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CRITICAL REVIEW

Engineered approaches to the stem cell microenvironment for cardiac tissue regeneration

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Micro- and nanoscale engineering approaches in medicine have the potential to recreate physiologically relevant stem cell microenvironments to enhance our understanding of stem cell behaviour and bring stem cell therapy closer to fruition. The realization of such advancements will impact a number of therapeutic applications, the most immediate of which may be the repair of heart tissue. Despite profound advances in creating physiologically relevant *in vivo* stem cell niches through the control of biochemical regulatory factors, further synergism of innovative techniques promise to elucidate the impact of a number of physical cues such as stem cell differentiation into cardiac cells, the electromechanical coupling among these cells, and the formation of bioengineered cardiac tissue grafts. This review examines the recent physiologically relevant micro- and nanoengineering efforts that have been made to address these factors. In Sections II and III, we introduce the traditional focuses of stem cell derived cardiac tissue: differentiation directed by transcription factors and structural cues within the stem cell niche. However, the majority of this review, Sections IV–VII, endeavours to highlight innovative and unconventional microscale engineering techniques that have employed topographic, biomaterial, microfluidic, mechanical, electrical, and optical stimulation for stem cell based cardiac tissue engineering.

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I. Introduction

Heart failure is the leading cause of death in most developed countries. Annually, it affects 25 million people worldwide and costs nearly $503.2 billion in medical management in the United

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States.[1] The occlusion of vital coronary arteries that supply blood and oxygen induces cardiomyocyte death, incapacitating the regenerative capabilities of the heart. Because of the limited proliferative capacity of cardiomyocytes and inability of heart tissue to regenerate after ischemia, the damaged heart undergoes a pathological process that results in the thinning of the heart wall, the reduction of pumping capacity, and consequent heart failure.[2,3] Heart transplantation is currently the only therapeutic modality for a failing heart; however, due to the ever-present shortage of donor organs it is important to search for alternatives such as the stem cell (SC) based engineering of cardiac tissue. The ability of SCs to differentiate into cardiac and cardiovascular lineages empowers SC therapy as a potential method for the generation of cardiac tissue that can replace the infarcted myocardium.[4–9]

Several types of SCs with variable degrees of differentiation capacity have been used to study cardiogenetic differentiation. Resident cardiac stem cells (CSCs) isolated from myocardium have the potential to differentiate into multiple myocardium cell types; however, it is not well understood why endogenous CSCs cannot more effectively regenerate myocardium (Fig. 1A). Potential SCs include embryonic SC (ESC), which have the highest level of pluripotency of all SCs, multipotent adult SCs (*e.g.* mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs)) and induced pluripotent SC (iPSC), which can be generated from fibroblasts by the retroviral transduction of transcription factors such as Oct3/4, Sox2, Klf4, Nanog, and c-Myc.[10–17] SCs are regulated by biophysical and chemical factors that dictate cell phenotypes, self-renewal, and differentiation during cardiac tissue development.[18] The cardiac constructs at the desired stage of development are transplanted into the injured heart yet the search for optimal transplantation modalities ranging from non-differentiated SCs to functional, beating cardiac tissue constructs is still ongoing.[19–22] In fact, there are several controversial issues regarding regeneration mechanisms and the condition of the infarcted myocardium upon employing SC-based cell/tissue therapy. For instance, inflammation and fibrosis are characterized as barriers that prevent the regeneration of cardiac tissue and thus reduce the success of exogenous cell therapies (Fig. 1A). However, some degree of fibrosis and inflammation might be necessary to prevent myocardial rupture and promote progenitor-cell recruitment, respectively.[4] Furthermore, the observation of bone-marrow (BM) derived cardiomyocytes in the heart indicates the migration, albeit a very low rate, of BM-derived progenitor cells to the heart and the differentiation of these cells into cardiomyocytes. Nevertheless, inadequate levels of mobilization and multipotency limit the efficiency of BM-derived SCs as a technique for cardiac regeneration.[4] From the collective research in the field, it is evident that multiple regulatory elements are present in the hostile microenvironment of injured myocardium and influence both endogenous and exogenous SCs (Fig. 1B). The extracellular cues delivered by support cells, neural cells, progenitor cells, and other cardiac cell types in close proximity of SCs are essential cell–cell factors to direct SC behaviour. Knowledge of such interactions is vital to the successful development of functional cardiac regenerative therapies.

The *in vivo* microenvironment of SCs, often referred to as the SC niche, plays an essential role in SC differentiation and self-renewal. Considerable efforts have been made to elucidate the mechanism of niches that maintain the ability of SCs to replenish themselves through survival, self-renewal, and differentiation toward particular cell types including cells that exist within a cardiac tissue milieu.[23] Native CT is composed of tightly packed myocytes, fibroblasts, and supporting vasculature along with the surrounding extracellular matrix (ECM).[20] An endomysial collagen network surrounds the layers of myocytes, typically 2–5 cells thick. Fibroblasts that lie within the endomysial collagen network form an interconnected network of cells and contract the endomysial collagen by application of mechanical forces on the myocytes. The high cell wall stress and mechanical deformation that the myocytes are exposed to are transduced into intracellular signals that regulate cell function and fate. CSC clusters accumulate within the atria and apex in adult hearts and differentiated myocytes surround the clusters of CSCs and other highly prolific, amplifying cells. The transcription factors of the cardiac (GATA-4), myocyte (MEF2C), smooth muscle cell (GATA-6), and endothelial cell (EC) lineages are expressed within these amplifying cells.[24]

The physical and chemical cues derived from ECM materials influence SC behaviour in a combinatorial manner, thus the separation of these cues during SC investigation is not possible. For this purpose, we classify the role of naturally derived scaffold and engineered materials as structural cues. As shown in Fig. 1C, structural and several other physical factors such as fluidic shear stress, mechanical cyclic loads, electrical fields, and optical signals have been studied to control stem cell fate. Additionally, further efforts might elucidate the role of other physical factors such as thermal gradients or magnetic fields for SC-based cardiac tissue engineering. Towards this direction, micro- and nanoscale engineering in medicine offer powerful tools to investigate such physiologically relevant microenvironmental factors and consequently may pave the way for the design and implementation of a fully automated biological system on-chip for cardiovascular regeneration.

Microscale technologies are well suited to assist SC research through the creation of innovative systems allowing the modulation of niche components.[25,26] For instance, micropatterns of ECM islands can control the diffusion of secreted growth factors, which affect a cell's shape and consequently the differentiated

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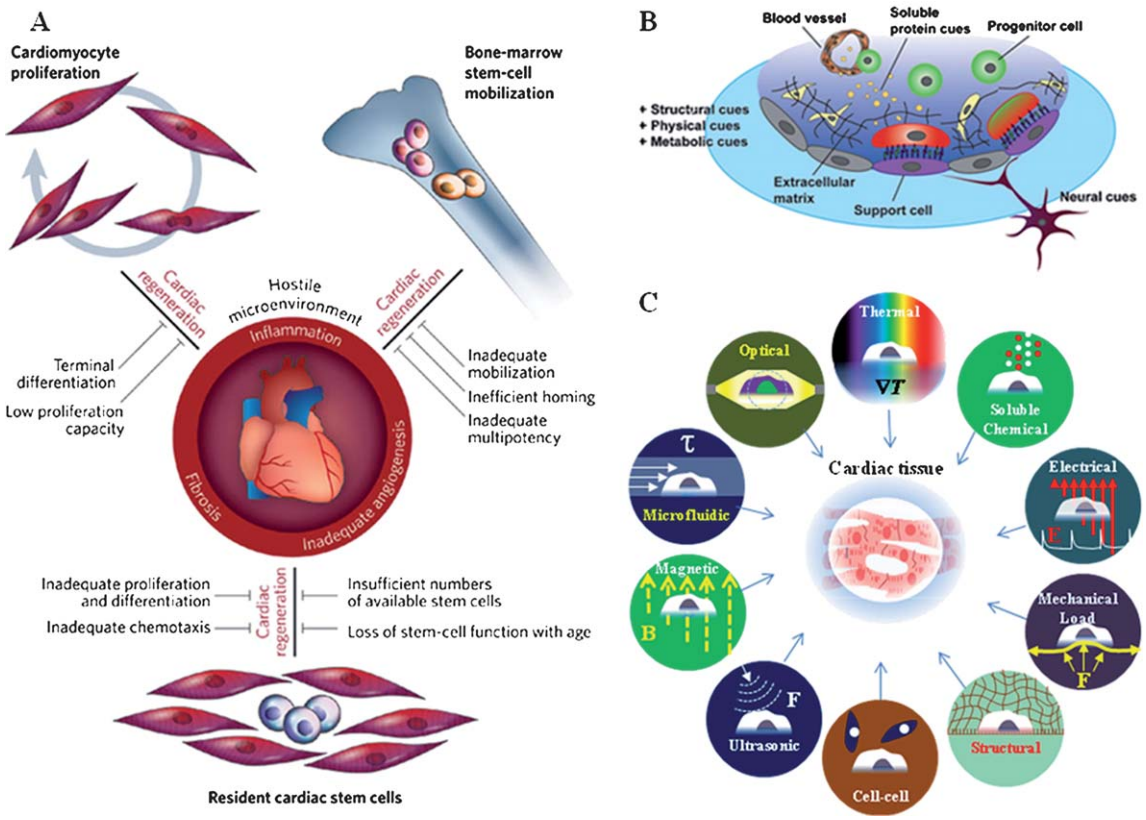

Fig. 1 Mechanisms of endogenous cardiac regeneration, *in vivo* stem cell niches, and exogenous stem cell stimulation methods. (A) Stem cells can be differentiated into cardiomyocytes and support native cardiomyocytes with limited proliferation/differentiation capability. (B) The architecture and composition of *in vivo* stem cell niches includes several factors and the mimicry of *in vivo* stem cell niches for cardiac tissue regeneration is a critical and challenging issue.[4] (C) *In vitro* stem cell stimulation with control of several physical and chemical factors can enhance our understanding of the mechanisms involved in stem cell niches.[42] Also, lesser-studied factors such as magnetic, thermal, or optical non-invasive methods may provide alternative means for directing cardiac tissue regeneration.

cell lineage.[27] Based upon these studies, the cells adhere to stiff substrates more strongly than to soft substrates.[28–30] Innovative protein-based microarrays have been developed through microscale technologies to examine protein–protein interactions (*e.g.* collagen I, human collagen III, human fibronectin) and are useful tools to explore the complexity of SC niches.[31] Additionally, microfluidic systems have been employed for the precise control and delivery of soluble biochemical factors with dynamic controls for the directed differentiation of SC cultures. High throughput, single cell analysis performed in a microfluidic system is an efficient means to study different cell behaviours including apoptosis and changes in cell-cycle kinetics. Multiplexing enables screening a large number of cells while minimizing the amount of culture medium and other chemical reagents used.[32] Furthermore, greater understanding can be obtained in highly integrated biological microprocessors on-chip, which allows systematic studies of combinatorial physical and biochemical factors to control SC fate.

The following sections of this review describe several innovative microscale techniques for the exogenous stimulation of SCs, with a particular focus on cardiomyocyte differentiation. In recent years, several papers have reviewed the common applications of physical or chemical stimuli in microscale devices for SC-derived cardiac tissue applications, with focuses on transcription factor regulation and engineered substrates.[33,34] Herein, after a brief review of the biological and chemical issues associated with cardiac tissue derived from stem cells in the next section, we focus on unconventional yet promising techniques for SC-based cardiac tissue engineering. We highlight engineered devices that utilize microfluidic, mechanical, electrical, thermal, and optical stimulants for cardiac development and introduce further points of exploration that might be applicable for the future research of SC-based cardiac tissue engineering.

II. Related biological and chemical background

Soluble biochemical factors, extracellular matrixes (ECMs), and physical stimuli such as mechanical, optical, and electrical cues have been identified as keys to SC fate determination.[18,35] The precise control of such physiologically relevant microenvironmental factors may enable the regulation of cell behaviours, including cell level phenotype, function, differentiation, and integration before and after implementation into a host's failing heart.[33]

The control of a multivariable biological system to achieve an optimum SC-based cardiac construct cannot be possible without a clear systematic understanding of SC regulation factors and their combinatorial effects. Such a system should be able to

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facilitate the regulation of SC factors post-implementation in order to induce coupling between the cardiac construct and host heart cells, and minimize adverse effects such as tumorigenesis and arrhythmia.[36,37] An optimal cardiac construct will exhibit similar contractile properties, architecture, and composition of native tissue.[22] Video analysis of spontaneous contractile cardiac tissue has been demonstrated to be a powerful tool to assess volume change and beating frequency.[38] Cellular markers have traditionally been used to identify cell specific differentiation; however, in many cases no unique marker is available for the identification of cell types. The cytoskeletal proteins β-myosin heavy chain (β-MHC), α-sarcomeric actin, cardiac troponin T (cTnT), and Nkx2.5 are common cardiac specific markers, and some markers are expressed in multiple cell types.[34] For instance, the transcription factor GATA1 is a protein that regulates genes involved in embryogenesis and myocardial differentiation. The markers specific to smooth muscle cells (SMCs) include smooth muscle α-actin (SMA) and smooth muscle myosin SM22.[34] These markers enable powerful biological immunostaining as a tool to investigate the role of certain stem cell niches. As previously mentioned, several biological, chemical, and physical niches affect SC fate and direct differentiation to a specific cell type. Among these, soluble biochemical factors are widely used for SC fate. In fact, these factors induce signal transduction to modulate cellular behaviours that include survival, growth, self-renewal, and differentiation.[33] For example, basic fibroblast growth factor (bFGF) is used to maintain the self-renewal of human ESCs (hESCs) and vascular endothelial growth factor (VEGF) is a signal protein for the differentiation of adult SCs into cardiovascular lineages.[39] A summary of important soluble factors applied on various SCs for directed cardiac differentiation, such as mesoderm derived from ESC and BM-derived MSCs, in addition to related markers, such as Bone Morphogenetic Protein 2 (BMP2), is shown in Table 1. In addition to bFGF and VEGF, other chemical factors including 5-azacytidine (a nucleoside in DNA and RNA), transforming growth factor-beta (TGF-β1), platelet-derived growth factor (PDGF) and CGA7, a marker for differentiated vascular smooth muscle cells, are widely used for cardiac stem cell stimulation.[39–43]

Soluble chemical factors are essential for the directed control of the stem cell microenvironment and with any effort to generate native like cardiac tissue, the selected tissue factors in Table 1 are critical to assess the effectiveness and accuracy of the employed reprogramming/differentiation cues. Additionally, there is a correlation between the physical factors of the stem cell microenvironment and the biochemical factors within the cell, which are traditionally manipulated in stem cell research. As we describe in the following sections, physical factors can be controlled with engineered approaches to manipulate both the stem cell niche and regulatory biochemical factors within stem cells.

III. Structural cues of engineered ECMs

Within any living organism, the ECM has multifunctional roles that influence the cells in which it surrounds. The functions of this porous polymeric structure include biochemical signalling, biomechanical signalling, supply of nutrients, and the removal of metabolites.[44] The architecture and molecular composition of the ECM mediate cellular activities by the precise spatial and temporal control of hydrodynamic, electrical, mechanical, and chemical cues, and the combinations of such. One challenge is to develop smart biomedical materials that have the ability to mimic the structural dimension, topography, porosity, and chemical composition of the ECM in a combinatorial manner. It is evident that the development of such materials requires a deep understanding of all ECM function. In order to enhance our understanding of ECM functions, it is important first to study each role of the ECM (*e.g.* mechanical, chemical, electrical, and topographical cues) individually using engineered materials and devices, and the combined cases. For instance, SCs can be cultured on substrates of various stiffnesses (in the presence or absence of other chemical factors) in order to elucidate the role of ECM stiffness and rigidity involved in the regulation of SC function.[45] In reality however, it is not always possible to isolate factors within the SC niche and study them individually. This section analyzes recent advances in systems that mimic and control the structural cues of SC niches. Table 2 highlights the structural cues of naturally derived ECMs, synthetically engineered ECMs, microchambers for EB formation, and micro- and nanoscale topographically patterned structures (TPS) for cardiac SC stimulation.[46–52]

Naturally derived ECMs have been widely used as scaffolds to regulate SC activity, including differentiation into cardiovascular cells. In such matrices, heart tissue is decellularized, populated with cardiogenic cells, and the adequate chemical or physical factors are applied for cardiac tissue development. The cells gradually remodel this initial matrix and replace it with their own.[22] Recent advances in SC biology have demonstrated the role of cell–Matrigel[53] interactions in the organization and differentiation of SCs.[54–56] Based upon these studies, hESCs on Matrigel with mouse embryonic fibroblast (MEF) conditioned medium can maintain pluripotency under a feeder-free condition.[55] When ESCs cultured on different types of naturally derived ECMs, including Matrigel, laminin-1, collagen I, and Cartrigel (without MEF-conditioned medium), they exhibited the highest differentiation potential on Matrigel.[56]

Despite the main advantages of naturally derived materials[57,58] used for SC stimulation, biodegradable synthetic materials such as poly-L-lactic acid (PLLA), polyglycolic acid (PGA), and poly (lactic-*co*-glycolic acid) (PLGA) have also attracted the attention of researchers. The mechanical properties of these temporary synthetic scaffolds can be better controlled and are more suitable for the study of several cellular activities including adhesion, migration, and the survival of transplanted and native cells.[59–62]

Micro- and nanoscale fabrication techniques have enabled the fabrication of substrates that replicate the native surface topography associated with extracellular matrix molecules.[63–65] The role of naturally occurring nanotopographic structures within the ECM has been demonstrated to effect cell migration, polarization, SC differentiation, and tissue organization.[66] Of particular interest in this review are topographically patterned structures (TPS) for SC-derived cardiac tissues. SC differentiation is influenced by cellular adhesion to TPS and the fundamental physics governing adhesion can be analytically modeled.[67] The process of cellular adhesion is associated with a decrease in free energy of the cell membrane–TPS system. The energy is estimated by summing the contributions of chemical

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Table 1 Role of soluble chemical factors for cardiac stem cell stimulation[a]

Factor	Cells	Effect	Ref
VEGF	Flk1+ mesoderm from murine ESC	Cardiac and EC differentiation: appearance of PECAM1+ sheets of endothelial cells and CGA7. A collagen scaffold was used	39
TGF-β1, BMP2	Murine ESC	Cardiac differentiation: up-regulated Nkx2.5 and myocyte-specific enhancer factor 2C (MEF2C). Significantly enhanced beating of ventricular myocytes in synchrony with host cells. A gelatin coat was used	40
5-Azacytidine	hMSC	Cardiac differentiation: high expression of β-MHC, desmin, α-cardiac actin, and cardiac troponin T. Cultured on SCs *ex vivo* then implanted	41
TGF-β1	CD117+ mouse BM-derived SC	Myogenic differentiation: up-regulated the expression of the transcription factor GATA-4 and Nkx2.5	42
PDGF	Mouse ESC	Cardiac differentiation: increase of cardiac MHC (cMHC) α/β expression and the percentage of beating EBs. A serum-free environment was used	43

[a] ESC: embryonic stem cells, EC: endothelial cells. hMSC: Human mesenchymal stem cell, EB: embryonic body. PECAM1: platelet endothelial cell adhesion molecule. MHC: myosin heavy chain, TGF-β: transforming growth factor-beta, BMP2: bone morphogenetic protein 2.

energy associated with the formation of ligand–receptor bonds, generation of nonspecific interfacial interactions, and the elastic energy stored within the deformed membrane bilayer. This simplified model assumes that the cell rests on a non-planar surface with a sinusoidal profile described as $h(x) = h[1 - \cos(2\pi x/\lambda)]/2$ where h and λ are the wave amplitude and wavelength of this profile, respectively. The dimensionless total free energy governing cellular adhesion is given by the summation of the three contributions (ligand–receptor binding, nonspecific interactions, and bilayer deformation) and defined as the function of the ligand density of the bilayer, the receptor density of the substrate, the density of new bonds formed (the binding energy factor, the cell membrane thickness, and the Young's Modulus of the cell membrane).[67] As is the case with many other events of the

Table 2 Role of structural cues for cardiac stem cell stimulation[a]

Factor	Structure	Cells	Application	Effect	Ref
Naturally derived ECM	GFF Matrigel	Mouse ESC	Myocardium restoration	Injected into the area of ischemia, restored the heart's geometry and function	46
Chemical-structural	RGD modified alginate	hMSC	Myocardium repair	Cells encapsulated with alginate prevented infarct wall thinning and negative LV remodeling	47
Engineered 2D ECM	Fibronectin on PLGA	hMSC	Myocardial differentiation	Up-regulated myogenic expression level of β-MHC and cTnT	48
EB size regulation	PEG concave cylindrical wells	Mouse ESC	SC fate *via* EB size control	Cardiogenesis and EC differentiation enhanced in larger and smaller EB sizes respectively	49,50
Mechanical-structural	PGS accordion-like honeycombs	NRHC	Regeneration of native-like cardiac tissue	Matched architecture and mechanical properties compared to native myocardium	20
Nanoscale topography	PEG nano-grooves and ridges	NRVM	Improvement of myocardium architecture	Well organized myocardium: improved cell–cell coupling and fast propagation of APs	51
Microsacle topography	Polystyrene, microgrooves and ridges	NRCM	Improvement of myocardium organization	Arrangement of gap junctions in heart tissue rather than the punctuate distribution in NRCM	52

[a] NRHC: neonatal rat heart cells, NRVM: neonatal rat ventricular myocytes NRCM: neonatal rat cardiomyocytes, GFF: growth factor free, LV: left ventricular, AP: action potential.

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cell cycle, the role of focal adhesions can determine SC fate, yet it is often assumed that ligand–receptor molecules are uniformly distributed over the cell surface. However, chemical energetic components should be taken into account by utilizing such simplified models to investigate how localized cell-surface adhesion can be controlled by topographical cues. It is evident that cell-surface adhesion can influence SC differentiation and the topographical cues that TPS substrates invoke on a cell should be considered.

Cell adhesion and cell spreading are influenced by the nano-topography of the substrate upon which a cell is cultured. This capability inspired the idea of using TPSs to induce cell–cell coupling and native-like gap junction distribution for myocardium repair. A demonstration of this concept was implemented with a TPS having PGA nanoscale grooves and ridges[51] (Fig. 2B, i). The neonatal rat ventricular myocytes (NRVMs) seeded on this TPS demonstrated cell and sarcomeric alignment in the direction of the grooves as shown in Fig. 2B,ii. This work demonstrates how nanoscale topographical cues influence contractility and anisotropic action potential (AP) propagation. Previous discussions detail how topographical cues enhance cell–cell coupling *via* an increase in mechanical coupling, electrical conduction, and consequently the propagation of APs. In another effort, cell alignment and elongation were observed on an abraded surface with a grain size of 1–80 μm.[68] As a follow up to this paper, a microscale polystyrene TPS with a periodicity of 1 μm and 400 nm deep grooves was developed using a hot embossing technique[52] (Fig. 2C,i and C,ii). Neonatal rat cardiomyocytes (NRCM) cultured on this substrate contained cross-striations in alignment with the topographical features of the substrate (Fig. 2C,iii). Two gold electrodes were microfabricated and incorporated on both sides of the culture system to simultaneously apply electrical cues parallel to the topographical cues. In general, the ability to apply two or more physical cues simultaneously on cardiac tissue cultures is crucial to the development of contractile constructs, prevention of de-differentiation, and enhancement of the electrical phenotype found in cardiac tissue.[28,52]

It has been found that the shape of an embryonic body (EB) is a significant factor in the regulation of embryonic

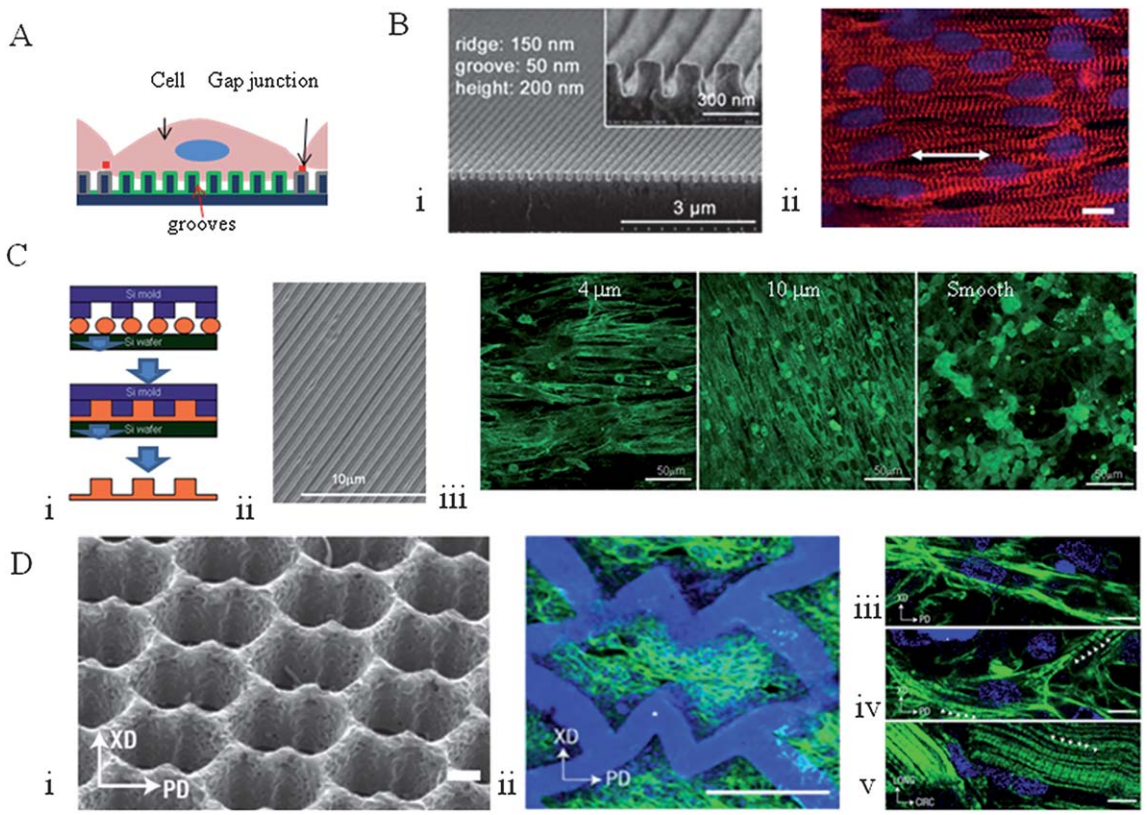

Fig. 2 Nano- and microscale topographical patterned structures for monolayer cell alignment. (A) Schematic of the interaction of a topographically patterned structure and cell membrane. (B) Anisotropically nanofabricated substratum (ANFS) PEG array (i) cross-sectional SEM image of the ANFS, (ii) immunofluorescent image of NRVMs cultured on the ANFS depicting sarcomeric α-actinin (red) and cell nuclei (blue)[51] demonstrating elongated cells in the direction of topographical cues (white arrow). (C) Microfabricated substratum polystyrene array (MSPA) (i) hot-embossing microfabrication process: (a) polystyrene pellets under press of Si mold (b) at evaluated temperature and (c) patterned substrate removal at low temperature (ii) SEM image of MSPA showing 1 μm period and 0.5 μm groove, and (iii) sarcomeric α-actinin (green) stained cardiomyocytes cultivated on MSPA with periodicities of 4 μm, 10 μm, and a smooth substrate.[52] (D) 3D scaffold for cardiac regeneration, (i) SEM image of accordion-like honeycomb design in PGS, (ii–v) NRHC cultured on scaffold fluorescently labelled for F-actin (green) and nucleus (blue). Images acquired by confocal microscopy to demonstrate cell morphology and alignment by Fast Fourier Transform (FFT) analysis using a circular mask. NRHCs cultured on MSPA demonstrate the presence cell elongation and cross-striations (white dots) similar, but less developed than (v) those in control specimens.[20] Scale bars: (B, ii) 1 μm, (D, i) 200 μm, (D, ii) 200 μm, and (D, iii–v) 10 μm.

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development[69,70] in the presence of the desired ECM and other soluble and physical factors. However, little attention has been paid to the development of microfabricated devices for the control of the shape, size, and homogeneity of EBs.[49,50] Among the published works is an array of polyethyleneglycol (PEG) hydrogel microwells implemented to generate SC aggregations with various diameter sizes ranging from 40 to 150 microns. An increased number of beating EBs in larger microwells indicated enhanced cardiomyogenesis in comparison to EBs developed in smaller microwells. The cardiac-specific marker sarcomeric α-actin was also identified in larger microwells yielding enhanced differentiation. Further gene expression analysis also revealed a correlation between the larger EBs and an increased expression of WNT11, a member of the noncanonical WNT pathway. Another effort in this direction was made to generate EBs of spherical geometry by developing concave microwell arrays.[50] The concave microwell more efficiently induced the aggregation of cells, the formation of single colonies, and incidentally facilitated ESC seeding.

Traditionally 2D cell culture results in a monolayer of cells and is a suitable model for the study of several biological functions including cellular adhesion, alignment, and cell–cell coupling. However, a common compliant against the conventional 2D tissue culture model is that normal cells in the body experience a 3D microenvironment surrounded by various ECMs and other cells and chemical compositions. Therefore, tissue regeneration for therapeutic applications such as cartilage and myocardium replacement requires the development of a 3D scaffold that mimics the mechanical and structural properties observed in normal tissue.[71]

A sophisticated 3D scaffold design has recently been reported for myocardium replacement with adequate cardiac anisotropy.[20] This design features an array of accordion-like honeycomb microscale structures developed in biodegradable poly (glycerol sebacate) (PGS) and has high porosity, elasticity, and controllable stiffness. Each accordion-like honeycomb well, fabricated by excimer laser microablation, consisted of two overlapping 200 μm × 200 μm PGS pores oriented at 45° axes (Fig. 2D,i and D,ii). NRHCs cultured on the 250 μm PGS membrane demonstrated cell alignment, elongation, and cross-striations (Fig. 2Diii,v).

From the works discussed throughout this section, it is clear that scaffolds need to mimic the complex union of mechanical, chemical, and topographical properties found in native tissue. Recently, an increasing number of artificial ECMs have employed advanced micro- and nanotechnologies to replicate and elucidate the role of structural cues. We anticipate further utilization of advanced fabrication technologies for the creation of functional smart materials in tissue engineering.

IV. Microfluidic cues

Endothelial cells (ECs) in blood vessels experience laminar shear stresses that modulate cellular signalling pathways and biochemical factors byway of mechanotransduction. The effect of shear stresses on EC and other cardiovascular cell functions is well documented,[72,73] but the ability to use shear stress to direct SC differentiation towards specific cell types has only recently begun to be investigated.[74] One active area of research is how ESCs are differentiated into cardiovascular lineages by regulating shear stress (Table 3). Among these efforts, mouse ESCs cultured on Matrigel-coated plates were exposed to 10 dyn cm^{-2} shear stress for 1 h. The expression of cardiac and smooth muscle markers such as α-SMA, MEF2C, and SM22 revealed enhanced cardiovascular development.[75] In another report, rat BM-derived MSCs experiencing fluidic shear stress revealed cardiomyogenic differentiation, indicated by the expression of cardiac specific markers including Nkx2.5. Additionally, Flk1+ cells derived from ESCs cultured on a type IV collagen-coated dish were subjected to a shear stress (1.5–10 dyn cm^{-2}) using a parallel plate chamber. This chamber consists of two flat surfaces and a 200 μm silicone rubber gasket. Cells were cultured on one side of the chamber with the perfusion of differentiation medium. The proliferation and differentiation of ESCs into vascular ECs were also modulated by chemical stimuli (*e.g.* VEGF and TGF-b) and laminar shear stress stimuli as well.[77] Based on this work and other successful efforts, shown in Table 3, shear stress has been determined to be an important factor for SC differentiation into endothelial and cardiac cells.

A parallel-plate channel can be used to model fluidic shear stress *in vitro* (Fig. 3A). The transient shear stress (τ) applied on cells is equal to $6\mu Q/wh^2$ where μ, Q, w, and h are the dynamic viscosity, flow rate, width, and height of the channel, respectively.[79] By altering the flow rate and the geometric attributes within a microfluidic channel, shear stress can be adjusted in microfluidic cell and tissue culture platforms. Micro- and nanofluidic techniques enable the creation of cell culture microenvironments in which physiologically relevant models of cells and tissues are exposed to fluidic forces that mimic those found *in vivo*. The fluidic cues generated in microfluidic devices can significantly affect chemical gradients, cellular alignment, mobility, morphogenesis, proliferation, and differentiation.[79–81] Microfluidic culture arrays with independent, multiplexed microbioreactors have been demonstrated as one successful tool to study cell and tissue cultures *in vitro*. Microfluidic culture arrays are advantageous as they can apply reproducible, steady-state conditions and individually address each bioreactor well. This capability enables a multitude of experimental variables to be investigated simultaneously such as cell density, medium composition, oxygen concentration, pH, flow profile, flow rate, and shear rate.[82,83]

Recent contributions to microfluidic technology have included the development of various techniques for the high-throughput screening of cultured cells, the applications of which include SC research.[84,85] Fig. 3B,i depicts an array of microfluidic culture wells under continuous perfusion, suitable for high-throughput SC-based assays. This microfluidic cell culture array integrates a linear gradient generator capable of delivering a spectrum of various reagent concentrations in each chamber. As shown in Fig. 3B,ii, each culture unit consists of two circular chambers, a perfusion inlet and outlet, along with two channels for cell loading and waste. Large fluidic resistance exists between adjacent chambers resulting in minimal lateral flow and cross-contamination between bioreactors. Such on-chip cell culturing technologies may facilitate further understanding of basic stem cell science by effectively multiplexing the delivery of potential reprogramming factors and other biochemical stimulants.

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Table 3 Role of microfluidic cues for cardiac SC stimulation[a]

Factor	Range	Cell	Effect	Ref
Shear stress	10 dyn cm^{-2}	Mouse ESC	Enhances the cardiovascular differentiation: induces up-regulation of smooth muscle actin, myocyte enhancer factor-2C (MEF2C), and sarcomeric actin	75
Perfusion	0.3 μl min^{-1}	hESC	Cardiovascular differentiation: indicated by α-SMA and Oct4, homogeneous cardiomyocyte distribution, enhanced cell viability	76
Shear stress	1.5–10 dyn cm^{-2}	Flk-1+ mouse ESC	Vascular EC differentiation: vascular endothelial (VE)-cadherin, PECAM-1, SM-actin, increases in proliferation, cell–cell adhesion	77
Shear stress	5–20 dyn cm^{-2}	RBM MSC	Cardiomyogenic differentiation, increased gene expression level of GATA-4, β-MHC, NKx2.5, MEF2c, cTnT, connexin-43, desmin, and a-sarcomeric actin	78

[a] SMA: smooth muscle actin, RBM: rat bone marrow, SM: smooth muscle.

The pulsatile perfusion of medium has been demonstrated as an influential factor in cardiovascular differentiation and cardiac regeneration.[86,87] In lab-on-a-chip systems, the pulsation of medium can be applied to culture microchambers using an array of controllable pressure-driven or voltage-gated microvalves.[45] Additionally, porous 3D scaffolds, such as hydrogels, can be combined with medium perfusion to influence the diffusion of gases, nutrients, and other macromolecules.[22,88,89] One report utilized such a combination of pulsatile perfusion and porous 3D scaffolds to facilitate the development of thick, compact cardiac grafts while providing excellent local oxygen levels with minimized hydrodynamic shear.[89] Recent microfluidic devices have demonstrated the capability to modulate the shear rate over a logarithmic range of values to enable the analysis of single SCs in perfusion cultures.[90–92] In another effort communicated, a poly (methyl methacrylate) (PMMA) device with an array of 900 miniaturized recesses was proposed to culture ESCs under various perfusion rates.[93] Each recess had a maximum volume of $300 \times 300 \times 300$ μm^3 and was implemented for 3D cell culture and differentiation analysis. The cardiac clusters derived from the ESCs contained spontaneously beating cardiomyocytes, thus validating the platform. Studies such as these exemplify the importance of controlling shear and perfusion rates in tissue engineering.

The ease of which miniaturized lab-on-chip systems can control the cellular microenvironment has been demonstrated in a number of cases. For example, a micro-bioreactor array capable of controlling multiple parameters that regulate cell differentiation mimicked *in vivo* conditions *via* the 2D and 3D cultivation of cells (Fig. 3C,i). This MFCA was engineered with 3 inlets (red), 12 microwells (orange), and 3 outlets (yellow), facilitating cell seeding, cultivation, and the study of differentiation on-chip. Various cell lines were investigated, including a mouse myoblast cell line (C2C12), primary rat cardiac myocytes, and hESCs (lines H09 and H13).[76] This device utilized gas exchangers to equilibrate the oxygen and pH levels in each of the three inlet streams and a splitter divided each of the three inlet streams into four equal partitions that separately flowed to the twelve micro-bioreactors. A multilayer poly (dimethylsiloxane) (PDMS) fabrication process was used to develop the 2D and 3D micro-bioreactors on the MFCA device (Fig. 3C,ii). hESCs exhibited higher cardiovascular differentiation and proliferation when media were perfused, as shown in Fig. 3Cv, and myoblasts differentiated into multinucleated myotubes in the direction of flow. Design and implementation of innovative MFCA devices with perfusion control and real-time analysis remains an active area of research with a variety of therapeutic applications, which include the cardiac tissue regeneration.[94]

We conclude that not only can microfluidic devices play an essential role in directing the precise volume of chemical/biological solutions within the SC microenvironment, but physiologically relevant microsystems can also effectively apply fluidic forces for SC differentiation. Further, scalable efforts should be made to develop sophisticated devices for SC differentiation into regenerated CT by utilizing the advantages of innovated micro- and nanofluidic technologies.

V. Mechanical cues

Cardiac cells in the heart are subjected to numerous mechanical forces. These forces are factors that help regulate gene expression and control cellular function.[95,96] For these reasons, the recreation of natural mechanical forces proves to be an important

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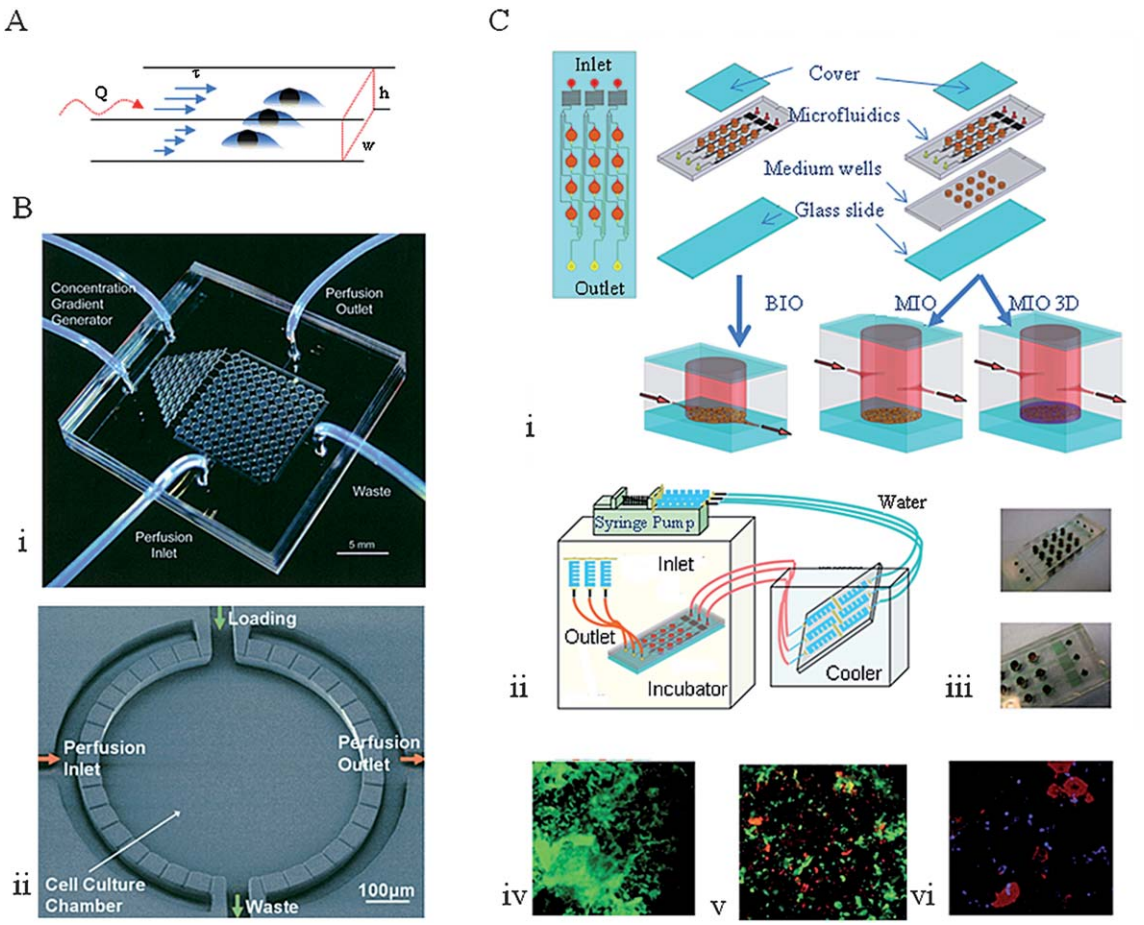

Fig. 3 Microfluidic devices for 2D and 3D cell cultures. (A) Illustration of culture cell experiencing a laminar shear stress, (B) optical and SEM images of microfluidic cell culture array (μFCCA) and an individual cell culture unit,[85] (C) A μFCCA for 2D and 3D cardiac tissue regeneration. (C) Illustration of a μFCCA (3 × 4) with 8 mm height and 7 mm diameter of each unit. Two configurations were used, bottom (BIO) and middle (MIO), for 2D and 3D cell culture models, respectively. The cultured cells in the (ii: illustrated, iii: fabricated) device were subjected to medium perfusion using three syringe pumps connected to the inlets of the incubated chip. Immunostained images of cultured hESCs after 4 days (iv) with and (v) without perfusion of culture medium (green: live cells; red: dead cell nuclei) reveal high cell viability under perfusion and (vi) vascular differentiation evident by the presence of α-SMA (in red).[76]

aspect for *in vitro* cardiac tissue regeneration. In current *in vitro* techniques, cells are cultured on a deformable substrate that is exposed to 2D mechanical strains with controllable magnitude and frequency. The most common 2D stretch models are uniaxial and equiaxial, where uniaxial strain is exerted along one axis and equiaxial strain is uniform strain in all directions. Air pressure (P) can be used to form hemisphere shape deformations on a membrane in order to apply an equiaxial stretch ($P > 0$) or contraction ($P < 0$) (Fig. 4A).

To date, several papers have reported the effects of strain on cardiovascular cell types, particularly in SMCs and cardiomyocytes due to their exposure to cyclic mechanical strain in native tissue.[94–99] Among these works, hMSCs subjected to cyclic equiaxial strain revealed down-regulation of Sec1/munc18-like proteins (SM) α-actin, and actin-binding protein (SM-22α), whereas cyclic uniaxial strain increased the expression of SM-22α and SM α-actin.[100] Based on this work, uniaxial strain invoked MSC differentiation when the surface of the membrane is patterned with microgrooves in the direction perpendicular to the mechanical strain. In another effort, equiaxial stretch with greater than 10% deformation was studied in the presence of conditioned and unconditioned media. This study found that mechanical equiaxial stretch could promote hMSC self-renewal and pluripotency, but only in the presence of conditioned media. A custom-made device for mechanical stimulation features an elastic and biologically compatible membrane such as silicone that is subjected to cyclic mechanical forces. Typically, a mechanical transducer controlled by a computer modulates the deformation amplitude, frequency, and exposure time (Table 4). To study the effect of mechanical stimulation on MSCs, a 250 μm thick silicon membrane was coated with collagen I (or elastin) to promote cell adhesion and growth.[100] In this study, the circumferential direction of mechanical strain in the blood vessel wall was postulated to promote the expression of smooth muscle cell (SMC) markers by applying uniaxial mechanical strain. In another effort, ESC-derived cardiomyocytes cultured on poly (lactide-*co*-ε-caprolactone) (PLCL) were subjected to mechanical strain for 2 weeks. During cardiomyocyte differentiation, exposure to mechanical strain enhanced myocardium regeneration and reduced apoptosis.

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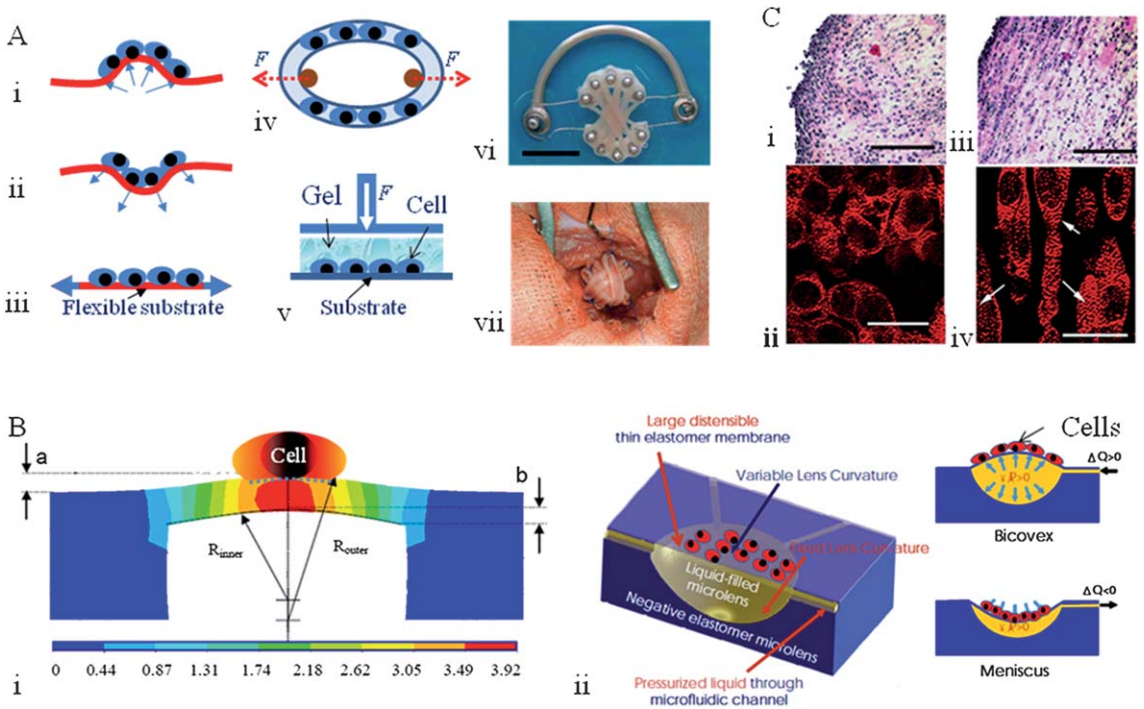

Fig. 4 Cyclic mechanical stimulation by substrate deformation. (A) 2D mechanical stimulation models, which include an elastic membrane upon which cells are cultured and subjected to (i and ii) equiaxial or (iii) uniaxial strain and 3D mechanical models, which include (iv) encapsulated cells exposed to mechanical (iv) strain and (v) compression. (vi) A custom-made device with five single engineered heart tissue (EHT) rings (vii) united with six single-knot sutures to form a complete cardiac graft. (B) (i) Finite element simulation of single PDMS micromechanical stimulation membrane exposed to deformation. (ii) Positive and negative mechanical strains can be induced by changing the air pressure within the cavity to $P > P_{atm}$ and $P < P_{atm}$, respectively.[106,107] (C) ESC-derived cardiomyocytes cultured on collagen and fibronectin scaffolds show improved cellular alignment using hematoxylin and eosin staining methods without (i) and (iii) with mechanical stimulation. Immunostaining of cells without (ii) and with mechanical stimulation (iv) depict cellular elongation.[104]

3D mechanical strain can be mimicked by embedding cells in synthetic ECMs and applying cyclic mechanical forces. Two common models of 3D tissue constructs under mechanical stimulation are ring-shaped and cylindrical-shaped constructs. In one work, neonatal rat heart cells (NRHCs) were cultured in circular molds with type I collagen and subjected to mechanic strain to form single engineered heart tissue (EHT) rings (Fig. 4A,iv). The study used a custom-made device to deliver an auxotonic load to fuse five individual EHT rings and create a united cardiac graft (Fig. 4A,vi). The engineered tissue graft was then implanted into a Wistar rat to regenerate the diseased myocardium.[103] The effects of oxygen, insulin, and load were investigated on the cultured cardiac graft and the construct was implanted onto the host's heart using six single-knot sutures (Fig. 4A,vii). Echocardiography, histology, and statistical analysis revealed that the mechanical strain under cyclic and static conditions enhanced electro-mechanical coupling of the graft to the native myocardium. A similar study was performed to elucidate the role of mechanical strain on the maturation of cardiac tissue and other related issues such as cell–cell coupling and gap junction distribution.[104] In this study, the ring shaped construct was prepared using ESC-derived cardiomyocytes and exposed to uniaxial stretch using two rods as shown in Fig. 4A,iv. Mechanical load with a 10% stretch was induced at 3 Hz for 3 days and the investigation proved sarcomeric structural organization, cellular alignment, and the up-regulation of cardiac specific gene expression (Fig. 4C). Another study revealed mechanical stretch on 3D tissue constructs increased cardiomyocytes proliferation, while proliferation was impaired by p38 MAPK inhibition.[105] These works emphasize the relationship between mechanical and chemical factors that control SC-based CT construction. Nevertheless, many more experiments remain to elucidate the role of mechanical forces on cellular behavior including the differentiation of SCs for cardiac tissue engineering. To achieve these goals, high-throughput mechanical stimulation systems will enable the study of physical stimuli in less time and with higher precision.

Polymeric microlens microdevices with tuneable curvatures have been developed for the integration of controllable optical networks within microfluidic systems.[106] These devices can additionally be employed for the micro-mechanical stimulation of cell cultures. For example, we have developed an array of pneumatically controlled curvatures using a PDMS microfabrication process. The cross-sectional view of the deformable membrane unit is shown in Fig. 4B. Air pressure can be applied within the microfluidic network to deform the microscale membrane and the cultured cells can be subjected to equiaxial strain. As a follow up to this work, a microscale deformable membrane array was fabricated using micromolding and photopolymer microdispensing procedures, creating PDMS

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Table 4 Cyclic mechanical strain for cardiac stem cell stimulation[a]

Factor	Cells	Device	Range	Effect	Ref
Cyclic uniaxial stretch	Human BM-MSC	Elastic silicone membrane	10% strain 1 Hz 1day	Up-regulated SM contractile markers: significant increase (2 fold) in SM-22α and α-actin	100
Cyclic equiaxial strain	Human BM-MSC	Elastic silicone membrane	10% strain 1 Hz 1day	Down-regulated SM contractile markers: expression levels of α-actin and SM-22α in MSCs were about 20% of that in human SMCs	100,101
Cyclic uniaxial stretch	Mouse ESC	Elastic PLCL scaffolds	20% strain 1 Hz, 2 weeks	Enhanced cardiomyocyte differentiation, higher level of VEGF gene-expression; enhanced morphology including myofibrillar bundles and Z-lines	102
Auxotonic load: static/phasic	NRHC	EHT fusion and contractions under auxotonic load	strain: 110% (static) and 110–100%, (phasic, 2 Hz), 7 days	Myocardium repair: electrical coupling to the native myocardium without evidence of arrhythmia induction	103
Cyclic uniaxial stretch	Murine ESC	Ring-shaped constructs placed around 2 rods	10% stretch at 1, 2, or 3 Hz	Cardiac tissue development: @ 3 Hz caused up-regulation of α-cardiac actin, α-skeletal actin, and more organized sarcomeric gap junction formations	104
Cyclic uniaxial stretch	Chicken embryonic cardiac cells	Collagen type I coated silicone membrane	0.5 Hz, 5% strain, 48 h	Proliferation enhanced by mechanical stretch and impaired by p38 MAPK inhibition	105

[a] BM: bone marrow, SMC: smooth muscle cell, EHT: engineered heart tissue. The terms "static" and "phasic" refer to the two static and dynamic mechanical stimulation methods.

cavities that can experience variable degrees of curvature (Fig. 4B).[107] Cells can be cultured on the microscale deformable membrane array and subjected to a positive or negative equiaxial strain, which corresponds to a positive or negative pneumatic pressure. The maximum deflection, W, occurs under the pressure drop $\Delta P_0 = Eta(W/0.662a)^3$ where a, t, and E are radius, thickness, and elastic modulus of a membrane. Assuming that the weight of the cells and media above the deflecting membrane are negligible (otherwise the drop pressure would be $\Delta P > \Delta P_0$), the radius of curvature (R_v) is obtained from $R_v = (W^2 + \varphi^2)/2W$ where φ is the radius of the deflected membrane. It should be noted that the profile of the deflected membrane in this system was modeled as a spherical cap. Therefore, by controlling the applied air pressure, the deflection of the membrane and the corresponding applied mechanical forces on the cultured cells can be controlled.

Pneumatic microdevices have been developed to exert mechanic force on cells for directed differentiation. One particular microchip consists of an array of 3 × 5 microwells, which deliver a compressive pressure to hMSCs to induce osteogenesis.[108] This five layer microfluidic system applies a mechanical load to hMSCs byway of a deformable membrane pressing downward onto the cells. This membrane is controlled by three factors: the diameter of the microwell, the air pressure, and the thickness of the PDMS membrane. This report demonstrated the use of mechanical stimulation to enhance proliferation and accelerate differentiation of hMSCs in the absence of growth factors. In another effort, a pneumatic microchip integrated expansion cavities below 3D cylindrical-shaped hydrogel wells seeded with mouse MSCs.[109] The encapsulated MSCs in each chamber were subjected to cyclic, compressive mechanical loads by pneumatically expanding the air cavity below each hydrogel well. The mechanical compression resulted in non-linear, cellular and nuclear deformations applicable for the 3D study of compressive stress on SCs.

In conclusion, microdevices have applied mechanical forces to cells seeded on 2D and 3D scaffolds in a variety of ways. Such microsystems have demonstrated myogenic differentiation and directional cell alignment without the use of biochemical factors or surface patterning. We will briefly describe how noninvasive, optical methods can be utilized to apply mechanical forces in Section VII.

VI. Electrical stimulation

The role of electric fields for the treatment of disease has been debated ever since the Leyden jar was first used to study the effect of electrical shocks to people in the mid-1700s.[110] An interesting application of electrical fields is for the purpose of wound healing. Endogenous wound healing was first demonstrated with electric fields more than 150 years ago, however the identification of the signalling pathways that govern electric field-induced therapy is an on-going and challenging issue. To date, it has been established that electric fields, when equal in strength to those detected endogenously, can significantly direct cell migration and affect wound healing. In one report, the speed of epithelial migration into the wound reached a maximum at electrical voltages in the range of 100–200 mV.[111] Despite advances in

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utilizing electrical fields for clinical and biological research purposes, the potential impact of utilizing electrical fields for the control of cellular activity and the development of regenerative medicine has largely been obstructed due to the poor technical control of the necessary variables to elucidate the underlying physical concepts.

Recent advances in micro- and nanofabrication technologies have demonstrated the effectiveness of employing electrical fields to precisely manipulate cell behaviour in wound healing, electrotaxis, neural stimulation, and SC differentiation into cardiac cell types.[112–114] Despite recent progress, the optimum use of these techniques for SC-based cardiac tissue engineering applications has been hindered by several barriers.[115] For instance, microelectrode properties and electrical field distribution in the proximity of living cells greatly influences cellular behaviour; however, few works have investigated the electrical factors involved for purposes of cardiac SC differentiation. Among these few works, one notable paper reported the characterization of electrodes for high efficiency electrical stimulation of cardiac tissue.[115] Based upon findings in this study, the electrode geometry, electrode material, and charge-transfer characteristics at the electrode–electrolyte interface are important factors for the design, implementation, and optimization of an electrical stimulation system. A biocompatible, toxic-free electrode can be analysed using electrical impedance spectroscopy in order to choose the optimal electrode material with a high constant-phase to increase charge injection and with high polarization resistance (R_d) to reduce harmful reactions (see Fig. 5A).

The present focus of this research is placed on designing innovative systems that can precisely mimic endogenous electrical signals in living CT and EBs. In this section, recently reported microfabricated devices for electrical stimulation of cardiac tissues and SC-derived cardiomyocytes are described. A common electrical stimulation model is shown in Fig. 5A and the equivalent impedance measured is expressed in the below equation. The applied voltage generates an electrical field between the electrodes that can electrically stimulate cells *in vitro*. In this model, the medium is represented by a resistance (R_m), which is in series with the capacitance of the double layer (C_d) and its parallel resistance (R_d). The total impedance $Z = R_m + R_d/(j2\pi f C_d R_d + 1)$ where $j = (-1)^{1/2}$ and f is the frequency. The effective electrical field on the membrane of the cell for controlling cell behaviour is a function of the parameters that appear in the above equation and the amplitude of the applied voltage. The selected material of the electrodes and culture medium assured that Z did not vary over the duration of the experiment, which lasted a couple of weeks.

The impact of electrical fields on cell behaviour, particularly in embryonic development, has been studied for many years using a number of techniques.[116] Previous investigations have probed the role of endogenous signals in tissue development by measuring unidirectional ion transport in the pA range in various animal models, including chicken and mouse embryos. Based on these studies, the electrical field distribution around the embryo is critical to the organization of the germinal epithelium (germ layers). The ability of endogenous signals to stimulate SCs for cardiovascular differentiation has only recently been investigated. Among these works, an effort was made to elucidate the role of electrical pulses for SC differentiation into cardiac cell types using 500 V cm^{-1} pulsatile electrical fields applied to EBs

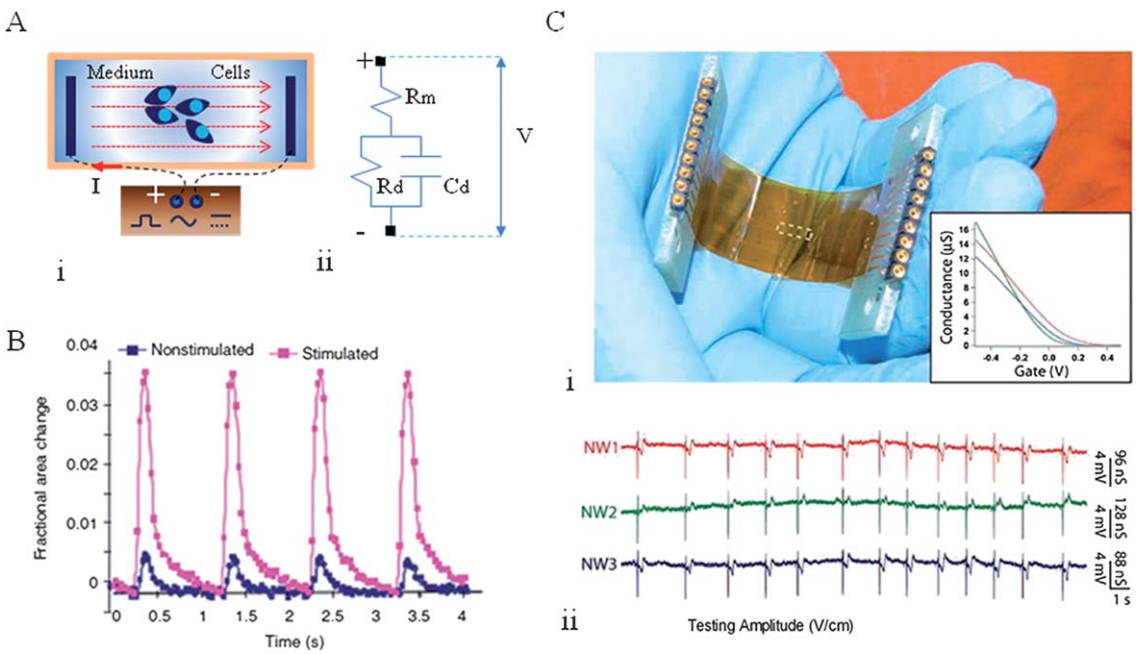

Fig. 5 Electrical stimulation for enhanced functionality of CT. (A) (i) A schematic of the (ii) equivalent model for electrical simulation for a biological sample. (B) Electrically stimulated CT leads to a 7-fold enhancement in the contractile properties of the graft. NRCMs cultured on collagen and exposed to a 1 Hz electrical field revealed enhanced electromechanical coupling as observed in the volume-time curve.[115] (C) An innovative cardiac recording device utilizing nanowire (NW) technology on a flexible polymeric substrate. (i) The drain-gate functions and (ii) recorded signals from three adjunct NW FET transistors.[132]

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for 90s.[117] In this report, the electrical fields on EBs grown from pluripotent ESCs significantly induced cardiomyocyte differentiation and increased the number and size of beating foci.

Intracellular reactive oxygen species (ROS) has been suggested as a possible promoter of early cardiac development. In one study, electrical field stimulation during the cardiomyocyte differentiation of pluripotent SCs increased the intracellular generation of ROS and activated transcription factor NF-kB.[117] Additionally, the integration of a 4 × 4 array of PDMS bioreactors with individual electrical conductors enabled the study of electrical field stimulation on ROS generation and cardiogenesis.[114] Fluorescent microscopy measurements revealed that electrical stimulation increased the generation of ROS and the study analyzed the effects of different electrode materials and electrical stimuli. Despite the importance of the role of electrical stimulation in embryonic development, few efforts have been made to investigate the potential of utilizing electrical fields during embryonic differentiation. Alternatively, more attention has been paid to the role of electrical fields in the development and enhancement of contractile cardiac constructs.

A cardiac construct with several million cardiomyocytes per cm is required to replace the average myocardial infarcted area of a heart and these cells must fully integrate with the remaining heart tissue. Cardiomyocytes are coupled to each other *via* intercellular gap junctions and electrical stimulation not only induces changes in the Ca^{2+} oscillations through these gap junctions, but also influences SC differentiation.[28,118] The expression levels of proteins associated with gap junctions (*e.g.* C × 40, C × 43), cardiac muscle contraction (*e.g.* troponin I and T), and sarcomers with thick and thin filaments (*e.g.* actin and myosin) have been evaluated with electrical stimuli applied to cardiomyocyte and skeletal muscle cultures.[119–127] In general, electrical stimulation increases the transcription levels of cardiac-specific genes, the mechanical–electrical coupling of the cells, and the integration of transplanted cells. Furthermore, the distribution of intercalated disks, which are responsible for force transmission during muscle contraction and the distribution of gap junctions, is enhanced by electrical stimulation.[126] Table 5 highlights some of the reported benefits of electrical stimulation. Overall, electrical stimulation has been determined to significantly enhance cardiac development as well as the electromechanical coupling between implanted cardiac constructs and damaged heart tissue.

Various types of electrodes have been used for stimulation. Among the latest efforts made to take advantage of electrical stimulation on cardiac constructs is a protocol for the stimulation of cardiac constructs in 2D and 3D cell culture platforms.[115] Two 1/4 inch diameter carbon rods were placed in a Petri dish with Matrigel and collagen scaffolds. NRCMs seeded on the scaffold were cultured and subjected to electrical pulses. The electrical field significantly increased the amplitude of the synchronous cardiac construct contraction by a factor of 7 (Fig. 5B). In another work, an array of ITO microelectrodes were fabricated on a glass substrate for the electro-stimulation of human adipose derived (HAD) SCs in a 2D cell culture. Interdigitated electrodes were fabricated by patterning ITO on glass using a laser ablation technique and the optically transparent ITO enabled the simultaneous capability to electrically stimulate and optically observe the plated cells.[129] Electrical stimulation was found to significantly increase the spontaneous beating area. It should be mentioned that the electrical and mechanical properties of cells in a living organism are interconnected. Among the studies highlighting this issue, a recent effort revealed that alterations in mitochondrial ionic balance are associated with the swelling of mitochondrial volume, and that the changes in mitochondrial volume can induce mechanical constraint and internal pressure on the myofibrillar and nuclear compartments.[130]

Despite the advantages of 3D cardiac constructs for therapeutic purposes, a 2D cell culture model integrated with stimulation electrodes was found to be a suitable platform to study the effect of SC regulatory factors during the early stages of embryonic development and colony formation. However, electrode fabrication and electrical setup does not need to be overly complex. One effort used a standard pacemaker for cardiac SC stimulation instead of a pulse generator. Gene expression analysis revealed the enhanced activation of the Myh6 promoter, determined by a luciferase reporter vector.[125] These works

Table 5 Role of electrical fields for cardiac SC stimulation[a]

Cells	Electrodes	Signal	Effect	Ref
NRHC	Carbon rods, D = 1.4 inch S = 1 cm	1 Hz, 5 V, 2 ms, 5d	Cell alignment, evaluation of the amplitude of synchronous construct contractions	28
hESC	Stainless steel rods, D = 1.3 mm S = 5 mm	DC, 1 s, 5 V	Cardiac differentiation, ROS generation	114
Murine ESC	Platinum circular electrodes A = 0.2–0.6 mm^2	1 Hz, 30 μA, 10 ms 7d	Cardiac differentiation, six-fold increase in troponin-T and twofold increase in β-MHC	127
NRHC	Polycarbonate wires, pacemaker (KAPPA 93), S = 11.7 mm	1 Hz, 7.5 V, 1.5 ms, 5d	Cardiac differentiation and spontaneous contraction	125
hAD SC	ITO interdigitated electrodes W = 180 μm, S = 200 μm	1 Hz, 0.5 V, 2 ms, 6d	Enhanced proliferation, cellular elongation alignment, higher number of Connexin-43-composed gap junctions	129

[a] D = diameter, S = space, W = width, A = area, d = days, hAD = human adipose derived, ROS: reactive oxygen species.

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Table 6 Role of optical stimulation on cardiac function and development[a]

Factor	Cell	Parameters	Recording	Effects	Ref.
Infrared	EQH	$\lambda = 1.875$ μm, $f = 2$ Hz laser pulse, $t = 20$ s, Du = 2 ms, $E_n = 2.64$ mJ pulse	Laser doppler velocimeter	Non-invasive cardiac pacing, rate control	148
Ultraviolet	ECH	$\lambda = 260$–310 nm, $t = 2.5$ min (2×10^6 cell μm^{-2}), DC	Intracellular electrical method	Increase in spontaneous beat rate	146
Visible	ECH	450–650 nm, (95–115 beats per min), $t = 10$ min,	Manually	Acceleration of beat rate	145

[a] EQH: embryonic quail heart, λ: wavelength, *f*: frequency of applied pulsed laser, Du: time duration per pulse, E_n: energy, ECHC: embryonic chick heart cells, DC: direct current.

demonstrate how various types of electrode configurations are needed for 2D and 3D cultures.

In myocardium, cells are depolarized by the local current flux and not by voltage, thus it might be advantageous to control electrical current instead of voltage. Recently this issue was discussed by mimicking a local-point current source.[127,128] During such experiments, current should be precisely controlled so that the voltage drops on the current source to within a safe margin. Murine ESCs were cultured with leukemia inhibitory factor (LIF) and mitomycin to inhibit differentiation and inactivate fibroblasts. EBs were formed *via* the hanging drop method, after which they were disassociated, plated on the platform, and subjected to electrical stimulation for 4 days.[127] Electrical stimulation significantly increased the population of spontaneous beating and affected the expression of cardiac markers, including troponin-T, which had a 25% higher than average concentration.

SC-derived cells injected into host cardiac tissue and exposed to a post-implantation, biphasic electric field replicating the native heart exhibited improved maturity, tissue contraction, and integration with the host myocardium.[131] The functionality of grafted cells in a 'native' culture environment was analyzed with quantitative molecular and electrophysiological techniques used to study the electrical signal propagation between the graft and host tissue. To date, several papers have studied the electrical properties of SC-derived cells *in vitro* using commercially available microelectrode arrays, but only a few papers have reported innovative electrical recording techniques to support *in vivo* electrical stimulation post-implantation.[132,133] Among these few works is an array of nanowire (NW) field-effect transistors (FETs) fabricated on a flexible polymeric substrate (see Fig. 5C).[132] In these NW FET devices, the drain and source conductor are passivated from direct contact with the NWs and the NW region acts as the channel of the FET. The NW FET, shown in Fig. 5C, demonstrates similar functionality for three different NW devices with close temporal relation between the signals. We expect the further development of such technologies to provide post-implantation recording and stimulation to cardiac grafts, thus enhancing functionality and regeneration.

Despite profound progress in the utilization of electrical fields for several biological applications, which include patch clamping and electro-transfection, the effect of an electrical field on cell behavior has not been well understood. Further efforts should be made to develop innovative techniques and tools in order to clarify the fundamental concept that underlies bio-electro-mechanics at the single cell level. Recent advances in large-scale single cell analysis have paved the way for new and exciting opportunities to study cellular behavior using various physical and chemical stimuli.[134,135] For instance, a single cardiac cell can be trapped and isolated in a microfluidic device and stimulated mechanically. The simultaneous electrical recording and mechanical stimulation provide much information about the mechano-electrical feedback of cardiac cells. The direct mechanical stretching of isolated cardiomyocytes can induce depolarization and provoke extra action potentials that might be suitable for the study of mechanically gated ion channels and mechanically sensitive whole-cell currents (Fig. 6A).[136] A simple model for stretch-induced depolarization is expressed as $I_{SAC} = G_{SAC} \times (V - E_{SAC})/(1 + e^{-\alpha(L-L_x)})$ where I_{SAC}, G_{SAC}, and E_{SAC} are the stretch activated channel (SAC) current, conductance, and reversal potential, respectively.[137] In this equation $(1 + e^{-\alpha(L-L_x)})$ is the length and tension dependence where α is the scale factor, L is the length, and L_x is the reference length. A precise differential current amplifier can measure I_{SAC}. Comparison of the recorded signals measured by electrodes located underneath the cardiomyocytes, before and after stretch, confirms the above model (Fig. 6B–C).[138,139] It is important that future single cell technologies probe a large numbers of cells such that subpopulations of cells can be identified. Currently, most quantitative techniques average out such subclasses when analyzing large populations of cells.

In summary, the integration of mechanical and electrical techniques for single cell analysis enables the opportunity to investigate single SC behaviour and enhance our understanding of control mechanisms necessary for cardiovascular development and regeneration. A vital importance to regenerative therapy is that the foundation of research upon which such techniques are built be accurate and meticulous.

VII. Other potential SC stimulation methods

Optical stimulation

While a large number of investigations have been performed to describe the molecular mechanisms responsible for optical excitability in nerves,[140–144] few efforts have been made to define these mechanisms in cardiac muscle.[145–148] Among these few works, visible and ultraviolet light were used to affect the spontaneous beat rate of embryonic chick heart cells.[145,146] One landmark paper investigated the excitability of cardiac tissue using a non-invasive optical method.[148] This study demonstrated for the first time the ability to pace heart cells with pulsed infrared (IR) laser light. Laser doppler velocimetry (LDV) was

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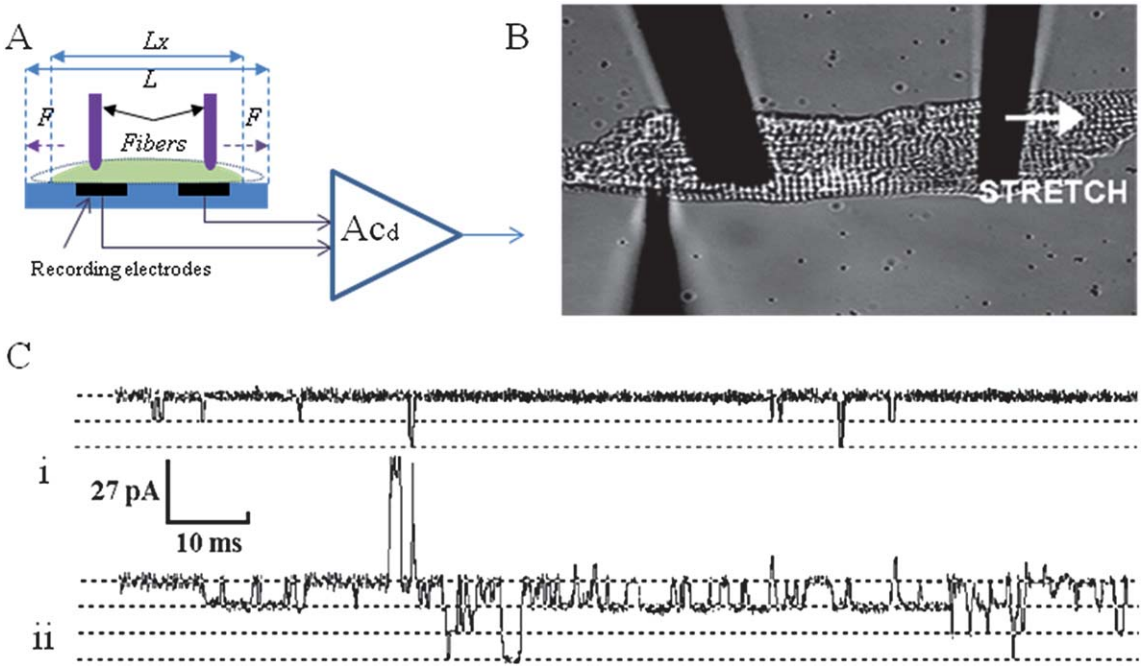

Fig. 6 Stretch induced depolarization. (A) Illustration of simultaneous mechanical stretch and electrical recording from a single cell and (B) insertion of a microelectrode to record the transmembrane voltage as the cell is stretched along its major axis with a supplementary fiber. (C) The cellular activity of single mechano-gated channels (MGCs) (i) without stretching (control) and (ii) during a stretch of length 8 μm. The holding potential was −45 mV.[136]

used as a non-invasive technique to record *in vivo* the heart beat of the quail embryos without tissue damage (Fig. 7A). This enabled the heart rate to be locked to the pulse frequency of the irritated IR light. The experimental results demonstrated that the heart rate of a 59 h quail embryo could be locked onto the pulse frequency of irritated IR laser light, $\lambda = 1.875$ μm (Fig. 7B and Table 6).

Several questions remain unanswered regarding the underlying physical mechanisms of these experiments. For instance, it is not well understood if the thermal effects of the laser light induce the extra heartbeats or if the light directly controls the heart beat *via* an unknown photo-sensitive process in the heart tissue.

Optical stimulation can also be suggested as a method for altering mechanical stresses and mechanically transduced signalling. The mechanical effect of light has widely been investigated; however, the utilization of these methods for controlling the SC behaviour has not yet been reported. Innovative optical techniques, such as optical stretching and optical cell rotating have emerged as powerful tools for manipulating single cells.[149–151]

As previously mentioned, mechanical stimulation of SCs is of paramount importance for cardiac tissue regeneration (Section IV). Opto-mechanical techniques offer several advantages over the previously described mechanical methods. These advantages include the generation of a non-contact and high-resolution mechanical force. Also, the compatibility of this technique with culture medium is very important for biological experiments. A single cell trapped in an optical reactor is irritated by two laser light sources as shown in Fig. 8A. The net optical force, $F_{optical}$, on a cell surface is equal to $n_m PQ/c$ where n_m, P, Q, and c are the index of refraction of the buffer medium, total laser power, dimensionless trapping efficiency, and speed of light in a vacuum,

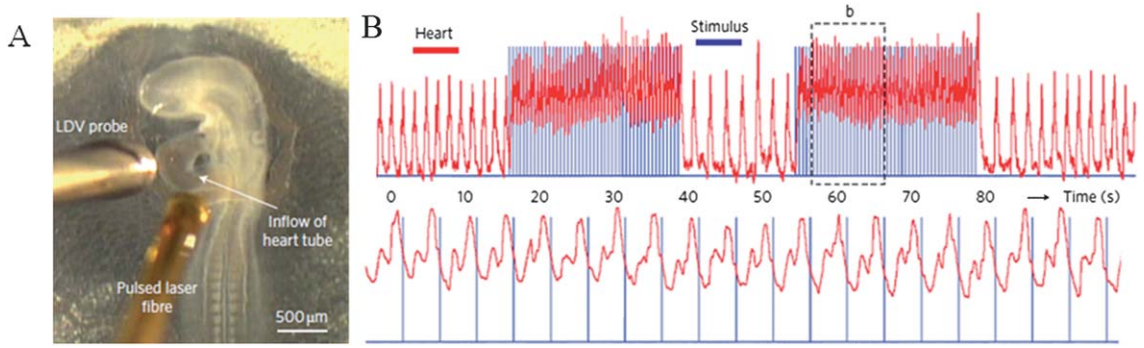

Fig. 7 Optical pacing of an embryonic quail heart. (A) Experimental setup for the pacing of a 59 h quail embryo. (B) The trigger pulse (blue) and heart rate response (red), which was measured using LDV.[148]

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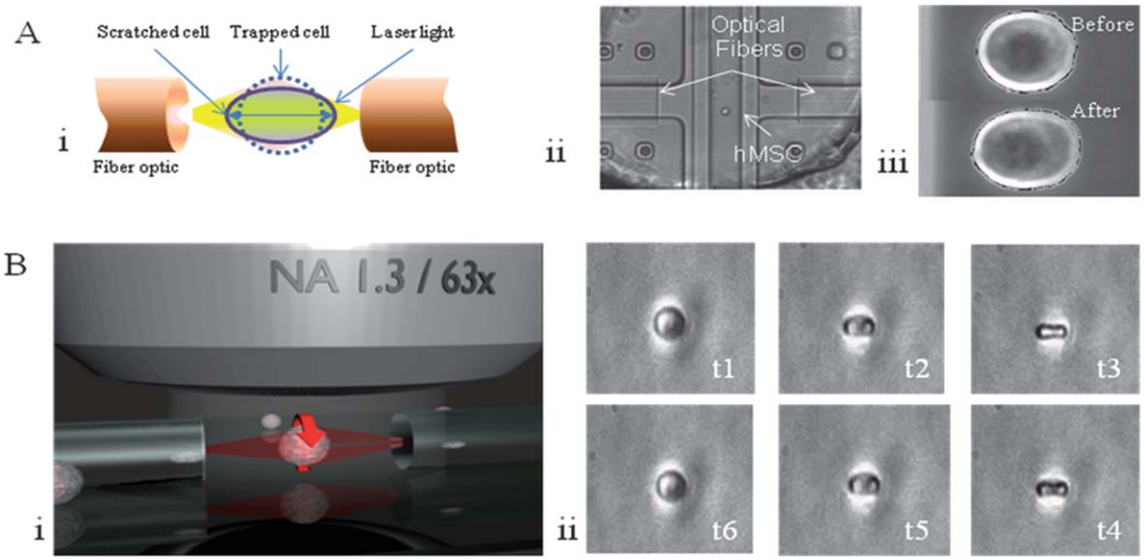

Fig. 8 Opto-mechanical methods for cellular deformation, stimulation, and manipulation. (A) (i) Illustration of an optical stretching method using two fiber optics to trap the cell, (ii) phase contrast photograph of a microfabricated capillary with opposing fibers to deform hMSCs, and (iii) an image of a suspended hMSC prior to and subsequently after optical stimulation/deformation.[150] (B) (i) Illustration of optical cell rotation *via* a dual-beam laser and (ii) the rotation of suspended red blood cells (time steps: 200 ms, $t_6 > t_5 > t_4 > t_4 > t_3 > t_2 > t_1$).[149,152]

respectively.[150] The change in refractive index at the cell edge causes a photon-induced surface stress and consequently deforms the suspended cell. In a recent attempt, dual counter propagating laser beams were adjusted to trap, center, and deform a single suspended hMSC, shown in Fig. 8Bi. A microfluidic channel was used to direct the suspended cells toward the optical stimulation site where the lasers were positioned.[153] The deformation of cells along the laser beam axis could clearly be observed in the modified microscopic images using an edge detection filter. Additionally, a modified dual-beam laser can optically rotate a suspended cell in a controlled manner. The manipulation of the cell is executed by the axial rotation of a dual-mode fiber emitting an asymmetric laser beam.[149] This technique allows the suspension and controlled rotation of viable cells for tomographic microscopy, which might be suitable for various applications such as blood cell analysis, shown in Fig. 8Bii. The ability to apply optical forces to suspend and manipulate cells could potentially be used to control SC behavior for cardiac tissue engineering with precise, non-invasive methods.

We have chosen to highlight the role of direct optical stimulation and optomechanical forces for cardiac SC studies; however, there are several questions regarding optical stimulation that remain to be answered. For example: what are the underlying biophysical concepts of optical stimulation in controlling heart beat rate and can optomechanical forces induce SC differentiation into cardiomyocytes? Further investigations are necessary into the issues mentioned above and the applicability of optical stimulation for SC-based cardiac tissue engineering should be assessed.

Magnetic stimulation

The effect of several physical factors on the differentiation of SC into cardiac cells has been discussed in the previous sections; however, there are a variety of techniques that can potentially be used for the purpose of cardiac SC stimulation. For instance, in addition to cyclic mechanical stretching and fluidic shear stress, mechanical stimulation can be induced on cultured cells using magnetic microparticles (MPs). Mechanical stimulation techniques with MPs have been applied to modulate osteogenesis in hMSCs.[153] In this technique, MPs were introduced into the cytoplasm of muscle cells and exposed to magnetic fields produced by two electromagnets. Cell alignment and elongation were observed along the axis of the magnetic poles. This method offers a very localized method of mechanical stimulation for SC differentiation in contrast to the large-scale techniques we have discussed. Furthermore, mechanical forces can be applied on cells *via* ultrasonic pressure waves.[154–158] In fact, suspended cells in ultrasonic wave fields experience non-zero time averaged radiation forces. The effect of ultrasonic forces has been demonstrated for the migration of cells, cell patterning, cell sorting, focusing of particles, and intercellular attraction or repulsion. SCs could also be stimulated through such non-invasive ultrasound techniques to control their behavior. Few efforts have also been made to investigate the effect of magnetic fields as a non-invasive tool for SC stimulation. Among these works included the application of pulsed electromagnetic fields (PEMF) on hMSCs to study proliferation and osteogenic differentiation using a custom-built magnetic stimulation system.[162,163] This system comprised of a large coil for magnetic field stimulation and a smaller coil for detection. Alkaline phosphate activity was measured in an osteogenic culture of hMSCs exposed to PEMF for 14 days. A similar magnetic stimulation system could be used to study the effect of magnetic fields on SC differentiation into cardiac tissue.

Thermal stimulation

Another important physical factor affecting the cellular activity is temperature.[159] Thermal stimulation of neonatal

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cardiomyocytes has been recognized as an important factor to enhance their survival after transplantation; however, there are no reported studies on the role of thermal stimulation as a physical factor to induce cardiomyocyte differentiation from SCs.[160,161]

VIII. Conclusion

Despite profound advances in biophysical and biochemical SC differentiation strategies for cardiac tissue development and regeneration, cardiac SC stimulation techniques remain quite primitive. Further efforts should be made to investigate other physiologically relevant physical (or biochemical) factors such as optical, thermal, and magnetic methods for SC-derived cardiac tissue construct generation. The ability to develop implantable SC-based constructs that are functional, scalable, and capable of electrochemically coupling to host tissue will be enabled by controlling a combination of multiple physical and chemical environmental cues. The development of a multivariable biological system on-chip will help elucidate the effects of electrical, magnetic, mechanical, topographical, chemical, and thermal signals. Towards this endeavour, multidisciplinary systematic approaches encompassing microfluidic, microelectronic, micromechanical, and biomaterial technologies promise to enhance our understanding of SC niches and bring SC-based cardiac tissue engineering closer to medical practice. However, the application of physical factors post-transplantation may remain to be the most vital obstacle yet to be faced. Even if cardiac tissue can be successfully generated *in vitro* under certain conditions, the transfer of those conditions to the cardiac tissue upon implantation into the host heart remains to be addressed.

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