Reconstructing the Differentiation Niche of Embryonic Stem Cells Using Biomaterials

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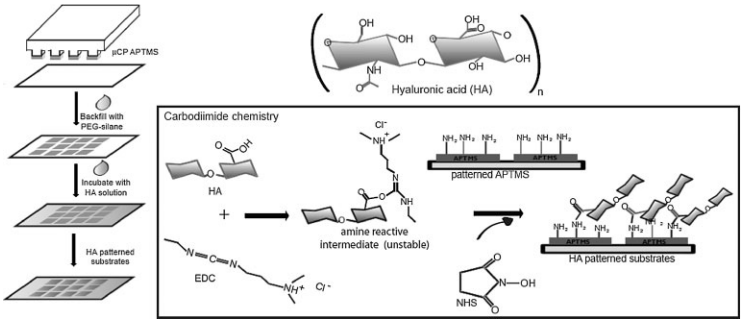
The biochemical cues and topographical architecture of the extracellular environment extensively influence ES cell fate. The microenvironment surrounding the developing embryo presents these instructive cues in a complex and interactive manner in order to guide cell fate decisions. Current stem cell research aims to reconstruct this multifaceted embryonic niche to recapitulate development in vitro. This review focuses on 2D and 3D differentiation niches created from natural and synthetic biomaterials to guide the differentiation of ES cells toward specific lineages. Biomaterials engineered to present specific physical constraints are also reviewed for their role in differentiation.



Introduction

The extracellular environment presents a myriad of biochemical cues and specific topographical architecture in a spatially and temporally distinct manner to influence cellular behavior, including the self-renewal and differentiation of embryonic stem (ES) cells. While an important function of the extracellular matrix (ECM) is to provide the structural framework to support cellular functions, this scaffold of proteins, proteoglycans, and glycosaminoglycans also provides cell adhesion sites and important signaling cues. ECM components and structure influence stem cell fate through integrin-mediated activation and downstream signaling events.

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ES cells have attracted attention as a promising source of cells for tissue engineering, as they can self-renew almost indefinitely in culture and have the capacity to differentiate into all cell types of the body (i.e., are pluripotent). ES cells are isolated from the inner cell mass of the developing blastocyst. In vitro, they can be maintained as undifferentiated cells via culture on mouse embryonic fibroblasts (MEFs) or via culture in feeder-free conditions using a defined medium composition to supply essential cytokines and nutrients to promote the undifferentiated phenotype.[1] Control over their differentiation has been extensively studied via manipulation of the parameters of the stem cell microenvironment, referred to as the stem cell niche. Thus, both soluble and insoluble cues of the niche which modulate the differentiation have been thoroughly investigated to determine the parameters amenable to inducing a particular cell fate.

The extracellular microenvironment surrounding the developing embryo presents a number of spatially and temporally instructive biochemical cues within a complex and interactive milieu that guide and govern the sequential development and cell fate decisions of embryogenesis.[2] Current stem cell research aspires to deconstruct this multifaceted embryonic niche, determine the influential elements involved in lineage differentiation, and recreate

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these environments for in vitro studies and future clinical applications. This has been examined in both two-dimensional (2D) and three-dimensional (3D) culture systems with both natural and synthetic biomaterials. While growth factors and other soluble signals largely direct ES cell differentiation, the extracellular environment plays an influential role. Utilizing ECM components in conjunction with advanced micro- and nanotechnology, researchers attempt to recapitulate the instructive architecture of the native microenvironment. In this review, we discuss how insoluble ECM molecules or scaffolds can provide instructive cues that influence the controlled differentiation of ES cells.

ECM Composition Effects: 2D Differentiation Environments

Embryoid body (EB) formation is a common method to study ES cell differentiation into desired cell lineages. This strategy yields clusters of spontaneously differentiating cells in suspension culture. However, studying this phenomenon in a 2D adherent culture may aid in elucidating the precise signaling effects which drive differentiation into a specific cell type. Examining the effects of various ECM substrate compositions and presentations may be used to reveal important signaling aspects of the developing embryo and to allow the high-resolution visualization of 2D spreading.

Various ECM components and compositions have been implicated for their role in guiding differentiation, including collagen, laminin, fibronectin, Matrigel, and decellularized matrices (Table 1). Research with knockout mice showed that the loss of function of even a single class of ECM molecules could yield embryonic or postnatal lethal phenotypes.[3] ECM components have also been patterned to manipulate spatial parameters and enhance the subsequent ES cell differentiation (Table 1).

Collagen

Collagens, ubiquitous proteins found throughout the body, are the most abundant ECM macromolecule (reviewed in ref. [4]). Approximately 28 types of collagen are distributed throughout the body. Among these, collagen IV is a network-forming collagen which has been largely implicated in mesodermal differentiation, including the hematopoietic,[5,6] endothelial,[6–8] and smooth muscle lineages.[6–9] In the absence of exogenous factors, mouse ES cells differentiated on collagen IV demonstrated greater enrichment of the mesodermal phenotype than those cultured on collagen I, fibronectin, or gelatin.[5] These mesodermal cells, indicated by their E-cadherin$^-$/Flk1$^+$ phenotype, were subsequently differentiated into mature blood cells. A following study demonstrated that collagen-type-IV-induced Flk1$^+$ cells from mouse ES cells could also be differentiated into endothelial cells (ECs) and smooth muscle cells (SMCs).[7] The mesodermal markers Flk1 and Brachyury were also expressed on mouse induced

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Table 1. 2D differentiation environments for embryonic stem cells.

2D Differentiation environment	Embryonic stem cell fate
collagen IV	hematopoietic cells,[5,6] endothelial cells,[6–8] smooth muscle cells,[6–9] trophoectodermal lineage[11]
collagen I	cardiomyocytes,[12,13] endothelial cells[14]
laminin	neural cells,[15] pancreatic cells[16–20]
fibronectin	mesodermal and ectodermal lineages,[32] endothelial cells[34]
Matrigel	hepatocytes,[42] neural progenitor cells[43]
decellularized ECM	osteocytes,[52] cardiomyocytes[53]
micropatterned 2D substrates	circular fibronectin patterns with 200 μm diameter[54]: cardiomyocytes circular Matrigel micropatterns[56,57] smaller diameters: endoderm larger diameters: mesoderm

pluripotent stem (iPS) cells after just 4 d in an adherent culture unsupplemented with exogenous growth factors.[6] Flk1$^+$ cells were isolated and subsequently differentiated into cardiomyocytes, SMCs, ECs, and hematopoietic cells. We previously determined that human ES cells grown on collagen-IV-coated dishes for 6 d and strained through a 40 μm filter could further differentiate into ECs when cultured in vascular endothelial growth factor (VEGF) or into SMCs when cultured in platelet-derived growth factor-BB (PDGF-BB).[8] A robust two-step method has also been developed to produce functional SMCs via culture on collagen IV in media supplemented with PDGF-BB and transforming growth factor beta-1 (TGF-β1).[9]

The differentiation of mouse ES cells has also been examined on collagen IV substrates modified with covalently immobilized VEGF.[10] Surfaces modified with VEGF promoted differentiation into ECs, whereas surfaces lacking VEGF promoted SMC differentiation. Together, these studies largely suggest that collagen IV influences mesodermal fate.

Additionally, Schenke-Layland and colleagues demonstrated that collagen IV – but not laminin, fibronectin, or collagen I – was able to direct mouse ES cells into trophoectodermal cells,[11] which previously could not be derived from mouse ES cells without genetic manipulation. This study clearly demonstrated that, under the appropriate culture conditions, mouse ES cells demonstrate totipotency without genetic alteration.

Collagen I, a fibrillar collagen, appears to play a role in guiding differentiation toward cardiovascular phenotypes in particular. Collagen is essential for cardiomyocyte differentiation from mouse ES cells;[12] the presence of collagen synthesis inhibitors significantly inhibited cardiac differentiation. Collagen I supplemented with Matrigel has been used to create engineered cardiac tissue when seeded with cardiomyocytes derived from mouse ES cells.[13] Collagen I has also been shown to play a role in EC morphology.[14] ECs seeded within collagen I gels are able to form tube structures intrinsically. Furthermore, this assay is used to confirm the functionality of human-ES-cell-derived ECs.

Laminin

Laminin, a trimeric protein found in the basal lamina, has also been investigated for its influence on ES cell differentiation. Accumulating studies suggest that laminin plays a critical role in differentiation toward ectodermal[15] and endodermal[16] cell fates. The effect of laminin on mesodermal differentiation is less understood.

Neural cells, which come from the ectoderm lineage, demonstrated preferential outgrowth on laminin and Matrigel (which is enriched in laminin, as discussed below) substrates.[15] Differentiating human EBs were plated on various substrates, including poly-(D-lysine) (PDL), PDL/fibronectin, PDL/laminin, collagen I, and Matrigel. PDL is a positively charged, synthetic molecule and was incorporated in these substrates to enhance cell attachment and ECM protein adsorption. Of these tested substrates, laminin and Matrigel yielded the greatest neuronal generation and outgrowth. Blocking α6 or β1 integrin subunits partially blocked the formation of the neural progenitors, implying that downstream signaling of these receptors affects neuronal differentiation.

Laminin has been implicated in the differentiation and maintenance of pancreatic cells, which arise from the endoderm.[17,18] Soluble laminin – but not collagen I, collagen IV, or fibronectin – was shown to induce the differentiation of islet cells derived from 13.5-d-old mouse fetus.[19] Laminin promoted the proliferation and differentiation of β-cells from precursor cells. Laminin has also been used as a substrate for inducing pancreatic cell

differentiation from mouse ES cells in vitro.[20] After EB formation, early progenitors of the pancreatic lineage were induced via culture on laminin substrates and in the presence of nicotinamide and insulin.[20]

Mouse cells genetically altered to lack laminin γ1 chain could not form a basement membrane.[21] In EBs generated from these knockout cells, ectodermal differentiation was greatly reduced and mesodermal differentiation was preferred and accelerated. Cardiomyocytes could still be differentiated from mouse ES cells deficient in laminin γ1 chain.[22] However, they were not able to support electrical signal propagation, implying that laminin may still be relevant for the functionality of cardiac cells.

Fibronectin

Fibronectin is an ECM molecule expressed during the early stages of development that is critical for proper development of the mesoderm and neural tube.[23] It is often used as a cell adhesive layer for biomaterials due to the presence of the peptide sequence arginine-serine-aspartic acid (RGD), which is widely involved in integrin-mediated cell adhesion[24–26] and is commonly tethered to biomaterials to improve cell adhesion.[27,28] Additionally, fibronectin has been implicated in enhancing the differentiation of stem cells to specific lineages.[29–31] Binding α5β1 integrin to specific fibronectin domains conferred differentiation into mesodermal and ectodermal lineages.[32] Blocking mouse ES cell interactions with a fibronectin substrate by the addition of anti-integrin β1 antibody maintained self-renewal.[33] Fibronectin has also been used to mature hES cells – and iPS-cell-derived $CD34^+CD31^+$ cells – into ECs.[34]

Matrigel

Matrigel is isolated from the natural basement membrane of mouse sarcoma cells and therefore contains a myriad of ECM components and growth factors[35,36] conducive to cell attachment, proliferation, and/or differentiation to all three lineages;[37–43] its efficacy is most likely due to it having many factors in optimal native concentrations. Though collagen IV and heparan sulfate proteoglycans are also present, laminin is its primary component.[36] Using Matrigel provides an environment that presents many of the appropriate extracellular biochemical and biophysical cues necessary for stem cell differentiation.[39,44] Whereas many fabricated hydrogels only present one major ECM component, Matrigel presents many; because it was derived from basement membrane, it instructs ES cell differentiation similarly to native basement membranes.[45]

Compared to collagen I and laminin, Matrigel was shown to be more effective at inducing hepatocyte differentiation from multiple human ES cell lines in the presence of activin A and hepatocyte growth factor.[42] Matrigel has also been used to support the growth of neural progenitors.[43] This study allowed mouse ES cells to differentiate on a mouse stromal cell layer for 8 d and then transferred them to matrices made from various ECM components. Of the tested matrices, which included Matrigel, gelatin, collagen IV, and laminin, Matrigel proved most effective in supporting the survival and differentiation of neural progenitor cells.

Decellularized ECM

Of note, Matrigel is isolated from murine species, presenting translational disadvantages. Decellularized matrices provide a bioinstructive template and can be isolated from various cell types for applications in tissue regeneration.[46–50] Extracted ECM has the potential to provide an inductive environment that guides undifferentiated cells to cell lineages supported in vivo.[47,48,51] ECM deposited by osteoblastic cells enhanced the osteogenic differentiation of mesenchymal stem cells (MSCs),[48] while a decellularized matrix of adipose tissue produced an inductive microenvironment for adipogenesis without exogenous growth factors.[47,51] The ability to extract intact ECM with conserved 3D structure and biochemical factors could allow the differentiation of ES cells to any lineage type.

Decellularized ECM has been studied as a potential substrate for ES cell differentiation.[52] Mouse ES cells were seeded onto decellularized matrices derived from osteogenic and non-osteogenic cells. Differentiation on either of these matrices was compared with differentiation on a collagen I matrix. Mineralized tissue formation and osteogenic gene expression revealed that the osteogenic cell-derived ECM was most favorable for osteogenic differentiation. Similarly, ECM derived from cardiac fibroblasts, which consists of collagen types I and III, laminin, fibronectin, proteoglycans, and glycoproteins, enhanced mouse-ES-cell-derived cardiomyocyte maturation and functionality better than Matrigel.[53]

Micropatterned 2D Substrates

Micropatterns allow the manipulation of spatial parameters involved in lineage commitment, and to this end, 2D patterned ECM substrates have been extensively examined for ES cell differentiation. Microcontact printing (μCP) is one method to achieve micropatterns. This technique uses a prefabricated silicon template to mold an elastomeric polymer, polydimethylsiloxane (PDMS), which is used to "stamp" molecules of interest to a substrate in the desired array. Fibronectin micropatterns have been shown to influence ES cell fate. When mouse ES cells were cultured

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on varying sizes of fibronectin circular micropatterns, they demonstrated variable differentiation to the cardiomyocyte lineage; the 200 μm diameters circles exhibited the greatest potential.[54] Additionally, MSCs cultured on smaller fibronectin islands acquired an adipocyte fate, and larger islands promoted more osteogenic cell lineages.[55] Clearly, the surface presentation of biomolecular cues is not the sole factor in cell differentiation, since fibronectin-coated substrates yielded varying cell lineages. Cell type, spatial dimensions, and topography are also crucial parameters in developing culture systems for controlled cell differentiation. Human ES cells cultured on circular micropatterns of Matrigel maintained their pluripotency or differentiated to endoderm on smaller geometries but differentiated to mesoderm on larger sized islands.[56,57]

We have developed a unique method to covalently immobilize micropatterns of hyaluronic acid (HA) by combining carbodiimide chemistry and μCP (Figure 1, Figure 2).[58] HA, a non-sulfated, linear polysaccharide composed of alternating monomers of (1-β-4)-D-glucuronic acid and (1-β-3)-*N*-acetylglucosamine, is known to regulate gene expression, adhesion, proliferation, and especially morphogenesis.[59–61] During early embryogenesis, the developing embryo is surrounded by a high concentration of HA, which decreases at the onset of differentiation. This indicates the crucial role of HA in development and its potential for use as a biomaterial for ES cell differentiation. We are currently investigating stem cell behavior on these patterns. Meanwhile, other researchers have utilized the non-fouling properties of HA in conjunction with other ECM components (fibronectin and/or collagen) to spatially control mouse ES cell cocultures that either maintain ES cell pluripotency,[62] or enhance neural differentiation.[63]

Two-dimensional micropatterns have also been employed to elucidate that cell-cell interactions have a profound effect on differentiation pathways.[64] Manipulating cell density on these carefully engineered micropatterns revealed varied differentiation products, demonstrating that this parameter plays an important role in differentiation. These studies, among many others (as cited in a recent review[65]), demonstrate the potential of microscale manipulation to control the early stages of differentiation and to influence specific lineage trajectories of stem cells.[64]

3D Differentiation Environments

It is evident that 2D substrates do not wholly reconstruct the biological intricacies of the native extracellular environment, the micro-/nanoarchitecture, and the complex physiochemical signals. Differentiation studies performed in 2D or 3D environments have been compared, and they indicate a disparity in stem cell differentiation between the two spatial conditions; increased proliferation and heterogeneous differentiation has been observed in 3D.[66,67] 2D cell substrate conditions constrain cells to a planar geometry, which in turn changes migration potential, morphology, and signaling pathways from those which are experienced in 3D. Even though all of the elements directing 3D in vivo differentiation may not necessarily be relevant in controlling differentiation in vitro, extensive research is focused on recreating and mimicking the critical conditions found in vivo. Engineering synthetic microenvironments that recapitulate the necessary elements is being widely investigated through biomaterial design. Fabricating 3D scaffolds using natural or synthetic ECM components can provide bioactive adhesion sites, signaling cues, and soluble factors instrumental in guiding ES cell differentiation.

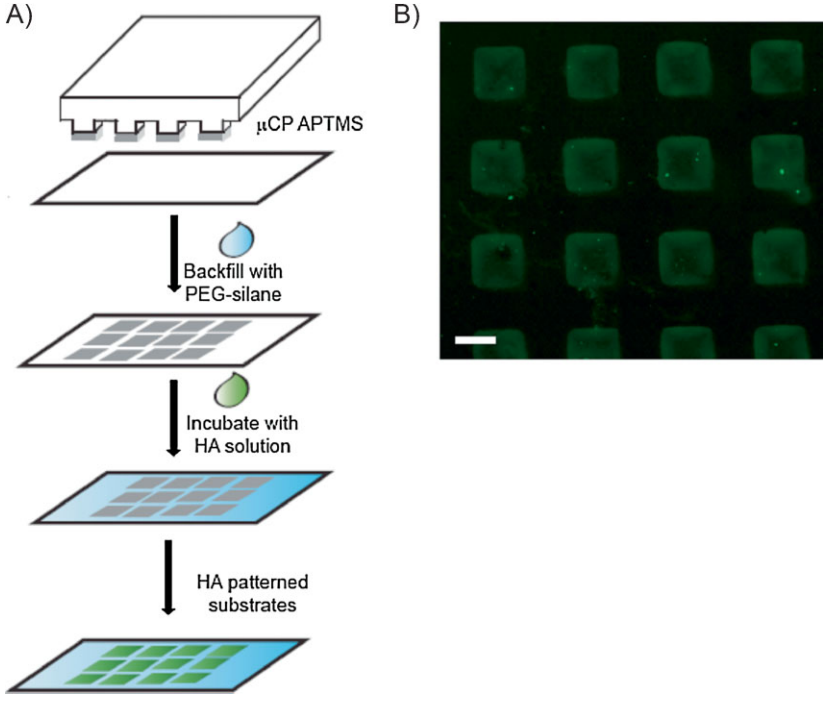

Figure 1. Patterned HA surfaces. (A) Schematic detailing the development of HA micropatterned surfaces. Aminopropyltrimethoxysilane (APTMS) is printed on glass substrates using microcontact printing, and unpatterned regions are backfilled using cell-adhesion-resistive PEG-silane. An HA solution is applied to substrates and incubated for 16 h. (B) Fluorescent image of fluorescein (FL)-labeled HA (green) immobilized on 80 × 80 μm² squares APTMS micropatterns. Scale bar: 50 μm.

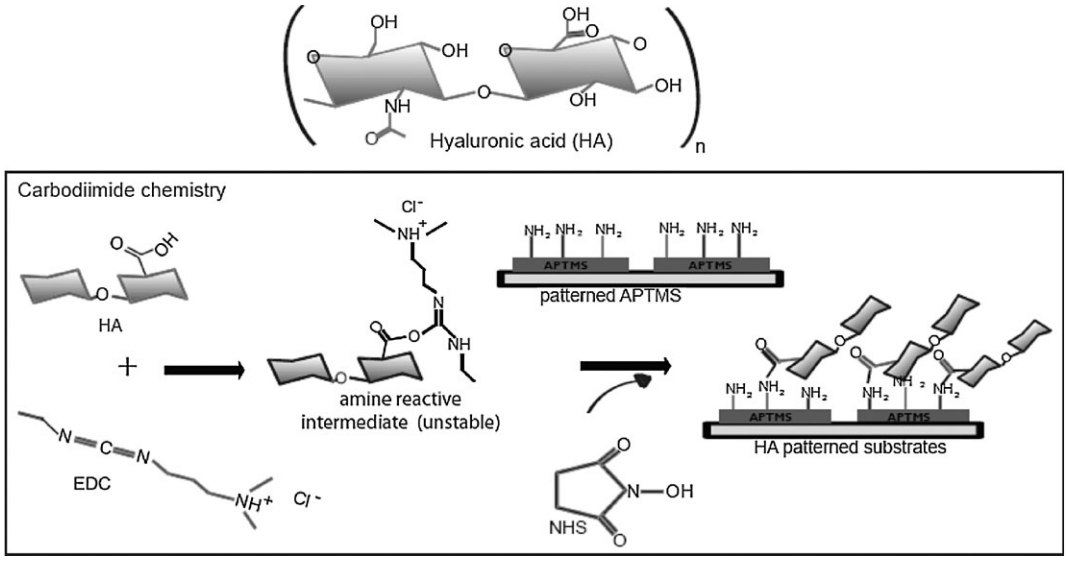

Figure 2. Carbodiimide chemistry immobilizing HA. HA carboxyl groups reacted with the linker molecule 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to form an amine-reactive intermediate. *N*-hydroxysuccinimide (NHS) increased the efficiency of this unstable intermediate; HA solution was added to glass substrates and reacted with patterned primary amines from APTMS.

Three-dimensional environments have been decorated with receptor-binding sites and protease-sensitive sites and subsequently encapsulated with cells.[68] The RGD tripeptide sequence of fibronectin has often been used to facilitate cell binding. Sites cleavable by matrix metalloproteinase (MMP), a protease used to degrade the basement membrane, have been used in conjunction with RGD sequences. These modifications allow improved cell spreading and growth and cell-controlled scaffold degradation.

Natural 3D Scaffolds

Hydrogels provide a highly controlled, synthetic 3D environment that is structurally and biomechanically similar to native ECM topology and provides a rich ligand landscape to influence cell behavior.[69,70] These 3D networks, formed by crosslinking natural polymers, are highly tunable biomaterials fabricated from a wide range of molecules using various synthesis methods to control cell behavior. The materials for such networks are not limited to ECM-derived components; natural macromolecules and polysaccharides not present in the ECM or even synthesized in vivo are being investigated and explored as practical biomaterials for stem cell differentiation. These materials are biocompatible[71,72] and easily modified with cell adhesive ligands.[73,74]

Matrix parameters, such as swelling, mesh size, or structural integrity, can be optimized by adjusting crosslinking properties.[75] Additionally, our ability to engineer these 3D microenvironments to present or deliver soluble biochemical factors makes them appropriate candidates for controlled differentiation studies.[76–78]

Collagen

Collagens are the most abundant ECM protein; with its ability to self-aggregate and crosslink, collagen is an attractive macromolecule for 3D biomaterials.[79] In fact, collagen has been extensively investigated in 3D ES cell encapsulation and differentiation studies.[30,66] 3D hydrogels, fabricated as semi-interpenetrating polymer networks of collagen I fibers, laminin, and fibronectin, provide an architecture similar to native ECM.[30] Matrices with higher concentrations of fibronectin preferentially initiated vascularization and differentiation of mouse EBs to ECs, whereas matrices with high concentrations of laminin supported differentiation to beating cardiomyocytes,[30] demonstrating the significance of matrix composition on lineage specification. Human EBs cultured within a 3D collagen scaffold exhibited specification towards hepatic cell types when cultured with additional hepatocytic growth factors in vitro.[66]

Fibrin

Fibrin, a large non-globular protein, forms a mesh-like network of fibrillar gel upon polymerization of the coagulation proteins thrombin and fibrinogen. Although primarily involved in blood clotting and coagulation cascades, this natural macromolecular polymer has been shown to regulate vasculogenesis in developing embryos[80] and to influence the differentiation of ES cells to endothelial and neuronal lineages.[67,81,82] Mouse EBs seeded within fibrin scaffolds differentiated to neuronal and astrocyte phenotypes within two weeks of culture;[67] the addition of supplemental growth

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factors enhanced their differentiation into neurons and oligodendrytes.[81] We have recently shown that patterned fibronectin surfaces, optimized in terms of width and substrate concentration and inverted onto a fibrin gel to create a 3D environment, guide the elongation and maturation of endothelial progenitor cells. The 3D environment also promoted tube formation, as indicated by the presence of vacuoles and Weibel-Palade bodies.[82b]

HA

Previously, we examined HA hydrogels capable of maintaining the pluripotency of human ES cells; upon modification, these hydrogels could induce differentiation.[83] Using photopolymerization, methacrylated HA was covalently crosslinked, and human ES cells were encapsulated within the hydrogel. Human ES cells encapsulated in HA hydrogels exhibited a preserved self-renewal state, full differential potential, and normal genetic integrity. Adding the proangiogenic growth factor VEGF to differentiation medium initiated human ES cell differentiation to vascular lineages within the HA hydrogel. Cell sprouting and elongation were observed within 48 h, and the presence of endothelial and smooth muscle markers confirmed their vascular cell fate.[83] By manipulating fabrication methods, Khademhosseini et al. fabricated micropatterned HA hydrogels for encapsulation that supported cell viability and controlled ES cell colonization.[84] Photocrosslinked HA hydrogels with spatially controlled matrix properties allowed the spreading and proliferation of MSCs,[85] and when enhanced with TGF-β3, permitted chondrogenesis of adult MSCs.[86] When HA hydrogels were crosslinked with MMP-cleavable peptides and coupled with RGD adhesive proteins, encapsulated MSCs demonstrated spatially controlled cell spreading.[87] Cell-secreted MMPs degraded ECM proteins, and crosslinking hydrogels with MMP-cleavable crosslinks allowed cell-dictated hydrogel degradation and remodeling, enabling further spreading, interaction, and invasion in the biomaterial.[88]

Dextran

Dextran is a branched polysaccharide with a molecular structure similar to that of the ECM macromolecule, HA. However, dextran is not distributed in the mammalian ECM. Its chemical structure is easily modified for cell adhesiveness.[72,89,90] Pendant functional groups, such as –OH, make dextran amenable to chemical modifications for greater flexibility in the formulation of dextran-based hydrogels. Modifying dextran-based hydrogels with cell adhesive ligands, such as laminin- or fibronectin-derived RGD peptides, increases their potential for use in tissue engineering applications by enhancing cell adhesion and survival.[89] Ferreira et al. designed a photopolymerized bioactive dextran-based hydrogel to enhance human ES cell differentiation. These dextran hydrogels, modified with such regulatory factors as the insoluble cell-adhesive peptide sequence RGD and the soluble angiogenic growth factor VEGF, initiated EB formation and vascular differentiation. Encapsulated EBs formed well-organized vascular networks, revealed upregulated vascular markers and, upon release from the hydrogel, proliferated towards the vascular lineage.[74] The microencapsulation of growth factors within the dextran hydrogel allowed distinct spatial, temporal, and biochemical signaling control over encapsulated cells.

Alginate

Alginate is another example of a naturally derived polymer well-suited for biomaterial scaffolds and the in vitro differentiation of ES cells. A hydrophilic polysaccharide isolated from seaweed,[91] alginate resembles native ECM in macromolecular structure and supports cell adhesion.[73,92] Biocompatibility properties have made alginate an appropriate biomaterial to investigate cell encapsulation[73,92] and the lineage differentiation of ES cells. Alginate hydrogels for cell encapsulation are formed when divalent cations, such as strontium and calcium, interact and ionically crosslink polymer chains.[91] Alginate-based hydrogels have been shown to support ES cell proliferation and viability,[93] as well as osteogenic, hepatic, and chondrogenic differentiation.[94–97] Much emphasis is placed on the influence of biochemical cues on stem cell differentiation; however, physical constraints, such as those imposed by pore diameter size, are also influential in lineage commitment. Encapsulation of undifferentiated human ES cells within 3D porous alginate scaffolds induced efficient and uniform EB formation in the absence of supplemental factors, with EBs exhibiting increased proliferation and vasculogenic differentiation (Figure 3).[96] These cellular responses were attributed to the ultrastructure of the alginate scaffold, namely pore size and scaffold degradation rate.[96] ES cells encapsulated in alginate microbead environments have controlled culture parameters, such as growth factor diffusion, and are restricted by the physical boundaries imposed by the microbeads. In this alginate-based microenvironment, ES cells differentiated to hepatic cell types.[94,95,98,99]

Hydrogels prepared with different ECM constituents have demonstrated distinctive effects on cell adhesion and differentiation pathways. Though naturally derived ECM components regulate and support the behavior, modification, and processing of native ECM molecules, photopolymerization, ionic crosslinking, and enzymatic reactions may disrupt or compromise their natural physical and biochemical properties. To this end, synthetic scaffolds have been investigated.

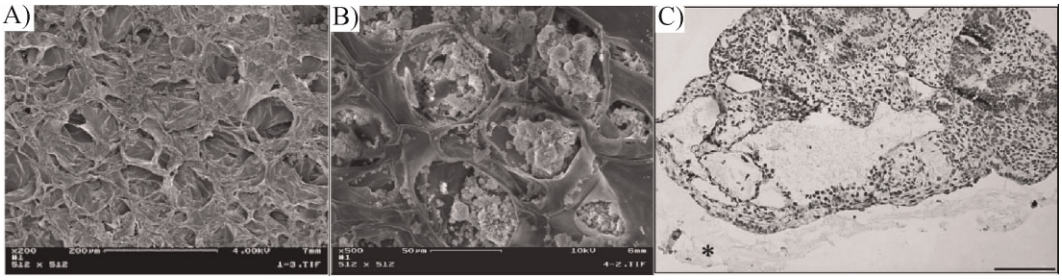

Figure 3. 3D alginate scaffolds for ES differentiation. (A) Scanning electron micrographs demonstrating the porous ultrastructure of alginate scaffolds. (B) EB formation within pores after 1 month of culture. (C) Vasculogenesis of EBs is demonstrated with $CD34^+$ staining and the formation of complex vascular structures. Scale bar: 100 μm. Adapted from ref.[96] with permission from John Wiley and Sons.

Synthetic 3D Scaffolds

Synthetic materials have been manipulated to mimic their natural counterparts and can provide instructional cues for ES cell differentiation.[77] Advantages of using synthetic materials include the manipulation of the scaffold's physical parameters, reproducibility, and ease of synthesis and processing. Synthetic materials can be modified with binding domains or sites responsive to cellular activity. These combinations of parameters have been demonstrated to influence the differentiation of ES cells.

Polyester-Based Scaffolds

Polyester-based scaffolds have been thoroughly investigated for tissue engineering, as they are biodegradable, biocompatible, and yield degradation byproducts which can be metabolized by the body. Poly(L-lactic acid) (PLLA) scaffolds have been used for hematopoietic cell differentiation.[100] A suspension of single mouse ES cells seeded onto the porous construct formed EBs which integrated into the scaffold and yielded reproducible amounts of hematopoietic cells. This was confirmed by protein expression and colony-forming ability. A following study systematically tested the effects of controllable properties of the PLLA scaffolds on the differentiation of mouse ES cells into hematopoietic cells.[101] The scaffolds were prepared via salt leaching; different pore sizes were obtained by the addition of varying sizes of salts. Once the polymer matrix was formed, these salt particles were removed via immersion in water, leaving behind a porous scaffold. When single cells of undifferentiated mouse ES cells were seeded on PLLA scaffolds, smaller pore sizes ($<$150 μm) with higher PLLA concentrations (20% w/v) were observed to generate significantly more hematopoietic cells than larger pore sizes and smaller polymer concentrations that were tested. Thus, this study systematically demonstrated that these physical properties of a 3D polymer can dictate hematopoietic differentiation.[101]

Composite scaffolds fabricated from poly[(lactic acid)-*co*-(glycolic acid)] (PLGA)/PLLA have been widely used in tissue engineering applications, since their composite structure imparts tunable degradation rates and mechanical properties.[39] The degradation rate can be tailored by altering the composition ratio, using polymers of different molecular weights, or varying porosity and pore size.[102,103] This composite polymer system has been widely used, because PLGA degrades faster than PLLA, allowing cellular ingrowth, while the PLLA component provides structure and support. The composite scaffold is fabricated by salt leaching, can achieve pore sizes between 150 to 500 μm and to facilitate attachment, is generally coated with fibronectin or Matrigel prior to cell seeding.

A single-cell suspension of EBs derived from human ES cells was seeded onto porous scaffolds made from a 50/50 blend of PLGA/PLLA.[39] Upon addition of the exogenous growth factors TGF-β, activin A, or retinoic acid (RA), the human ES cells differentiated into cartilage, liver, or neural tissue, respectively. Furthermore, histological analysis revealed EC-lined vessels in the constructs, except for those treated with RA. This scaffold was also used specifically to induce neural differentiation upon addition of nerve growth factor and neurotrophin-3 and yielded vascular structures throughout the construct.[104] Porous PLGA/PLLA scaffolds with pore sizes between 212 and 600 μm have also been seeded with human-ES-cell-derived cardiomyocytes.[105] The engineered cardiac muscle was vascularized the most when ES-cell-derived cardiomyocytes were co-encapsulated with mature or human-ES-cell-derived ECs and embryonic fibroblasts. When the triculture system was implanted into a rat model, the vascularized construct was functional and increased tissue perfusion.[106] A PLLA/PLGA scaffold has also been used to engineer skeletal muscle tissue. ECs derived from human ES cells were co-seeded with myoblasts on a porous, biodegradable PLLA/PLGA scaffold, mimicking skeletal muscle tissue.[107] In vitro and in vivo analyses confirmed that co-seeding promoted stable vessel formation consisting of tube structures of ECs surrounded by myoblasts. The addition of embryonic

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fibroblasts increased vascularization dramatically, as validated by increases in EC number, lumenal area, and VEGF expression.

Another study demonstrated that porous poly(glycerol-*co*-sebacate)-acrylate (PGSA) scaffolds support the growth and differentiation of encapsulated human ES cells.[108] In vivo experiments indicated that porous PGSA, and not non-porous PGSA, promoted tissue ingrowth and vascularization.

Hepatic differentiation has been studied on fibrous scaffolds[109] and spheroid foams[110] made from polyesters. Non-aligned, fibrous scaffolds have been created from a 50/50 blend of PLLA and poly(glycolic acid) (PGA) and have been employed in the hepatic differentiation of mouse ES cells.[111] EB-derived cells were mixed with Matrigel, seeded onto a scaffold, and allowed to differentiate for 20 d. The cells showed aggregate growth into the pores of the scaffold, as well as along the fibers of the scaffold, and demonstrated differentiation into hepatocyte-like cells.[109] Porous spheroid foams made from polyurethane have also been seeded with mouse ES cells, which differentiated into mature hepatocytes, as evidenced by marker expression and cell morphology and functionality (ammonia removal) in the presence of specific growth factors.[110]

Modified Poly(ethylene glycol)-Based Scaffolds

Poly(ethylene glycol) (PEG)-based hydrogels have also been modified and studied with respect to ES cell differentiation. PEG is actually a cell-resistant material which is still biodegradable and biocompatible; thus, functionalization of PEG can impart appropriate scaffold properties and responsiveness to cell behavior specifically due to its modifications. For example, PEG-diacrylate (PEGDA) hydrogels micropatterned with Arg-Gly-Asp-Ser (RGDS) in 50 μm stripes were able to reorganize ECs into structures reminiscent of capillaries.[111]

MMP-sensitive sites and RGD sites incorporated into PEG hydrogels have been shown to promote the migration of ECs when embedded into embryonic chick aortic arches.[112] Intriguingly, altering the MMP sensitivity and the presence of adhesion peptides was able to tune capillary sprouting and cell migration.

PEGDA hydrogels formed by photopolymerization promoted chondrogenic differentiation from ES cells.[113,114] Compared to monolayer culture, EBs cultured in the hydrogels in chondrogenic media expressed higher levels of cartilage mRNA and contained cells with a chondrocytic phenotype. A separate study demonstrated that 5-d-old mouse-ES-cell-derived EBs, when differentiated in PEG hydrogels using media supplemented with glucosamine, yielded enhanced chondrogenic differentiation.[115] Furthermore, when these PEGDA hydrogels were modified with RGD, a significantly greater amount of cartilage-specific genes was observed than with unmodified hydrogels.

PEG-based scaffolds may also be incorporated into natural materials. Hydrogels created from dextran modified with PEG promoted chondrogenic differentiation from mouse ES cells.[116] The hydrogel degradation rate could be controlled by altering the degree of substitution of dextran. Intact EBs derived from mouse ES cells were added to the polymer solution prior to gelation, and 16 d after culture, cartilaginous tissue was observed in hydrogels with any of the studied degradation rates.[116] We have developed dextran-based hydrogels crosslinked with PEGDA to enhance vascularization.[117] Varying the functional groups on the polymeric backbone (Figure 4) yielded different hybrid hydrogels that exhibited different biocompatibility, degradation rates, and VEGF release. By systematically

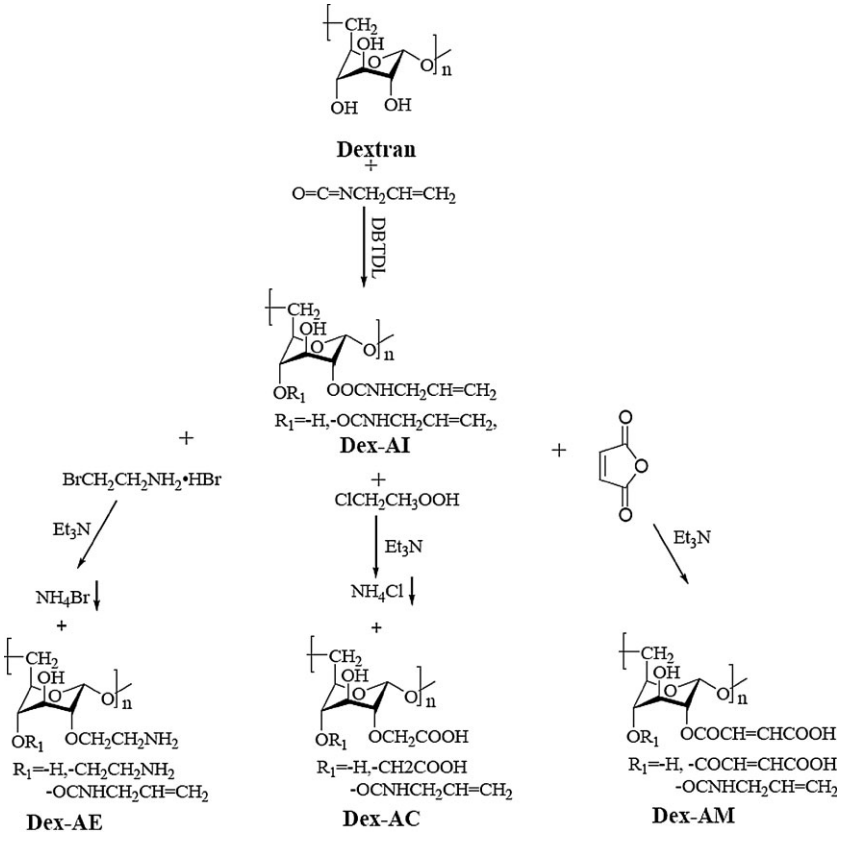

Figure 4. Chemical modifications of dextran backbone in dextran-PEGDA-based hydrogels. Adapted from ref.[118] with permission from John Wiley and Sons.

examining various functional groups and their concentrations, we determined that incorporating amine groups favorably affected mechanical properties and in vivo vascularization.

Physical Constraints

Other methods to control ES cell differentiation using engineered biomaterials have focused on physical constraints, such as nanotopographical cues, EB size, and mechanical stiffness.

Nanotopographical Cues

The ECM presents an abundance of macromolecules with feature sizes at the nanometer scale. For example, collagens, the most abundant ECM macromolecule, exhibit dimensions among the nanoscale regime, with single collagen monomers approximately 300 nm in width and 1.5 nm in diameter[118,119] and self-assembled fibrillar structures extending several micrometers in length and hundreds of nanometers in diameter.[120] Cells interact with these nanometer-sized molecular structures[121,122] via contact guidance, the widely known principle that cellular behavior and function is influenced by physical topography.[123,124] This is thought to occur through the reorganization of membrane-bound integrins, initiating the alteration of the cytoskeleton and modulating the formation of associated focal adhesion proteins that ultimately activate specific signaling cascades, thereby inducing such cellular events as adhesion, proliferation, migration, and differentiation.[125–129]

It is well known that cells receive instructive cues from their topographical environment;[123,130] with the progress of micro- and nanotechnology, many studies have investigated specific cellular behavior in response to engineered micro- and nanoscale substrates[123,131–133] and have demonstrated that topographical geometry and dimension clearly correlate with cellular response. 2D patterned cues have been fabricated in the nanoscale regime, and have demonstrated a profound effect on cellular response, including adhesion,[134,135] motility,[136] and stem cell function.[137] Although there have been recent advances in nanopatterning techniques and complexities,[138,139] these designs have yet to be utilized to study stem cell differentiation. Culturing human ES cells on fibronectin-coated PDMS substrates with nanogratings enhanced such morphological changes to ES cells as elongation and alignment, demonstrating distinct cytoskeletal reorganization via contact guidance while also reducing ES cell proliferation.[127]

Nanofibrous 2D films made of PLLA demonstrated enhanced osteogenic differentiation of mouse and human ES cells in the presence of pro-osteogenic growth factors, as compared to flat substrates.[140,141] In the absence of growth factors, osteogenic differentiation was still observed on nanofiber films, although to a lesser extent than when supplemented with growth factors. ES cell lineage differentiation is strongly affected by the cells' physical interactions with their environmental topography, and coupling these biophysical cues with supplemental signaling molecules, such as growth factors or adsorbed proteins, may have a more pronounced effect on ES cell fate decisions and produce more homogeneous populations of differentiated cell types. Human ES cells cultured on gelatin-coated polyurethane nanogratings differentiated to neuronal lineages after only 5 d in a culture lacking additional differentiation-inducing agents.[142] Clearly, the presence (or absence) of different soluble and immobilized biochemical factors, cooperatively with nanotopographical features, guide lineage specification. These studies demonstrated that the determination of stem cell lineage is affected by the dimension, geometry, and composition of nanosized features.[143]

Electrospinning is another technique that achieves the complex nanoscale features that essentially mimic native ECM topography. This technology produces fibrous networks from either synthetic or natural polymers. An electrical field is used to extrude a charged polymer solution from its source to a grounded collection material, and in doing so, deposits nanosized fibers.[144] Manipulation of operating parameters allows distinct control over specific properties, such as nanofiber diameter and alignment. These environments recreate extracellular topographies and structures in vitro, maintain the ability to present functionalized bioactive molecules, and offer more sites for cell adhesions and interactions due to a high surface-area-to-volume ratio.[145,146] Because of these properties, electrospun materials have already been investigated to determine their effects on ES cell differentiation.

Poly(ε-caprolactone) (PCL), a biodegradable polyester with a slow degradation rate and the ability to maintain its architectural integrity,[143,147] or PLLA are commonly utilized polymers for electrospun matrices.[51,143] Uniaxially aligned PCL nanofibrous scaffolds support the neuronal differentiation of mouse ES cells with directed neurite outgrowth, while randomly organized nanofibrous scaffolds support mouse ES cell adipogenesis.[145,148] Cells are capable of sensing their surrounding environment. An oriented template, such as aligned nanofibers, instructs cell differentiation through contact guidance. However, culturing multipotent adult MSCs on nanofibrous PCL constructs yields either chondrogenic or osteogenic lineages, demonstrating cell-dependent sensitivity to the nanoscale scaffolds.[51,143]

Other methods to create a topographically complex environment include cell electrospinning,[149,150]

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bio-electrosprays,[151,152] and biojetting.[153,154] These techniques can be used to create oriented scaffolds in a controlled manner, allow cell seeding to occur simultaneously, and thus hold great potential for future studies in ES cell differentiation.

EB Size

In vitro differentiation of ES cells occurs through the spontaneous formation of EBs.[155] Differentiation based on EB formation mimics in vivo embryonic development and is being used as a template to investigate the effects of spatial organization on the differentiation to specific cell types. One critical factor of this differentiation strategy is EB size, which has been demonstrated to play a role in specific lineage commitment.[156] A known number of human ES cells aggregated by centrifugation form uniformly sized EBs and exhibit reproducible hematopoietic differentiation. Efficient blood formation required initial EBs formed by more than 500 human ES cells, with optimal erythropoiesis occurring with initial aggregations of 1 000 hES cells.[156] Control over the formation of EBs can lead not only to a more homogeneous cell population, but also to efficient differentiation.[75]

Microfabrication technologies are now being harnessed to focus specifically on spatially controlling EB size to determine its correlation with cell fate.[157,158] Methods such as photolithography are utilized to fabricate polymeric microwells that restrict the spatial microenvironments of ES cells and control the uniformity of EB size.[157–163] Microwells for this purpose are generally fabricated from PDMS, a biocompatible elastomeric polymer amenable to surface functionalization. In fact, different surface functionalizations prove to have varying effects on EB development. PEG microwells coated with fibronectin and subsequently seeded with MEFs, the feeder layer on which human ES cells maintain their self-renewal properties, preserved their viability and undifferentiated state,[162] whereas coating with a cell-repellant and inert material, such as photocrosslinked PEG, actually initiated the formation of homogeneously sized EBs.[159] Coating the bottom of PDMS microwells with a manufactured ECM, such as Matrigel, and then surrounding the walls with a cell resistive molecule, provided an ES cell culture microenvironment which was also conducive to uniform EB formation and possibly regulated differentiation to cardiomyocytes.[160] With the ability to form homogeneous EBs *in vitro*, it was recently determined that larger aggregates enhanced cardiogenesis;[164] also, smaller EBs from more spatially restrictive microwells were found more likely to differentiate to endothelial lineages.[163] With such a size-dependent response of EBs to lineage differentiation, microwell substrates provide an easy and controllable method of forming uniform EBs for high-throughput applications and ES cell differentiation studies.

Stiffness

The mechanical properties of a scaffold regulate cell fate. Synthetic biomaterials are capable of being tailored with controllable mechanical properties, thus impacting differentiation. Synthetic biomaterials are advantageous for cardiac tissue repair, as their mechanical properties can be tailored to withstand repetitive stress and the activity of the infarct without obstructing electrical conduction.[165] A PEG-ylated fibrinogen hydrogel is among the materials developed for cardiac tissue engineering.[166] Cardiomyocytes derived from human ES cells were encapsulated in a photopolymerizable PEG-ylated fibrinogen hydrogel, which induced cell maturation and functionality in two weeks. The hydrogel was fabricated to achieve precise control of its mechanical properties and degradation and was designed to photopolymerize in situ, which would allow the even dispersal of cells and scaffold when used in vivo.

Another study examined the mechanical stiffness of microporous polyurethane tubes on the differentiation of ES cells into ECs.[167] The material properties were tailored to achieve compliance similar to that of a human artery. Under static conditions, the seeded cells expressed smooth muscle actin (SMA) after 4 d. However, when incubated in the presence of a pulsatile flow, the cells oriented in the direction of the flow and expressed endothelial markers. Additionally, cells of the deeper layers of the tube were SMA^{+}, indicating that these conditions were permissive for segregated differentiation into vascular lineage cell types.

Future Perspectives

As clearly demonstrated, not just a single parameter of the surrounding milieu impacts and guides ES cell differentiation; rather, differentiation results from the coordinated actions of various critical parameters, such as scaffold composition, ligand presentation, ultrastructural topography, physical properties, and chemical cues. Indeed, both 2D and 3D environments with micro- and/or nanoscale dimensions, along with immobilized or exogenous factors, profoundly affect the ES cell fate decision, all of which have been developed in an effort to reconstruct the native environment as closely as possible. Future studies to elucidate the effects on the ECM of a wide range of molecules could incorporate developed combinatorial arrays for the high-throughput screening of cell fate.[168]

Emerging technologies in micro-/nanofabrication, drug release, and materials science are enabling the development of instructive environments. Recent achievements emphasize that the fabrication of micropatterned and gradient-based ECM hydrogels with innate nanoscale topography can spatially control cell attachment and

orientation and still maintain the ability to be functionalized with exogenous adhesion molecules.[85,169–171] With the advent of tunable hydrogels, researchers are gradually succeeding in synthetically recreating the native microenvironment.

One important factor to consider is that tissue development occurs in a transient environment with bidirectional signaling between cells and their ECM.[172] However, many of the scaffolds developed so far may not be conducive to incorporating this dynamic behavior to truly mimic the extracellular environment.[173] Cell behavior is constantly governed by instructive biochemical and biophysical cues from the ECM; likewise, cells are continuously remodeling the ECM. Furthermore, the ECM is variable across different cell types,[174] and synthetic environments should be individually constructed with distinct cell lineages in mind.

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