From fetus towards adult : maturation and functional analysis of pluripotent stem cell-derived cardiomyocytes

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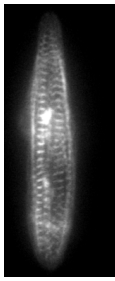
Chapter 2

Mimicking the *in vivo* microenvironment in a cell culture dish



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Abstract

The *in vivo* microenvironment plays a major role in the regulation of cell homeostasis and function. This microenvironment is extremely complex which makes it difficult to mimic *in vitro*. Polydimethylsiloxane (PDMS) and polyacrylamide are currently used as cell compatible substrates with tunable stiffness capabilities. We assessed differences in differentiation efficiency of human embryonic stem cell derived cardiomyocytes (hESC-CM) on PDMS with stiffness resembling the native tissue in combination with different extracellular matrix proteins. Furthermore, we combined the tunable substrates with μcontact-printing technique to pattern cardiomyocytes into a more physiological relevant shape and developed it to a high throughput system. This combination was further used to assess the contraction force output of single hESC and human induced pluripotent stem cell derived cardiomyocytes (hPSC-CM) and its response to adrenergic stimuli.

In this study we show that *in vivo* stiffness and cellular patterning can be mimicked *in vitro* and used to measure contractile output of single hPSC-CM.

Introduction

The current standard materials for cell culture use polyethylene (plastic) or silica (glass) based surfaces for cell culture due to an efficient and low-cost manufacturing procedure. Nevertheless, these substrates are very different from the natural environment that cells encounter in the human body. In the plastic or glass culture dish, cells are in contact with a 2 dimensional (2D) surface that has a Young's modulus of 5 to 6 orders of magnitude stiffer than the human tissue[1]. Moreover, under these conditions cell-to-cell contact is predominantly restrained to the peripheries of cells. Furthermore, cells within the *in vivo* situation are in general part of a highly organized 3D structure consisting of multiple interacting and/or communicating cell-types. These differences are more profound in organs with mechanical functions such as the heart, since the mechanical loading and electrical stimulus, fundamental for proper cardiomyocyte function, are absent or underdeveloped in standard cell or tissue cultures. Various methods have been applied to mimic the *in vivo* environment, including the development of biocompatible polymer scaffolds[2] for 2D or 3D cultures, which have been used in combination with μcontact-printing for cell shape regulation and orientation[3], mechanical[4] or electrical[5] stimulation and cell co-cultures[6].

Biocompatible scaffolds can be made from natural or synthetic sources and have different characteristics with respect to properties such as architecture, cyto-compatibility, bioactivity and mechanical properties[7]. The most widely used scaffolds for cell culture are Polydimethylsiloxane (PDMS) and hydrogel (e.g. Matrigel or Polyacrylamide) based due its versatility, low cost, biocompatibility, tunability over a wide range of Young's modulus, light transparency and low light scattering.

PDMS is a silicon-based organic polymer comprised of repeating units of -O-Si(CH3)2-. PDMS is considered to be extremely useful for microfabrication and biological applications based on the following: i) it can produce features at the micron scale and be released from the template without damaging it; ii) it is optically transparent down to the 280nm; iii) it cures at low temperatures; iv) it is nontoxic to cells *in vitro* or *in vivo* (biocompatible); v) the chemistry of its surface can be altered, vi) it can be reversibly deformed[8]. Although PDMS is nonreactive due to its closed chemistry composition, exposing it to air plasma induces a transition of its surface from hydrophobic to hydrophilic by introduction of polar groups (Si-OH) at the expense of the methyl groups (Si-CH3), allowing protein anchorage such as extracellular matrix components (ECM) to the surface in a specific pattern. Such characteristic enabled the development of the μcontact-printing technique that is comprised by the patterning of ECM proteins with features at a scale of 1μm onto an activated PDMS surface. When attached the cells acquire the printed shape[3]. Furthermore, the Young's modulus of PDMS can be varied from 5 kPa to 1.72 MPa by mixing versions of PDMS with different composition (Sylgard 527 and Sylgard 184)[9]. This combination of technologies creates a relatively inexpensive cellular environment with similar stiffness and cell architecture to the *in vivo* situation that can be applied to the standard cell culture.

Polyacrylamide is a synthetic hydrogel-based polymer formed by acrylamide repeats ((C3H5NO)n) co-polymerized with bis-acrylamide (N,N-methylene-bis-acrylamide). Although this polymer is not used generally for microfabrication it has similar cell culture

applicabilities as PDMS, as it is suitable for cell culture, μcontact-printable and its Young's modulus can be varied between 5 and 100kPa[10],[11]. One advantage of polyacrylamide over PDMS is that it is a liquid prior to polymerization and is hydrophilic as a polymer, allowing the encapsulation of cells or water-soluble molecules. These characteristics enabled development of model to measure traction force during the dynamic process of cell migration[12] and have been recently used for measuring traction force of stem cell-derived cardiomyocytes[13],[11]. Cell's attachment on a substrate is implemented by the anchorage of an assembly of membrane proteins - focal adhesions, to the ECM proteins of the substrate. The anchorage of the focal adhesions is a dynamic process that changes over time, allowing the cells to change position or migrate through the substrate. Attachment and locomotion are processes that can be described as forces applied by the cell thought the focal adhesions onto the substrate. If the substrate is elastic and has a low Young's modulus, the cell is able to deform it during attachment or migration.

In this chapter we describe the development and adaptation of soft substrates, μcontact-printing and contraction force techniques onto human pluripotent stem cell derived cardiomyocyte differentiation, culture and analysis.

Materials and Methods

Human embryonic stem cell culture and differentiation

The human embryonic stem cell (hESC) line HES3-NKX2-5-eGFP, described previously[14] in which one allele of the cardiac transcription factor NKX2-5 was replaced by GFP, was used to allow selection of cardiomyocytes after differentiation. Human induced pluripotent stem cells (hiPS) were generated from skin fibroblasts obtained from a healthy female individual, after written informed consent and following approval by the Leiden University ethics committees, using a non-integrating Sendai virus to deliver the four reprogramming factors OCT4, SOX2, KLF4, and MYC [15]. Both cell-lines were maintained on irradiated mouse embryonic fibroblasts (MEFs) and differentiation was performed by two different methods, 1) Monolayer differentiation was performed in a 6-well plate, coated with Matrigel where 300.000 hESC were seeded at day 0 in BSA polyvinylalcohol essential lipids medium (BPEL) [16] together with 20ng/ml BMP4 (R&D), 20ng/ml Activin A (Miltenyi), 1.5μM chir99021 (Axon Medchem). The medium was refreshed after 3 days with BPEL supplemented with 5μM XAV-939. 2) For spin embryoid body (EB) differentiations 30.000 hESC were seeded per well in a V-shaped 96-well plate in BPEL containing 35ng/ml BMP4 (R&D), 30ng/ml Activin A (Miltenyi), 30ng/ml VEGF (Miltenyi), 40ng/ml SCF (Miltenyi), 1.5μM chir99021 (Axon Medchem)[14]. At day 3, medium was refreshed with BPEL. GFP-positive spin EBs started beating between day 7 and 10 of differentiation. Dissociation of both monolayer and spin EB-derived cardiomyocytes was performed at day 20 with 1x TryplE select for 10 mins at 37 ^{o}C.

Gene expression analysis

HESC-derived cardiomyocytes (15 days of differentiation of HES3-NKX2-5-eGFP) were purified by FACS sorting for GFP expression. RNA was isolated from purified cardiomyocytes

using the RNeasy-MicroKit (Qiagen) and reverse transcribed to cDNA using the Iscript cDNA kit (BioRad). Gene expression analysis was performed by q-PCR using IQ SYBR Green (BioRad). The samples were normalized to the house keeping gene human Acidic Ribosomal Protein (RPLP). The primers used are listed in supplementary Table 1.

Spin Coating

Spin coating was performed using spin coater WS-650MZ-23NPP (Laurell) for coating surfaces with solutions/polymers at high-speed rotation. A drop of freshly mixed PDMS was added to the center of the coverslip and we used the following protocol for optimal and evenly spreading of the polymer evenly.

Time (s)	RPM	Acceleration
5	500	100
5	1000	200
10	3000	300
60	4000	400
15	2000	200
10	1000	100
5	500	100

Fluorescent conjugation of fibronectin

The fibronectin was conjugated with Alexa Flour 546 Maleimide (Invitrogen) in a 8M Guanidine Hydrochloride (Sigma) solution for 1h, followed by dialysis overnight in PBS using a Slide-A-Lyzer Dialysis cassette.

μcontact-printing technique

The procedure was started by coating an in-house available PDMS stamp containing line features of 20μm thick spaced 20μm apart (20x20) with 50μg/ml Fibronectin for 1h. The liquid was aspirated and the stamp was dipped on PBS for a short wash, and dried with a nitrogen gun before stamping the fibronectin onto the PDMS surface activated by UV-Ozone treatment. The quality of the stamping was checked under a fluorescence microscope.

Cardiomyocyte isolation and culture

Chick cardiomyocytes (CMs) were isolated from day 7–8 (HH stages 30–32) White Leghorn embryos and are not considered live vertebrate animals under US Public Health Service policy. For each isolation, 7–15 hearts were dissected, cut into small pieces and digested in 3 mL of 0.05% Trypsin-EDTA (Sigma-Aldrich) for 7 min. The supernatant was carefully collected, added to seeding medium (M199 medium, 10% heat-inactivated FBS, 1% penicillin/streptomycin) and filtered through a 40-μm cell strainer to isolate individual cells. The 7-min incubation was repeated three times so that the hearts were completely digested while minimizing cytotoxicity by long exposure to trypsin. The cell solution was then centrifuged at 50g for 10 min and resuspended in 30 mL of seeding medium. Fibroblasts were removed by selective adhesion by pre-plating successively for 45-min incubations in T75 cell culture flasks. The supernatant, enriched in CMs, was centrifuged at 50g for 10 min then resuspended in seeding medium at 7.5 × 105 cells/mL. PDMS substrates were placed in a 6-well plate, 200 μL of the cell suspension was pipetted on top and incubated for 2 h to

allow cells to attach and then the wells were filled with additional seeding medium. On the next day, the medium was replaced with a maintenance medium containing a reduced level of 2% heat-inactivated FBS to minimize fibroblast proliferation.

Immunocytochemistry and sarcomere structure evaluation

Cells were fixed with 4% paraformaldehyde for 20mins and washed three times with PBS. Cells were permeabilized with 0.1% Triton for 8mins and blocked with 4% swine serum for 1h at room temperature (RT). For identification of sarcomeres and cell boundaries the samples were incubated with the primary antibodies mouse anti α-actinin (Sigma-Aldrich A7811) diluted 1:800 and rabbit anti pan-cadherin (Sigma-Aldrich C3678) diluted 1:200 for 1h at RT. The samples were washed three times 10 mins with PBS with 0.05% tween20 and incubated with the secondary antibodies donkey anti mouse IgG Cy3 (Jackson ImmunoResearch Europe 715-165-150) and donkey anti rabbit IgG A488 (Invitrogen A-21206) both diluted 1:200 for 1h at RT

Traction force technique

The traction force microscopy technique makes use of fluorescent carboxylate-modified microspheres embedded in the polyacrylamide gel to track such substrate deformation. By taking into account the displacement of the microspheres, the Young's modulus of the polyacrylamide and the thickness of the gel, Dr. Micah Dembo together with Dr. Yu-li Wang developed the LIBTRC software package that translates the substrate deformation into a force.

1% gelatin was made from porcine skin powder (Sigma Aldrich) and autoclaved. The gelatin was further oxidized with 3.5mg/ml Sodium Periodate (Sigma Aldrich) for 45mins at room temperature and used to coat the 20x20 lines stamp for 1h. The stamp was then dried with a nitrogen gun and used to stamp a 15mm glass coverslip. The polyacrylamide solution was prepared with a final concentration of 0.1% bis-acrylamide (Bio-Rad), 5% acrylamide (Bio-Rad) and 10mM HEPES pH 8.5 in distilled water, followed by centrifugation for 1 min at 10.000RPM for degassing. 0.006% (m/v) of ammonium persulphate (Sigma-Aldrich) and a 1:1000 dilution of 0.2-μm fluorescent beads (Ex/Em: 660/680nm - Molecular Probes) were added to the solution and briefly vortexed. The gel polymerization was initiated with TEMED (Bio-Rad) and 9.2μl of the final solution was added to a 25mm coverslip treated with plus Bind-Silane solution (GE Healthcare). The μcontact-printed 15mm coverslip was applied on top of the drop with the gelatin lines facing the gel. After 20 min of polymerization the 15mm coverslip was removed and the 25mm coverslip was mounted onto a well of a glass bottomed 6-well plate (Mattek), replacing the initial glass. The polymerized gel has a Young's modulus of 5.8 kPa [17]. The plates were sterilized under UV light for 30mins and the gels were rehydrated with 1xPBS for another 30mins. Before cell seeding the gels were equilibrated in culture medium for 15mins.

Statistics

The different groups were compared using one-way A-NOVA followed by Bonferroni. Significance was attributed to comparisons with values of $P < 0.05$*; $P < 0.01$**; $P < 0.001$***. All error bars represent SEM.

Results

Previous studies have shown the effect of substrate stiffness on stem cell differentiation[18]. In this chapter we studied the effect of substrate stiffness on human pluripotent stem cell-derived differentiation to cardiomyocytes. In order to culture cells on substrates with a stiffness which is comparable to the fetal heart *in vivo*, we combined two different PDMS formulations, Sylgard 527 and Sylgard 184 (Dow Corning), as described by Palchesko et.al[9]. The elastic modulus (y in kPa) in function of the percentage of the polymer Sylgard 184 (x) in a 184/527 mixture can be calculated by the following equations:

For low percentages of Sylgard 184 (0-20%) the regime is non-linear:

$$y=0.3236x^2+2.0606x+5 \quad 1)$$

For higher percentages of Sylgard 184 (20-100%) the regime is linear:

$$y=18.591x-156.87 \quad 2)$$

Previously, the embryonic heart has been described to generate a maximal contractile work output on matrixes with elasticity E≈11-17kPa[19]. In order to mimic the embryonic conditions *in vitro* PDMS with an estimated Young's modulus of 15kPa, was made using a combination of 96.78% of Sylgard 527 and 3.22% of Sylgard 184 and used as a substrate for cardiac differentiation. Plastic 6-well plates were coated with a layer of 15kPa PDMS and cured for 3h at 60ºC. The PDMS was then activated using a plasma cleaner and coated with various the respective ECM components. In order to additionally assess the best ECM for cardiac differentiation on this substrate each PDMS well was coated with either with Matrigel, 50µg/ml Fibronectin (BD), 50µg/ml Laminin (Sigma) and 0.1% Gelatine (Sigma). The monolayer differentiation was started at day 0 in every substrate. At day 1 and 2 the wells with PDMS coated with Fibronectin, Laminin and Gelatine were clumped in suspension whereas the cells on Matrigel coated PDMS and plastic control were attached and displaying normal morphology. As the differentiation progressed the cells in suspension attached as all conditions showed cell attachment and proliferation at day 3. At day 7 cells were visibly expressing GFP in all conditions. However, at day 14 there were obvious differences in monolayer morphology. A flat uniform beating cell layer was observed in both Matrigel conditions, whereas Laminin-coating resulted in beating cell layers with more evident three-dimensional structures on PDMS substrates in comparison with the plastic control. The cells on the Fibronectin coated PDMS displayed a string-like web on the PDMS that was only attached to the surface on the edges. Gelatine surface conditions resulted in floating beating spheroids (**Figure 1A**). At day 15 the cells were dissociated and FACS sorted for GFP. FACS analysis showed a modest increase in differentiation efficiency from 57% in the Matrigel coated plastic to 71% in the Matrigel coated PDMS (N=2). There was also an increase in cardiomyocyte differentiation efficiency to 65% in Laminin coated PDMS in comparison to Matrigel coated plastic control, but no change in Gelatine coated PDMS (55%) and a decrease in Fibronectin coated PDMS (19%). Despite no study clearly reporting it, there is generally accepted that hPSC differentiation protocols to cardiomyocytes are inconsistent through time. Due to the differences in colony morphology the RNA from GFP+ sorted cells were isolated for gene expression analysis. Preliminary Q-PCR analysis indicat-

ed no changes in expression of genes encoding for structural, sarcomeric and/or contractile proteins (ACTN2 and MYBPC3) in any conditions. MLC2V expression was decreased in PDMS/Matrigel conditions, indicating a decrease in ventricular specification on PDMS with Matrigel or a decrease in levels of maturation. The same holds true for Laminin. In contrast Gelatine-coating displayed an increase in MLC2V expression. As a marker of calcium handling CASQ2 expression is elevated in cardiomyocytes differentiated in Laminin, Fibronectin and Gelatine. On the other hand cardiomyocytes in these conditions also displayed increased expression of the hypertrophic marker ANF (**Figure 1B**).

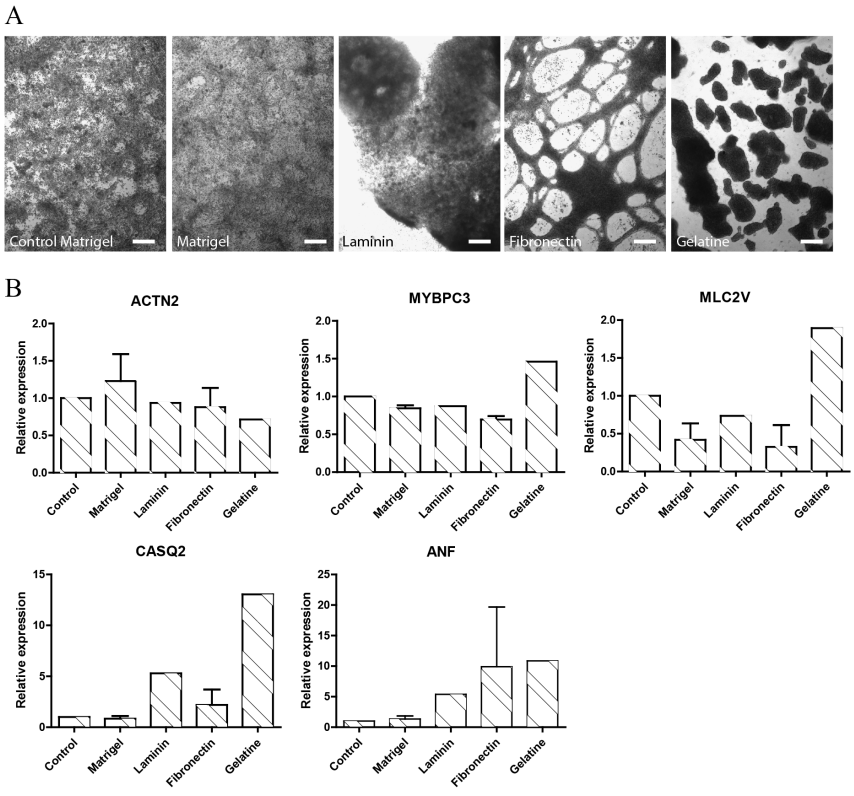


Figure 1 – Cardiomyocyte differentiation on different substrates. **A.** Representative pictures of hESC-CM culture morphology in plastic coated with Matrigel (Control Matrigel) and with PDMS coated with Matrigel, Laminin, Fibronectin and Gelatin (Bar-100μm). **B.** Relative expression levels of ACTN2, MYBPC3, MLC2V, CASQ2 and ANF in cardiomyocytes cultures in different substrate conditions (Control, Matrigel and Fibronectin N=2, Laminin and Gelatine N=1).

Since cell shape is known to be important for cardiomyocyte morphology and function [3,20] we applied μcontact-printing of ECM substrates on PDMS. To increase the applicability of this procedure we prepared coverslips coated with PDMS with and without the use of a spin-coater, which is not readily available in a standard cell biology lab. For this 100μl of PDMS was added onto the center of a glass 25mm coverslip and incubated overnight at room temperature. The next day the coverslips were fully covered with PDMS and were cured for 2h at 60ºC. Fibronectin was used for μcontact-printing since it has been

used frequently for this procedure[21]. In order to immediately visualize the outcome of the fibronectin was conjugated with Alexa Flour 546 Maleimide (Invitrogen). Two different stiffnesses were tested during this experiment ranging between the softer 5 kPa (0% Sylgard 184) and the stiff 1.72 MPa (100% Sylgard 184). On the stiff PDMS the μcontact-printing was successful in both spin-coated and non-spin-coated conditions, displaying uniform line pattern (**Figure 2A**). On the soft PDMS the μcontact-printing was only successful on spin-coated condition, whilst on the non-spin-coated PDMS the lines were not present or with large gaps in between (**Figure 2B**). The same μcontact-printing pattern was observed on PDMS with Young's modulus of 24kPa (data not show), indicating that μcontact-printing without a spin coater cannot be successfully applied to PDMS substrates with a lower Young's modulus range. The patterned lines on the spin coated PDMS were subsequently tested for cell culturing. First we used cardiomyocytes of chicken embryos, which were isolated and seeded on patterned coverslips at a density of 200.000 cells per cm2. The following day the cells were nicely attached and acquired the shape of the lines in both conditions (Figure 2C and D).

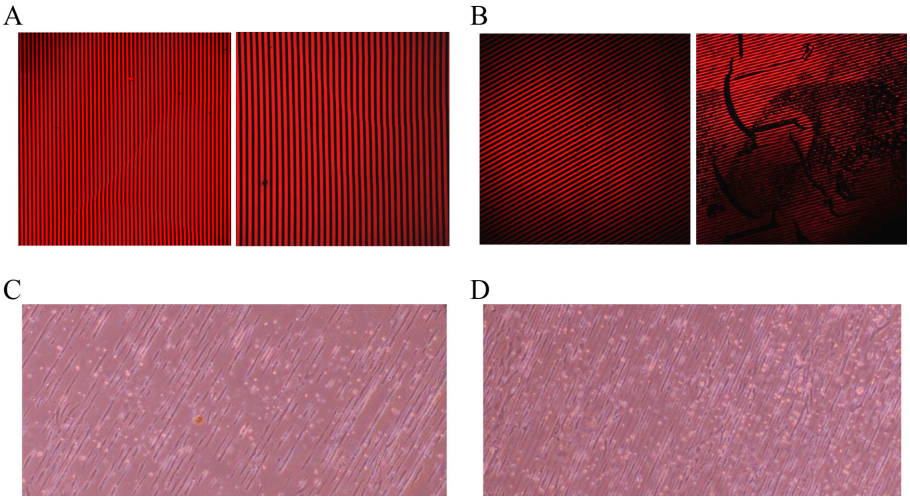


Figure 2- μcontact-printing of fibronectin-546 on PDMS with different Young's Modulus. **A.** μcontact-printed fibronectin lines 15 and 20μm wide onto spin coated (left-15x magnification) or non-spin coated (right-15x magnification) PDMS with ≈1.72MPa (stiff). **B.** μcontact-printed 15 and 20μm wide fibronectin lines onto spin coated (left-10x magnification) or non-spin coated (right-5x magnification) PDMS with ≈5kPa (soft). **C.** Chick primary cardiomyocytes aligned on stiff spin coated PDMS with μcontact-printed lines displayed on **2A** (10x magnification). **D.** Chick primary cardiomyocytes aligned on soft PDMS with μcontact-printed lines displayed on **2B** (10x magnification).

In order to test whether human-derived cardiomyocytes would attach and function, we cultured hESC-CMs on PDMS with a stiffness which is comparable to the adult heart (Young's modulus of 24kPa) was prepared, mimicking the elastic stiffness described in the adult heart muscle[1]. Alexa Fluor 548 labeled fibronectin was successfully μcontact-printed on PDMS in a configuration of 20μm wide 20μm interspaced lines and 50μm x50μm squares, although it was not uniformly distributed in the latter situation (**Figure 3A** and **B**). hESC-

CM were seeded on the patterned PDMS at a density of 200.000 cells per cm2. After 16 hours, although cardiomyocytes were nicely attached on the lines, there was a clear difference when compared to primary chick cardiomyocytes: the majority of the hESC-CM aggregated and bridged between lines (**Figure 3C**). On squares cell attachment was not very effective and hESC-CM formed spheroids instead of spread out single cells (Figure 3D). After 8 days of culture on the lines most of the hESC-CM remained attached and beating. Nevertheless, the aggregates had a more compact morphology and more single cells were aligned. Attachment of the hESC-CM on squares decreased and cells maintained a spheroid-like morphology at 8 days of culture (**Figure 3E** and **F**). The cells were then fixed in 4% paraformaldehyde and immunostained for α-actinin. HESC-CM on the lines showed aligned sarcomeres perpendicular to the direction of the patterned lines (**Figure 3G**). Cardiomyocytes that bridged the lines displayed a more disorganized sarcomere structure (**Figure 3G**). In an attempt to reduce the bridging effect by hESC-CM, fibronectin was μcontact printed on PDMS with patterns of 20μm lines interspaced at 100μm. Although the lines were successfully printed on the PDMS, the distance between lines in combination with the elasticity of the PDMS caused undesirable contact between the PDMS surface and the ceiling of the stamp, transferring a visible amount of fibronectin on the surface between lines (**Figure 4A**). Next, in order to avoid such contact μcontact-printing was performed without pressing the stamp on the surface, which indeed resulted in a clean space between lines but unfortunately also resulted in a decreased quality of the printed lines (**Figure 4B**). Following seeding of hESC-CM on these patterns of spaced lines, we observed that attachment was reduced and restricted to the edges of the coverslip (**Figure 4C**). Moreover, removal of aggregates by filtering cardiomyocytes with a 70μm cell strainer before seeding, still resulted in formation of aggregates and bridging 100μm space between the lines (**Figure 4C**).

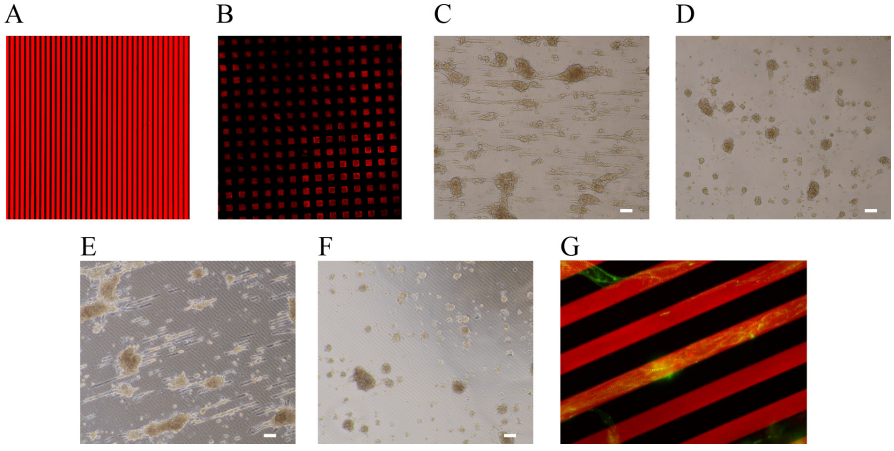


Figure 3- Culturing hESC-CM on μcontact-printed substrate with tissue-like stiffness. **A.** μcontact-printed 20μm wide fibronectin lines on spin coated PDMS with Young's Modulus of 24kPa. **B.** μcontact-printed 50x50μm fibronectin squares on spin coated PDMS with Young's Modulus of 24kPa. **C.** and **D.** hESC-CM seeded on fibronectin lines and squares from **3A** and **3B**, respectively. **E.** and **F.** hESC-CM cultured for 8 days on fibronectin lines and squares from **3A** and **3B**, respectively. **G.** α-actinin staining (green) of hESC-CM cultured on 20μm wide fibronectin lines (red). White bars- 50μm

Even with the undesirable bridging between the lines, the μcontact-printing technique still has potential added value when applied to high throughput screenings, as in this set up the percentage of cardiomyocytes effectively aligned can be picked and further analyzed. In order to prepare this μcontact-printing procedure for high throughput screening, instead of spin-coating single coverslips, 96-well format plates were used combined with 55mm x 75mm x 0.15mm glass (half the size of the bottom of a 96 well plate) purchased from Grace-bio and spin-coated with PDMS using a 50mm round chuck. The PDMS coating was initially only uniformly distributed in the 55mm diameter circle at the center of the coverslip and displayed a stringy pattern on the edges due to air bubbles trapped within the PDMS (**Figure 5A**). Different approaches such a decreasing the spinning speed, increasing the time of the top speed step, or pre-coating the entire coverslip with PDMS did not eliminate the stringy pattern. Despite the differences in uniformity the μcontact-printing was successfully performed on the uniformly coated circle in the center of the coverslip (**Figure 5B**). The μcontact-printed coverslip was glued to a bottomless 96 well plate (Nunc) using high vacuum grease (Dow Corning) (**Figure 5C**). HESC-CM attached to the lines as shown before (**Figure 5D**). In addition, no visible leak was observed between the different wells, demonstrating the feasibility to further develop this platform for high throughput assays.

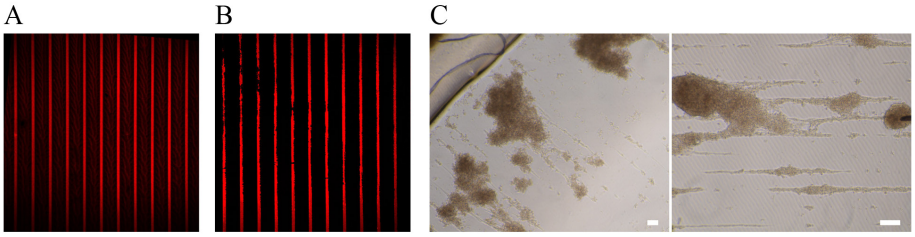


Figure 4 – Culturing hESC-CM on 100μm spaced lines. **A.** μcontact-printed 20μm wide fibronectin lines interspaced by 100 μm applying pressure on the stamp. **B.** μcontact-printed 20μm wide fibronectin lines interspaced by 100 μm without applying pressure on the stamp. **C.** hESC-CM cultured on lines showed in **4A** and **4B** with visible cell aggregates and bridging between lines (top-left shows the edge of the gel). White bars- 50μm

Measuring contractile function of cardiomyocytes is important for obtaining a better understanding of heart development and disease, and drug-induced cardiotoxicity. As introduced earlier traction force microscopy has been applied to assess the displacement of the substrate upon contraction of single cardiomyocytes[11],[13]. Displacement is recorded by video imaging and following software analysis computed to force applied by the cell per unit of area (mN/mm2), which are known as units of stress. In order to adapt this technique to our current set up we combined it with the μcontact-printing technique. Polyacrylamide (5%) gels embedded with fluorescent beads and gelatin printed lines were seeded with hESC-CM (**Figure 6A** and **B**). Nevertheless, cell attachment of hESC-CM to gelatin printed lines was poor if cardiomyocytes were derived from spin EBs or from monolayer differentiations on Matrigel. These problems were solved by dissociating these hESC-CM and pre-plating as a monolayer on 0.1% gelatin coated wells for 5 days or/and by equilibrating the gels in medium containing 10% fetal calf serum (Sigma) for 30mins prior to cell seeding. Initial contraction measurements revealed forces of 0.271±0.02mN/mm2 and 0.253±0.02mN/

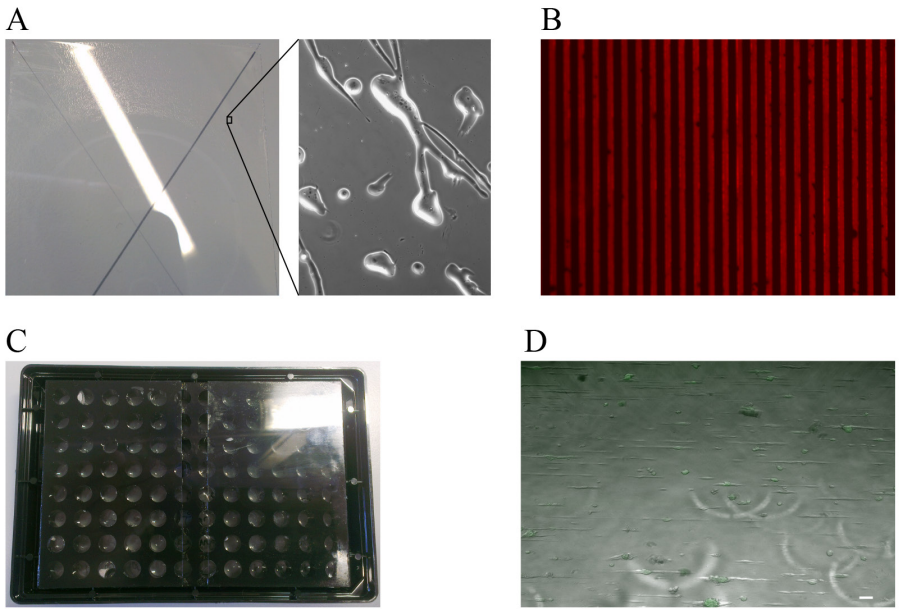


Figure 5- Up scaling μcontact-printing to 96 well format. **A.** 55mm x 75mm glass coverslip spin coated with PDMS displaying a flat uniform coating in center circle. The higher magnification (right side) shows the air bubbles trapped in the PDMS around the circle. **B.** μcontact-printed 20μm wide fibronectin-546 lines on the uniform PDMS coated center circle. **C.** Two spin coated 55mm x 75mm glass coverslip glued to the bottom of a 96well bottomless plate using high vacuum grease. **D.** hESC-CM cultured on the μcontact-printed 20μm wide fibronectin-546 lines (Green-GFP expression in cardiomyocytes). White bar- 50μm.

mm2 for hESC-CM and hiPSC-CM, respectively (**Figure 6C**). In order to measure contractility on substrates with a stiffness comparable to the heart, the acrylamide was increased to 10%, producing a polymerized gel with a Young's modulus of 18.4 kPa[11]. Contraction stress of hESC-CM and hiPSC-CM increased with the substrate stiffness (**Figure 6C**), indicating a correlation between contractile stress and substrate stiffness. Of note, cell alignment on the 18.4kPa polyacrylamide gels was poor in three consecutive experiments (**Figure 6D**), limiting the usage of this substrate for further experiments. Further characterization of hESC-CM was performed by analyzing their contraction performance after activation of the α and β adrenergic receptors with phenylephrine and isoprenaline, respectively. Phenylephrine induces an increase in beating rate and isoprenaline induces and increase in beating rate and contraction force in the adult heart [22,23]. Although both phenylephrine and isoprenaline induced an increase in beating frequency, no change in contraction force was observed as the net variation in contraction stress is close to null (**Figure 7A** and **B**).

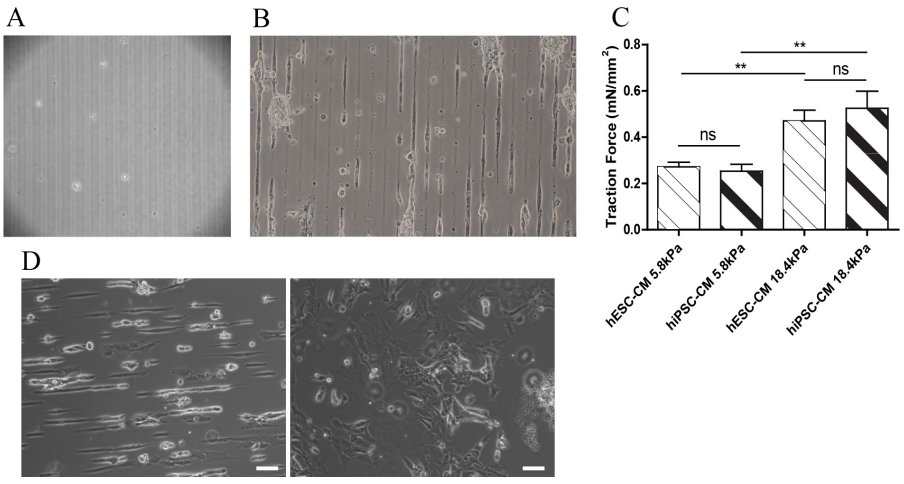
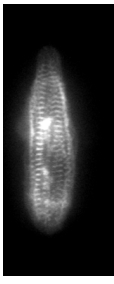


Figure 6- Contraction stress measurements using patterned polyacrylamide gels. **A.** 20 μm wide gelatin lines contact-printed onto 5.8kPa polyacrylamide gel. **B.** hESC-CM cultured on lines as shown in **6A. C.** Mean contraction stress generated by hESC-CM and hiPSC-CM in 5.8kPa (n=29 and n=12 respectively) and 18.4kPa (n=24 and n=13, respectively). **D.** hESC-CM aligned on a patterned polyacrylamide gel (5.8kPa) (left) but not aligned in patterned 18.4kPa polyacrylamide gel. White bar-50μm. ns = non-significant; **P<0.01



Discussion

This chapter describes the use of synthetic polymers as soft substrates for hESC-CM differentiation, maintenance and contractile analysis. These soft substrates were used in combination with μcontact-printing of different ECM proteins for cardiomyocyte alignment during culture and contraction force measurements. Additionally, we made the first steps for development of a 96 well-automated high throughput platform for functional analysis of human cardiomyocytes.

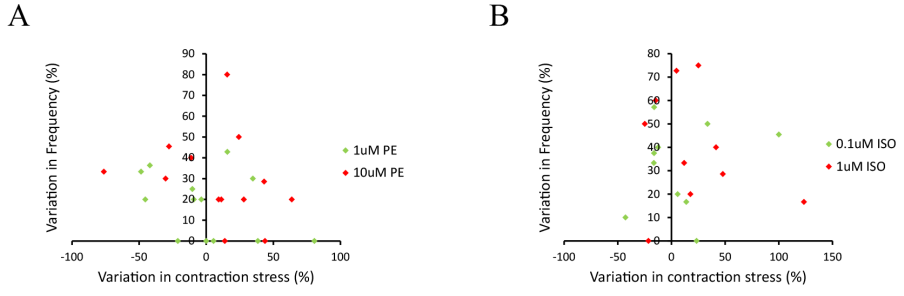


Figure 7- Chronotropic and inotropic effects of phenylephrine and isoprenaline on hESC-CM. **A.** Variation in beating frequency of each cell relative to its baseline frequency is plotted in function of the variation in contraction stress of that same cell, relative to its contraction stress baseline upon stimulus with 1μM or 10 μM phenylephrine. **B.** Variation in beating frequency of each cell relative to its baseline frequency plotted in function of the variation in contraction stress of that same cell relative to its contraction stress baseline upon stimulus with 0.1μM or 1 μM isoprenaline.

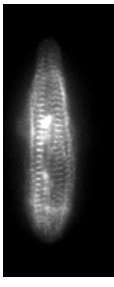
Standard cell cultures are realized on plastic substrates with a stiffness of several fold higher than tissues in the human body. Moreover, in general the stiffness of tissues during embryonic development are even softer than the adult counterparts[1],[19]. In order to consider this aspect for mimicking *in vivo* cardiac development, we differentiated hESC on PDMS with different stiffness by combining Sylgard 182 and 527. Results showed a modest increase in differentiation efficiency (from 57% to 71% GFP+ cells) under conditions, which are more comparable to the stiffness of the heart, although no obvious difference in morphology or gene expression were observed. These results are in disagreement with the decrease in cardiac differentiation efficiency on softer substrates described by Hazeltine et al.[24]. This discrepancy could be explained by the use of different substrates (polyacrylamide versus PDMS) and differences in cardiac differentiation and maintenance protocols. Furthermore, the soft PDMS substrate was also tested in combination with different ECM proteins for optimization of cardiac differentiation and maintenance. The most striking differences observed the lack of hESC attachment to the substrate until day 3 of differentiation when laminin, gelatin or fibronectin were used. This observation could be due to changes in conformation or availability of ECM protein upon interaction with activated PDMS, since the hydrophilic part of ECM proteins are bound to the PDMS leaving the hydrophobic part, less favorable, as the major anchor for cardiomyocytes. Moreover, the different ECMs induced different cardiomyocyte monolayer morphology by day 15, demonstrating the important role of ECM proteins in cardiac differentiation. Despite all ECM proteins promoting cardiogenesis, the efficiency was highest in combination with Matrigel. Recently Burridge et al. used Vitronectin as ECM protein for cardiac differentiation with better outcome than Matrigel[25], which may represent a good candidate for further improvement of this procedure. Although ACTN2 and MYBPC3 were not changed in any condition, preliminary results indicated an increase in MLC2V expression on gelatin, suggesting a more ventricular specification or maturation. Additional analysis would be required in order to make solid conclusions.

Besides the substrate stiffness, cell shape and geometry are among other parameters that influence cell differentiation and function. HPSC-derived cardiomyocytes are very similar to primary human fetal cardiomyocytes based on morphological (as well as molecular and functional) aspects, which is in contrast to the elongated, brick-like morphology of adult cardiomyocytes with length to width ratio of 7:1. In order to constrain the adult cardiomyocyte morphology *in vitro* we made use of μcontact-printing. We were able to show that this technique can be applied to spin-coated PDMS with Young's modulus ranging from 5kPa to 1.72MPa, but not on non-spin coated PDMS surfaces with low Young's modulus (5kPa). Human hESC-CM successfully attached and contracted on fibronectin printed lines, although bridging of hESC-CM between lines was frequently observed. This is in contrast to primary embryonic chick cardiomyocytes and neonatal rat ventricular cardiomyocytes[21], illustrating the difference between hESC-derived and primary cardiomyocytes.

The combination of culturing on soft transparent polymers (such as PDMS) with μcontact-printing has great potential advantages for high throughput screening since it allows imaging of cells in a micro-environment with an elasticity and geometry comparable to the *in vivo* situation and with higher degree of standardization and consequently a lower

degree of variation. This is crucial for successful implementation of hPSC-based assays for disease modeling, drug discovery and safety pharmacology. In line with this, it is encouraging that we have shown that it is feasible to use this technology in a custom-made 96-well plate format, allowing high throughput screening.

The combination of a soft flexible substrate embedded with fluorescent beads and μcontact-printing was applied for traction force measurements of single hPSC-CM. The initial values of contraction stress measured in hPSC-CM were congruent with previous studies on similar substrate stiffness[13]. We also demonstrated that contraction stress of both hESC-CM and hiPS-CM increased with the increase of substrate stiffness, which is in accordance with previous observations[11]. This observation is inherent to the complexity of the contraction machinery depicted by the Frank-Starling Law. Although there is no preloading stress (pressure generated by the blood volume in the ventricles at the end of diastole) in this 2D system, the afterload stress (pressure applied by the contracting ventricle walls during ejection) is mimicked by the elastic modulus of the substrate. At lower elastic modulus the pressure exerted by the substrate to the cell at the maximal contraction is lower than the stress applied by the cell on the substrate[19], meaning that in this setup the contraction stress measured is only influenced by the sarcomere dynamics and by cell size. Although this technique outputs an absolute value of stress, the dependence of this contraction stress value on the stiffness of the substrate limits the its comparison with other studies using different contraction force measurement setups (e.g. Force transducers). This limitation is inherent to the assumptions made on the cell attachment, substrate properties and bead location in order to translate the substrate deformation into a force/stress value using a mathematical algorithm, which are technically different from the assumptions made on the translation of the spring deflection into a force/stress in a force transducer apparatus. Measuring contraction stress in single cardiomyocytes will be important for functional analysis of specific cardiac disease and the effect of drugs on heart function. In this context, it has been previously shown that agonists of α/β adrenergic receptors, which are known to affect cardiac inotropy[22,23], did not affect contractile force in single hESC-CM[11], which may result from a low level of maturity of hESC-CM under the current *in vitro* conditions.



Conclusion

In this chapter we show that the tunability of different polymers can be used to mimic native tissue stiffness *in vitro*. Moreover, we showed prove of principle of combining tunable substrates with μcontact-printing and use it in a high throughput set up. Furthermore, this combination of techniques was used to measure contraction force of single hESC-CM and hiPSC-CM in different substrate stiffness together with its response on adrenergic stimuli. To conclude we established a 2D platform for culturing and analyzing hPSC-CM in a more native tissue-like environment.

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