturally occurring amino acid with the exception of proline.

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

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

5.8. Biomaterials with ECM-Mimicking Oligopeptides

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6. CONCLUSION

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

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

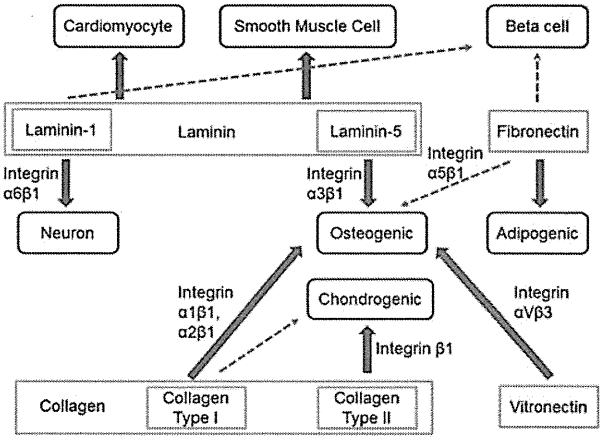

Figure 10. ECM proteins guide stem cell fate through integrin and nonintegrin binding.

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

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

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

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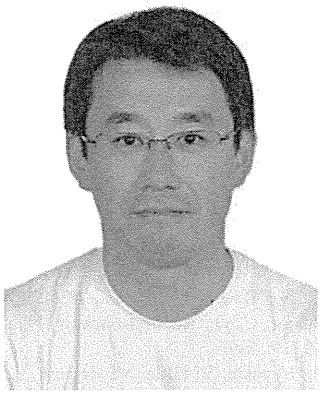
Notes

The authors declare no competing financial interest.

Biographies



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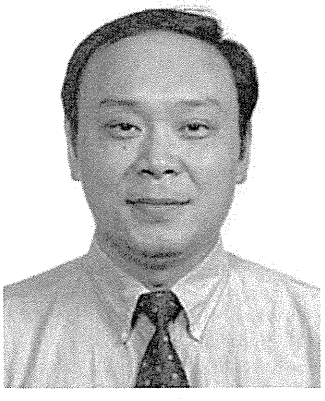

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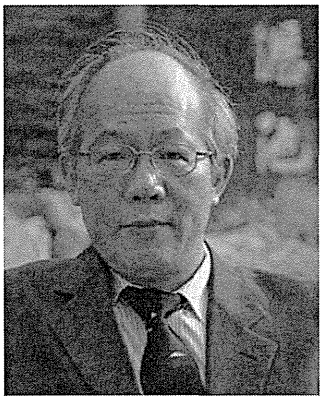

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Feeder-Free and Serum-Free Production of Hepatocytes, Cholangiocytes, and Their Proliferating Progenitors from Human Pluripotent Stem Cells: Application to Liver-Specific Functional and Cytotoxic Assays

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Abstract

We have established a serum- and feeder-free culture system for the efficient differentiation of multifunctional hepatocytes from human embryonic stem (ES) cells and three entirely different induced pluripotent stem (iPS) cells (including vector/transgene-free iPS cells generated using Sendai virus vector) without cell sorting and gene manipulation. The differentiation-inducing protocol consisted of a first stage; endoderm induction, second stage; hepatic initiation, and third stage; hepatic maturation. At the end of differentiation culture, hepatocytes induced from human pluripotent stem cells expressed hepatocyte-specific proteins, such as α-fetoprotein, albumin, α1 antitrypsin and cytochrome P450 (CYP3A4), at similar or higher levels compared with three control human hepatocyte or hepatic cell lines. These human iPS/ES cell-derived hepatocytes also showed mature hepatocyte functions: indocyanine green dye uptake (~30%), storage of glycogen (>80%) and metabolic activity of CYP3A4. Furthermore, they produced a highly sensitive hepatotoxicity assay system for D-galactosamine as determined by the extracellular release of hepatocyte-specific enzymes. Hepatoprotective prostaglandin E1 attenuated this toxicity. Interestingly, bile duct-specific enzymes were also detected after drug treatment, suggesting the presence of bile-duct epithelial cells (cholangiocytes) in our culture system. Electron microscopic studies confirmed the existence of cholangiocytes, and an immunostaining study proved the presence of bipotential hepatoblasts with high potential for proliferation. Differentiated cells were transferrable onto new dishes, on which small-sized proliferating cells with hepatocyte markers emerged and expanded. Thus, our differentiation culture system provides mature functional hepatocytes, cholangiocytes, and their progenitors with proliferative potential from a wide variety of human pluripotent stem cells.

Introduction

THE LIVER PLAYS CRITICAL ROLES for regulating metabolic homeostasis, because it is responsible for the metabolism, synthesis, and storage of nutrients. It is also well known that the liver is a central organ for the detoxification of drug compounds and other toxic substances taken into the human body. Thus, dysfunction of liver results in serious conditions, and liver/hepatocyte transplantation is the major therapeutic option for patients with chronic end-stage liver disease (Miro et al., 2006). However, the major limitation of cell-based therapies for liver disease is the availability of human hepatocytes.The use of embryonic stem (ES) cells may be the most effective strategy to develop hepatocytes that may be valuable in regenerative medicine and for pharmacological studies. Human ES cells proliferate infinitely *in vitro* while maintaining their potential to differentiate into almost all cell types (Thompson et al., 1998), and thus provide a potential source for obtaining hepatocytes. Several studies have demonstrated the capacity of human ES cells to differentiate into

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hepatocyte-like cells (Agarwal et al., 2008; Cai et al., 2007; Chiao et al., 2008; Duan et al., 2007, 2010; Hay et al., 2008a, 2008b; Ishii et al., 2008; Mfopou et al., 2010; Sasaki et al., 2009; Touboul et al., 2010; Zhao et al., 2009). However, most reports performed limited phenotypic and functional tests on the differentiated cells, and most differentiation culture methods include steps using animal-derived components unsuitable for clinical application. In addition, detailed studies on individual diversity of metabolism and toxicity for drugs, leading to tailor-made medicine, cannot be performed readily using only limited numbers of human ES cell lines.

Induced pluripotent stem (iPS) cells were generated directly from somatic cells as a result of the introduction of four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc (Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007), and shared many characteristics with ES cells, including multilineage differentiation potential, and intensive proliferation *in vitro*. In addition, the establishment of human iPS cells is ethically acceptable and does not require human oocytes. Thus, we may be able to obtain patient-specific functional cells for research into diseases, apply these cells to the regenerative medicine for therapeutic use, and use these cells for *in vitro* testing to satisfy industrial requirements, including drug discovery. However, as is the cases with human ES cells, it is not easy to regulate the differentiation of human iPS cells toward endoderm cells such as hepatocytes (Inamura et al., 2011; Liu et al., 2010; Si-Tayeb et al., 2010; Song et al., 2009; Sullivan et al., 2010). In addition, unlike human ES cells, most of human iPS cells have been generated via retrovirus/lentivirus vector systems, resulting in genomic integration of viral components into iPS cells (Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007).

Here we report the establishment of a serum- and feeder-free method for hepatocyte differentiation of human iPS/ES cells (including virus-free iPS cells established using Sendai virus vector) (Fusaki et al., 2009), providing excellent tools for the evaluation of drug metabolism and hepatotoxicity. We succeeded in producing cholangiocytes and bipotential hepatoblasts and developed minimally invasive subculture methods of proliferating hepatocyte stem/progenitor cells under feeder-free conditions without using cell-sorting techniques. Our system will contribute to drug discovery and tailor-made medicine with the aim of dispensing the safest drugs for each individual.

Materials and Methods

Generation and culture of human iPS cells

A human iPS cell line, 253G1, was established by transducing human adult skin fibroblasts with retrovirus containing Oct3/4, Sox2, Klf4, and/or c-Myc, as described previously (Takahashi et al., 2007). Human iPS cell line #40 was generated from human fetal lung fibroblasts (MRC-5 cells), via procedures described by Yamanaka and colleagues (Takahashi et al., 2007) with slight modifications (Nagata et al., 2009; Toyoda et al., 2011).

We also established human iPS cell line SeV5 without integration of viral vector components from human neonatal fibroblasts using Sendai virus (SeV) vectors, as described previously (Ban et al., 2011; Fusaki et al., 2009). Human fibroblast cell line BJ from neonatal foreskin (ATCC, Manassas, VA) were infected with SeV vectors containing Oct3/4, Sox2, Klf4, or c-Myc and were then incubated for 6 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories GmbH, Linz, Austria). Then the cells were cultured on dishes coated with γ-irradiated murine embryonic fibroblasts (MEFs) in Primate ES cell medium (ReproCELL Inc., Tokyo, Japan) for 3 weeks. Human ES cell-like colonies were picked up using a micropipette and were further cultured on dishes coated with γ-irradiated MEFs. These human ES-like cells expressed several multipotent markers such as SSEA4, Oct3/4, and Nanog. SeV and transgenes in human iPS cells were diluted to undetectable levels during repeated passage for approximately 2 months and/or were deleted by high-temperature cultivation (at 39°C) for 7 days (Ban et al., 2011).

All human iPS cells were maintained on dishes coated with γ-irradiated MEFs as described previously (Gokoh et al., 2011).

Culture of human ES cells and normal human hepatocyte cell lines

The use of human ES cells was performed in accordance with the Guidelines on the Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology of Japan, after approval by the institutional review board of the National Center for Global Health and Medicine. Human ES cells (KhES-1) (Suemori et al., 2006), provided by Kyoto University (Kyoto, Japan), were maintained as described previously (Nakahara et al., 2009a, 2009b; Saeki et al., 2009).

We used two human hepatic cell lines as positive control. HepG2 cell was purchased from Research Resources Bank of Japan Health Science Foundation (Osaka, Japan) and cultured in DMEM supplemented with 10% heat-inactivated FBS (PAA Laboratories). HepaRG cells (Gripon et al., 2002) was purchased from BIOPREDIC INTERNATIONAL (Rennes, France) and cultured on collagen IV-coated dish in General Purpose medium 670, Maintenance and Metabolism medium 620, or Induction medium 640 (BIOPREDIC INTERNATIONAL), according to the protocol of the supplier.

Hepatocyte differentiation of human iPS and ES cells in nonfeeder and serum-free culture

Before the induction of differentiation, human iPS and ES cells maintained on MEF were detached with dissociation liquid containing 0.25% trypsin (Invitrogen Corp., Carlsbad, CA), 1 mg/mL collagenase IV (WAKO Pure Chemical Industries, Osaka, Japan), 20% Knockout™ Serum Replacement (KSR) (Invitrogen), and 1 mM $CaCl_2$. Detached cells were collected into conical tube, and were then allowed to stand at room temperature to sediment iPS/ES cells. After appropriate periods (approximately a few minutes), MEF in the supernatant of the tube were aspirated and iPS/ES cells at the bottom of the tube were collected. Collected human iPS/ES cells were then cultured for 2–3 days on matrigel-coated dish in DMEM/F12 medium supplemented with 20% KSR (Invitrogen), 1% nonessential amino acid solution (Invitrogen), 1 mM sodium pyruvate solution, 100 μM 2-mercaptethanol, 2 mM L-glutamine, 20 U/mL penicillin, and 20 μg/mL streptomycin.