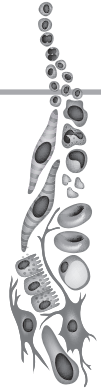

Engineering microenvironments for embryonic stem cell differentiation to cardiomyocytes

Embryonic stem cells and induced pluripotent stem cells have the potential to be a renewable source of cardiomyocytes for use in myocardial cell replacement strategies. Although progress has been made towards differentiating stem cells to specific cell lineages, the efficiency is often poor and the number of cells generated is not suitable for therapeutic usage. Recent studies demonstrated that controlling the stem cell microenvironment can influence differentiation. Components of the extracellular matrix are important physiological regulators and can provide mechanical cues, direct differentiation and improve cell engraftment into damaged tissue. Bioreactors are used to control the microenvironment and produce large numbers of desired cells. This article describes recent methods to achieve cardiomyocyte differentiation by engineering the stem cell microenvironment. Successful translation of stem cell research to therapeutic applications will need to address large-scale cardiomyocyte differentiation and purification, assessment of cardiac function and synchronization, and safety concerns.

KEYWORDS: bioreactor ▪ cardiomyocyte ▪ embryonic stem cell ▪ extracellular matrix ▪ induced pluripotent stem cell ▪ scaffold

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The inability of the human heart to repair itself after trauma renders it vulnerable to ischemia, viral infection or other pathologies. Significant loss of cardiomyocytes is irreversible and may progress to heart failure. Congestive heart failure is endemic; one in three American adults suffers from one or more types of cardiovascular disease [1]. Lack of a sufficient organ supply to fulfill demand coupled with donor compatibility and organ rejection leaves a dire requirement for new therapeutic alternatives. Cell-replacement therapies may be the solution to this deficit.

Many regenerative efforts focus on embryonic stem cells (ESCs) as a possible method for organ repair. ESCs are derived from the inner cell mass of blastocysts and have the potential to regenerate indefinitely and differentiate into virtually any cell type. Because of these capabilities, human ESCs (hESCs) may be a renewable source of differentiated cells for regenerative medicine. However, embryonic differentiation is poorly understood. Of particular interest are robust cardiomyocytes for cardiovascular repair.

Cardiomyocytes are an ideal cell type for implantation to replace diseased or necrotic heart tissue because of their unique electrical, mechanical and biological characteristics. ESC-derived cardiomyocytes display pacemaker-, atrial- and ventricular-like characteristics [2], and spontaneously beat *in vitro*. These cardiomyocytes can integrate, *in vivo*, into the myocardium and improve the function of ventricles after infarction in animal models [3–5]. For example, porcine hearts exhibiting complete blockage were injected with microsectioned hESC-derived cardiomyocytes. Electrocardiogram analysis revealed that the injected cardiomyocytes successfully coupled with native heart tissue and were able to conduct electrical activity [3–5]. Concerns regarding implantation remain, including beating synchrony between implanted cells and native cells, and inflammation at the injection site.

In addition to integration concerns, sufficient hESC populations are required to repair damaged tissue. It is estimated that on the order of 10^9 replacement cells would be necessary to treat a cardiac infarct [6]. Attention has turned to the expansion of hESCs and new strategies for their differentiation. Early work focused on the use of signaling hESCs through the addition of morphogens. Met with limited success, the hESC microenvironment has recently been shown to increase cardiac cell generation. Here, we review strategies that give rise to increased cardiac differentiation by controlling the stem cell microenvironment.

Differentiation strategies & limitations

Common culture methods for propagating undifferentiated hESCs entail the use of a feeder layer of mitotically inactivated mouse embryonic fibroblasts (iMEFs) or feeder-free techniques

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that employ the use of Matrigel™. The feeder layer serves as a support system for stem cell colonies through secretion of various factors and matrix proteins to maintain hESCs in an undifferentiated state in conjunction with basic FGF supplementation [7]. Matrigel is a nonhuman basement membrane extract on which cells are commonly cultured [8]. Derivation and propagation of hESCs under good manufacturing practices without animal products or feeders will be necessary for clinical applications [9].

Human embryonic stem cells are often induced to differentiate by generating 3D suspended cellular aggregates called embryoid bodies (EBs) in the absence of iMEFs and basic FGF (FIGURE 1) [10,11]. EBs recapitulate many aspects of embryonic development and can serve as a model for studying organ development. The complex 3D environment provides temporal and spatial cues that control differentiation into all three germ layers.

Recent progress has been made differentiating hESCs under 2D, serum-free culture by adding exogenous factors to direct differentiation to cardiomyocytes, allowing for better control of the microenvironment [5]. Passier *et al.* employed a 2D co-culture method to induce differentiation of hESCs towards cardiomyocytes. Replacing the typical iMEF feeder layer with mitomycin C-inactivated endoderm-like cell line (END-2), in the presence or absence of serum, led to cardiomyocte formation. Furthermore, the absence of serum enhanced hESC differentiation towards cardiomyocytes [12].

Many studies have examined cellular responses to growth factors. In particular, studies have employed serum-free media, conditioned media or used media additives, such as activin A [5], ascorbic acid [13], dimethyl sulfoxide [14], 5-azacytidine [15], retinoic acid [16] and VEGF [17], to promote differentiation to cardiomyocytes. The *in vitro* functionality of these cardiomyocytes is assessed by visual observation of spontaneous contractions, measurement of the action potential and measurement of intracellular Ca^{2+} oscillations [18]. Numerous signaling pathways, such as TGFβ, bone morphogenic protein (BMP), Wnt and Notch, are involved in cardiomyogenesis [19]. Behfar *et al.* reported that TGFβ and BMP-2 activated cardiac promoters in mouse ESCs (mESCs), leading to the expression of cardiac transcription factors NK2 transcription factor related, locus 5 (Nkx2.5) and myocyte enhancer factor 2C, and cardiomyocyte differentiation [20]. mESC diffentiation methods are often translated to hESCs, but differentiation of cardiomyocytes from hESCs is often less efficient. More recently, translation of differentiation strategies from hESCs to induced pluripotent stem cells (iPSCs) has been investigated.

Induced pluripotent stem cells are a promising alternative source of autologous cells and are derived by reprogramming somatic cells, such as fibroblasts, to an embryonic-like state by inserting, often by virus vectors, factors such as Oct4, Sox2, c-Myc and Klf4, or Oct4, Sox2, Nanog and Lin28 [21]. Cardiomycoytes have been derived from both mouse [22–24] and human [25] iPSCs that have nodal-, atrial- and ventricular-like action potentials and have similar cardiac gene expression, protein expression and spontaneous contraction rates compared with ESC-derived cardiomyocytes. Some reports found the rate and efficiency of differentiation comparable to ESCs [22], and others, slower [23] and less efficient [23,25]. Persistent expression of the transgene c-Myc, a proto-oncogene that presents a potential tumorigenic risk, was observed when using a cardiomyocyte differentiation protocol [25].

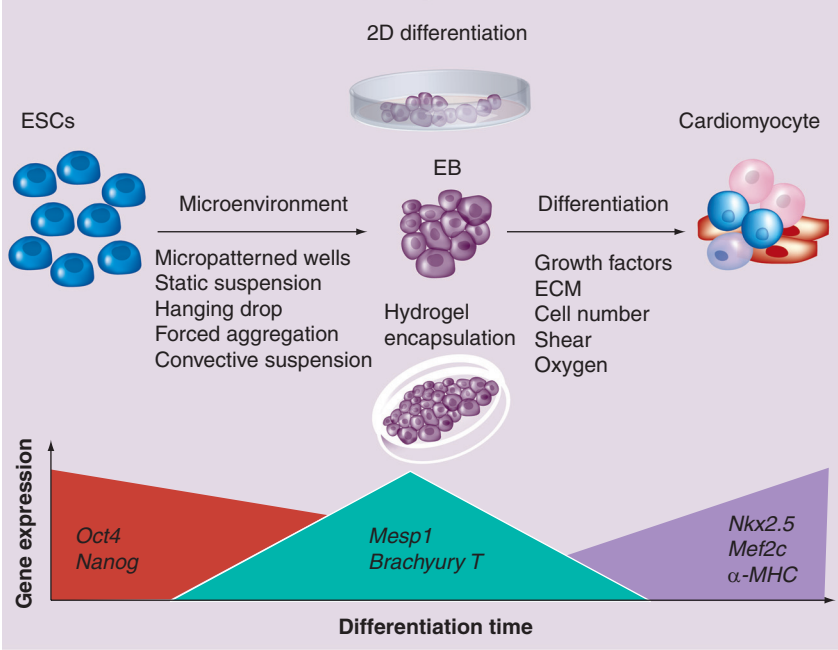

Figure 1. Embryonic stem cell differentiation strategies. ESCs aggregate into EBs via hanging drop, static suspension, convective suspension, micropatterned wells or by forced aggregation. ESCs may also be cultured in 2D plates on various ECM matrices or micropatterned substrates. ESCs may also be embedded in scaffolds. These microenvironments may be subjected to shear and morphogens, for example, to promote differentiation. During differentiation, expression of pluripotency markers, such as Oct4 and Nanog, decreases, early mesodermal markers, such as Mesp1 and Brachyury T, are transiently expressed and expression of late-stage cardiac markers, such as Nkx2.5, Mef2C and α-MHC, increases.
EB: Embryoid body; ECM: Extracellular matrix; ESC: Embryonic stem cell.

Other cardiovascular cell types have been reported, including endothelial, smooth muscle and hematopoietic cells [22,24].

Methods for quantifying cardiomyocyte generation from ESCs vary. The most basic approach is to count the fraction of resulting EBs containing cells that spontaneously contract, providing crude quantification of the effects of an experimental treatment on generating cardiomyocytes compared with a control. This method does not provide information on the cells within an EB. Better quantification can be achieved by measuring the fraction of cardiomyocytes in the total cell population by immunostaining cells for cardiac-restricted proteins, such as sarcomeric myosin heavy chain, cardiac troponin T, α-sarcomeric actin and α-MHC. The number of cells expressing these proteins may be counted with a flow cytometer. This information can be combined with a measurement of the total cell number to calculate the fraction of cardiomyocytes or, when the number of initial ESCs is known, the number of cardiomyocytes generated per input of ESCs – the most useful comparison. However, most or all of this information is seldom provided, limiting rigorous comparisons between different studies.

Numerous challenges need to be addressed before hESCs can be used in clinical and therapeutic applications, such as the ability to generate cardiomyocytes in clinically relevant numbers, purification of the desired cell type and elimination of tumor-formation risk. Differentiation typically yields a mixture of cell lineages of unsuitable purity for regenerative therapies. Residual undifferentiated hESCs within differentiated populations have the potential to form tumors upon implantation. If these challenges can be overcome, hESC- or iPSC-derived cardiomyocytes could be used to treat heart disease, opening a door to a new era of regenerative therapies.

Extracellular matrix

The extracellular matrix (ECM) is a network of macromolecules consisting of proteoglycans and fibrous membrane proteins such as collagen, fibronectin and laminin. The ECM is important in stem cell differentiation, attachment, proliferation and migration during early embryo development [26,27]. In addition, interactions between stem cells and the ECM are important for cell function and maintenance. The ECM evolves during cell differentiation and these changes may cue tissue development.

In considering the influence of the ECM, the role of the basement membrane must also be considered. The basement membrane is in direct contact with cells such as endothelial, cardiac and cardiovascular cells. While its exact role is not fully understood, it likely influences hESC differentiation. The differentiation of EBs towards mesodermal cells can be enhanced through the disruption or absence of the basement membrane [28]. Mesodermal markers brachyury and cadherin 11 were accelerated in basement membrane-deficient EBs with a five- and twofold increase, respectively. In addition, expression of TGFβ, which has been linked to increased differentiation of cardiomyocytes from mESCs [29], increased more than fourfold in basement membrane-deficient mESC-derived EBs in comparison to EBs that maintained their basement membranes [28].

Analyzing ECM deposition during cellular differentiation within hESC-derived EBs reveals that the ECM infiltrates voids between cells as the EB remodels, eventually creating a smooth outer ECM encasing (Figure 2). This ECM shell drastically hinders diffusion by more than 80% for molecules of comparable size to retinoic acid (300 Da), a neuronal cell-lineage promoter [30]. This suggests that the diffusivity of the ECM affects the efficiency of differentiation methods that rely on biomolecular activation. Because typical differentiation methods entail the addition of soluble factors into cell culture medium, the ability of molecular factors to diffuse across the ECM of the EB is important in stem cell differentiation [30].

The heart has a specific ECM composition, which modulates cellular interactions. For example, fibrosis can alter cell signaling, which affects differentiation and plays a role in the progression of myocardial infarction to heart failure [8]. The basement membrane surrounding cardiomyocytes consists primarily of collagen types I and IV and laminin [8]. Cardiogel, ECM extracted from cardiac fibroblasts, consists of collagen types I and III, laminin, fibronectin, proteoglycans and glycoproteins [31]. Cardiogel enhances structural maturation and beating rates of mESC-derived cardiomyocytes when compared with cardiomyocytes cultured on Matrigel or in the absence of a matrix coating [8].

Cardiomyogenesis within mESC-derived EBs is promoted by an ECM protein that is expressed by many different types of cells called secreted protein, acidic and rich in cysteine (SPARC). SPARC is associated with development, remodeling, cell turnover and tissue repair [32]. Studies demonstrate that SPARC treatment resulted in nearly 100% of EBs containing beating

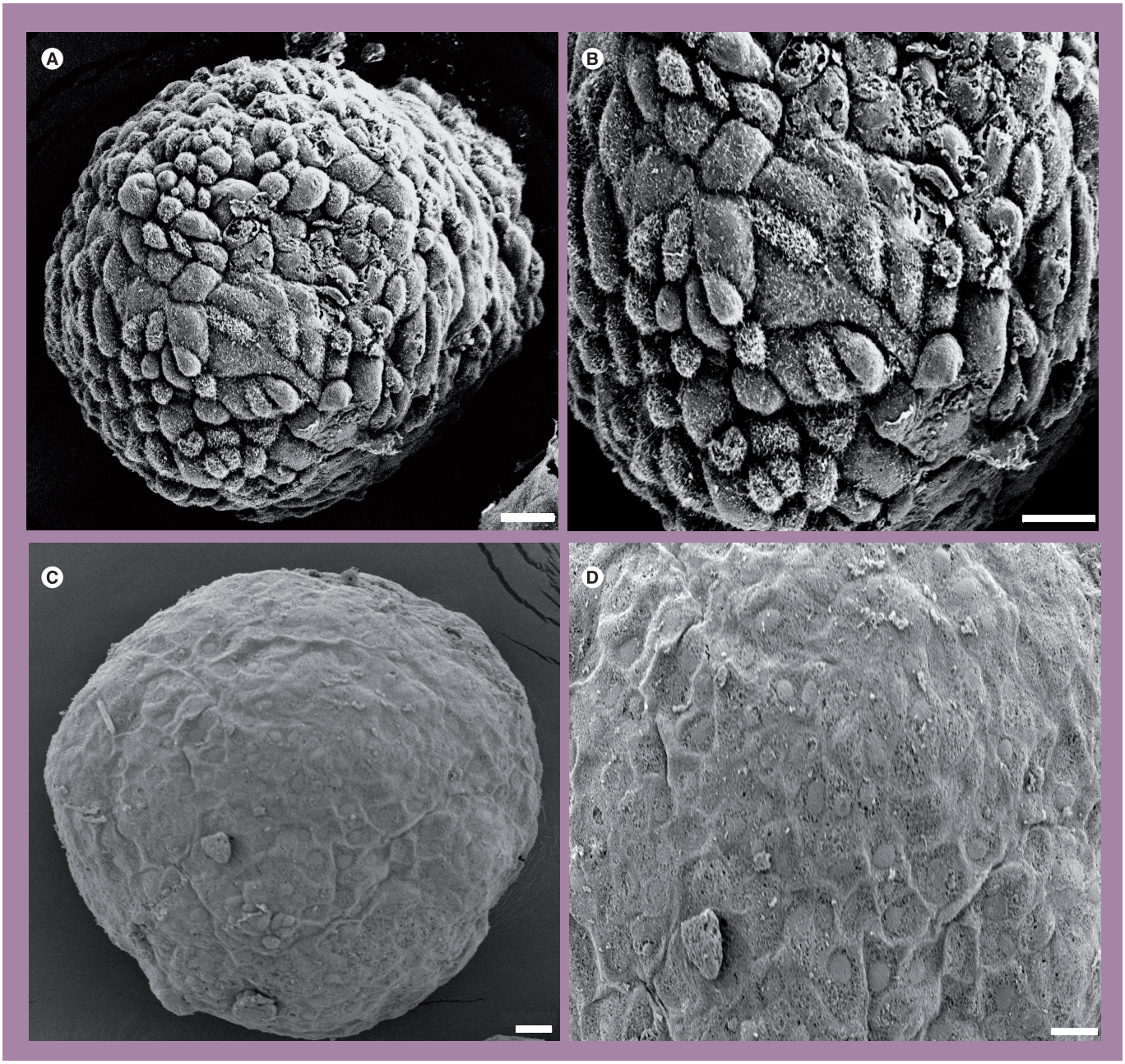

Figure 2. Scanning-electron microscopy images of extracellular matrix deposition on human embryonic stem cell-derived embryoid bodies. (A) Day 3 embryoid bodies (EBs): EBs exhibit distinct cell boundaries. **(B)** Magnification of day 3 EBs. **(C)** Day 11 EBs: cells are virtually indistinguishable as a result of extracellular matrix deposition leading to a smoother outer surface. **(D)** Magnification of day 11 EBs. Scale bars = 20 µm.

cardiomyocytes and an increase in Nkx2.5, BMP-2 and MHCα expression [33]. EBs cultured in recombinant baculovirus-produced SPARC protein also resulted in an increased expression of the cardiac genes Nkx2.5, MHCα and BMP-2, ECM genes collagen IVα3, collagen IVα5 and laminin-α1, and appearance of beating cardiomyocytes [34].

ECM proteins have been examined to determine their role in stem cell-fate decisions. Manipulation of the ECM and the introduction of ECM components into the cell culture environment can provide insight into the role of diffusion and cell–matrix interactions responsible for stem cell differentiation.

ECM components

The ECM is composed of soluble and insoluble components, primarily proteins and proteoglycans. The effect of fibronectin, laminins and collagens on differentiation to cardiomyocytes and other cardiovascular cell types has been

explored (TABLE 1). ECM expression changes as EBs differentiate as a result of cell-fate decisions (FIGURE 3). Collagen, laminin and fibronectin have important roles in hematopoiesis [35]. We will briefly highlight the roles of the major ECM proteins, fibronectin, laminin and collagen, in differentiation, with particular interest in their roles in cardiac and cardiovascular lineages.

Collagen supports cell attachment, provides mechanical stability and influences stem cell differentiation. Collagen type I is found widely in cardiac tissue and blood vessels [36], and has been found to enhance cardiac-cell engraftment [37]. The collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$ have been implicated in angiogenesis [38]. Collagen has been used in a number of regenerative efforts. Collagen scaffolds alone [39,40] and in combination with Matrigel have been used to engineer cardiac tissue constructs in mESCs [41]. Ascorbic acid, a collagen stimulator, has been shown to increase beating areas in hESCs [42], suggesting that enhancing collagen expression improves beating occurences. Exposing mESCs and mESC-derived EBs to ascorbic acid revealed a dose-dependent increase in beating areas [43]. Introducing collagen inhibitors, L-2-azetidine carboxylic acid and *cis*-4-hydroxy-D-proline, to treated EBs led to cardiac differentiation inhibition, suggesting that collagen is essential for cardiac differentiation [43].

Although collagen may offer mechanical stability necessary for contractility, fibronectin is critical in signaling for vasculogenesis. Fibronectin and related integrins mediate cell migration, proliferation, adhesion and signal transduction [27]. Fibronectin receptors ($\alpha_5\beta_1$ and $\alpha_4\beta_1$) and ECM αv receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) have been linked to the promotion of the angiogenesis [44]. Studies conducted using α5- and fibronectin-null mouse EBs revealed a lack of primitive vasculature formation. Fibronectin deficiency in mice proved to be lethal owing to defective cardiovascular and neuronal systems.

Similarly, the role of laminins in cardiac differentiation has been investigated as a possible enhancing factor of cardiomyogenesis. Laminins are a vast group of ECM proteins that are a component of basement membrane. Like fibronectin, laminin promotes cell adhesion, proliferation and differentiation [38]. Studies employing knockout laminin determined that it is dispensable in vasculogenesis, however, the absence of laminin dramatically altered the basement membrane composition [10,45]. Laminin-γ1-deficient cardiomyocytes with poor basement membrane formation resulted in defective electrical signal propagation between neighboring cardiomyocytes. Taken together, laminin is not essential for cardiomyocyte development and differentiation, but is required for the normal deposition of ECM proteins and adequate electrical signal propagation [46].

Scaffolds

Scaffolds have been widely employed in tissue engineering to repair the heart, vessels and other organs. They influence cell adhesion, organization, migration and differentiation. Embedding stem cells into 3D scaffolds and adding various growth factors have been shown to induce differentiation. Ideal scaffolds mimic characteristics of the ECM and support cell integration and function within the host tissue. However, the dynamic ECM makes it difficult to mimic its properties with high efficiency.

Most scaffold materials are biomimetic and biodegradable, such as collagen [47] and poly(lactic-co-glycolic acid) [47], and often

Table 1. Overview of studies examining the effects of extracellular matrix proteins on cell differentiation.

ECM protein	Cell type/model	Result	Ref.
Laminin	Laminin-deficient CM	CM lack BMs and have defective electrical signal propagation. Laminin is not essential for CM development and differentiation	[46]
Laminin–collagen	mESC-derived EBs	Collagen–laminin SIPNs enhanced cardiac differentiation in a dose-dependent manner based on laminin concentration	[26]
Collagen	mESCs and mESC-derived EBs	Ascorbic acid increases beating areas. Collagen is essential for cardiac differentiation and is upregulated by ascorbic acid	[43]
Fibronectin		Fibronectin receptors ($\alpha_5\beta_1$ and $\alpha_4\beta_1$) have been linked to promotion of angiogenesis	[38,44]
BM	mESC-derived EBs	mESCs extracted from laminin γ1-deficient mice. BM disruption resulted from laminin deficiency	[28]
Cardiogel	mESC-derived CM	Enhances structural maturation and beating rates of CM	[8,31]
SPARC	mESC-derived EBs	Enhances and accelerates mesodermal, cardiac and ECM gene expression profiles	[33,34]

BM: Basement membrane; CM: Cardiomyocyte; EB: Embryoid body; ECM: Extracellular matrix; mESC: Mouse embryonic stem cell; SIPN: Semi-interpenetrating polymer network.

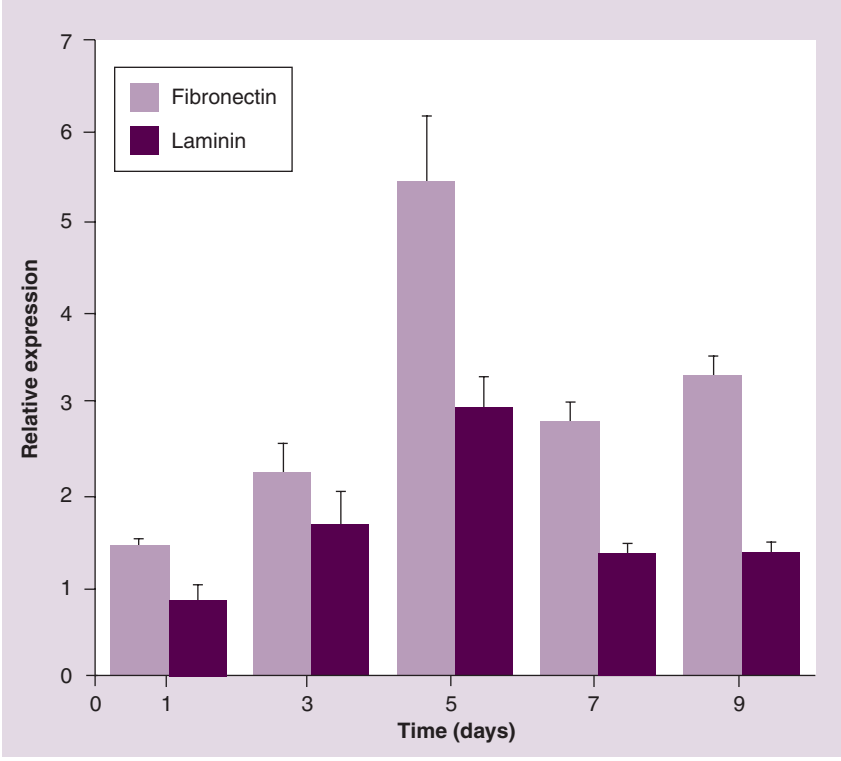

Figure 3. Extracellular matrix component expression versus time.
Fibronectin and laminin expressions within human embryonic stem cell-derived embryoid bodies were measured with real-time PCR and increase initially during differentiation.

incorporate ECM components to enhance cell attachment and influence differentiation, such as arginine–glycine–aspartic acid (RGD) and tyrosine–isoleucine–glycine–serine–arginine (YIGSR). The addition of growth factors and differentiation factors, such as BMPs and activin A, are also used to enhance cell attachment, proliferation and differentiation yields [5,13,15,16]. Battista *et al.* investigated the effect of the ECM on EB differentiation by modifying the fibronectin and laminin concentration within semi-interpenetrating polymer networks (SIPNs). mESC-derived EBs were introduced into SIPNs with different collagen/fibronectin ratios or collagen/laminin ratios. With a fixed collagen concentration (1.2 mg/ml), fibronectin and collagen concentrations varied between 5 and 100 µg/ml, and 1 and 100 µg/ml, respectively. A dose-dependent EB vessel formation enhancement was realized in collagen–fibronectin SIPNs, with vascular vessel outgrowth observed faster at a concentration of 100 µg/ml compared with 5 µg/ml. The collagen–laminin SIPNs enhanced cardiac differentiation in a dose-dependent manner based on laminin concentration with the highest cardiac differentiation observed at 100 µg/ml [26]. These results suggest that ESC differentiation is influenced by chemical cues on the scaffold.

Stiffness, a function of scaffold composition, can be critical for cell integration, adhesion and cell fate. Mature cardiomyocytes aggregate preferentially on surfaces with elasticities comparable to the developing myocardium (11–17 kPa), suggesting that soft materials are optimal for cardiomyocyte scaffolds [48]. In order to create effective and efficient scaffolds, a better understanding of the cell microenvironment is needed.

While polymer scaffolds aim to mimic native ECM, efforts are shifting towards using natural ECM. Acellularized scaffolds have been explored for vessel regeneration and directed differentiation. Acellularized matrices are unique in the sense that they are a mixture of naturally secreted products obtained from various lineage specific cells. These matrices are separated from host cells through physical or chemical methods such as lyophilization or detergent treatments, respectively [49,50]. These matrices are used to house undifferentiated hESCs with expectations of yielding cells from the same lineage as the native cells of the matrix. Acellularized matrices hold promise for applications in tissue regeneration [51] and may provide more control over hESC differentiation with a higher efficiency. Unlike polymer scaffolds, acellularized matrices are able to provide a native structure for seeded cells that contains complex and potentially organ-specific physiological cues.

Embryoid body

Matrix–cell and cell–cell interactions are important in stem cell differentiation and are affected by EB size. Conventional methods used to generate EBs often result in heterogeneous size and shape. Microcontact printing [52], forced aggregation [53–55] and microwells [56] are common methods for addressing heterogeneity.

Microcontact printing is a well-established technique that restricts cell-growth surface area, forcing cells to conform to the patterned protein regions. ECM proteins are 'inked' onto a polymer stamp containing an array of geometric designs and then printed onto a substrate. Areas without ECM proteins are treated to inhibit cell adhesion, yielding a fixed array of protein islands. A cell suspension is washed over the substrate, and cells only attach to regions where ECM proteins are present. EBs are formed by detaching the resulting colonies from adherent surfaces and continuing culture in suspension. This technique allows for the control of cell number, which is dictated by the adhesive area, resulting in colony-size control [57,58].

Altering colony and EB size influences cell-fate decisions. Bauwens *et al.* demonstrated that hESC colony size, controlled using the microcontact printing technique, influences cellular gene expression [52]. Using real-time PCR quantification, they demonstrated that the Gata6 (endodermal marker)/Pax6 (neural marker) ratio increases with decreasing colony size and hESCs with a high Gata6/Pax6 ratio preferentially differentiate to cardiomyocytes. The endoderm secretes procardiogenic factors whereas neural signals, such as Wnt, inhibit embryonic heart development. hESC colony size and EB size are inter-related parameters that influence cell fate by altering gene expression. In another study, Niebrugge *et al.* combined EB size and oxygen control. Using microcontact printing, uniform hESC-derived EBs were generated and exposed to hypoxic (4% oxygen) conditions, resulting in a 3.5-fold increase in overall cardiomyocyte output when compared with traditional EB formation methods [59].

Another technique for controlling EB size is forced aggregation, which entails the centrifugation of a defined number of cells into multiwell plates. Forced aggregation was used to deposit hESCs into round-bottom multiwell plates to examine the effects of size on EB differentiation patterns. Ng *et al.* found that efficient blood cell formation required at least 500 hESCs per well, whereas optimal erythropoiesis was achieved in wells containing 1000 hESCs per well [55].

Control of colony size, the number of ESCs comprising EBs and the gene expression profiles of input cells within EBs are important in influencing cell-fate decisions. Furthermore, controlling the size of EBs can allow for better control of biomolecular and oxygen gradients [60], which may affect differentiation mechanisms. Because many differentiation methods rely on factors to induce differentiation, molecular diffusion and gradient concentrations within cell colonies can produce heterogeneous differentiation.

Bioreactors

Bioreactors allow for the scalable production of cardiomyocytes and other cardiovascular cell types in a controlled microenvironment (TABLE 2). Cardiomyocyte expansion is essential for use in clinical therapies, in which 10^9 cells are needed for therapeutic use [6]. Media in bioreactors is commonly agitated by stirring, rotation and perfusion. Agitation rates can be used to control the shear experienced by the cells. Coupled with process control, important parameters, such as pH, metabolite concentration and oxygen concentration, of the bulk phase can be maintained at a set point.

Convective bioreactors can be beneficial for generating larger numbers of cells and higher fractions of cardiomyocytes compared with static suspension culture. A study with hESCs found that, after 21 days of differentiation, stirred culture at 80 rpm produced 3.8-times more total cells than static suspension culture [61]. EBs from the stirred bioreactor had homogeneous morphology and size [61]. Sargent *et al.* found that a rotary bioreactor increased the fraction of cardiomyocytes generated from mESCs compared with hanging-drop and static-suspension culture [62]. mESCs subjected to the rotary bioreactor at 40 rpm for 10 days produced a final cell population of which 13% immunostained positive for α-sarcomeric actin, a higher fraction than when differentiation was carried out in hanging-drop (6.7%) and static-suspension cultures (3.5%). However, other published work [5] and unpublished work of some of the authors (CK Colton and JR Millman) have achieved higher fractional yields with static-attachment culture, although a comparison with convective reactors was not made. The benefits of the bioreactor were attributed to the shear introduced by the rotation. Lu *et al.* also reported that convective bioreactors were beneficial for generating spontaneously beating cardiomyoyctes [63]. Differentiation of mESCs derived by somatic cell nuclear transfer in the presence of ascorbic acid in a slow-turning lateral vessel at 10 rpm led to spontaneous contraction within 91% of the generated EBs, compared with 71% in static culture.

Combining the advantages of convective bioreactors and a morphogen, Niebruegge *et al.* generated a therapeutically relevant number (4.6×10^9) of nearly pure cardiomyocytes derived from mESCs [64]. These cardiomyocytes expressed α-MHC and cardiac troponin T. They were produced in a 2-liter bioreactor using all-*trans* retinoic acid to promote differentiation to cardiomyocytes and genetic selection to remove other cell types. No teratomas were observed when these cells were implanted into infarcted murine hearts. The cells improved left ventricle function.

Bioreactors have also been used to generate large numbers of undifferentiated ESCs for differentiation to cardiomyocytes. Zur Nieden *et al.* expanded undifferentiated mESCs for 28 days in a stirred bioreactor and in 2D culture, then differentiated the resulting cells in hanging drops [65]. Cells propagated from the bioreactor

Table 2. Overview of studies producing cardiovascular cells in bioreactors.

Species	Cell line(s)	Bioreactor type	Cell yields	Notes	Ref.
Mouse	NT1	STLV	90.6% of EBs beat	STLV increased fraction of beating EBs that are more uniform with no large necrotic centers; SCNT cell line	[63]
	D3	Perfused; stirred	3.17 CM per input ESC	Genetic selection; agarose encapsulation; 4% O_2 and encapsulation increased number of cardiomyocytes	[68]
	R1	Stirred	14.7–17.7% of EBs beat	28 days ESC expansion in bioreactor, then differentiation in hanging drops; bioreactor increased fraction of beating EBs	[65]
	D3	Stirred	N/A	CM differentiation in bioreactor, then seeded them on 3D scaffolds; agarose encapsulation	[69]
	D3	Rotary	13.1% CM	Bioreactor generated more CM	[62]
	R1 and YC5	Stirred	N/A	EBs agglomeration E-cadherin mediated; agarose encapsulation prevented agglomeration	[54]
	CM7/1	Perfused; stirred	23 CM per input ESC; 4.6×10^9 CM	Genetic selection; perfusion increased cardiomyocyte yield	[64]
Human	H9 and HES2	Stirred	49.7% of EBs beat	4% O_2 increased total cell and beating EB number; EBs initially made in micropatterning wells, then transferred to bioreactor	[59]
	H1 and H9	Stirred	Four times increase in total cell number	Stirred flask increasd total cell number and had more homogenous EB size and morphology	[61]
	H9 and H13	Perfusion-fed micro-bioreactor array	23% vascular lineage	Bioreactor size of microscope slide; encapsulation in hydrogels	[70]

CM: Cardiomyocyte; EB: Embryoid body; ESC: Embryonic stem cell; N/A: Not applicable; SCNT: Somatic cell nuclear transfer; STLV: Slow-turning lateral vessel.

produced a final population of EBs of which 15–18% were spontaneously beating, a higher fraction than 8.3% for cells from the 2D culture. The authors theorize this is because cells from the bioreactor were better able to retain pluripotency, expressing higher levels of Oct4.

Hypoxia

Oxygen concentration is an important physiological parameter because of its significance in development [66]. Cells in the early developing embryo are exposed to low-oxygen levels [67]. In static culture, oxygen delivery within the incubator occurs only by diffusion. In bioreactors, convection combined with process control of the oxygen level can virtually eliminate oxygen concentration gradients in the medium, although concentration gradients can exist within cellular aggregates and EBs. Heterogeneity of cellular aggregate size, shape and/or cell number will cause the oxygen gradients to be inconsistent. Fortunately, maintaining convection in the bioreactor can produce homogenous EBs [61] and encapsulating EBs can prevent agglomeration [54].

Low-oxygen culture has been shown to increase the yield of cardiomyocytes. Using genetic selection, 3.8 sarcomeric myosin heavy chain-expressing cells per input mESC were generated under hypoxia (4% oxygen), a factor of 1.5 higher than under normoxia (20% oxygen) [68]. However, the final fraction of sarcomeric myosin heavy chain-expressing cells between the two oxygen conditions were similar – 66.9% for normoxia and 69.3% for hypoxia.

In a separate study with hESCs, differentiation under hypoxia resulted in a 30–47% increase in total cell number and increased expression of some cardiac markers compared with normoxia [59]. However, there was no difference in the fraction of beating EBs observed between hypoxic and normoxic cultures. These two observations suggest that low oxygen increased the number, but not the fraction, of cardiomyocytes in this bioreactor system. In addition, hypoxia increased the fraction of beating EBs by a factor of two compared with culture without oxygen process control [59].

Combination with other strategies

In addition to oxygen control, differentiation in bioreactors can be combined with other strategies for cell production. EBs are often encapsulated to prevent EB agglomeration, which is E-cadherin mediated [54]. Encapsulation allows for controlled EB development such as controlling cell number and EB size. Bauwens *et al* generated 20-times more sarcomeric myosin heavy chain-expressing cells when EBs were encapsulated in agarose compared with unencapsulated EBs [68].

Bioreactors have been used as part of a multistage approach to ESC differentiation. Fromstein *et al.* produced agarose-encapsulated EBs in a bioreactor to generate cardiomyocytes, then seeded them on porous 3D scaffolds [69]. Two scaffold

methodologies were investigated: electrospinning and thermally induced phase separation. Cardiomycoytes on both scaffolds spontaneously contracted; electrospun scaffolds had elongated morphology, whereas thermally induced phase separation scaffolds were rounded.

Bioreactors can be disadvantageous in benchtop research because of their large size and reagent requirements, which makes it difficult and time consuming to explore multiple conditions and perform multiple biological replicates, unlike static culture on multiwell plates. Figallo *et al.* developed a micro-bioreactor array, combining the benefits of multiwell plates and perfusion bioreactors to produce a microfluidic device bioreactor [70]. To demonstrate the usefulness of the setup, hESCs were differentiated to α-smooth muscle actin-expressing vascular cells. Differentiation was more efficient with lower cell densities and increasing shear. Because of the small size scales, microfluidic devices offer the ability to exquisitely control the microenvironment to better understand ESC behavior, but are not suitable for large-scale production [71].

Conclusion

Although ESCs have the potential to differentiate into cardiomyocytes, the lack of efficiency has hindered therapeutic application. Recent advances demonstrate the importance of the microenvironment on the differentiation of ESCs to cardiomyocytes and other cardiovascular lineages. The ECM contains fibronectin, laminins and collagens that influence differentiation, which can be deposited on scaffolds to influence cell adhesion, organization, migration and differentiation. Cell–cell interactions within EBs can regulate differentiation; control of EB size and cell number influences differentiation to cardiac progenitors. Bioreactors can be used to control the microenvironment. Convection and control of soluble oxygen within these systems can increase the yield of cardiomyocytes. Incorporating ECM cues and microenvironmental controls in differentiation strategies will allow for the development of more effective methods for the expansion and differentiation of cardiomyocytes from hESCs.

Future perspective

In order for stem cell-based therapy to advance, differentiation mechanisms for ESCs and iPSCs must be understood and better controlled. Bioreactors, engineered substrates and native ECM scaffolds are current strategies to obtain uniform microenvironments.

There are many challenges to mimicking organ-specific ECM. Additional research on cell–material interaction and mimicking native 3D ECM environments is needed to facilitate cardiac regeneration.

Desirable cell types must be produced in therapeutically relevant populations. Differentiation of pluripotent cells to cardiomyocytes is still

Executive summary

Differentiation strategies & limitations

- Several strategies have employed serum-free media, conditioned media or used media additives, such as activin A, ascorbic acid, dimethyl sulfoxide, 5-azacytidine, retinoic acid and VEGF, to promote differentiation to cardiomyocytes.
- Induced pluripotent stem cells are promising alternatives to human embryonic stem cells (hESCs) and are able to differentiate towards cardiomyocytes.
- While embryoid bodies (EBs) are able to recapitulate embryogenesis, differentiation occurs spontaneously and is poorly understood, yielding a mixture of cell lineages of unsuitable purity for regenerative therapies.
- Residual undifferentiated hESCs within differentiated populations have the potential to form tumors upon implantation.

Extracellular matrix

- The extracellular matrix provides structural support for cells and provides important cellular cues that influence differentiation. Fibronectin, laminin and collagen have been proven to enhance cardiac differentiation.
- Scaffolds provide structural support for cell implantation in regenerative applications. Scaffolds mimic extracellular matrix properties with the hope of enhancing cell survival, cell integration and influencing cell differentiation. Acellularized scaffolds have the potential to direct differentiation based on the native cell type.

Embryoid body

- A common differentiation technique involves creating 3D aggregates termed EBs, which can recapitulate embryogenesis.
- Cell–matrix and cell–cell interactions are important in stem cell differentiation, and are affected by EB size.
- Control of colony size and the number of hESCs comprising EBs plays a role in cell-fate decisions.

Bioreactors

- Bioreactors are a useful tool for maintaining a homogeneous microenvironment and producing large numbers of cells.
- Hypoxic culture conditions and exposure to shear increases the yield of cardiomyocytes.
- Therapeutically relevant numbers of cardiomyocytes can be generated. Bioreactors can be useful in the maintenance of the undifferentiated state, large-scale expansion and differentiation.

highly inefficient. A better understanding of the microenvironmental cues that regulate differentiation is essential for increasing differentiation efficiency and engraphment.

The risk of residual, undifferentiated pluripotent cells within differentiation populations that may form tumors upon implantation needs to be addressed by improving purification methodologies.

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- ▪ of interest
- ▪▪ of considerable interest

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