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Cardiac Tissue Engineering Using Stem Cells

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The limited clinical success of stem-cell injections for the treatment of myocardial infarction [1], [2] has been mainly attributed to the low retention and survival of injected cells. Alternative methods for improving the efficiency of cell delivery include the following: 1) injection of a mixture of bioactive hydrogels and cells followed by cell-hydrogel polymerization in situ [3] and 2) the epicardial implantation of a tissue-engineered cardiac patch [4]. Although surgically more complex than cell or cell-hydrogel injection, the patch implantation is also expected to yield improved survival of delivered cells, and potentially, a more efficient structural and functional tissue reconstruction at the infarct site.

The tissue engineering of a functional biomimetic cardiac patch in vitro is a highly challenging problem because of the following: 1) the limited proliferative potential and high metabolic demand of differentiated cardiac cells, 2) the requisite presence of functional intercellular connections, and 3) the complex anisotropic architecture and electromechanical function of heart tissue. Since the late 1990s, research in the cardiac tissue engineering field has primarily involved the use of neonatal or embryonic cardiomyocytes to create three-dimensional (3-D) heart tissue equivalents for use in in vitro experimental studies [5]–[9] and, more recently, for the treatment of myocardial infarction in animal models [10]–[12]. These studies have shown that the structure and function of a cardiac tissue patch depend on the animal species from which the cells are derived [13]–[15], the composition of the seeded cells [6], [16], [17], the initial seeding density [6], [14], [18], [19], the scaffold characteristics [20]–[26], the composition of the culture medium [22], [27], the bioreactor type [18], [22], and the nature of the applied physical forces [13], [28], [29]. Although these studies have established a number of useful design rules for the engineering of a functional cardiac tissue patch, it is well recognized that the use of differentiated cardiomyocytes dissociated from heart tissue will remain limited to in vitro model systems and proof-of-concept in vivo studies. On the other hand, tissue patches made of stem cells offer a potential for translation to clinical practice and as such have been recently utilized in several studies for the functional repair of heart injury. Therefore, this short review is aimed at describing recent advances in the emerging field of stem-cell-based cardiac tissue engineering, with an emphasis on the potential use of cardiogenic stem cells for the construction of electrically conducting and contractile cardiac tissue patches. For the first time, the ability of genetically selected embryonic stem-cell-derived cardiomyocytes (ESC-CMs) to support continuous action potential propagation over a few-square centimeter area will be demonstrated using optical mapping of membrane potentials.

Noncardiogenic Stem-Cell Tissue Patches for the Repair of Myocardial Infarction

Autologous stem cells currently used in clinical trials lack significant potential to differentiate into functional cardiac myocytes [30]. Nevertheless, these cells represent a natural first choice in the engineering of a tissue patch for treatment of myocardial infarction. Recent studies have thus utilized tissue patches made of skeletal myoblasts [31]–[35], bone marrow-derived stem cells [36]–[42], or endothelial progenitor cells [43] for the repair of heart damage. Compared with the injection of a cell suspension, the implantation of tissue sheets composed of skeletal myoblasts has been proven more advantageous for the treatment of myocardial infarction in rats [32] and dilated cardiomyopathy in hamsters [31]. In particular, implantation of the

engineered myoblast sheets over an infarction site yielded improved neovascularization, attenuated left ventricular dilatation, decreased fibrosis, improved fractional shortening, and prolonged animal survival compared to the delivery of the same number of myoblasts by cell injection. These benefits were mainly attributed to the improved survival of cells when implanted as a tissue sheet, and consequently, to the increased secretion of different paracrine factors including stromal-derived factor 1, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) [32]. In a follow-up study, the functional benefits of implanted myoblast sheets were also demonstrated in a pacing-induced canine heart failure model [33]. In an independent study, Siepe et al. compared the implantation of skeletal myoblasts seeded on polyurethane scaffolds with direct cell injection in the treatment of rat myocardial infarction [34]. Although cell survival was significantly higher in tissue patches compared with direct injection (80% vs. 10%–20%), both treatment groups exerted similar improvements of contractile heart function relative to untreated controls. In their most recent study, the same group demonstrated significantly improved fractional shortening in infarcted rat hearts after implantation of a tissue patch made of differentiated skeletal myoblasts and a mixture of collagen gel and matrigel [35]. In all described studies, the functional improvements were attributed to paracrine action of the implanted myoblasts.

Similar to the skeletal myoblast studies, several groups have attempted to treat myocardial injury by implanting a tissue patch made of either whole population or just the mesenchymal fraction of bone marrow cells (BMCs). In particular, in studies by Duplàa et al., abdominal muscle patches were formed into a cup shape to hold collagen gel mixed with either unfractionated BMCs [36], BMCs and mesenchymal stem cells (MSCs), or BMCs and endothelial progenitor cells [43], and implanted over one-month-old murine cryoin-farcts. Two weeks after patch implantation, angiogenesis, cell survival, scar thickness, and pressure waveforms were improved in all treatment groups, but only MSCs were found to invade the scar. Fukuhara et al. have implanted polyglycolic acid scaffolds impregnated with BMCs and collagen I gel, with or without basic fibroblast growth factor (bFGF) in one-month-old rat infarcts [37]. Four weeks after implantation, the capillary growth as well as the amplitudes and slopes of pressure waveforms were most improved in the bFGF/BMC group (relative to cell-free scaffolds), whereas no BMCs were found to invade the scar area. Using the cell sheet engineering approach, Miyahara et al. have implanted tissue sheets made of adipose-derived MSCs or dermal fibroblasts over four-week-old rat infarcts [38]. Eight weeks after implantation, MSC, but not fibroblast, tissue sheets reversed wall thinning, promoted angiogenesis, and significantly improved cardiac function and survival compared to untreated controls. Because implanted MSCs were found to express endothelial and smooth muscle rather than cardiac markers, the observed functional benefits were attributed to MSC secretion of VEGF and HGF. In a series of studies by Sung et al., decellularized bovine pericardia crosslinked with genipin were seeded with a concentrated suspension of 5-azacytidine-activated MSCs [39] or sandwiched with multilayered MSC sheets [40], [44], and implanted over a surgically created right ventricular defect and a four-week-old infarct in rats. Twelve weeks after implantation, MSCs within the patch predominantly differentiated into either smooth muscle cells or myofibroblasts, to a lesser extent into endothelial cells, and only occasionally into early but not mature cardiomyocytes. The expression of angiogenic (bFGF and platelet-derived growth factor beta polypeptide) and cardioprotective (insulin-like growth factor 1 and HGF) factors by MSCs was found to be the main contributor to significant patch vascularization and improved heart function [44]. In a recent study by Simpson et al., a tissue patch made of human MSCs embedded in collagen I gel was implanted 10 min after coronary ligation in rats [41]. Despite the improved ventricular remodeling and fractional shortening relative to treatment with a nonviable patch, no MSCs were found at the implantation site four weeks after infarction. These results suggest that the initial paracrine actions of implanted cells may be sufficient to exert at least a short-term positive effect on cardiac function. Finally, in the most recent study by Potapova et al., urinary bladder extracellular matrix was seeded with

a suspension of human MSCs or with 3-D MSC spheroids and implanted over a surgically created right ventricular defect in canine hearts [42]. Interestingly, MSCs in spheroids, but not in regular monolayer cultures, exhibited increased expression of cardiac markers with 16% of the cells containing L-type Ca currents resembling those of adult ventricular myocytes. Eight weeks after implantation, MSC spheroid-seeded patches improved regional systolic contraction and stroke work compared to MSC suspension-seeded and cell-free patches.

> The expression of angiogenic and cardioprotective factors by mesenchymal stem cells was found to be the main contributor to significant patch vascularization and improved heart function.

Tissue Patches Made of Cardiogenic Stem Cells

Although a noncardiogenic patch is expected to exert functional benefits mainly through concentrated paracrine actions, cardiogenic stem cells capable of generating large numbers of contractile cardiomyocytes can additionally augment heart function through direct electromechanical coupling with host cells. The resulting therapeutic benefits in this case would be mainly derived from significant remuscularization of the infarct or peri-infarct area rather than revascularization alone. Ideally, a functional cardiac tissue patch made for safe, efficient, and sustained repair of myocardial damage should comprise the following: 1) support the anisotropic architecture and electromechanical function characteristic of native cardiac muscle and 2) rapidly vascularize and integrate upon implantation. Among the cardiogenic sources being considered for therapy [45]–[49], recently identified autologous resident cardiac progenitor cells with a restricted potential to differentiate into cardiomyocytes, endothelial, and smooth muscle cells [45], [47], [49] appear to be an ideal candidate for the engineering of a functional cardiac patch. However, the ability of these cells to generate sufficient numbers of functional cardiomyocytes in vitro and without the contact with differentiated cardiomyocytes (e.g., from neonatal rat) is still questionable. ESC-CMs, on the other hand, can be generated in relatively large numbers in vitro [50], [51], and upon implantation, electromechanically integrate and successfully form new cardiac muscle [46], [52]. In light of the recent discovery of induced pluripotent stem cells [53]–[55], the ethical and immunogenic concerns related to the use of ESCs could be eliminated, and the future of cardiac tissue engineering therapy may involve the reprogramming of a patient's own cells into stem cells suitable for cardiogenic differentiation [56], [57] followed by the construction and implantation of functional cardiac patch.

To date, only a handful of studies have described attempts to engineer embryonic stem-cell-derived tissue patches for cardiac repair. Ke et al. have implanted polyglycolic acid scaffolds seeded with undifferentiated mouse ESCs 15 min after coronary ligation in mice [58]. Eight weeks after implantation, scar size and ventricular dilatation were reduced, whereas hemodynamic functional indices and survival rate were improved relative to the use of cell-free patches. Interestingly, no tumor formation was reported despite the use of undifferentiated cells. In studies by Guo et al., differentiating mouse ESCs were enriched for cardiomyocytes using Percoll gradient, embedded in matrigel-supplemented collagen rings, and statically cultured for five days followed by seven days of 2-Hz cyclic stretch [59]. The resulting tissue constructs contained aligned and cross-striated cardiomyocytes, as well as neural, endothelial, and fibroblastic cells, and exhibited twitch amplitudes and pharmacological responses similar to those previously measured in constructs made of neonatal rat cardiomyocytes. No signs of tumorigenesis were found after four weeks of subcutaneous implantation. In studies by Caspi et al., microdissected beating areas from differentiating human ESCs were enzymatically dissociated into single cells, mixed with human umbilical vein endothelial cells and mouse embryonic fibroblasts, embedded in matrigel, and seeded in porous poly-L-lactic/polyglycolic acid scaffolds [60]. After two weeks of culture, a synchronously contracting cardiac tissue patch with endothelial vessel networks was formed. The three cocultured cell types acted in

synergy to promote cell survival, proliferation, and stabilization of blood vessels. In a recent study by Gwak et al., mouse ESC-derived beating cells were enzymatically dissociated from embryoid bodies, seeded on porous, elastic poly(lactide-*co*-caprolactone) scaffolds, exposed to cyclic stretch for two weeks, and implanted over three-week-old cryoin-farcts in rats [61]. Six weeks after implantation, the cyclically strained patches exhibited reduced fibrosis and apoptosis, higher VEGF expression and capillary formation, and upregulation of cardiac markers compared to unstretched ESC control patches made using nonelastic poly(lactide-*co*-glycolide) scaffolds. No assessment of tumorigenicity or cardiac function was performed. Finally, in the most recent study by Shimko et al., mouse ESC-CMs were purified based on -myosin heavy chain promoter driven resistance to neomycin, embedded in fibronectin-supplemented collagen gel rings, and after seven days of static culture, cyclically stretched at different frequencies for three days [62]. Cyclic stretch at 3 Hz, but not 1 Hz, upregulated the expression of sarcomeric cardiac genes and yielded improved cell density and alignment as well as the distribution of the gap junctional protein connexin-43. Despite the enhanced cardiac gene expression, no spontaneous beating was observed in these cultures.

Future Challenges of Stem-Cell-Based Cardiac Tissue Engineering

It may not be necessary or desirable to culture a noncardiogenic tissue patch in vitro for extended time periods before implantation, whereas the use of cardiogenic cells to create tissue patches with structure and function resembling those of native cardiac muscle will require specialized and well-controlled culture conditions over a period of several weeks. Fortunately, the experience gained with the engineering of tissue patches made of differentiated (neonatal or embryonic) cardiomyocytes can be utilized to promote in vitro cardiogenic stem-cell survival, differentiation, alignment, and electromechanical coupling to yield the formation of a cardiac tissue patch that supports fast action potential propagation and large contractile forces. Achieving this goal will be contingent upon establishing a well-defined and reproducible culture practice that will be based on our thorough understanding of the key cellular, biochemical, and physical determinants of cardiac growth and differentiation.

For example, although ethical and immunogenic issues related to use of ESCs may be resolved by the discovery of induced pluripotent stem cells, tumorigenic risks from implantation of undifferentiated cells still remain [63]. To date, the most efficient methods to obtain pure populations of differentiated or already committed ESCs involve selection based on genetically acquired resistance to antibiotics driven by the activation of cardiogenic promoters [64]–[67]. However, which cardiogenic promoter will yield a tissue patch with the optimal electrical and mechanical function remains unknown. Although pure ESC-CMs selected using different cardiac promoters (e.g., -MHC, NCX1, MLC-2v, ANF) may prove adequate as a source for future cell injection therapies, it is not clear if on their own they are capable of sufficiently differentiating and remodeling the surrounding matrix to form a functional tissue patch in vitro. On the other hand, ESC-derived cardiovascular progenitor cells (ESC-CPCs) selected for the combination of mesodermal (Brachyury or T) and cardiovascular transcription factors (Isl1, Nkx2-5) and cell surface receptors (Flk1, c-Kit, CXCR4) can differentiate into a mixture of cardiomyocytes, smooth muscle, and endothelial cells [68]–[70]. In theory, these cells could reconstitute both a cardiac muscle patch and its vasculature by providing a more natural cardioinductive environment. Eschenhagen et al. demonstrated that an increased proportion of nonmyocytes (mostly fibroblasts, but also endothelial and smooth muscle cells) relative to neonatal rat cardiomyocytes improved the mechanical function of their engineered heart tissues [13]. However, we showed that an excess of nonmyocytes deteriorated the electrical properties of a neonatal rat cardiac patch leading to an increased occurrence of arrhythmic activity [6]. Therefore, although the ESC-CPC derived vascular cells in the cardiac tissue patch may be beneficial for the differentiation and contractile function of individual cardiomyocytes [71], [72], they may also hamper the establishment of electrical coupling and successful formation

of a functional syncytium. Taken together, the optimal balance of electrical and mechanical function within a cardiac tissue patch may require a specific proportion of cardiomyocytes and noncardiomyocytes. Ideally, these different cell types would be obtained from a common cell source rather than multiple tissues.

In addition, the development of safe and efficient cardiac tissue engineering therapies will be dependent on our ability to promote the functional maturation of stem-cell-derived cardiomyocytes in vitro. In essence, the functional competence of the cardiac patch is expected to directly correlate with the ability of derived CMs to conduct action potentials with a high velocity and exert a strong contraction through a Ca^{2+}-dependent process of excitation–contraction (E-C) coupling. Relatively mature E-C coupling has been demonstrated in 20-day-old mouse ESC-CMs [73], [74], whereas one- to two-month-old human ESC-CMs exhibited immature sarcoplasmic reticulum (SR) function including negative force–frequency relationship, dependence of Ca^{2+} transients on sarcolemmal calcium inflow rather than SR release, lack of postrest potentiation, and no phospholamban or calsequestrin expression [75], [76]. Other studies, however, demonstrated more mature Ca^{2+}-handling properties of the same age human ESC-CMs [77]. Although a longer culture time, 3-D cardiac patch environment, or postimplantation environment in vivo may promote maturation of the SR function in human ESC-CMs, the genetic manipulation strategies may be necessary to significantly improve the ability of a cardiac tissue patch to effectively contribute contractile forces in an infarcted heart.

In addition to the challenges of developing mature contractile properties of the ESC-CM patches, the establishment of efficient electrical conduction will also be crucial. The main determinants of conduction velocity in cardiac tissue are the availability of sodium current for propagation (which depends both on sodium conductance and cell resting potential), strength of gap junctional coupling, and cell size. Interestingly, although studies by several groups have shown that single human and mouse ESC-CMs exhibit sodium current densities comparable to those of neonatal or fetal ventricular myocytes [78], [79], conduction velocities of ES-CMs within the beating embryoid body outgrowths were measured to be only 1–5 cm/s in both human and mouse cells [52], [79]–[82]. For comparison, isotropic cultures of neonatal rat ventricular myocytes exhibit conduction velocities of 20 cm/s, comparable to those of neonatal rat ventricles [22], [83]. In human ESC-CMs, low-conduction velocities can be partially explained by limited expression of inward rectifier K^+ currents and partial sodium current inactivation due to depolarized resting potential [79], [84], whereas in mouse ESC-CMs, the resting potential after a few weeks of differentiation is comparable to that of neonatal rat cardiomyocytes [65], [78], [85]. Alternatively, low-conduction velocities in outgrowth ESC-CMs may be caused by their small size or potentially strong coupling with surrounding nonmyocytes.

Our recent studies, however, suggest that a very important determinant of conduction velocity in mouse ES-CMs is the distribution and functionality of gap junctions. Specifically, pure ES-CMs selected based on their puromycin resistance driven by an α-myosin heavy chain promoter were dissociated into single cells after ten days of embryoid body differentiation and seeded on fibronectin-coated coverslips. After seven days of culture, the obtained confluent monolayers consisted of pure, interconnected, and cross-striated cardiomyocytes [Figures 1(a) and (b)]. Unlike the previous conduction measurements in ESC-CMs that utilized extracellular microelectrode arrays with only an approximately 2-mm^2 field of view, propagation of action potentials in the resulting ESC-CM monolayers was optically mapped over an approximately 3-cm^2 area [Figures 1(c) and (d)] using a 504-channel photodiode array [86]. As seen in Figure 1, we demonstrate, for the first time, that large numbers of pure ESC-CMs can establish functional gap junctions to support relatively uniform and continuous action potential propagation over a few-square centimeter area. This result suggests that the formation of a relatively large and functional ESC-CM patch is feasible. Furthermore, it is important to note

that two very similar cell differentiation and isolation procedures from the same ESC clone have yielded significantly different conduction velocities after the same number of days in culture. The observed conduction velocity of 17.6 cm/s [Figure 1(d)] is comparable to those reported in isotropic monolayers of neonatal rat cardiomyocytes [83] and significantly higher than previously reported velocities in ESC-CM outgrowths [52], [79]–[82]. Although other differences between the two ESC-CM populations in Figures 1(a) and (b) may exist, one obvious difference is the spatial distribution and abundance of connexin-43 gap junctions. Sporadic and mainly punctate distribution within and between cardiomyocytes in slower cultures [Figure 1(a)] is contrasted with the presence of long intercellular gap junctional plaques (also characteristic of neonatal cardiomyocytes [87]) in faster cultures [Figure 1(b)]. These results also indicate that specific details of cell differentiation, selection, isolation, and culture procedure may play a crucial role in our ability to reproducibly engineer mature electrophysiological properties in ESC-derived cardiac patches.

Along with the choice of cardiogenic cell source and factors that can promote the functional maturation and 3-D assembly of derived cardiomyocytes in vitro, other important issues for the future of cardiac tissue engineering therapy relate to the transition from in vitro to in vivo environment after implantation. In particular, the extent to which the architecture or biochemical environment (including incorporation of bioactive molecules) of a tissue patch can be manipulated to promote patch vascularization, survival, and electromechanical integration has yet to be fully addressed. Additional questions that need to be answered include the following: 1) What time after infarction will the implantation of an ESC-derived cardiac tissue patch exert the most benefit? 2) Which surgical procedure will allow optimal survival and integration of the patch? 3) Is the alignment of cells within the patch and their specific orientation relative to the host tissue during implantation relevant and ultimately beneficial to successful cardiac repair? Although obtaining definite answers to these and other important questions will require a systematic and long-term effort from multiple research groups, only through the establishment of well-defined and reproducible design rules and practices for the engineering and implantation of stem-cell-derived cardiac tissues will we be able to realize the full therapeutic potential of cardiac tissue engineering.

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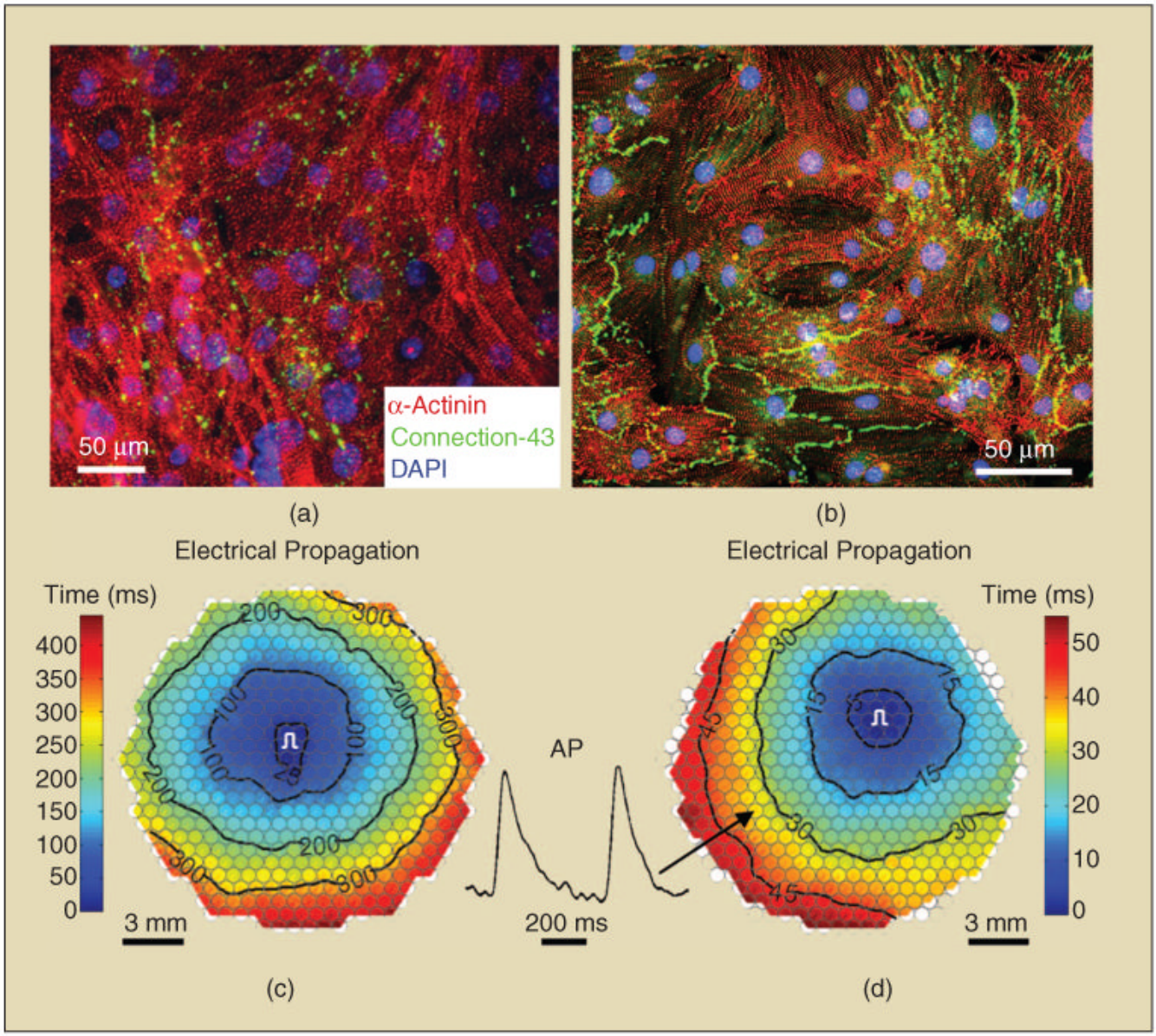

Fig. 1.

Mouse embryonic stem-cell-derived cardiomyocyte monolayers. (a) and (b) Two different cardiomyocyte preparations with distinctly different patterns of connexin-43 expression. (c) and (d) Corresponding isochrone maps of action potential propagation. Note a significantly lower conduction velocity (longer propagation time) in (c) (2.5 cm/s) compared with (d) (17.6 cm/s). Black circles in propagation maps denote 504 recording sites. Hexagonal field of view has a diameter of approximately 20 mm. Pulse signs denote the sites of electrical stimulation. AP inset shows optical action potential traces from one of the recording sites during the 3-Hz stimulation.