Culture Conditions and ECM Surfaces

Utilized for the Investigation of Stem Cell Differentiation

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INTRODUCTION

Stem cells are a natural choice for cellular therapy and regenerative medicine because of their potential for self-renewal and differentiation into a variety of cell lineages. The ability to efficiently drive stem cell differentiation to the lineage of choice will be critical for the success of cellular therapies. To achieve this objective, it will be necessary to investigate the biological properties of diverse stem cell populations, and expand our understanding of the dynamic microenvironment within tissues that governs stem cell behavior.

While great progress has been made in developing methods for expanding undifferentiated stem cells (a promising source for cell-based therapies), there are limited methods currently available for differentiating certain stem cell types in a defined environment. By manipulating the culture conditions in which stem cells differentiate, it has been possible to control differentiation pathways to generate some lineage-specific precursors *in vitro*. This review will focus on the *in vitro* culture systems currently utilized for lineage-specific differentiation of embryonic, induced pluripotent and adult-derived stem cells. Specifically, we will focus on the use of extracellular matrix molecules and a unique peptide-based hydrogel to mimic the stem cell microenvironment and provide conditions that enable a variety of stem cell types to execute specific differentiation pathways.

STEM CELL TYPES

Stem cells are defined by their ability to self-renew (duplication without loss of developmental potential) and to give rise to multiple cell types (differentiation). They can be further defined as pluripotent stem cells and multipotent adult stem cells.

Embryonic stem (ES) cells are derived from the inner cell mass of the embryonic blastocyst. ES cells were first isolated and characterized in mice[1] and subsequently in humans[2,3,4]. ES cells can differentiate into cells from all three germ layers and give rise to most cell types[5]. The pluripotency of stem cells is controlled by a conserved regulatory network of transcription factors and multiple signaling cascades[6,7]. These regulatory networks maintain ES cells in a pluripotent and undifferentiated state. Thus, ES cells have great potential for clinical use, but the mechanisms that direct ES cell differentiation *in vitro* and *in vivo* are not well understood. Drawbacks to the clinical use of ES cells are the potential for immune rejection and ethical controversy surrounding the use of cells extracted from human embryos.

Recent progress with induced pluripotent stem (iPS) cells derived from differentiated cells has potentially expanded the source of therapeutically relevant stem cells. iPS cells are generated by the forced expression of transcription factors such as Oct3/4, Sox2, c-Myc, and KFL-4[8,9,10,11,12]. iPS cells are thought to be similar to ES cells in many respects, including pluripotency and differentiation potential. Recent work has differentiated both ES and iPS cells under identical methods, on Corning® Matrigel® matrix, laminin, or vitronectin to obtain the same end-product[13,14]. One notable difference between ES and iPS cells is the cellular epigenetic memory, which may bias the differentiation potential of iPS cells toward lineages of the donor cell[15,16,17,18].

CORNING

iPS cells have unique advantages. They are fairly easy to produce, they have been obtained from every human somatic tissue[5], and they have been made from somatic cells from patients with neurological, metabolic, haematological, cardiovascular and immunodeficiency diseases[17]. Thus, iPS cells provide an *in vitro* model suitable for studying specific diseases using patient derived cells and for drug discovery[19,20,21]. However, there are several barriers to using iPS cells for clinical applications. The first obstacle, which is common to ES cells as well, is the potential for teratoma formation. Even a small number of undifferentiated stem cells can result in germ cell tumors. Thus, a key goal is to differentiate ES or iPS cells to the required cell type as efficiently as possible and minimize or eliminate a residual population of undifferentiated cells[11]. Secondly, reprogramming of adult somatic cells via viral transfection alters many of the iPS cells to be unstable and thus limits their use. Furthermore, the efficiency of generating iPS cells is low[7]. However, some of these obstacles have been overcome. Recent work has shown that it is feasible to induce iPS cells without viral integration using adenoviruses, miRNAs, or small molecules that demonstrably increase the stability and transduction efficiency of iPS cells[22,23].

Adult stem cells are found in adult tissue and organs including the heart, nervous system, gut, and skin. Somatic stem cells are present in most tissues of the adult body, and they play a critical role in response to stress and injury. Adult stem cells are more difficult to study than pluripotent stem cells because they are maintained physiologically in a nearly arrested or non-proliferating state within tissues and organs. Growing them *in vitro* requires them to divide, which is contrary to the quiescent state. Evidence for coexistence of quiescent and active adult stem cells in
mammals is found in hair follicle, gut and bone marrow[24]. A potential disadvantage of adult stem cells is that their ability to differentiate and proliferate decreases with age. They have a more restricted differentiation potential as compared with ES cells[25]. However, adult stem cells present few ethical concerns and have low immunogenecity[26].

The most characterized adult stem cells are blood-derived hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC), which are required to maintain bone, cartilage and other tissues[27,28,29]. MSCs are rapidly expanded *in vitro* and sustain differentiation potential to several lineages. The lineage potential and ease of accessibility of MSCs has made them an attractive choice as a cell source for tissue engineering and regenerative medicine[30]. MSCs have been shown to possess the capability to differentiate *in vitro* into a variety of cell types, including adipocytes, osteoblasts, chondrocytes, myoblasts and neuron-like cells. They have also been shown to differentiate into tissue-specific cells *in vivo* after systematic infusion to treat osteogenesis imperfecta and myocardial infarction[31,32]. They can be delivered together with various natural and synthetic biomaterials or scaffolds such as tricalcium phosphate and hydroxyapatite ceramics[33,34], and are currently utilized in preclinical and clinical studies[26,35].

More recently, researchers have reprogrammed adult cells without first passing the cells through a pluripotent state. Fibroblasts have been directly reprogrammed into cardiomyocytes[36], blood cell progenitors[37] and neurons[38]. Directed reprogramming may be capable of generating cell-type diversity that is comparable to that derived from pluripotent stem cell intermediates. One unique therapeutic strategy using direct reprogramming is the *in situ* conversion of cell fate. This was demonstrated when pancreatic endocrine cells were converted *in vivo* into insulin producing endocrine cells in the pancreas of a mouse[39]. A further potential advantage of direct reprogramming is the overall speed and simplicity of the differentiation conditions[40]. Interestingly, reprogrammed cells still need *in vitro* assays that require ECM surfaces to characterize the survival and growth of the newly differentiated cells[36,37,38,41,42].

Stem cells have also been isolated from the heterogeneous mixture of cells within tumors and from cancer cell lines. These cancer stem cells (CSC) have the ability to perpetuate through self-renewal and to differentiate to produce diverse mature cell types[43, 44, 45]. Like stem cells, CSC are controlled by interactions with their microenvironment. Thus, the ECM plays a role in both normal growth and differentiation of stem cells, and in cancer progression[46,47,48]. For a review on the variety of extracellular matrix substrates utilized in culture systems for growing and studying CSC, see REVIEW, March 2012[251].

IMPORTANCE OF MICROENVIRONMENT and ECMs for STUDYING STEM CELL DIFFERENTIATION

Classically, the control of stem cell fate has been attributed to genetic and molecular mediators (growth factors, cytokines, and transcription factors). Increasing evidence has revealed that the self-renewal and differentiation of stem cells is controlled by their surrounding environment, which is known as the stem cell niche. In this niche, stem cells communicate with each other via cell junctions, through interactions with the extracellular matrix (ECM), via receptors, and by engaging in dynamic interactions with hormones and soluble factors. The ECM is composed primarily of glycoproteins (laminin, fibronectin, vitronectin), collagens, proteoglycans, and elastin[49,50]. This material serves to stabilize tissues, promote cell attachment, and modulate cell functionality by specifically interacting with cell surface receptors and activating the associated signaling pathways[51,52,53]. Integrins are the principal cellular receptors that interact with ECM proteins. ECM proteins may directly bind specific growth factors and morphogens to integrate signals within the niche microenvironment[54]. The ECM plays structural, biochemical and mechanical roles in normal growth and differentiation of stem cells[55]. The solid state of the ECM, the nanometer scale geometry, matrix elasticity, and the mechanical signals transmitted from the ECM to the cells have been recently shown to direct stem cell differentiation[30,56,57,220,221,229].

Recent studies have expanded the range of cell types contributing to the stem cell niche, and include mesenchymal stem cells and macrophage. In mammals, the stem cell niche has been experimentally identified in the bone marrow[57,58], nervous system[60,61], intestine[62,63] and skin. While specific components that constitute a particular niche may vary in different tissues, the extrinsic signals that effect self-renewal and differentiation of stem cells appear

to be common, and able to regulate cells at the molecular level through common genetic networks[6].

Along with the plasticity and multilineage potential of stem cells comes an increased need for regulating their growth and differentiation. However, the mechanisms that enable stem cells to respond with flexibility to their environment have not been well defined[65,66,67]. Although it is often represented as a static environment, recent studies have demonstrated that the stem cell niche is spatially and temporally dynamic[12]. This allows the stem cells to integrate long term developmental signals with short term cyclical and injury mediated responses. Because of this complexity, the precise and efficient differentiation of stem cells into distinct cell types and tissues is still a major challenge despite rapid advances in stem cell biology. It will be important to better define the interplay of the ECM in the stem cell niche in order to harness the potential of stem cells for treating human disease.

STRATEGIES for STUDING STEM CELL DIFFERENTIATION

Expansion

In order to realize the potential of stem cell therapies, there is a trend to moving toward more defined systems to reduce the risk of contamination from pathogens common to animal sourced materials and to reduce variability and cost[68,69,70,71,72]. As stem cell research strategies move away from growing stem cells on feeder layers and in non-defined animal-based liquid media, there is also a need to change the culture substrate on which stem cells are grown to make it more chemically defined and less variable[73].

The original culture systems designed for embryonic stem cell expansion utilized mouse embryonic feeder (MEF) layers and non-defined media including serum[1,2,74]. These systems are inefficient for iPSC generation and not applicable to defined, scalable culture for clinical use. Feeder free culture of human embryonic stem cells (hESCs) was first established using Corning® Matrigel® matrix and laminin in conjunction with conditioned media from MEF[75]. Corning Matrigel matrix is a gelatinous, reconstituted basement membrane extract from Engelbreth Holm Swarm mouse sarcoma. More defined protocols with Corning Matrigel matrix were later established to remove conditioned media or serum from the culture systems [70,76,77].

Extracellular Matrix proteins (Laminin, collagen IV, vitronectin and fibronectin) were found to support hESC proliferation in the absence of serum[78] and without Corning Matrigel matrix[76]. Laminin[79], fibronectin[80,81], vitronectin[82,83], and laminin/entactin complex[84] have all been utilized to expand human-derived stem cells. More recently, recombinant Laminin-511 was shown to permit self-renewal of hESCs for at least four months with normal karyotype and the ability to form cells from all three germ layers[85]. Now, we are realizing that there is a synergistic effect of medium, matrix and exogenous factors on human ESC[83].

Li and colleagues were the first to produce a completely synthetic ECM surface[86]. These synthetic polymer matrixes provided xeno-free culture, but only allowed for short term propagation[87]. Using a high throughput screening approach, Brafman et al identified a synthetic polymer that supports the long term self-renewal of pluripotent stem cells[88]. More recently, synthetic peptides have been attached to acrylate surfaces and shown to support proliferation as well as differentiation of hESC into cardiomyocytes[89]. Biomimetic Peptides such as Corning® Purecoat™ ECM Mimetic Cultureware (Fibronectin and Collagen I Mimetic Surfaces) serves as animal-free surfaces for expansion of mesenchymal stem cells[90] as well as other primary and progenitor cell types. These Biomimetic surfaces contain the active cell binding domains of ECM proteins, synthetically synthesized and covalently bound in a functional orientation.

Finally, synthetic hydrogels supplemented with ECMs or growth factors have been shown to maintain short-term pluripotency and the undifferentiated state of hESC in xeno-free culture systems[91,92]. However, such hydrogel systems have not been able to maintain the long-term pluripotency of hESCs using xeno-free culture medium. In contrast, Corning Matrigel matrix coated surfaces combined with a chemically defined medium containing xenogenic proteins provides a feeder-free system that is capable of supporting long-term proliferation of hESC[70].

Differentiation

Traditionally, embryonic stem cells have been induced to differentiate via three fundamental methods, embryoid body (EB) formation, using a feeder layer comprised of stromal cells, or cultured on ECM-based culture substrates[93,94]. EBs spontaneously differentiate into derivatives of the three embryonic germ layers[95] upon removal of factors that maintain the undifferentiated state[96]. EBs can be formed using different methods such as dissociated suspension culture, methylcellulose culture, or hanging drop culture[97,98]. The heterogeneity of EBs and the difficulties associated with producing EBs of uniform size often result in heterogeneous differentiation profiles[3].

EBs have been further terminally differentiated using 2D tissue culture plates coated with gelatin followed by supplementation with growth factors and other bioactive factors that induce differentiation. In addition to gelatin, EBs have been differentiated on ECM culture surfaces such as laminin[15,99],Corning Matrigel matrix[100,101,102], and matrix of human origin from decellularized foreskin fibroblasts[103]. Differentiation of EBs can also occur in 3D hydrogels composed of Collagen or Corning Matrigel matrix[104]. Interestingly, the addition of fibronectin to a collagen-based hydrogel was shown to drive the differentiation of the EBs toward endothelial cells, whereas supplementation of collagen with laminin was shown to produce cardiomyocytes[105].

Differentiation

Co-culturing ESC on stromal cells can provide the advantage of growth factors, but these same factors may lead to the differentiation of stem cells to an undesired cell lineage and may also result in contamination from animal-derived components[106]. In fact, sialic acid contamination has been found in stem cells grown in the presence of animal products[68]. Recently, researchers have utilized human-derived stromal cells as feeder layers, such a human foreskin fibroblasts, to isolate ESCs under xeno-free culture conditions[107]. This strategy does not address other drawbacks when using feeder layers systems for stem cell expansion, including high cost and variability.

Differentiation on monolayers comprised of known ECM-based substrates is one of the simplest protocols. With this method, the type of matrix protein(s) can be well defined. ECM-based surfaces offer considerably more control over the culture micro-environment, and may better mimic the stem cell differentiation niche. In fact, the use of diverse culture surfaces may be an ideal way to
promote differentiation into specific lineages. For example, substrates of different shape, nanotopography, or stiffness have been found to direct MSC differentiation into either adipose or osteogenic lineages, even in the absence of biochemical factors[55,57,108,109,110,111].

In addition to 2D culture, stem cells can be differentiated in 3D culture environments. Tissue engineering approaches have been used to design synthetic and natural scaffold materials[112,113]. Considerable research has focused on identifying biomaterials/scaffolds that can provide a 3D culture system that is conductive to stem cell growth and differentiation, and ultimately is capable of mimicking the *in vivo* microenvironment[25]. A synergistic approach to studying stem cells has brought together cell biologists, biomaterials specialists, and engineers. This interdisciplinary field of stem cell research and bioengineering is focused on creating novel 3D culture systems that promote stem cell differentiation and tissue regeneration. Nanobiomaterials have been developed as substrates for stem cell differentiation[114,109] and approaches such as electospinning, micropatterning and
microengineered cell adhesive substrates[114,115] are being utilized to create artificial stem cell niches[12,61,117]. For these tissue-engineering strategies to be successful, the dynamic relationship between stem cells and the ECM must be understood[50]. This knowledge will enable researchers to more effectively design artificial ECMs that can control stem cell behavior, and further expand our capabilities to produce therapeutically relevant differentiated cell types *in vitro* from adult or pluripotent stem cells[30].

DIFFERENTIATION of PLURIPOTENT STEM CELLS

Many laboratories have utilized purified ECM proteins (e.g., laminin, fibronectin, and collagens) or a more physiological material such as Corning® Matrigel® matrix as a surface for pluripotent stem cell differentiation. Other studies have utilized synthetic hydrogels or scaffolds that are functionalized with ECM proteins or peptides. **TABLE 1** provides an overview of culture surfaces utilized for the differentiation of a variety of cell types (and tissues) derived from pluripotent stem cells.

Importance of 3D Differentiation

The optimization of stem cell differentiation protocols have focused primarily on the timing of administration and concentration of growth factors. However, stem cell fate choices are also critically impacted by ECM: stem cell interactions[30,54,93,97,111]. Although many studies have described ECM preparations, including synthetic surfaces, for the effective attachment and culture of undifferentiated ESCs and iPSCs, the ability of ECM molecules to promote differentiation is less understood. The following two examples highlight the use of Corning Matrigel matrix as a 3D surface for the differentiation of cardiomyocytes, and 3D organogenesis of intestinal crypts.

Recently, Zhang and colleagues have described a novel protocol for the robust differentiation of cardiomyocytes using an overlay system comprised of Corning Matrigel matrix[160]. In this protocol, the sequential application of growth factors onto ESC or iPSC cultures in a Corning Matrigel "Matrix Sandwich" generated cardiomyocytes with high purity, and allowed for the epithelial-mesenchymal transition required for the generation of precardiac mesoderm (FIGURE 1A). The mesoderm differentiation of iPSCs grown in the matrix sandwich was determined by examining the expression of Brachyury. Brachyury positive clusters were markedly increased by Corning Matrigel matrix overlays as compared with controls grown without the Corning Matrigel overlay (FIGURE 1B). Furthermore, the sarcomeric organization of differentiated cardiomyocytes isolated from the matrix sandwich was analyzed by immunolabeling of myofilament proteins alpha-actin and myosin light chain, and confirmed cardiomyocyte differentiation (FIGURE 1C). This novel Matrix Sandwich approach suggests that the addition of ECM in the form of Corning Matrigel matrix provides a favorable microenvironment, complementing the sequential addition of growth factors, to promote a robust differentiation process.

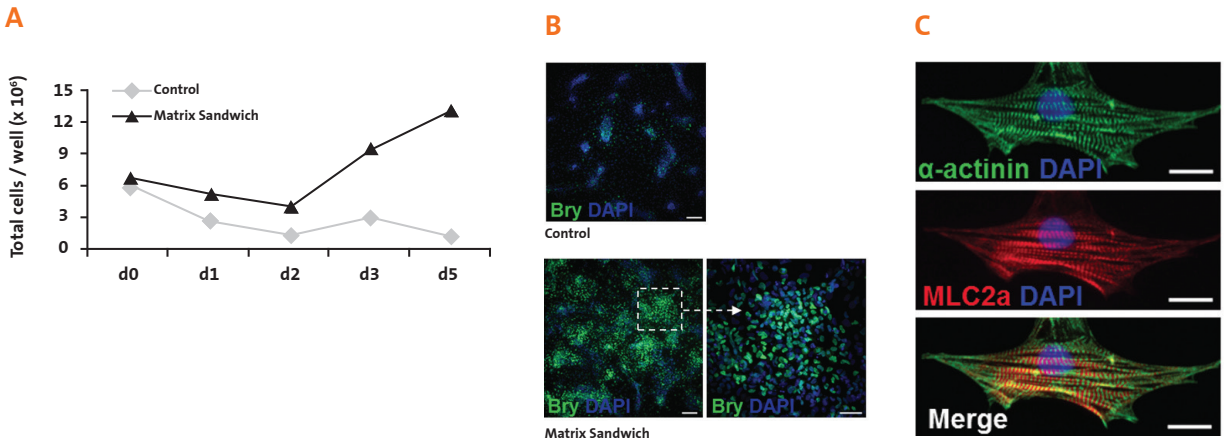
Another example of a study that utilized Corning Matrigel matrix for 3D differentiation of pluripotent stem cells *in vitro* comes from Spense et al[14]. These researchers generated a complex 3D organ from ESC (and iPSC) *in vitro* using Corning Matrigel and a temporal series of growth factor manipulations to mimic embryonic intestinal development (FIGURE 2). Figure 2A shows the 3D intestine-like organoids that formed highly convoluted epithelial structures surrounded by mesenchyme after 13 days grown in Corning Matrigel matrix. Figure 2B-E show the expression of intestinal transcription factors after 14 and 28 days of differentiation and reveal that the epithelium matured into columnar epithelium with villus-like involutions that protrude into the luman of the organoid. This 3D Corning Matrigel culture system has been previously used to differentiate adult stem cells into 3D tissue of the intestine[189], stomach[190] and colon[191]. In addition, this differentiation system has also been shown to support the differentiation of cancer stem cells[192]. Interestingly, recent reports have demonstrated that Corning Matrigel matrix can be used *in vitro* to generate optic cup[169], pituitary[175], and thyroid tissue[174] from embryonic stem cells. Therefore, this differentiation system has broad utility for pluripotent, adult-derived and cancer stem cells.

In addition to Corning Matrigel matrix, individual ECM proteins have been utilized to elucidate the differentiation process with pluripotent stem cells. Battista and colleagues used fibronectin and laminin

interspersed in semi-interpenetrating polymer networks comprised of collagen type I fibers to investigate the influence of the physical and structural properties of the scaffold on mouse ESC differentiation[105]. Interestingly, both the composition and strength of the matrix were found to play a role in the formation of EB from ESCs. The presence of fibronectin in the 3D collagen constructs stimulated endothelial cell differentiation and vascularization of the stem cells. On the other hand, the presence of laminin increased the ability of the ESCs to differentiate into beating cardiomyocytes. These findings highlight the importance of scaffold-mediated biological signals in guiding EB differentiation to specific lineages *in vitro*.

A more recent example of ECM proteins used for stem cell differentiation comes from studies generating retinal pigmented epithelium (RPE). Using a novel co-culture system with an osmolarity gradient, Nistor et al generated 3D retinal progenitor tissue constructs from hESCs[168]. Specifically, hESC derived neural retinal progenitors were co-cultured with hESC derived RPE cells on a cell culture insert coated with a 3D mixture of collagen type I and laminin to derive organized retinal tissue. In a separate study, Rowland and colleagues tested various purified mouse and human ECM proteins (laminin-111, laminin-332, fibronectin, collagen, vitronectin, elastin, and Corning® Matrigel® matrix) to generate RPEs from both hESCs and iPSCs[171]. This work demonstrated that a laminin-111 substrate generated the highest yields of functional RPEs, which was greater than that observed with Corning Matrigel matrix. The identification of a key ECM may help in the future development of a defined, xeno-free, GMP-compliant culture system for the derivation of RPEs that are clinically relevant.

Figure 1. (**A**) Total cardiomyocytes (CM) present per 35 mm well of the control (without Corning Matrigel overlay) or the matrix sandwich culture for 1-5 days with Activin A (100 ng/mL) added on day 0 followed by bone morphogeneticprotein 4 (BMP4) (10 ng/mL) and basic fibroblast growth factor (bFGF) (5 ng/mL) added on days 1 to 5. (**B**) Fluorescent images of day 2 cells differentiated under control conditions (without Corning Matrigel overlay) or the matrix sandwich culture immunolabeled with Brachyury (Bry) antibody. Scale bars are 200 μm for the left and middle panels and 50 μm for the right panel. (**C**) Epifluorescence images of singe CM isolated from the matrix sandwich culture shows sarcomeric organization. Replated CMs form the matrix sandwich culture were colabeled with anti-α-actinin antibody, which marks the Z-lines and anti-myosin light chain (MLC) 2a antibody that shows the A bands in the sarcomere. Scale bars are 20 μm (Zhang et al 160).



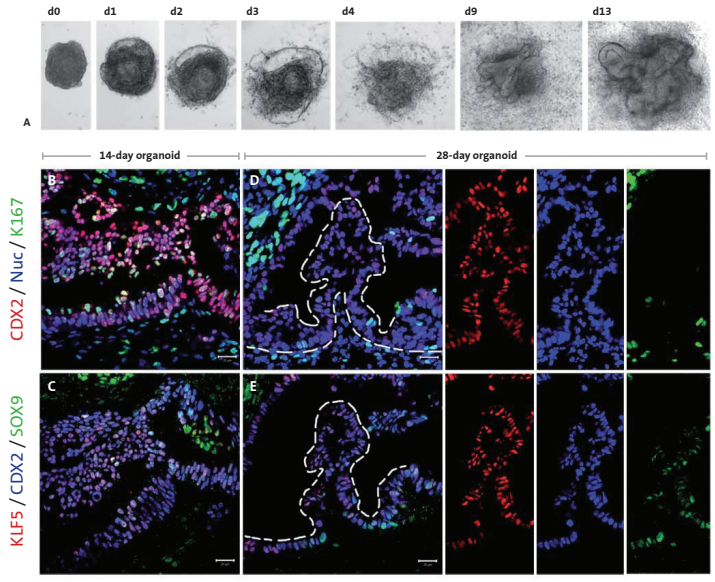


Figure 2. Human ES cells and iPSCs form three-dimensional intestine-like organoids. (**A**) A time course shows that the intestinal organoids formed highly convoluted epitheial structures surrounded by mesenchyme after 13 days. (**B-E**) Intestinal transcription factor expression (KLF5, CDX2, SOX9) and cell proliferation on serial sections of organoids after 14 and 28 days (Spence et al 14).

TABLE 1: SURFACES for *in vitro* DIFFERENTIATION of PLURIPOTENT STEM CELLS

Stem Cell Source	Differentiated Cell Type/Tissue	Surface	Reference
mESC,hESC	Germ Cell Layers:ectoderm, mesoderm and	MEF Feeder Layer	1,2,3,106
hESC	endoderm	EB-Gelatin	118
mESC	Germ Cell Layers:ectoderm, mesoderm and	EB-Laminin	99
hiPSC	endoderm	Corning® Matrigel® Matrix, Vitronectin	119
hpSC	Germ Cell Layers:ectoderm, mesoderm and	3-D Collagen I Gel	120
hESC	endoderm	Corning Matrigel Matrix	121
mESC	Germ Cell Layers:ectoderm, mesoderm and	Laminin-111	122
mESC,hESC	endoderm	Collagen IV	123
hESC	Endoderm	Hydrogels with MEF conditioned media	124
ESC and iPSC	Endoderm	Corning Matrigel Matrix or Feeder Layer	125
hESC	Mesoderm	2-D and 3-D Synthetic Fibrous Scaffolds coated with Corning Matrigel	126
	Mesoderm	Matrix, Laminin, Fibronectin, Vitronectin, or Poly-D-Lysine	
mESC	Smooth Muscle	Corning PuraMatrix™, Corning Matrigel Matrix, Collagen and Laminin	127
hESC	Smooth Muscle	Corning Matrigel Matrix, serum-free	77
hESC and hiPSC	Smooth Muscle	Corning Matrigel Matrix	13
hESC		Collagen, 2-D and 3-D HA Matrix	128
hESC	Neuronal tissue	Poly-D-Lysine/Laminin	129, 130
mESC	Neurons	Poly-D-Lysine	131
hESC and mESC	Neruonal tissue	SFEB Culture	132, 133
mESC	Neural precursors	SFEB Culture	134
mESC	Neural precursors	3-D Collagen Scaffolds	135, 136
mESC	Neural progenitor cells	PLGA and PLLA Scaffolds coated with Corning Matrigel Matrix or	102
mESC	cortical neurons, cortical tissue	Fibronectin	137
mESC	rostral hypothalomic differentiation	EB, 2-D and 3-D Fibrin Scaffold	138
rESC	neurons	Gelatin, Poly-D-Lysine	139
hESC and hiPSC	neurons	Laminin	15
hESC	neurons and astrocytes	EB-Laminin	126
	cortical pyramidal neurons	2-D and 3-D Synthetic Fibrous Scaffolds coated with Corning Matrigel	
hESC and iPSC	Neural epithelial progenitors	Matrix, Laminin, Fibronectin, Vitronectin, or Poly-D-Lysine	13
hESC	Neuronal tissue	Corning Matrigel Matrix	140
mESC	Neurons	EB, Poly-D-Lactide scaffold	141
mESC		Nanofiber Scaffold	142
hESC and hiPSC	Osteogenic	Corning PuraMatrix	14
mESC	Osteogenic	Corning Matrigel Matrix	143
mESC	Osteogenic	Laminin, Corning Matrigel Matrix, Corning Cell-Tak™	144
mESC	Osteogenic	EB-coculture on OP9 feeder layer	145
hESC	Intestinal	3-D tantalum Scaffold	146, 147
mESC	Hematopoietic	Corning Matrigel Matrix	105
hESC	Hematopoietic	Collagen Gel with Fibronectin	101
mESC	Hematopoietic	EB-Corning Matrigel Matrix	104
hESC and hiPSc	Endothelial	Corning Matrigel Matrix	148
mESC	Endothelial	Corning Matrigel Matrix, serum-free	143
hESC and hiPSC	Endothelial	Laminin, Corning Matrigel Matrix, Corning Cell-Tak	125
mESC and miPSC	Endothelial	Corning Matrigel Matrix, Feeder Layer	149
hESC	Endothelial	Corning Matrigel Matrix, Feeder Layer	77
hESC	Endothelial	Monolayer, Corning Matrigel Matrix	150
mESC	Endothelial	GF-reduced Corning Matrigel Matrix	151, 152
hESC	Endothelial	EB-gelatin	151, 153, 154
mESC	Cardiomyocytes	EB-gelatin	105
mESC	Cardiomyocytes	Collagen Gel with Laminin	155
mESC	Cardiomyocytes	EB-Collagen-gel with Corning Matrigel Matrix supplement	156
Chicken ESC	Cardiomyocytes	Laminin, Fibronectin, Collagen on microarray	157
hESC and hiPSC	Cardiomyocytes	Collagen on haluronic acid hydrogels	158
hESC	Cardiomyocytes	Coculture with endodermal cells	89
hESC and hiPSC	Cardiomyocytes	Synthetic peptide-acrylate surface	159
hESC and hiPSC	Cardiomyocytes	Monolayer, Corning Matrigel Matrix	160
hESC	Cardiomyocytes	Corning Matrigel Overlay	161
hESC	Cardiomyocytes	Corning Matrigel Matrix	162
	Cardiomyocytes	EB-gelatin	
	Cardiomyocytes		
	Lung Aveolar epitheial		
	Lung Aveolar epitheial		

TABLE 1: SURFACES for *in vitro* DIFFERENTIATION of PLURIPOTENT STEM CELLS

Stem Cell Source	Differentiated Cell Type/Tissue	Surface	Reference
mESC	Lung Aveolar epitheial	Decellurized Matrix (mouse lung), Corning® Matrigel®, and Collagen I	249
mESC	Lung Epithelium	EB-gelatin	163
mESC and patient specific iPSC	Respiratory epithelium (Tracheospheres)	ECM Matrix	164
hESC	Retina	Corning Matrigel, Laminin, Feeder layer	165
hESC	Photoreceptors	Corning Matrigel Matrix	166
hESC	Retinal Pigment Epithelium (RPE)	Serum-free Adherent Culture, Corning Matrigel Matrix	167
hESC	3-D Retina	Collagen/Laminin or Corning Matrigel Matrix	168
hESC	3-D Optic Cup	3-D Corning Matrigel Matrix	169
hiPSC	RPE	EB-Gelatin	170
hiPSC	RPE	Corning Matrigel Matrix, Laminin-111	171
hiPSC	Photorecptors	Corning Matrigel Matrix	166
siPSC	Photorecptors	EB-Corning Matrigel Matrix, LN, FN	100
hiPSC	RPE	Corning Matrigel Matrix	172
hiPSC	RPE	Serum-free adherent culture, Corning Matrigel Matrix	167
hESC	Thyroid	3-D Corning Matrigel Matrix	173
mESC	Thyroid epithelium	EB-gelatin	163
mESC	Thyroid Follicular Cells	3-D Corning Matrigel Matrix	174
mESC	Adenohyphysis tissue (Pituitary)	Aggregate culture	175
hESC	Hepatic progenitor cells, Cholangiocyte	Collagen I, Corning Matrigel Matrix	177
hESC	like cells	Collagen I	177
hESC	Biliary lineage	Laminin, Collagen I, Corning Matrigel Matrix	178
rESC	Hepatic endoderm	Corning Matrigel Matrix	179
hiPSC	Hepatocyte	Corning Matrigel Matrix	180
hiPSC	Hepatic endoderm	Corning Matrigel Matrix	181
hiPSC	Hepatocyte	GF-reduced Corning Matrigel Matrix	18
mESC	Hepatocyte	EB-Gelatin	182
hESC	Hepatocyte-like cells	Corning Matrigel, conditioned media from HepG2-CM	183
hESC	Hepatocyte-like cells Hepatocyte-like cells	2-D and 3-D Synthetic Fibrous Scaffolds coated with Corning Matrigel Matrix,	126
hESC		Laminin, Fibronectin, Vitronectin, or Poly-D-Lysine	184
hESC	Pancreatic exocrine cells	EB-gelatin	77
hESC	Pancreatic endoderm	Corning Matrigel Matrix	185
hESC, hiPSC	Pancreatic progenators	Feeder Layer	186
hESC	Insulin producing pancreatic cells	Corning Matrigel Matrix	103
mESC	Adipose	Human Foreskin Fibroblast ECM Extract	187
mESC	Chondrocytic	Alginate encapsulated EB	188
mESC	Chondrocytic Chondrocytic	2-D and 3-D Scaffolds of Polyethylene glycol Cartilage extract	104

DIFFERENTIATION OF Adult Stem Cells

In contrast to ESC and iPSCs, there have been no reports to date of adult stem cells differentiating into tumorigenic cells. Consequently, adult-derived stem cells are considered to be a very promising source of cells for tissue engineering and cell-based therapies[7]. Similar to the work described above for pluripotent stem cells, many different ECM surfaces have been utilized to investigate the differentiation of adult stem cells. Both naturally occurring and synthetic molecules have been used. **TABLE 2** provides an overview of the culture surfaces utilized for the differentiation of a variety of cell types (and tissues) derived from adult stem cells.

Physical and Mechanical Cues for Differentiaton

Stem cell differentiation has become increasingly linked to mechanobiological concepts such as cell generated physical force and nanotopological structure. By examining the differentiation behavior of MSCs cultured *in vitro* on different substrates, scientists are beginning to understand the dynamic physical and mechanical properties that control the stem cell niche.

Killian et al have elegantly demonstrated the effect of cell shape on MSC differentiation. This study used fibronectin as a coating on synthetic surfaces of different geometries constructed using a microcontact printing strategy[57]. The shape of the individual synthetic substrate, either star shaped or flower shaped, strongly influenced the cytoskeletal organization within these adherent cells (FIGURE 3A-E). Furthermore, the star shape directed differentiation into osteoblasts while the flower shape directed differentiation into adipocytes (FIGURE 3F). This work established that even subtle shape changes play a significant role in promoting stem cell differentiation. Furthermore, it highlights the critical need for functionalization with ECM or peptides for optimal behavior of the synthetic substrates. Similarily, polyacrylamide gels of varying stiffness were functionalized with collagen and found to drive the differentiation of MSCs to muscle, bone or neuronal lineage depending on the stiffness of the substrate[108,239], and PEG hydrogels of varying stiffness functionalized with laminin affected adult mouse stem cell differentiation[56]. These findings are further supported by recent differentiation studies of MSCs on nanotubes of differing diameters[109]. Oh and colleagues found that as the nanotube diameter increased, there was an increase in MSC differentiation into osteoblastic lineages.

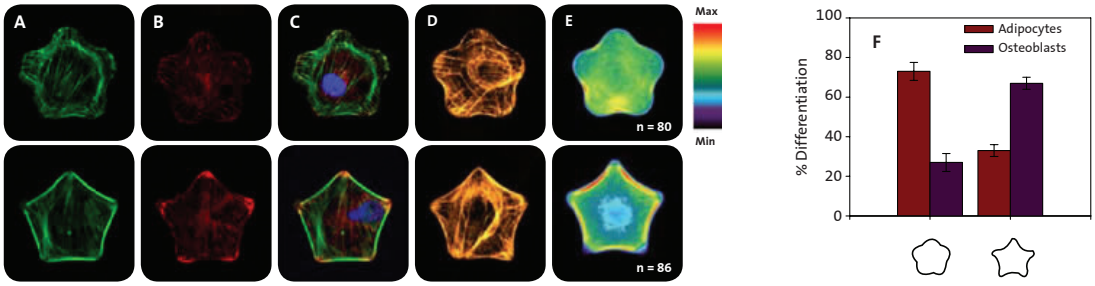


Figure 3 A-E. (**A-C**) Immunofluorescent images of cells in flower and star shapes stained for F-actin (green), vinculin (red) and nuclei (blue). (**D**) Immunofluorescent images of cells in flower and star shapes stained for myosin IIa. (**E**) Fluorescent heatmaps of >80 cells stained for myosin IIa as a quantitative mearsure of contractility (Scale bar, 20 microns). (**F**) Percentage of cells differentiating to adipocytes or osteoblasts when cultured on patterns of the same shape. p- value < 0.01 (Kilian et al 57).

TABLE 2: SURFACES for *in vitro* DIFFERENTIATION OF ADULT STEM CELLS

Stem/Progenitor Cell Type	Differentiated State	Extracellular Matrix or other Surface for Differentiation	Reference
INTESTINAL	Intestinal organioids	Corning® Matrigel® Matrix	193
	Crypt villus structure	Corning Matrigel Matrix	189, 194
	Crypt villus structure	Collagen	195
COLON	Epithelial organoids	Corning Matrigel Matrix	191, 196
	Epithelial organoids	Collagen I	197
STOMACH	Gastric Units	Corning Matrigel Matrix	190
MAMMORY	Functional mammary glands	Corning Matrigel Matrix	198, 199, 200
EPIDERMAL	Keratinocytes	Fibroblast feeder layer	201
	Keratinocytes	Fibronectin, Collagen I, Laminin on micropatterned	202
HAIR FOLLICLE	Neurons, smooth muscle	surfaces	203
	Melanocytes	Corning Matrigel Matrix	203
PANCREATIC	Endocrine cells	Fibronectin	204
	Endothelial	Corning Matrigel Matrix	205
	Pancreatic endocrine and exocrine cells, neurons and glial cells,	Laminin, Collagen IV, Netrin 1, Netrin 4	206
MUSCLE	stellate cells	Corning Matrigel Matrix	56
CARDIAC PROGENITOR	Skeletal muscle	Laminin covaltenly attached to PEG Hydrogel with differ-	207
	Cardiomyocyte	ent stiffness	157
ADIPOSE	Cardiomyocyte	Corning Matrigel Matrix, Collagen I, and Poly-D-Lysine	209, 210
HEPATIC OVAL	Endothelial	Collagen coated HA Hydrogels of varying stiffness	211
HEPATIC PROGENITOR	Pancreatic	Corning Matrigel Matrix	213
	Hepatic or bilary cells	Serum-free Suspension	212
LUNG	Endothelial cells and osteocytes	Laminin, Synthetic polyurathane	214
SALIVARY GLAND	Bronchioalveolar epithelium	Collagen	215
RETINAL	Salivary epithelium	Corning Matrigel Matrix	216
	Retinal neuron	Corning Matrigel Matrix, Corning PuraMatrix™	217
NEURONAL	Rod-like cells	Corning PuraMatrix	218
NEURAL PROGENITOR	Neuron, neuroblasts	Retrinal Neurospheres-ECM	220
	Neuron, astrocyte	Neurospheres, Poly-L-Ornathine, Laminin	223
	Neuron	Neurospheres, Poly-D-Lysine/Laminin	226
	Neuron	IKVAV peptide linked to nanofibrils	228
NEURAL STEM CELLS	Neuron	Corning PuraMatrix + Laminin	220
	Neuron	Corning PuraMatrix	224
	Neuron	3-D culture on Alignate hydrogels	225
	Neuron	Corning PuraMatrix	227
	Neuron Neuron, glial and astrocyte	Corning PuraMatrix, Corning Matrigel Matrix Diff in 2-D and 3-D self assembling peptide hydrogels	229
PRENATAL RAT CELLS		Hydrogel with RGD peptides, Variable moduli of the	221
MSC	Neuron and glial cells	interpenetrating plymer networks (vmIPNs)	231
	Cardiomyocyte	Laminin attached to acrylamide gels of differing stiffness	108
	Cardiomyocyte	Collagen IV	250
	Cardiomyocyte	Collagen I linked to polyacrylamide gels of varying stiff-	232
	Cardiomyocyte	ness	233
	Cardiomyocyte	Corning Matrigel Matrix, collagen, laminin, fibronectin	234
	Cardiomyocyte	Corning Matrigel Matrix	235
	Chondrocyte	Fibronectin on PLGA Thin Film	236
	Chondrocyte	Hydrogel + Laminin	237
	Osteocyte	Fibronectin	108
	Osteocyte	Corning PuraMatrix	238
	Osteocyte	Corning PuraMatrix	108
	Osteocyte	TiO2 nanotubes	239
	Adipocyte	Corning PuraMatrix	209
	Adipocyte	Collagen I linked to polyacrylamide gels of varying stiff-	240
	Osteocyte, adipocyte	ness	57
	Osteocyte, adipocyte	Collagen modified PDMS and polyacrylamide gels	241
	Hematopoetic	Corning Matrigel Matrix	242
	Hematopoetic	ECM peptides conjugated to synthetic polymers	230
	Neuron	FN printed onto PDMS substrate	222
	Neuron	Co-culture with HSCs	108, 110
	Neuron	Corning PuraMatrix	243
MSC + ENDOTHELIAL PROGENITORS	Hepatocyte Microvascular endothelial network	Collagen , LM, FN Corning Matrigel Matrix, Fibronectin, Laminin, Collagen Collagen I linked to polyacrylamide gels of varying stiff- ness FN, Corning Matrigel Matrix	244

Nanobiomaterials

Native ECM is constructed through self-assembly of many nanofibrillar proteins secreted by cells. Thus, the normal cell environment is comprised of a network of ECM molecules with nano- and micro-scale architecture. Although many synthetic hydrogels are successful at providing 3D support for stem cells, most fail to mimic the native dimensionality and functional diversity of the ECM environment.

A peptide-based hydrogel has been developed to study stem cells in a defined and 3D environment, which has been shown to support the growth and differentiation of a variety of cell types when supplemented with known bioactive molecules (e.g., growth factors, ECMs)[224,245,246,247]. This material, Corning® PuraMatrix™ Peptide Hydrogel, contains a synthetic, self-assembling peptide that exhibits a nanometer scale fibrous structure under physiological conditions (average pore size of 50-200 nm). The peptide component is comprised of 16 amino acids with a repeating sequence of Arg, Ala, Asp and Ala [$(RADA)_4$ or RAD16] and the complete material is composed of 1% peptide and 99% water[245]. Corning PuraMatrix gives rise to the spontaneous assembly of a water-soluble beta sheet bilayer structure, providing cells with an attachment substrate that exhibits a structure that is analogous to that exhibited by proteins.

While Corning PuraMatrix has been shown to support cell proliferation, attachment, and neurite outgrowth using established cell lines or primary cell types[248], more recently it has also been used as a substrate for adult stem cell differentiation of neuronal precursors and MSCs. Thornhoff et al, 2008 found that Corning PuraMatrix was the most optimal hydrogel for human fetal neural stem cells, supporting both cell migration and neuronal differentiation. These cells were isolated and cultured as neurospheres, primed with poly-D-lysine and then mixed with different hydrogels including Corning PuraMatrix and Corning Matrigel® matrix[225]. A neural stem cell-like cell type, Muller Glia of the retina, has been shown to differentiate on Corning PuraMatrix[216]. Muller cells exhibited differential expression of neuronal genes when cultured in a 3D system comprised of Corning PuraMatrix, in contrast to cells cultured in 2D on uncoated plates under identical media and growth factor supplementation conditions.

More recently, Ortinau and colleagues have demonstrated the direct influence of a 3D Corning PuraMatrix-based scaffold on the neuronal differentiation of human neural progenitor cells[226]. Cells were grown in Corning PuraMatrix supplemented with laminin (PML 0.25 %). Using transmission light microscopy (FIGURE 4A + B) and scanning electron microscopy (FIGURE 4C + D) to follow the induction of differentiation, these researchers could directly show that neural progenitor cells started to develop a dense network of neuronal processes (FIGURE 4B + D). Immunocytochemistry revealed that after 7 days of differentiation in the Corning PuraMatrix-laminin scaffold, the neuronal precursor cells began to express βIII-tubulin and tyrosine hydroxylase (TH) (FIGURE 4E + F). They concluded that functionalized Corning PuraMatrix peptide hydrogel may be a valuable tool for the generation of defined cell types, and could be utilized for in vitro assays of stem and progenitor cell differentiation.

Moreover, Hamada et al have evaluated the osteogenic differentiation of MSCs, and analyzed the spatial distribution of mineralized bone in 3D using Corning PuraMatrix[237]. Their results were confirmed by biochemical, gene expression and microscopic analyses. In addition to *in vitro* studies, recent work has characterized *in vivo* MSC differentiation into osteoblasts using Corning PuraMatrix. Furthermore, the Corning PuraMatrix complexes were implanted
subcutaneously into rats and demonstrated feasibility for osteogenesis in an animal model system[238].

Corning PuraMatrix has also been used to mimic the functional hematopoietic stem cell niche *in vitro* by co-culturing human mesenchymal stromal cells (MSCs) with human bone marrow derived CD34+ cells[242]. Instead of artificially tailoring the composition of the matrix with the addition of purified ECM molecules, the researchers formulated the matrix naturally by culturing MSCs in Corning PuraMatrix. When grown with the Corning PuraMatrix hydrogel, the MSCs were found to secrete high levels of ECM proteins, which interacted with matrix fibers and formed a meshwork-like structure similar to the *in vivo* marrow microenvironment.

Moreover, recent studies have evaluated whether human MSCs and human endothelial precursors can form microvascular networks on scaffolds composed of collagen type I, fibronectin, or Corning PuraMatrix *in vivo*. Corning Matrigel matrix was used as a control scaffold based on previous *in vitro* and *in vivo* studies. The results demonstrated that vascular density was greatest in more compliant ECM scaffolds comprised of Corning PuraMatrix (5 Pa) and Corning Matrigel (80 Pa), as compared to stiffer substrates composed of collagen and fibrinectin (385-510 Pa)[244]. These findings are consistent with related studies, which show that substrate stiffness directly influences stem cell differentiation *in vitro*[57,108].

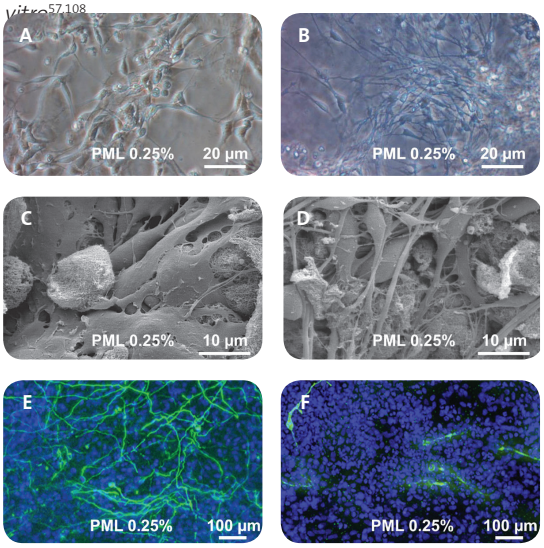


Figure 4. Proliferation and differentiation in 3D scaffold. (**A + B**) Transmission light picture of proliferating cells in PuraMatrix with 0.25 % laminin (PML) and differentiating cells in PML 0.25% (**C + D**). Scanning electron microscope picture of proliferating cells and differentiated cells in PML 0.25%. (**B + D**) Upon induction of differentiation one observes the development of a dense 3 dimensional network of processes. (**E**) Immunocytochemistry for βIII-tubulin and TH of uninduced cells in PML 0.25% and (**F**) Cells after 7 days of differentiation revealed a dense network of βIII-tubulin positive cells. TH+ cells were found to possess processes, but without building up a dense network (Ortinau et al 226).

FUTURE DIRECTIONS

The multilineage potential of pluripotent and adult-derived stem cells presents a challenge as well as an opportunity. Differentiation at the wrong time or in the wrong place or to an undesirable cell type may lead to pathophysiological conditions. Ultimately, scalable animal component–free cell culture surfaces will be essential for the translation of basic research into the clinic. However, these defined surfaces must allow not only for expansion, but also directed differentiation. The convergence of two important disciplines, biomaterials engineering and stem cell research, will allow us to better mimic the physiological complexity of the stem cell niche and ultimately provide the multitude of required cell types for
clinical therapies in humans.

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