Microstructured Cocultures of Cardiac Myocytes and Fibroblasts: A Two-Dimensional In Vitro Model of Cardiac Tissue

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Abstract: Cardiac myocytes and fibroblasts are essential elements of myocardial tissue structure and function. In vivo, myocytes constitute the majority of cardiac tissue volume, whereas fibroblasts dominate in numbers. In vitro, cardiac cell cultures are usually designed to exclude fibroblasts, which, because of their maintained proliferative potential, tend to overgrow the myocytes. Recent advances in microstructuring of cultures and cell growth on elastic membranes have greatly enhanced in vitro preservation of tissue properties and offer a novel platform technology for producing more in vivo-like models of myocardium. We used microfluidic techniques to grow two-dimensional structured cardiac tissue models, containing both myocytes and fibroblasts, and characterized cell morphology, distribution, and coupling using immunohistochemical techniques. In vitro findings were compared with in vivo ventricular cyto-architecture. Cardiac myocytes and fibroblasts, cultured on intersecting 30-μm-wide collagen tracks, acquire an in vivo-like phenotype. Their spatial arrangement closely resembles that observed in native tissue: Strands of highly aligned myocytes are surrounded by parallel threads of fibroblasts. In this in vitro system, fibroblasts form contacts with other fibroblasts and myocytes, which can support homogeneous and heterogeneous gap junctional coupling, as observed in vivo. We conclude that structured cocultures of cardiomyocytes and fibroblasts mimic in vivo ventricular tissue organization and provide a novel tool for in vitro research into cardiac electromechanical function.

Key words: microfluidics, micropatterning, in vivo, cell culture, rabbit, rat

INTRODUCTION

The complex structural makeup of myocardium contains cardiomyocytes, arranged in clusters, strands, and sheets, which are surrounded by a dense network of connective tissue. Communication between cardiomyocytes is via gap junctions, and the distribution of cardiomyocyte subtypes and their coupling via various connexins has been reviewed in detail elsewhere (Kanter et al., 1995; Severs, 1995). The connective tissue that surrounds them is a mix of fibrous extracellular matrix proteins and nonmuscle cells. Although cardiomyocytes constitute the majority of the cardiac tissue volume, they contribute only about a third of total cardiac cell numbers (Vliegen et al., 1991).

Nonmyocytes, in particular cardiac fibroblasts, account for the majority of cells in the heart (Vliegen et al., 1991). Some cardiac regions are exceptionally rich in fibroblasts, such as healthy sino-atrial node (SAN) pacemaker tissue or scars in the infarcted heart (Davies & Pomerance, 1972; Shiraishi et al., 1992). Fibroblasts are located throughout the heart, alongside individual myocytes and between layers of myocardium. They are understood to contribute to cardiac development and the biochemical, metabolic, and structural properties of myocardium (MacKenna et al., 2000; Long & Brown, 2002; Sun et al., 2002). Their possible involvement in electrical signaling, in contrast, has been a subject of considerable debate (Kohl, 2003). Fibroblasts have been known, for many years, to readily form functional gap junctions with other fibroblasts and myocytes in vitro (Goshima, 1970; Rook et al., 1989, 1992). In vivo, previous studies had yielded largely circumstantial electrophysiological evidence for such coupling (Kohl et al., 1994), and it was only recently that we confirmed the presence of structural and functional coupling between fibroblasts and between fibroblasts and cardiomyocytes in native cardiac tissue (Camelliti et al., 2004a, 2004b, 2005).

The structural and functional interrelation of cardiac fibroblasts and myocytes in vivo is generally not well replicated in standard cardiac cell cultures models. In most cases, fibroblast presence and growth are deliberately inhib-
ited. In addition, most cell cultures are grown as confluent monolayers, which reduces tissue-specific properties (such as myocyte orientation), supports myocytes dedifferentiation (loss of sarcomeric organization), and alters functional characteristics (e.g., electrophysiological properties).

In recent years, cell culture micropatterning (mainly surface etching/scratching or deposition of a growth matrix) allowed the design of predetermined layouts of cell attachment and distribution. These advanced cell cultures have yielded more in vivo-like models of cardiac morphology and organization (Rohr et al., 1991, 2003; Singhvi et al., 1994; McDevitt et al., 2002; Gopalan et al., 2003). A prime example, in the context of heterogeneous cocultures, is the study by Gaudesius et al. (2003) that highlighted the ability of fibroblasts (and of connexin-43 transfected HeLa cells) to bridge electrical conduction between cardiac myocytes across gaps of up to 300 μm in length. By simulating localized fibroblast accumulation, this model mimics pathological conditions involving regional fibrosis and supports the notion that cardiac fibroblasts may not only serve as an obstacle to electrical impulse conduction, but—in certain circumstances—as a facilitator/conductor.

Cocultures of cardiac myocytes and fibroblasts, representative of in vivo structures, require alignment of cardiomyocytes and fibroblasts throughout the in vitro model. This can best be achieved by plating cells on tracks of up to 40 μm in width (Gopalan et al., 2003; Motlagh et al., 2003a, 2003b). In addition, the recent successful combination of linear growth patterns with the use of elastic growth substrates (for control of the mechanical environment and application of deformation) has provided a significant further enhancement of cardiac cell culture properties (Gopalan et al., 2003).

Here, we present a novel two-dimensional (2D) structured cardiac tissue model, which combines the management of myocyte and fibroblast content and alignment with control over multicellular structure, as well as the biophysical (including mechanical) and biochemical environment. The model implements intersecting lanes of micropatterned substrate—extracellular matrix proteins, deposited on elastic membranes, at a width equivalent to 2–3 cardiomyocytes (30 μm)—that intersect at prescribed angles to mimic predominant fiber orientation in the myocardial model.

Using antinymesmin antibodies to identify myocytes and antivimentin antibodies to mark fibroblasts (for detail, see Camelliti et al., 2004b), we identify myocyte and fibroblast morphology and spatial interrelation in the in vitro cell culture model, and compare that data to native in vivo myocardial tissue structure. We show that cardiac myocytes and fibroblasts in our model acquire a more adult-like phenotype, including highly organized myocyte striation patterns. The spatial interrelation of the two cell types in vitro closely resembles that observed in native ventricular and atrial tissue: Strands of oriented and aligned myocytes are surrounded by parallel threads of fibroblasts. In addition, we show that fibroblasts form numerous contacts with other fibroblasts and myocytes, both in native cardiac tissue and in the cell culture model. Such contacts have recently been shown to be the site of homogeneous and heterogeneous gap junctional coupling of fibroblasts in rabbit right atrium (Camelliti et al., 2004b) and underlie functional coupling between fibroblasts and myocytes in normal cardiac tissue.

We conclude that the present two-dimensional in vitro model mimics a number of essential structural properties of in vivo myocardium, thereby providing an advanced tool for fundamental and applied cardiac research.

**Materials and Methods**

**In Situ Studies**

**Tissue Preparation**

Rabbit SAN and ventricular tissue were obtained as described previously (Camelliti et al., 2004b). Briefly, hearts were dissected from young New Zealand white rabbits after cervical dislocation were Langendorff-perfused with modified Tyrode solution (containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 8 mM HEPES, 1.8 mM CaCl₂, 10 mM glucose at pH 7.4). The SAN was dissected, embedded in Tissue-Tek compound (Miles Inc., IN), flattened between two glass slides, and rapidly frozen in liquid nitrogen. After removing 40–60 μm of the endocardial SAN tissue surface to expose nodal myocytes beneath the endocardial connective tissue layer, 16-μm cryosections were cut in the plane of the flattened SAN, collected on SuperFrost® slides (Menzel-Glaser, Germany), and stored at −80°C. Ventricular tissue samples were collected and handled in a similar manner.

All experiments were performed in accordance with UK Home Office regulations.

**Immunolabeling**

Detailed immunolabeling protocols have been previously reported (Camelliti et al., 2004b). In brief, tissue sections, fixed in cold acetone for 10 min, were blocked in 10% serum/0.3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; Oxoid, UK) containing 0.1% Triton X-100 for 1 h at room temperature. Myocytes were identified using mouse antinymesmin antibodies (clone B4 [Grove et al., 1984; Agarkova et al., 2000], kindly provided by Dr. H.M. Epenberger, ETH Zurich, Switzerland), which label the M-lines in sarcomeres of myocytes.

Fibroblasts were labeled using a monoclonal mouse antivimentin antibody (clone V9 [Osborn et al., 1984; Bohn et al., 1992], Sigma Aldrich, USA), which specifically reacts with the intermediate filaments that are abundant in fibroblasts.

For double labeling of myocytes and fibroblasts, sections were exposed, overnight at 4°C, to a mix of mouse
monoclonal antimyomesin (1:100) and mouse monoclonal antivimentin antibodies (1:1,000) diluted in PBS containing 0.3% BSA and 0.1% Triton X-100, followed by the secondary goat anti-mouse Cy3-conjugated antibody (1:500, 2 h at room temperature; Jackson Immuno Research Laboratories Inc.). A washing step in PBS was performed between antibody incubations. Identification of the individual cell types in double-labeled preparations was based on their markedly different staining: sarcomeric patterns in myocytes and whole-cell staining of intermediate filaments in fibroblasts (revealing brightly labeled “solid” cells and their processes; for detail see Camelliti et al., 2004b).

Scrape Loading of Lucifer Yellow

Scrape loading of Lucifer yellow was performed as previously described (Camelliti et al., 2004b). Hearts, excised from young New Zealand white rabbits, were Langendorff-perfused at 37°C with oxygenated Tyrode solution for 5–10 min. The SAN was dissected out and a droplet of a PBS solution, containing Lucifer yellow (gap junction permeable dye, 2.5 mg/ml; Sigma, MO) and Texas red dextran (gap junction impermeable dye, 0.5 mg/ml; Molecular Probes) was placed onto the endocardium of the Crista terminalis. Dye penetration was induced via fine insect pin holes (100 μm), punched transmurally through the atrial wall. Dye exposure continued over a period of 2 min. The preparation was then washed with Tyrode solution, taking care that the septal side of the Crista terminalis and the SAN surface were not in contact with the dye at any time. After a further 2–5 min, the tissue was fixed in 4% paraformaldehyde for 1 h at room temperature. Finally, the fixed preparation was washed for several hours in PBS, mounted in CitiFluor antifading medium and the SAN surface were not in contact with the terminalis in PBS, mounted in CitiFluor antifading medium. A washing step in PBS was performed between anti-body incubations. Identification of the individual cell types in double-labeled preparations was based on their markedly different staining: sarcomeric patterns in myocytes and whole-cell staining of intermediate filaments in fibroblasts (revealing brightly labeled “solid” cells and their processes; for detail see Camelliti et al., 2004b).

Confocal Microscopy

Immunolabeled sections were examined with a TCS SP2 confocal laser scanning microscope (Leica Microsystems, Germany), using 458 nm and 476 nm excitation and 489–600 nm emission for Lucifer yellow labeling, and 543 nm excitation and 555–700 nm emission for Cy3 and Texas red dextran labeling.

In Vitro Studies

Micropatterning of Collagen Tracks

Structuring of cell cultures was achieved using the microfluidic matrix patterning technique described previously (Bhatia, 2002; Gopalan et al., 2003). In brief, a silicon wafer is photo-lithographically surface-etched to produce the cell culture target pattern in the shape of highly accurate surface protrusions (here parallel lines, 30 μm wide, 2 cm long, and spaced 300 μm apart). The patterned silicon wafer is used as a master to cast polydimethylsiloxane (PDMS, Sigma) replication molds with matching surface indentations (parallel microchannels). These blocks are tightly sealed onto the cell culture dish surface, and the microchannels are flushed with solution containing extracellular matrix proteins (here collagen type I, 1 mg/ml, Sigma) to form a substrate for preferential cell adhesion (see Fig. 1 for a schematic representation of the procedure).

This patterning can be performed on hard and soft substrates, such as elastic membranes (here 0.25 mm thick silicon sheets; Specialty Manufacturing, Saginaw, MI). In this study, membranes were held in custom-made cell culture chambers (Lee et al., 1996) and sterilized with 70% ethanol, then rinsed with sterile PBS and subsequently exposed to UV light overnight. Between applications, replication molds were incubated in 0.1 M acetic acid for 15 min to remove any collagen residue from the microchannels, then sonicated in alcohol for 10 min, and exposed to UV light for 10 min.

After the sealing of a mold to the culture surface and the flushing of channels with collagen solution, the complete culture chamber and mold were placed in an incubator for 1 h at 37°C, to allow membrane protein adsorption. After curing, the replication mold was carefully removed, and the culture substrate with collagen tracks returned to the incubator at 37°C overnight. For criss-cross patterning, a double deposition of parallel collagen lines was applied: The mold was resealed on to the elastic membrane so that lines intersected at a prescribed angle, and a second set of collagen lines was deposited. Finally, the culture surface was exposed to 2% Pluronic F-108 (BASF) solution for 12 h at 37°C. Pluronic F-108 is a tri-block polymer with polyethylene oxide side chains that reduces protein and cell adhesion outside the collagen-covered areas. Excess Pluronic solution was removed with several washes in sterile PBS, and the culture chamber was ready for cell plating.

Cell Culture

Primary neonatal cardiac cell suspensions were obtained using a modification of the method by Bogoyevitch et al. (1995). Hearts were removed from 1–2-day-old neonatal Wistar rats after cervical dislocation. Atria and fat tissue were trimmed away, and ventricles were minced with dissecting scissors. Cells were dissociated with a series of digestion steps at 37°C in a shaker bath, using a combination of collagenase type 2 (0.5 mg/ml, Lorne Laboratories) and
pancreatine (0.8 mg/ml, Sigma). After each digestion step, excluding the first, cells were collected and resuspended in a mixture of 1:1 fetal calf serum (FCS, Autogen Bioclear) and newborn calf serum (NCS, Autogen Bioclear) to stop enzyme activity, and centrifuged for 5 min at 90 g. The supernatant was discarded, cells were resuspended in fresh FCS/NCS, and kept in the incubator (37°C, 5% CO₂). At the end of the digestion series, all collected cells were centrifuged for 6 min at 90 g. The cell pellet was resuspended in Dulbecco’s modified Eagle medium (DMEM; Sigma), containing 17% medium 199 (M199, Sigma), antibiotics (100 units/mL penicillin, 50 μg/mL streptomycin; GibcoBRL), 10% horse serum (Life Technologies), and 5% fetal calf serum (Autogen Bioclear). The cell suspension was preplated in patterned or nonpatterned culture chambers and incubated for 15 min or 2 h at 37°C in an incubator, to reduce fibroblast content in the supernatant. The suspended nonattached cells (myocytes and fibroblasts, or mainly myocytes, depending on preplating step duration) were collected and seeded at a density of 1 million cells per culture dish onto microstructured or unstructured membranes.

Twenty-four hours later, preparations were washed with cell culture medium to remove nonattached cells and weakly attached cells between target collagen lines, and fresh medium was added. Cells were cultured for 3 days before being processed for immunohistochemistry. For any experiments involving mechanical stimulation, Streptomycin has to be removed from the culture medium at least 24 h prior to use, because this aminoglycoside is a potent blocker of stretch-activated ion channels in isolated cells and may affect the physiological response to stretch (Belus & White, 2003).

Purified fibroblast cultures (99% fibroblast content) were obtained from the first 15-min preplating step (where predominantly fibroblasts attach to the cell culture dish), kept in culture under the same conditions as the myocyte–fibroblast cocultures, and then processed for immunolabeling.

**Immunolabeling and Microscopy**

Cells were washed in sterile PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After a further PBS wash, cells were blocked for 1 h at room temperature in 10% goat serum/0.3% BSA in PBS containing 0.1% Triton X-100. Small areas of the membranes were removed from the stretchers and attached to microscopy slides. Myocytes were identified with antimyomesin antibodies and fibroblasts with antivimentin antibodies. A Cy3-labeled goat anti-mouse secondary antibody was used at a dilution of 1:300 for myomesin and at 1:500 for vimentin, diluted in PBS containing 0.3% BSA and 0.1% Triton X-100.

Preparations were mounted in CitiFluor mounting medium for fluorescence to prevent fading and viewed with a Leica NT confocal laser scanning microscope (Leica Microsystems, Germany).

**RESULTS**

**Native Cardiac Tissue: Rabbit Ventricular Myocardium and SAN**

Morphology and spatial interrelation of fibroblasts and myocytes in native rabbit ventricle and SAN were investi-
gated using immunohistochemical labeling of cell types and confocal microscopy. Myocytes are clearly identifiable using anti-myomesin antibodies, and fibroblasts using antibodies against intermediate filaments (antivimentin antibodies; Fig. 2A,B). By combining the two antibodies, the spatial interrelation of the two cell types can be studied.

Fibroblasts in ventricular and atrial myocardium are organized in longitudinal threads or sheets that run in parallel to strands of cardiomyocytes or form bridge-like structures between myocyte layers (Fig. 2A,E). Fibroblasts in the central SAN have a more complex organization and can be found interspersed with myocytes or forming islandlike aggregates between myocyte clusters (Fig. 2B,F). Thus cardiac tissue cytoarchitecture, and in particular fibroblast spatial organization, shows significant regional differences in the heart.

Fibroblasts form numerous contacts with other fibroblasts and with myocytes, both in the ventricle and in the SAN. Such contacts have recently been shown to contain homogeneous and heterogeneous gap junctions (Camelliti et al., 2004b), supporting the concept of electrical coupling between the two cell types.

The use of Lucifer yellow scrape loading confirmed that fibroblasts are functionally coupled, giving rise to clear cell labeling of fibroblast threads, organized in parallel to cardiomyocytes (Fig. 2C). The preferential staining of fibroblasts suggests that fibroblasts form a highly coupled network. This is confirmed by combining Lucifer yellow scrape loading with myomesin antibody labeling, which reveals that both atrial tissue and SAN contain complex networks of highly interconnected (dye-loaded) fibroblasts (Fig. 2E,F).

Furthermore Lucifer yellow spread reconfirms our recent observation of functional coupling between fibroblasts and myocytes (Camelliti et al., 2004b), as illustrated by the labeled fibroblast/myocyte cell strands in Figure 2D.

**In Vitro Cardiac Model**

To mimic, *in vitro*, key aspects of *in vivo* tissue architecture, we have developed 2D structured cardiac tissue models, which include both myocytes and fibroblasts (to mimic myocyte–fibroblast intermingled regions) or mainly fibroblasts (to mimic high density fibroblast areas). The model consists of criss-cross patterns of extracellular matrix protein tracks (collagen lines), deposited on elastic membranes, with variable intersection angles (Fig. 3C,D). In this highly structured cell culture system, cells have a more adultlike phenotype and multicellular structural organization mimics that of adult ventricular and atrial myocardium. The model also addresses cardiac tissue inhomogeneity, mimicking anisotropic fiber orientation and, by growing the cells on elastic membranes, offers the possibility of controlling the mechanical environment. One can, therefore, independently control cell orientation, geometry, cell-to-cell contacts, extracellular matrix composition, biochemical environment (culture medium, drugs), and biophysics (strain). For functional studies, 2D patterns with an adaptable intersection angle allow one to mimic varying ratios of apparent conduction velocity along the long (*x*) and short (*y*) diagonals of the resulting cell culture “diamonds” (between 1:1 for an angle of 90°, isotropic model, and 10:1 for an angle of approximately 11°, anisotropic model). The apparent conduction velocity ratio in the *x* and *y* directions is 1.7:1 for an intersection angle of 60° (Fig. 3C), and 2.4:1 for an angle of 45° (Fig. 3D). Such models lend themselves to the study of reentrant excitation and mechanical modulation of arrhythmogenesis or arrhythmia sustenance.

**Cell Morphology and Organization**

Cell suspensions containing either predominantly fibroblasts, predominantly myocytes, or a mixture of myocytes and fibroblasts were cultured on collagen-patterned surfaces. For comparison, the same cell suspensions were cultured under matching conditions but on nonpatterned collagen substrates.

Cells seeded onto the patterned and nonpatterned surfaces attach on the collagen-coated substrate in the first 24 h after plating. Cells plated on patterned substrate adhere preferentially to the collagen-coated lines (or migrate from noncollagen-covered surface areas), and then spread along the growth tracks. Gaps between individual cells on the collagen tracks exist during day 1 of culture, but after 2 days lines are almost completely filled with cells. On the third day in culture, cells are closely approximating each other, and myocyte beating is synchronized. After 6–7 days in culture, cell growth cannot be accommodated anymore on the restricted space offered by collagen-plated areas, and the designed cell culture pattern is progressively wiped out.

Immediately after seeding, all cells have a spherical aspect; they acquire a more physiological shape after attachment onto the collagen-patterned substrate: Both fibroblasts and myocytes expand into elongated structures (Fig. 3B). If seeded onto collagen-coated nonpatterned membranes (Fig. 3A, top half), fibroblasts and myocytes are randomly oriented, with no preferential alignment, and both cell types acquire a multipolar shape with numerous extended cell processes. Both in structured and unstructured cultures, it is difficult to distinguish with certainty cell types by light microscopic analysis of optical features only. Also, because entire tracks of cells contract in synchrony, it is impossible to distinguish individual cell types by their mechanical activity.

Purified fibroblast cultures, both structured and unstructured, were obtained during the preplating step. Fibroblasts attach very quickly to the collagen substrate and fill the collagen tracks in 3–4 days of culturing (1–2 days longer than required for cells in cocultures). To validate the presence of cell types in the various cell cultures (myocyte-dominated, fibroblast myocyte cocultures, or fibroblast-enriched), we used standard immunolabeling techniques. We found that myocyte-dominated
suspensions contain 75% myocytes (i.e., there is a significant fibroblast “contamination”), myocyte–fibroblast cosuspensions contain both cell types with a slight prevalence of fibroblasts, whereas fibroblast-enriched suspensions contain 99% fibroblasts.

Immunohistochemical identification of cell types also supported the study of cell morphology and cytoarchitectural spatial organization in both structured and unstructured culture models. Figure 4A,B compares fibroblasts grown on an elastic membrane coated with nonpat-
terned collagen by fibroblasts grown on collagen-patterned substrate. Fibroblasts in nonpatterned cultures are randomly oriented, nonelongated, with a multipolar shape and multiple cell processes. The total cell area appears larger than expected. Fibroblasts in patterned cultures are aligned along the collagen tracks and have an elongated shape with predominantly bipolar membrane extensions.

Figure 4C,D compares myocytes in nonpatterned and patterned cultures. Myocytes growing on a patterned culture substrate have an elongated (rodlike) shape, are highly organized, and have regular cross-striation patterns. Sarcomere arrangement is "in register" even between neighboring cells (Fig. 4D), which also share extended contact regions along the entire length of their cell bodies. In comparison, cells on nonstructured growth substrates have a more random shape and irregular striation patterns. They, too, form contacts with neighboring cells, although mainly via multiple nonstriated cell extensions.

Overall myocyte appearance is significantly improved in patterned cultures, and resembles that of myocytes in native myocardial tissue (see myocardial tissue in Fig. 5A compared to cultured myocytes in Fig. 5B). Immunolabeling of cell types in cocultures grown on lines shows furthermore that fibroblasts and myocytes, forced to coexist in a confined space, coalign and form elongated, well-oriented, and polarized chains with exten-
sive intercellular contacts. Myocytes occupy the central area of growth tracks, whereas fibroblasts attach preferentially to the edge of lines, forming parallel threads along the contractile muscle strands, like in native myocardium (Fig. 5, compare A and B).

Thus, it would appear that structured in vitro cocultures mimic a number of essential aspects of in vivo cell morphology, tissue architecture, cell–cell interaction, and contractile function.

**DISCUSSION**

We present a 2D structured in vitro model of cardiac myocyte–fibroblast interrelation that mimics important aspects of in vivo cyto-architecture, such as the highly organized striation patterns of cardiomyocytes, both within individual and between neighboring cells, as well as the intimate interrelation of fibroblasts with the densely packed myocyte strands.

Fibroblasts are essential structural and functional components of the heart (for review, see Camelliti et al., 2005). Their content increases with development and during aging, as well as in pathological conditions such as rheumatic heart disease, sick sinus syndrome, ischemia, and infarction (Davies & Pomerance, 1972; Adler et al., 1981; Shiraiishi et al., 1992; MacKenna et al., 2000). Fibroblasts are involved in the maintenance of myocardial structure in the normal heart (by balancing synthesis and degradation of connective tissue components), and in myocardial remodeling during cardiovascular disease (e.g., via excessive proliferation, [Whitaker, 1995], synthesis of extracellular matrix components, and elevated release of humoral factors [Booz & Baker, 1995; Sun & Weber, 2000]). In this capacity, they affect the electrophysiological properties of cardiac tissue indirectly (paracrine effects) or by posing as an obstacle to orderly impulse propagation. On the other hand, recent research has reinforced the possibility that cardiac fibroblasts may act as conductors of excitation, both in vitro (Goshima & Tonomura, 1969; Gaudesius et al., 2003) and in vivo (Kohl et al., 1999; Kohl, 2003). In the SAN, in particular, fibroblasts have been found to be functionally coupled by Cx45 to homo- and heterogeneous cell types including cardiac myocytes (Camelliti et al., 2004b). Thus, representative in vitro models of cardiac tissue function need to include both fibroblasts and myocytes, and to control their structural and functional interrelation.

Myocytes in the ventricle form highly organized sheets, three to four cells thick, that are surrounded by connective tissue and bridged by branches of myocytes and fibroblasts (Fig. 2, see also Sommer & Scherer, 1985; LeGrice et al., 1995). Within these bundles, sarcomeres are arranged in register, even between neighboring cells, as can be seen from the cell-specific labeling used in this study (see, e.g., Fig. 2A,E).

This highly regular arrangement is reproduced in our structured tissue cultures. Cells acquire a more in vivo-like cell morphology (Figs. 3, 4), highly organized striation patterns (Fig. 4), and end-to-end gap junctional coupling of...
myocytes (Gopalan et al., 2003). This is vital for the development of representative in vitro models of ventricular structure and function. In addition, sarcomere structures of neighboring cells show similar coordination to that seen in vivo (Figs. 4D, 5B).

The present model furthermore combines the advantages of pseudo-1D models with important aspects of 2D functionality. By superimposing two sets of intersecting parallel lines, we create a model that mimics prevalent fiber orientation to address cardiac tissue inhomogeneity. By varying the intersection angle, ratios in apparent conduction velocity of between 1:1 and 10:1 can be created.

In addition, using the microfluidic technique illustrated in Figure 1, cell structuring can be conducted on elastic membranes, offering control over the mechanical environment. Because the heart is a mechano-sensitive organ, and stretch affects gene expression, protein synthesis, paracrine activity, and cell electrophysiology (to name but a few; Yamazaki et al., 1995; Kohl et al., 1999; Mackenna et al., 2000; Ruwhof & van der Laarse, 2000; Wang et al., 2000), this aspect of cardiac functionality needs to be captured to build faithful in vitro models. The cell culture presented here is a further step towards more representative in vitro models of cardiac structure and function.

Future research is directed at describing comprehensively the homo- and heterogenous coupling behavior of myocytes and fibroblasts in structured cell cultures, including such aspects as the presence or absence of intercalated disks between myocytes. Nonstructured cocultures of neonatal myocytes and fibroblasts express connexin43 (Rook et al., 1989, 1992), whereas myocytes and fibroblasts in structured cultures have been described to express both connexin43 and connexin45 (Gaudesius et al., 2003). A systematic study of in vitro fibroblast–myocyte and fibroblast–fibroblast coupling requires a combination of connexin labeling and cell type identification. Another important facet of further research is the study of environmental influences on drug efficacy in highly structured cardiac cultures, which, in isolated cells, has been suggested to be affected by mechanical factors (Wright & Rees, 1997).

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