Cell culture chips for simultaneous application of topographical and electrical cues enhance phenotype of cardiomyocytes†

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In vivo, cardiomyocytes are exposed to multiple biochemical and physical cues including topographical and electrical cues. During prolonged *in vitro* cultivation in standard tissue culture set-ups, cardiomyocytes are known to de-differentiate due to the lack of appropriate micro-environmental cues. Most currently available cell culture systems provide only a single biophysical cue, thus development of advanced cell cultivation systems incorporating multiple cues is urgently needed. We report here the development of a microfabricated system, incorporating topographical and electrical cues on a single chip, which enables cultivation of differentiated cardiomyocytes. The cell culture chips were created by hot embossing of polystyrene, to create microgrooves and microridges of precisely defined depth, width and periodicity. Substrates consisting of 0.5 μm-wide grooves and 0.5 μm-wide ridges (1 μm period) and those consisting of 3 μm-wide grooves and 1 μm-wide ridges (4 μm period) were investigated, with smooth surfaces used as controls. The depth of the microgrooves was 400 nm. The two gold electrodes were electrodeposited 1 cm apart such that the microgrooves in-between were oriented either parallel or perpendicular to the electrodes, enabling studies of interaction between topographical and electrical cues. Neonatal rat cardiomyocytes cultivated on microgrooved substrates for 7 days were elongated and aligned along the microgrooves forming a well developed contractile apparatus, as evidenced by sarcomeric α-actinin staining, with a more pronounced effect on substrates with 1μm compared to 4 μm periodicity. Importantly, simultaneous application of biphasic electrical pulses and topographical cues resulted in gap junctions confined to the cell-cell end junctions rather than the punctate distribution found in neonatal cells. Electrical field stimulation further enhanced cardiomyocyte elongation when microgrooves were oriented parallel to the electric field. Due to the compatibility of the described cell culture chips with fluorescence and optical microscopy as well as the ability to independently control field stimulation parameters, biochemical and topographical cues on each chip, this system may in the future become a useful tool in drug development and maturation of cardiomyocytes derived from stem cells.

Introduction

In vivo, cells are exposed to multiple biochemical and physical cues including topographical and electrical cues. The cell phenotype, including its orientation and elongation, is ultimately determined by the interaction amongst these multiple cues and may change with time as the type and magnitude of the cues changes. In contractile tissues such as myocardium, functional properties can directly be correlated to the orientation and elongation of cardiomyocytes[1] that occupy 90% of the volume of native myocardium. Thus, our ability to create high-fidelity cultures of cardiomyocytes *in vitro* critically depends on the availability of advanced cell culture systems that combine multiple physical and biochemical cues. These efforts are focused on both two-dimensional systems (2D) where cultures of cardiomyocytes may serve as platforms for drug development and three-dimensional systems (3D) where cultivation of functional cardiac patches is the ultimate goal.

Fully differentiated adult cardiomyocytes are elongated and rod-shaped, containing well developed contractile apparatus, identified as a characteristic cross-striation pattern in cells stained for contractile proteins, with intercalated discs as electro-mechanical cell end-to-end coupling. Gap junctions, located in intercalated discs at the ends of cells in adult myocardium,[2] are responsible for rapid propagation of electrical signals between the cells. When cultivated on simple two-dimensional substrates (2D) as monolayers or single cells, cardiomyocytes are known to de-differentiate due to the lack of appropriate micro-environmental cues.[3,4] In this process the defined contractile apparatus disappears and the cells slowly assume irregular oval or star shapes. Mechanical stimulation,[5–8] pharmacological[9] or electrical[10–15] induction of contractions as well as cultivation on patterned substrates[16,17] were utilized previously in order to provide one of the micro-environmental cues required for maintenance of cardiomyocyte phenotype and contractile function *in vitro*.

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The mechanically activated signaling pathways were implicated in the response of cardiomyocytes to the electrical field stimulation,[18] as the presence of contraction following electrical stimulation was required for the observed beneficial effect on cell elongation, contractile properties and hypertrophy. Cardiomyocytes were also reported to have a preferred orientation in response to field stimulation,[19] so that they were more excitable when the long axis of the cell was oriented parallel to the electrical field.

Most currently available cell culture systems provide only isolated micro-environmental cues such as independent application of either topographical,[16] electrical,[10] adhesive[20,21] or biochemical cues.[22] Thus development of advanced cell cultivation systems incorporating multiple cues is urgently needed. In our previous study[23] we used abraded polyvinyl surfaces that were placed between two parallel carbon electrodes to combine electrical and topographical cues into a single system. Although this approach allowed us to gain valuable insight in the response of fibroblasts and cardiomyocytes to combined electrical and topographical cues, the system contained several shortcomings: a) since topographical cues were created by abrading the surface using a fine lapping paper, there was variability in abrasion shape, width and depth on the same surface as well as between the surfaces; b) polyvinyl is not a preferred material for cell culture; and c) multiple surfaces had to be placed between two parallel carbon electrodes, thus forcing us to apply identical electrical stimuli and soluble factors (biochemical cues) to different surfaces.

We report here the development of a microfabricated system, incorporating topographical and electrical cues on a single chip, which overcomes all of the above shortcomings and enables cultivation of highly differentiated cardiomyocytes. The cell culture chips were created by hot embossing of polystyrene, which is a standard tissue culture plastic material, in order to create microgrooves and microridges of precisely defined depth, width and periodicity. The gold electrodes were electrodeposited such that the microgrooves were oriented either parallel or perpendicular to the electrodes, enabling studies of interaction between topographical and electrical cues. Most importantly, the electrical stimulation parameters and biochemical cues can be independently controlled on each chip. These features will ultimately make the described system a useful and unique platform for studies of stem cell differentiation or drug testing.

Methods

Hot embossing mold fabrication

The mold for hot embossing was fabricated by standard photolithography for the surfaces with 4 μm period grating and deep-UV lithography for the 1 μm period grating. The trench was etched by RIE (PlasmaLab 80 Plus, Oxford Instruments, UK) using a mixture of gas of 20 sccm CF_4 and 2 sccm O_2 at 10 mTorr and 100 W, leading to an etching rate ~20 nm/min. The depth of the trench was 0.4 μm.

Hot embossing for preparation of microstructured polystyrene surfaces

We used hot embossing (EVG, Austria) to create a polystyrene replica of the silicon mold. We dispersed the polystyrene pellets (120 kg/mol) evenly over an area of 30 cm^2 on top of the mold and covered it with another flat wafer. Both the mold and the flat wafer were treated with an anti-adhesion silane layer (1H,1H,2H,2H-perfluorooctyl-trichlorosilane) to facilitate the separation. We heated up the stack to 160 °C, applied a force of 1500 N, waited for 2 minutes and then evacuated the system to below 1 Torr, followed by applying the final force of 10000 N for 5 minutes. The system was then vented and cooled down to 90 °C before removing the force. The thickness of the polystyrene sheet was 300–400 μm.

Electrode fabrication

The grating at the electrode area (the area between electrodes covered with Si pieces to protect it from etching) was first etched by O_2 plasma with a power of 100 W, 20 sccm O_2 and 100 mTorr (etching rate 100 nm/min) for 10 minutes, so that the sidewall of the grating ridges became inclined. This was essential to ensure a continuous metal film. Otherwise, metal film would break at the vertical ridge-edges. Next, the electrode area was coated with 30 nm Cr adhesion layer and 70 nm Au conducting layer. Electroplating using Au electrolyte from Tecknic Inc was subsequently carried out to deposit ~800 nm Au film on the electrode area. The chips were fabricated such that the microgrooves were oriented either perpendicular or parallel to the electrodes. As a control, smooth polystyrene surfaces were used between the electrodes.

O_2 Plasma treatment

For improved cell adhesion, all substrates were treated with oxygen plasma with a power of 100 W, 20 sccm O_2 and 10 mTorr for 20 seconds.

Scanning electron microscopy

The microgrooved surfaces were examined using a Hitachi S4800 (Japan) field-emission scanning electron microscope (SEM). At the end of cultivation, cell culture samples were fixed in a glutaraldehyde/formaldehyde solution supplied by the Microscopy Imaging Laboratory (Faculty of Medicine, University of Toronto). The samples were washed consecutively in 30% (1×), 50% (1×), 70% (1×), 90% (2×), and 100% (3×) ethanol. The samples were then subjected to critical point drying followed by gold coating. The samples were imaged in a Hitachi S3400 scanning electron microscope.

Cell source

We obtained cardiomyocytes from 2-day old neonatal Sprague-Dawley rats as described in previous studies[23] and according to a protocol approved by the University of Toronto Committee on Animal Care. Briefly, the hearts were quartered and subjected to an overnight digest at 4 °C in 0.06% (w/v) solution of trypsin (Sigma, Canada) in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) (Gibco, Canada). A series of five 4–8 min digestions in collagenase II (Worthington, USA 220 units/mL) in HBSS followed. The resulting cell suspension was pre-plated for 1 hr in T75 flasks to enrich for cardiomyocytes.

Cell culture

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Canada) containing 4.5 g/L glucose, with 10% (v/v) fetal bovine serum (FBS, Gibco, Canada), 1% HEPES and penicillin-streptomycin (100 units/mL and 100 μg/mL, respectively, Gibco, Canada). Sheets of microgrooved polystyrene were cut into pieces of 1 cm^2 area, and were each placed in one well of a 12-well tissue culture dish with the side of the microgrooves facing up. The pieces were then soaked in 70% ethanol overnight and exposed to UV light in a biosafety cabinet (Labconco, VWR, Canada) for 2 hours. Each piece of polystyrene was coated with 60 μL of fibronectin (25 ng/μL, Sigma, Canada) in phosphate buffered saline (PBS) for 90 min.

For experiments without electrical stimulation, 500000 cardiomyocytes in 60 μL of culture medium were seeded onto the fibronectin coated surfaces for 1 hr to allow the cells to attach, then 2 mL of culture medium were added into each well. The cells were cultivated for 4 or 7 days as indicated, at which point they were fixed in 4% paraformaldehyde (PFA) solution in PBS (made from powder, Sigma, Canada). In order to assess the effect of contraction on elongation and orientation of cardiomyocytes, cells were cultivated under non-stimulated conditions on the cell culture chips with 1 μm grating period for 4 days. On day 4, verapamil was added to the culture medium to block any spontaneous contractions at the concentrations of 2, 5 and 10 μM (Sigma V4629, Canada) and maintained for 20 hours. The control sample at 0 μM of verapamil was maintained in the regular cardiomyocyte medium for 20 hours.

For experiments with electrical stimulation, chips with built-in electrodes were used. A platinum wire was wrapped around each electrode in a secure manner to ensure consistent contact with the electrode. The chip was then placed in a well of a 12-well dish, with the wires extending to the outside of the well. The chips were secured such that they did not touch the bottom of the well; such an arrangement was made to prevent the cells from attaching to the tissue culture wells instead of microgrooved substrate itself. The surfaces were treated with 70% ethanol and UV irradiation followed by fibronectin coating as described above. Cells were seeded and cultivated for 24 hrs in the same manner as in the experiments without electrical stimulation. After 24 hrs, the platinum wires were connected to a programmable electrical stimulator (Grass s88x, Astromed, Canada). Electrical field stimulation was applied in the form of symmetric biphasic pulses at a frequency of 1 Hz, amplitude of 1.15 V/cm per phase and duration of 1 ms per phase. The desired stimulation regime was verified with an oscilloscope with this particular setup in place. With respect to the electric field direction, there were at least $N = 3$ samples within each group (parallel, perpendicular, smooth). Electrical field stimulation was stopped at day 7 and cells were fixed with 4% PFA as described above.

Cell staining and assessment

To visualize morphology and phenotype of cardiomyocytes cultured under the different conditions, immunofluorescent staining using the following primary antibodies was performed as previously described:[23] monoclonal anti-sarcomeric α-actinin made in mouse at dilution factor of 1:50 (Sigma, Canada) and polyclonal anti-connexin-43 antibody made in rabbit at 1:100 (Chemicon, USA). Live-dead staining was performed as described previously using CFDA and PI according to the manufacturer's protocol (CFDA = carboxyfluorescein diacetate, succinimidyl ester, 10 μM; PI = propidium iodide, 75 μg/mL Molecular Probes, USA).[23] Images were taken using a fluorescence microscope (Olympus IX2-UCB, Canada) or a confocal microscope (Olympus FV5-PSU confocal with IX70 microscope, Canada).

To visualize actin cytoskeleton and the presence of focal adhesions with respect to the topographical cues, a sub-set of cell culture chips (non-stimulated, 1μm grating period, 4 days of culture) was stained with Tetramethyl Rhodamine Isothiocyanate conjugated phalloidin (phalloidin-TRITC) or immunostained for vinculin. To visualize actin cytoskeleton, the cells were fixed in formalin for 30 min at room temperature, rinsed in PBS and incubated in 50 μg/mL of phalloidin-TRITC conjugate (Sigma P1951, Canada) for 40 min at room temperature according to a protocol we described previously.[23] The samples were then washed in PBS to remove unbound conjugate and imaged using a fluorescence microscope (Olympus IX2-UCB, Canada). For vinculin staining, samples were permeabilized in 0.1% Triton-X-100 (Sigma, Canada) for 10 min, then blocked with 10% normal horse serum (Vector Laboratories, Canada) for 30 min. Mouse monoclonal Anti-Vinculin (Clone hVIN-1, Sigma, Canada) was added at a dilution of 1:200, and incubated at 4 °C overnight. Samples were then stained with fluorescein conjugated goat anti-mouse IgG (Sigma, Canada) at a dilution of 1:64 and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Canada) at a dilution of 1:100 at room temperature for 40 min. Samples were attached to a glass coverslip and imaged using confocal microscopy. Imaging was performed at the Department of Cell and Systems Biology using a Zeiss LSM 510 (Zeiss, USA) confocal microscope.

Image analysis

ImageJ software was used to determine cell alignment and elongation as described previously.[23] Briefly, cell elongation was defined as the ratio of the long axis of the cell compared to its short axis. Cell alignment was determined by measuring the angle of deviation of the long axis of the cell from the microgroove line. For cells cultured on the control surfaces without microgrooves, the angle was taken to be the angle between the long axis of the cell and the electric field lines or a fixed horizontal line in non-stimulated samples.

Excitation threshold and maximum capture rate

Functional properties of cardiomyocytes were determined by measuring excitation threshold (ET) and maximum capture rate (MCR). For non-stimulated samples, the ET and MCR were measured on day 7, in an electrical stimulation chamber consisting of two parallel carbon electrodes spaced 1cm apart and connected to an electric stimulator (Grass s88x).[23–25] Measurements were taken with the sample placed both parallel and perpendicular, respectively one after the other, to the carbon electrodes. For experiments with electrical stimulation, ET and MCR were determined using the electrodes on the substrate

itself, at day 1 and on day 7 just prior to fixing the cells. ET was defined as the minimum voltage required to induce synchronous contractions of at least 75% of the cells in the field of view, by monophasic pulses of duration of 2 ms and frequency of 1Hz. MCR was the maximum beating frequency that could be induced at 200% of the ET.

Statistical analysis

SigmaStat 3.0 software was used for statistical analysis. Tests for normality and equal variance were performed on all quantitative data sets. If the data sets satisfied the assumptions of normal distribution and equal variance, one-way ANOVA in conjunction with the Student-Newman-Keuls test was used to compare multiple groups and t-test was used to compare two groups. Otherwise, we performed the Kruskal-Wallis one-way ANOVA on ranks in conjunction with Dunn's method for pairwise comparisons. The groups were considered significantly different if $p < 0.05$.

Results and discussion

Hot embossing of polystyrene for fabrication of microgrooved surfaces with built-in electrodes

The main objective of this study was to create a microfabricated cell culture substrate that will enable integration of precise topographical and electrical cues into a single system and their independent control. For this purpose, we chose to utilize hot embossing as a high throughput molding process of low cost and high pattern transfer fidelity. Unlike photolithography which can pattern polymers by lithography followed by pattern transfer *via* etching, hot embossing creates a pattern inside a polymer within a single step. It is suitable for patterning a broad range of thermoplastic polymers including those that are biodegradable, biocompatible or semiconducting. Hot embossing has been used previously to create micron scale features in polymethyl methacrylate,[26–28] polyimide,[26] polyethylene[29] and polycarbonate.[29] In addition, hot embossing is a "dry" process, which is essential for patterning polymers susceptible to degradation by solvents, water or other chemicals. Auger, Veres and colleagues were the first to use polystyrene surfaces with micron-scale features created by hot-embossing for cell culture.[30] Polystyrene is a standard plastic material used in tissue culture, thus motivating its use in this study as well.

The microgrooves fabricated in polystyrene *via* hot embossing technique were used to introduce topographical cues to the cell culture (Fig. 1), while the electrodes made *via* gold electroplating were used to introduce electrical cues. The distance between electrodes was chosen as 1cm, consistent with our previous work,[23,24] as well as to enable easy calculation of the electric field strength, which is measured in units of V/cm. (Fig. 1D,E). Two different geometries were assessed independently of electrical field stimulation: 1) substrates consisting of 0.5 μm-wide grooves and 0.5 μm-wide ridges (1 xμm period) (Fig. 1B), and 2) substrates consisting of 3 μm-wide grooves and 1 μm-wide ridges (4 μm period) (Fig. 1C). The depth of the microgrooves was 400 nm and identical in both cases. Scanning electron microscopy images indicated that the microgrooves were of uniform size and spacing and mostly defect-free (Fig. 1B,C). While the groove

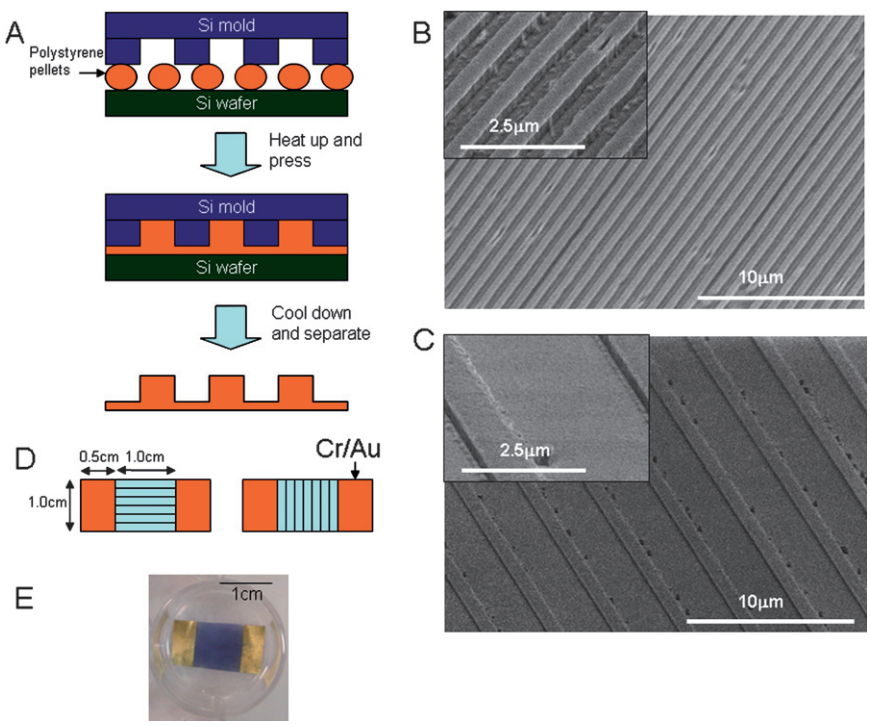

Fig. 1 Microgrooved surfaces prepared by hot embossing of polystyrene. A) Schematics of hot-embossing process. Scanning electron micrograph of the microgrooved surface with B) 1μm period, 0.5μm groove width, 400 nm groove height and C) 4 μm period, 3μm groove width, 400nm groove height. D) Schematics of the orientation of grooves with respect to the electrodes. E) Photomicrograph of a cell culture chip with cardiomyocytes between electrodes (blue) visualized by Giemsa staining.

width was significantly smaller than the width of V-shaped abrasions used in our previous studies (1 μm compared to 12 μm),[23] the depth was on the same order of magnitude. The oxygen-plasma treatment, as well as the additional coating of fibronectin prior to cell culture, facilitated cell adhesion to the surfaces during seeding and culture. High cell seeding density and efficient cell adhesion is important for achieving confluent monolayers, as cardiomyocytes do not proliferate.

This approach enabled key advances over the set-up used previously for simultaneous application of electrical and topographical cues.[23] In our previous studies, the V-shaped microchannels were fabricated on polyvinyl surfaces *via* manual abrasion, resulting in an uneven surface of varying abrasion dimensions. Previously, in order to subject the samples to an electric field stimulus, all substrates had to be placed inside an electrical stimulation chamber, between two parallel carbon electrodes. These carbon electrodes were in turn connected to the programmable electrical stimulator.[23] In this new setup, each chip was directly connected to the stimulator, eliminating difficulties involved in securing samples at the correct position between the electrodes and enabling independent application of the electrical cues to each substrate. In addition, each substrate was placed in its own culture medium well, ensuring that no cross-talk *via* soluble factors occurred between the substrates.

Cardiomyocyte phenotype and function on microgrooved polystyrene surfaces

Cells subjected to topographical cues without electrical field stimulation were significantly more elongated than those on smooth surfaces (Fig. 2A, C) with the aspect ratio in the range of 4–6, in comparison to the aspect ratio of 3 for non-abraded surfaces. In addition, the 1μm grating resulted in a significantly higher elongation than the 4 μm grating.

Cardiomyocytes were significantly more aligned on surfaces with topographical cues as compared to those on smooth surfaces (Fig. 2B, C). Cells on surfaces with grating period of both 4 μm and 1 μm had an average angle of deviation of 4°, whereas those on smooth surfaces had an angle of deviation of approximately 42°. An angle of deviation of zero indicates perfect alignment, whereas an angle of 45° indicates maximal misalignment. Moreover, narrow distribution in orientation for cells on surfaces with microgrooves indicated the consistency of the applied topographical cues, relative to a wide distribution on the smooth surfaces, indicating lack of guidance for alignment (Fig. 2B). In our previous studies,[23] the uneven surfaces made *via* manual abrasion resulted in a wider distribution of cell orientation angles, where the average angle was in the range of 10–12°, in comparison to the current narrower distribution and smaller average angle of 4°. Thus, the uniform channels obtained by hot embossing provided topographical cues in a more reliable manner.

The fact that similar responses in cellular alignment and elongation were observed, in this study and in our previous studies,[23] is consistent with a previously reported finding that the depth of the channels,[31] rather than the width, governs these cellular processes. Cellular orientation may be achieved by confining focal adhesion molecules within the ridges of the microgrooves since the adhesion clusters and the ridges are on the same order of magnitude (~1–2 μm).[32,33] The adhesion molecules are coupled to the cell's cytoskeleton, thus resulting in the overall orientation of the cell along the direction of the ridges.

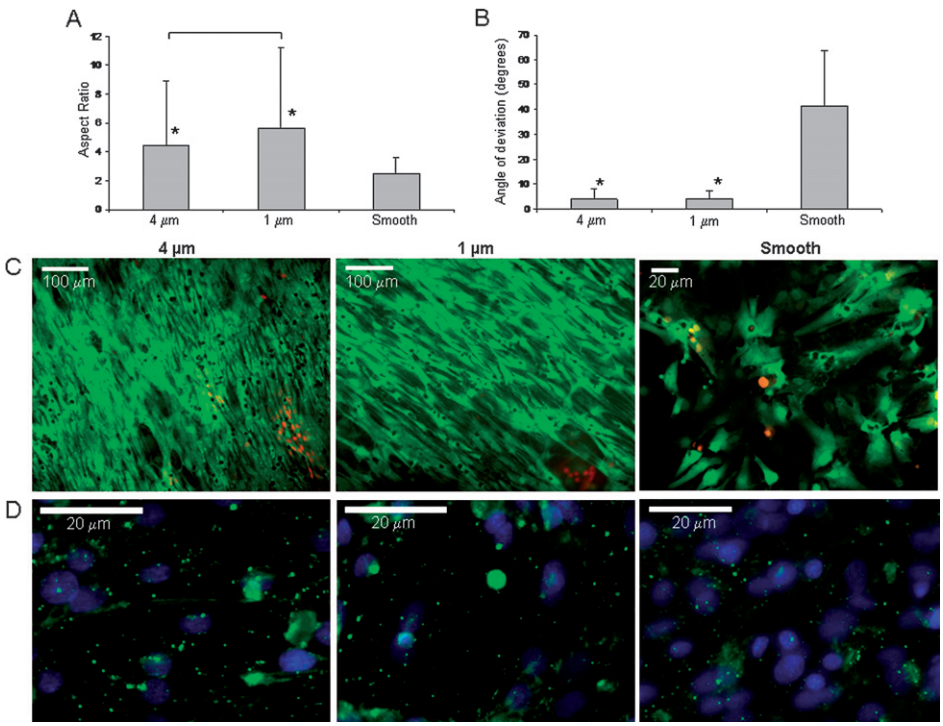

Fig. 2 Cardiomyocytes cultivated on microgrooved polystyrene surfaces without electrical field stimulation. A) Aspect ratio, B) Orientation angle, C) Live/dead staining, and D) Fluorescent micrographs of cells immunostained for Connexin-43 (green dots). Nuclei were counterstained blue with DAPI.

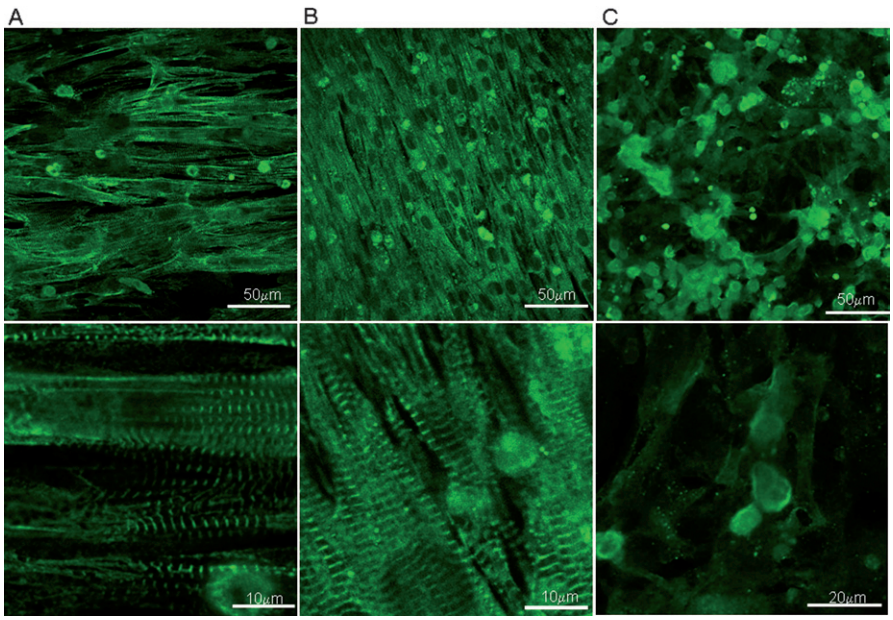

Fig. 3 Sarcomeric-α-actinin staining of cardiomyocytes cultivated on the microgrooved polystyrene surfaces with A) 4μm period, B) 1μm period, and C) Smooth controls. Images were obtained by confocal microscopy.

Live/dead staining indicated that the great majority of cells were alive in all of the experimental groups (Fig. 2C). Immunofluorescence staining was used to validate the phenotype of the cardiomyocytes. Staining was performed for cardiac gap junction protein Connexin-43 (Fig. 2D) and contractile protein sarcomeric α-actinin (Fig. 3). Connexin-43 (Cx43) is required for electrical communication between the cells and as such it is essential for contraction of both engineered and native cardiac tissue. Immunostaining for Cx43 revealed that cells in the non-stimulated groups developed gap junctions necessary for intercellular communication (Fig. 2D). However, the distribution of gap junctions was punctate, characteristic of the neonatal myocardium where gap junctions are distributed over the entire surface of ventricular cardiomyocytes.[2] In addition, there were no significant differences between the cells cultivated on microgrooved and smooth surfaces.

Sarcomeric α-actinin is responsible for attaching actin filaments to Z-lines thereby cross-linking thin filaments in adjacent sarcomeres. It is found in both neonatal and mature cardiomyocytes. Contractile proteins such as α-actinin become increasingly more organized in the heart as development progresses.[34] Staining for sarcomeric α-actinin, revealed that more than 90% of the cells at day 7 were indeed cardiomyocytes and not other cell types (Fig. 3 and Fig. 4E). Confocal microscopy revealed the large extent to which the cell monolayers were ordered by topographical cues and cross-striations (perpendicular to the long axis of the cells), indicative of extremely well-developed contractile apparatus (Fig. 3).

Spontaneous contractions of the monolayers were apparent as early as day 4 in culture. Next, we assessed the monolayer's electrical excitability on the substrate by measuring excitation threshold (ET) and maximum capture rate (MCR). The excitation threshold is defined as the minimum voltage required to induce synchronous beating of 75% of the cells in the field of view. It is indicative of the stimulus amplitude required to achieve contraction of a cell or a cell monolayer. Generally, healthy and easily excitable cardiomyocytes have low ETs. MCR, another measure of electrical excitability, is defined here as the maximum beating frequency of the cells measured at 200% of the ET. MCR values indicate the contractile versatility of the cells and ability to beat at high frequencies. Low ET and high MCR values are desirable for the purposes of devising *in vitro* models for drug testing as well as the tissue engineering of cardiac patches.

ET was measured at the end of the culture period (day 7) by placing the microgrooved samples first parallel then perpendicular to the direction of the electric field. At day 7, the average ET was higher if the measurement was taken with the aligned cells placed perpendicular to the electric field (Table 1). When placed parallel to the electric field, however, the same sample would exhibit a lower ET value consistent with the previous studies of Tung and colleagues.[35] Previously, the average ET value for cardiomyocytes *in vitro* was documented to be about 2.3 V/cm.[25] Average ET values measured in the parallel direction and those on smooth surfaces were approximately equal to or slightly lower than this value, whereas cells placed perpendicular to the electric field had values higher than 3.0 V/cm. This implies that the orientation of the cell monolayer with respect to the electric field is an important factor in determining and enhancing electrical excitability of the cells. For the non-stimulated samples, there was no significant difference in MCR when the measurements were taken with the cells oriented parallel or perpendicular to the field lines (Table 1). In addition, the values for 4 μm, 1 μm period grating and smooth surfaces were comparable.

Electrical stimulation during culture of cardiomyocytes

We hypothesized that electric field stimulation applied *via* electrodes integrated within the tissue culture substrate will yield cells with a differentiated phenotype. By placing the cells on the same surface on which the electrodes reside, the electrical stimuli may

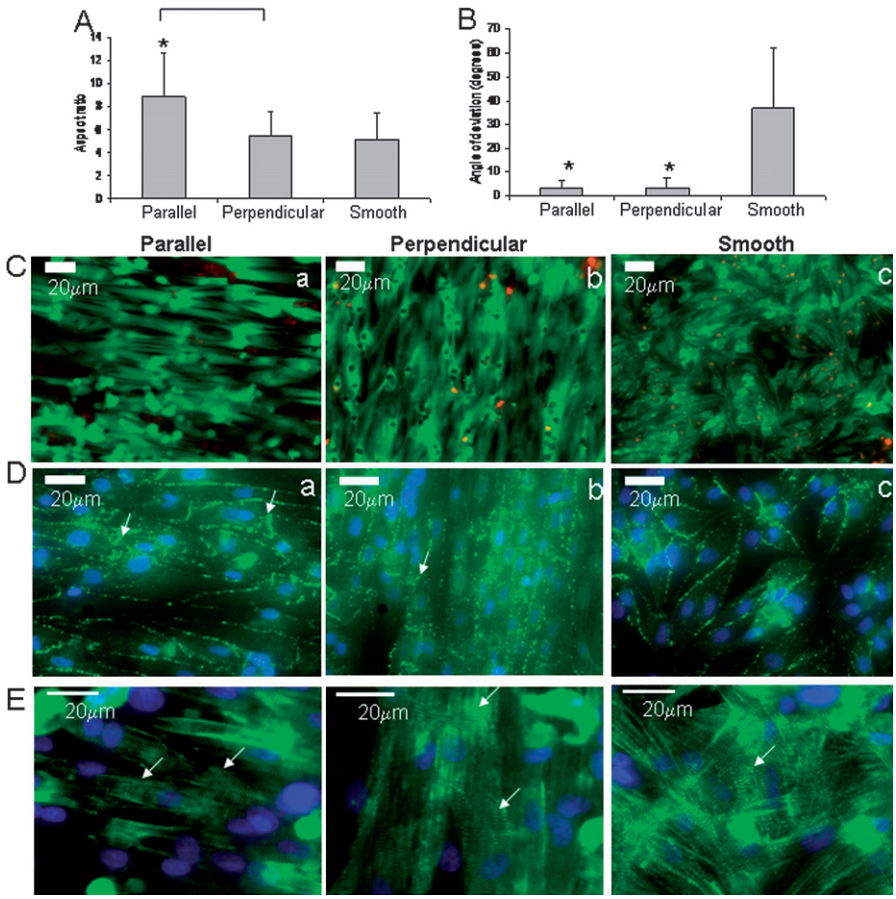

Fig. 4 Cardiomyocytes cultivated on microgrooved surfaces (1μm period) in the presence of electric field stimulation. Electrical stimulation was achieved *via* symmetric biphasic square pulses at the frequency of 1 Hz, pulse duration of 1ms per phase and amplitude of 1.15V/cm per phase. A) Aspect ratio, B) Orientation angle, C) Live/dead staining, and D) Fluorescent micrographs of cells immunostained for Connexin-43 (green dots). Nuclei were counterstained blue with DAPI. Arrows indicate the localization of Connexin-43 at cell-cell junctions. E) Sarcomeric-α-actinin staining (green). Arrows indicate cross-striations. Nuclei were counterstained blue with DAPI. Images were obtained by fluorescence microscopy.

be applied more directly with less resistance, while the electric field strength remains the same. This setup may also eliminate resistances to electrical current that are inherent to the bioreactor consisting of carbon electrodes placed in a glass chamber[23,24] The chosen field amplitude of 1.15 V/cm, delivered in biphasic pulses, was equivalent to the 2.3 V/cm, monophasic pulses, used in the previous studies.[23] During the culture period, pH in the medium was maintained and no gas formation was observed indicating an absence of electrolysis.

Effects of electric field stimulation, in addition to simultaneous topographical cues, on cardiomyocytes alignment and elongation are illustrated in Fig. 4. Cardiomyocytes cultured on microgrooves parallel to the electric field were significantly more aligned than those cultured perpendicular to the electric field as well as those cultured on smooth surfaces (Fig. 4A). This is consistent with previous findings that cardiomyocytes prefer to align in the direction of the electric field,[24,35] and hence may also have developed a phenotype closer to that found in the native environment.

Comparison between stimulated samples (Fig. 4A) and non-stimulated samples (Fig. 2A) indicated that electrical field stimulation significantly improved cellular elongation on smooth

Table 1 Functional properties of cardiomyocytes cultivated on microgrooved surfaces without electric field stimulation. At the end of cultivation (day 7) samples were field-paced using monophasic pulses of 2 ms duration to assess their electrical excitability parameters. Brackets indicate the number of samples that could be induced to contract by stimulation, over the total number of samples tested in that group

Periodicity	4 μm		1 μm		Smooth
Direction with respect to electric field during test	Parallel	Perpendicular	Parallel	Perpendicular	
ET (V/cm)	2.8 ± 1.8 (3/3)	3.4 ± 1.4 (3/5)	1.9 ± 0.5 (3/3)	3.9 ± 1.0[a] (4/5)	2.1 ± 1.0 (3/3)
MCR (Hz)	2.5 ± 0.3 (3/3)	2.2 ± 0.2 (3/5)	2.5 ± 0.3 (3/3)	2.4 ± 0.3 (4/5)	2.5 ± 0.9 (3/3)

[a] Significantly different than the non-abraded control.

surfaces (aspect ratio of 2.5 ± 1.1 for non-stimulated vs. 5.2 ± 2.2 for stimulated cells, $p < 0.05$), and on surfaces where the gratings of 1μm period were oriented parallel to the electric field (aspect ratio of 5.6 ± 5.6 for non-stimulated vs. 8.9 ± 3.8 for stimulated, $p < 0.05$). This suggests that electric field stimulation may further improve cellular elongation when topographical cues are also used to guide cellular alignment in the preferred direction (parallel) with respect to the electric field.

For the stimulated samples, the average angle of deviation was significantly smaller in the cases where topographical cues were present, compared to the random cellular orientation on the smooth surfaces (Fig. 4B). Comparison of stimulated samples (Fig. 4B) to the corresponding non-stimulated samples (1μm period and smooth surface, Fig. 2B) indicated that electrical stimulation had no significant effect on cell orientation, although the average orientation angle was slightly smaller on the stimulated smooth surfaces ($37 \pm 25°$) compared to the non-stimulated smooth surfaces ($42 \pm 22°$). Taken together, these findings indicate that topographical cues are a stronger determinant of cellular orientation than the electric field stimulation, consistent with previous findings.[23]

Live/dead staining indicated that the great majority of cells were alive in all of our experimental groups (Fig. 2C and Fig. 4C) with no significant differences in cell viability between respective stimulated and non-stimulated samples (Fig. 2C: a,b, and Fig. 4C: a,b). An exception was the group cultivated on smooth surfaces in the presence of electrical field stimulation (Fig. 4C: c) where a larger number of red nuclei (dead cells) were observed in comparison to the cells cultivated on microstructured surfaces (Fig. 4C:a,b).

Electrical field stimulation clearly resulted in the abundance of gap junctional protein Cx43 (Fig. 4D) indicating that electrical stimulation may facilitate cell-cell communication. Interestingly, cardiomyocytes cultured on microgrooves and subjected to electric field stimulation demonstrated a higher abundance of gap junctional proteins at the ends of the elongated cells (arrows in Fig. 4D: a,b) consistent with the appearance of Cx43 in adult cardiomyocytes. This effect was not noted in any other sample groups. Although electrical field stimulation clearly induced the presence of Cx43 to change from punctate (Fig. 2D) characteristic of the neonatal heart to the one confined to cell periphery, some Cx43 was observed at the lateral sides of the cells, especially on smooth surfaces (Fig. 4D: c), indicating that further maturation was required.

Cross-striations characteristic of healthy cardiomyocytes were observed in the stimulated groups (sarcomeric α-actinin staining, Fig. 4E). On microgrooved surfaces, the cross-striations were perpendicular to the cell's long axis as well as the microgroove direction; while on the smooth surfaces, there was no preferred directionality as multiple overlapping cross-striations were observed (Fig. 4E).

Upon application of electrical field stimulation, ET of the cells cultured on smooth surfaces increased, but remained the same for cells cultivated on the grooves oriented parallel to the electrical field and decreased significantly for cells cultivated on the grooves orientated perpendicular to the electric field (Table 2). ET values for the parallel group and smooth controls were comparable to that reported in our previous studies,[23] whereas those for the perpendicular group were significantly lower than what we reported previously and comparable to the ET found in the neonatal rat heart.[25] No significant differences in MCR were observed for the stimulated samples, although at Day 7 a trend towards a higher MCR in the parallel group was observed (Table 2).

Rationale for the selection of topographical cues and cellular elements involved in contact guidance in cardiomyocytes

The size of the microgrooves was selected based on our previous studies with micro-abraded surfaces.[23] In that study, we used V-shaped abrasions with the average width of 3, 10 and 13 μm and average depth of 140, 640 and 700 nm, respectively. All abrasion sizes were capable of inducing cellular orientation at significantly higher levels than non-abraded controls, including the abrasion size that was smaller than the cell width, *i.e.* 3 μm. Therefore, we knew that the topographical cues of the depth of several hundred nm were sufficient to exert contact guidance on neonatal rat cardiomyocytes, and the value of 400 nm was chosen for this study.

In this study, we decided to use the groove/ridge width smaller than the cell width in order to enable high-density culture of cardiomyocytes which is critical for their contractile function. In order to contract in unison cardiomyocytes need to form gap junctions and be in direct contact with one another. Thus, if very wide and deep grooves are used, so that each cell is sitting within the groove, the elongation and orientation response of cardiomyocytes may improve; however the cells would not be able to make direct contact with the neighboring cell on each side, which is required for proper electromechanical coupling of the cell sheet.

Table 2 Electrical excitability of cardiomyocytes cultivated on microgrooved surfaces of 1μm period in the presence of electric field stimulation. Electrical field stimulation during culture was achieved *via* symmetric biphasic square pulses at the frequency of 1 Hz, pulse duration of 1ms per phase and amplitude of 1.15 V/cm per phase. At the end of cultivation (day 7) samples were field-paced using monophasic pulses of 2 ms duration to assess their electrical excitability parameters. Brackets indicate the number of samples that could be induced to contract by stimulation, over the total number of samples tested in that group

Time point	Day 1			Day 7		
Direction with respect to the electric field during culture and test	Parallel	Perpendicular	Smooth	Parallel	Perpendicular	Smooth
ET (V/cm)	2.4 ± 1.3 (3/4)	3.7 ± 0.3 (2/3)	2.1 ± 1.5 (3/3)	2.4 ± 1.2 (4/4)	1.2 ± 0.5^a (3/3)	3.8 ± 1.9 (2/3)
MCR (Hz)	4.2 ± 0.6 (3/4)	3.9 ± 0.6 (2/3)	3.9 ± 1.0 (3/3)	3.6 ± 1.4 (4/4)	2.6 ± 0.4 (3/3)	2.6 ± 0.8 (2/3)

[a] Significantly differently than the same group at day 1.

Confining the entire cell body within the groove, may not be required for successful contact guidance. Scanning electron microscopy indicated that the cells oriented and elongated in the direction of ridges (ESI Figure 1†). The cell bodies were longer and narrower when cultivated on finer grooves, *i.e.* the surfaces of 1 μm period (groove width 0.5 μm) in comparison to the surfaces of 4 μm period (groove width 3 μm). Consistently, image analysis of live/dead stained images demonstrated significantly improved elongation on finer grooves (Fig. 2A).

According to one hypothesis, grooved surfaces enable cellular orientation by confining cell adhesion molecules (*e.g.* focal adhesions) to the ridges in an orientation parallel to the groove/ridge direction.[32,33] As the ridge size is decreased to an order of 1μm, focal adhesions which are also on the order of 1 μm in width can assume only one possible orientation on the surface: the one that is parallel to the groove/ridge direction. In addition, ECM proteins orient parallel to the groove direction on micro-structured surfaces, affecting the orientation of integrins. Since adhesion molecules are coupled to the actin cytoskeleton, this causes the orientation of the actin filaments parallel to the groove/ridge direction.[32,33] Numerous previous studies demonstrated that polymerization of F-actin is critical in cell motility and response of fibroblasts to surface topography.[32,36,37]

Our data are consistent with this hypothesis. For example, the surfaces with both 1μm and 4μm periodicity induced similar levels of cellular orientation (Fig. 2B), while cellular elongation was slightly but significantly higher on the surface with finer periodicity, 1μm, compared to the periodicity of 4 μm (Fig. 2A). Immunostaining for vinculin, a focal adhesion protein that links actin cytoskeleton to integrin receptors, indicated the presence of 1–2 μm wide clusters following the ridges of the microfabricated surfaces (Fig. 5A). This is consistent with the observed cell membrane projections attaching to the ridges in SEM images (ESI Figure 1†). Actin cytoskeleton of cardiomyocytes was oriented parallel to the groove/ridge direction (Fig. 5B).

Cardiomyocytes are contractile cells, which makes them unique in comparison to many other non-contractile cell types. In the current set-up, the cell culture substrate is made of non-

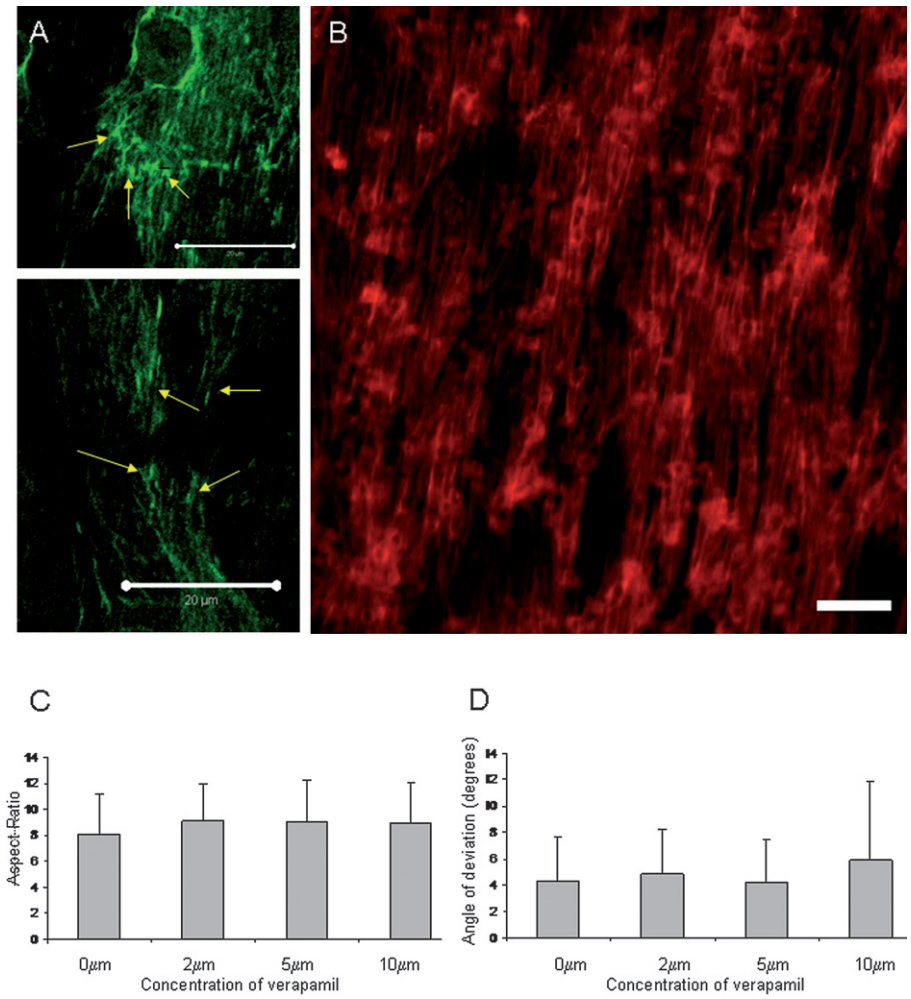

Fig. 5 Contact guidance in cardiomyocytes. Cells were cultivated under non-stimulated conditions on the cell culture chips with 1 μm grating period for 4 days in standard cardiomyocyte culture medium. For C and D, in order to assess the effect of beating on cardiomyocyte orientation and elongation, the cells were incubated with different concentrations of verapamil for 20 hr. A) Immunostaining for focal adhesion protein vinculin. Scale bar 20 μm. B) Staining for actin cytoskeleton. Scale bar 20 μm. C) Elongation and D) Alignment of cardiomyocytes treated in various concentrations of verapamil. There were no significant differences between the treatment groups and the control in terms of both cellular orientation and elongation.

biodegradable and rigid material, polystyrene. Due to the high stiffness of the substrate, the cells are not able to move the material and change the orientation of the grooves/ridges with each contraction. In addition, the substrate is non-biodegradable and as such the cells cannot remodel it during culture. Thus, in the current configuration it is unlikely that the beating of cardiomyocytes had an effect on cell orientation.

To investigate the importance of cardiomyocyte beating on orientation and elongation on cell culture chips, we blocked the beating using verapamil in a range of concentrations (2–10 μM). Verapamil is an L-type Ca^{2+} channel blocker and it is used to prevent contractions while maintaining excitability of cardiomyocytes[10,24] Since spontaneous contractions of cell monolayers usually do not appear in this set-up until day 4 of culture, the cells were cultivated under non-stimulated conditions for 4 days prior to addition of verapamil. Immediately upon addition of verapamil, the spontaneous contractions of cardiomyocytes ceased. After 20 hr of cultivation in the presence of verapamil, the viability of the cells in all groups was maintained (ESI Figure 2†) and beating was absent. There were no significant differences in the orientation and elongation response between the verapamil treated groups and the control (Fig. 5C, D). These findings indicate that on the polystyrene cell culture chips cardiomyocyte beating does not have a significant effect on cellular orientation and elongation. As such, the orientation and elongation response was controlled primarily by the properties of the cell culture chip itself.

Advantages over previous cell culture set-ups and further improvements

Many previous studies were focused on independently providing either electrical cues or contact guidance cues to cardiomyocytes during culture. For example, chronic supra-threshold electrical field stimulation of cardiomyocytes provided by parallel electrodes placed around standard 2D cell culture substrates was shown to preserve contractility of cardiomyocytes,[10] maintain calcium transients,[11] promote hypertrophy,[12] increase protein synthesis[13,14] and maintain action potential duration and maximum capture rate.[15] Topographical cues or micro-contact printing of extracellular matrix components were previously used to introduce contact guidance cues. Entcheva and colleagues[38] used electrospinning to fabricate oriented biodegradable non-woven polylactide (PLA) scaffolds for cultivation of neonatal rat cardiomyocytes which oriented along the fiber direction and acquired a remarkably well developed contractile apparatus. Attachment and alignment of cardiomyocytes has been modulated using grooved and pegged silicone membranes.[17] Abraded polyvinyl coverslips were used to create oriented cardiomyocyte monolayers for studies of electrical impulse propagation and arrhythmia.[39] Spatially organized cardiomyocyte cultures were created on biodegradable polyurethane films with micro-patterned laminin lanes.[20] Elongated cardiomyocyte phenotype was achieved by patterning non-adhesive photoresist lanes on a glass substrate.[40,41]

In our previous study, we used abraded polyvinyl surfaces placed between two parallel electrodes to simultaneously apply topographical and electrical cues to the monolayers of 3T3 fibroblasts and neonatal rat cardiomyocytes. The main finding of that study was that surface topography more strongly determined orientation of fibroblasts and cardiomyocytes; yet pulsatile electrical field stimulation had appreciable effects on cellular elongation. This response was completely abolished by inhibition of actin polymerization and only partially by inhibition of PI3K pathway. However, the system described in our previous study had several shortcomings including the non-uniform nature of abrasions as topographical cues as well as the inability to independently control the electrical stimulation modes on different surfaces.

We described here a microfabricated cell culture chip with integrated topographical and electrical cues. The precise topographical cues were enabled by hot embossing of polystyrene while electrical stimulation was provided by electrodeposited gold electrodes. Our results here demonstrate that the phenotype of cardiomyocytes and their contractile properties can be modulated in a facile manner using these microfabricated chips. The general findings regarding the interactive effects of electrical field stimulation and topographical cues are in agreement with those in our previous studies.[23] The current results also demonstrated that topography was a stronger determinant of cellular orientation, while cell elongation could be enhanced using electrical field stimulation provided that the topography acted in concert with electrical field. In the case of cardiomyocytes, for enhanced elongation the topographical cues had to be orientated parallel to the electric field lines.

We also demonstrated that the microfabricated cell culture chips maintained and enhanced the differentiated phenotype of cardiomyocytes. As such the system is superior to the standard tissue culture wells that yield de-differentiated cardiomyocytes with time in culture. In addition, no pharmacological agents were required to maintain the contractility and differentiated phenotype of cardiomyocytes.

Due to the stated advantages, in our future studies we will evaluate the described chips as tools in drug testing and studies in maturation of stem cell derived cardiomyocytes. The base material for these chips, the tissue culture polystyrene, was selected due to its compatibility with culture of cells derived from many different sources. The chips could be integrated into an array with independent wells for drug testing, where electrical stimulation parameters to each chip are independently controlled. The simplest approach to achieve this would be to place the cell culture chips into wells of a standard multi-well plate (*e.g.* 24 well plate) and connect each cell culture chip to an independent channel of a multi-channel electrical stimulator. A more sophisticated approach would include microfabrication of two layers: 1) a polystyrene based bottom layer with hot-embossed topographical cues and gold electrodes (connected to a multi-channel electrical stimulator) deposited around the topographical cues in a desired configuration, and 2) a PDMS or polystyrene based top layer containing wells of desired sizes. The layers 1 and 2 would then be superimposed and bonded. The cover for this array could be a standard multi-well plate cover or a custom-made polystyrene cover of desired shape.

The transparent nature of the base material also ensures compatibility with fluorescence and optical microscopy enabling real-time monitoring of gene expression (in conjunction with fluorescent reporters) and cell morphology. Non-invasive imaging of the electrophysiological properties in response to

a pharmacological agent would be possible on-line using optical mapping.

A limitation of the current design is that the electrodes are always confined to a particular orientation (parallel/perpendicular) to the grooves. As such, if perpendicular functional testing is required on a chip with a parallel field to groove configuration, the same electrode cannot be used. An alternative design would include a movable microfabricated substrate that could be inserted between the electrodes in either parallel or perpendicular fashion as desired. An example of dynamic cell culture substrate was recently described by Bhatia and co-workers.[42] A similar micromachined silicon substrate with moving parts could be utilized for insertion of electrodes in either parallel of perpendicular orientation on-demand.

We assessed the feasibility of cultivating cardiomyocytes on cell culture chips with grooves and field oriented in parallel, followed by testing with the field oriented perpendicular to the grooves. Cardiomyocytes were cultivated on one of our standard "parallel" cell culture chips in the presence of electrical field stimulation as described in the methods. At the end of cultivation, the built-in electrodes were insulated using electrical tape and the chip was placed between two carbon electrodes (1/8 in diameter,[24]) to perform testing with electrical cues applied perpendicular to the grooves. The sample had an ET of 4.7 V/cm, which was higher than either perpendicular or parallel stimulated samples reported in Table 2, and MCR of 3.8 Hz which was comparable to the values reported in Table 2. The movable microfabricated substrate enabling testing of the functional properties at the angle of 90° relative to the stimulation position would enable more detailed studies of functional properties of cardiomyocyte monolayers.

We demonstrated here that the described system enables maturation of cardiomyocytes derived from neonatal rat cardiomyocytes. Embryonic stem (ES) cells are capable of generating bona fide cardiomyocytes in large numbers.[43–45,46,47] However, most studies agree that the developmental level of these cardiomyocytes corresponds to those found in the neonatal heart, and the maturation of ES cell derived cardiomyocytes to an adult phenotype has not been achieved yet.[48] The described cell culture chips may in the future become a useful tool in these maturation studies, due to the integrated application of electrical and topographical cues and ability to independently apply biochemical cues (*e.g.* growth factors) to different substrates.

Conclusions

Hot embossing enabled fabrication of polystyrene cell culture chips with precise topographical cues consisting of microgrooves and microridges with periodicity of 1 and 4 μm. Electroplating of gold enabled integration of the electrodes for electric field stimulation into the microgrooved cell culture chips. Simultaneous application of biphasic electrical pulses and topographical cues enhanced the phenotype and maturation level of neonatal rat cardiomyocytes as evidenced by a well developed contractile apparatus (sarcomeric α-actinin staining) and gap junctions confined to the cell-cell end junctions rather than the punctate distribution found in neonatal cells. Biphasic electric field stimulation of cardiomyocytes cultivated on microgrooves oriented parallel to the field lines, significantly enhanced the levels of cellular elongation beyond those found on smooth surfaces as well as non-stimulated microgrooved surfaces. Cellular orientation was strongly determined by the topographical cues.

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