

Induction of Anisotropic Orientation and Enhancement of Gene Functional Expression of Human Pluripotent Stem Cell-Derived Cardiomyocytes Cultured on Nanofabricated Substrates Consisting of Micron Planar Lines and Nano Dot Structures.

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Abstract

Human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) are expected to have applications in the fields of regenerative medicine and drug discovery. However, the immaturity of hiPS-CMs is an issue to be considered, and in order to replicate *in vivo* responsiveness, there have been several attempts to induce maturation in hiPS-CMs, including methods to induce differentiation of hiPS-CMs by changing culture medium and culture substrate. In particular, anisotropic culture, in which the cultured cells are aligned in one direction, induces the cellular morphology resembling that of *in vivo* cardiomyocytes and is expected to be a useful method for maturation of hiPS-CMs.

We tried forming a nanostructure on the surface of the cell culture substrate using our original nanofabrication technology, with the aim of aligning cardiomyocytes and inducing maturation. Our newly developed nanostructure for anisotropic culture (line/dot structure) comprises a region of cone-shaped nanopillars with pitch distance of several hundred nm and a planar region, alternating in a striped pattern with intervals of several tens of μm , arranged on the surface of the cell culture substrate.

The hiPS-CMs cultured using the line/dot structure showed anisotropic orientation, and increased mRNA expression was observed in myocardial structural protein genes, genes relating to ion channels, and the gene for Cx43. These results suggest that the line/dot nanostructure is effective for anisotropic culture and cell maturation of hiPS-CMs.

1. Introduction

Stem cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have pluripotency, the ability to develop into various different cell types. This characteristic can be used to induce differentiation of stem cells into cells such as nerve cells, cardiomyocytes, hepatocytes, and pancreatic cells, and these differentiated stem cells are therefore expected to have applications in regenerative medicine, identification of the causes of diseases, and evaluation of drug efficacy and side effects. They are also increasingly being used as an alternative to conventional animal experiments (1–2).

Research using cardiomyocytes derived from iPS cells (human-induced pluripotent stem cell-derived cardiomyocytes, hiPS-CMs) is progressing in the fields of regenerative medicine, tissue engineering, disease modeling, and drug toxicity testing. The main advantage of hiPS-CMs is that they can avoid species-dependent differences because they contain the human genome (3). However, prior studies have reported that the drug responsiveness of hiPS-CMs differs from that of *in vivo* cardiomyocytes due to the immaturity of the cells, which poses a challenge for the use of hiPS-CMs in the regenerative medicine and drug discovery fields (1, 3–4).

Mature cardiomyocytes *in vivo* exhibit rod-shaped morphology, with dimensions of approximately 10 μm in diameter and 150 μm in length (ventricular cells). The aspect ratio is in the range from 5:1 to 9:1, and 25–57% are multinucleated (5–7). In comparison, hiPS-CMs are smaller (5–10 μm in diameter) than mature cardiomyocytes, circular or polygonal in shape, and are generally mononuclear (5–7). In addition,

hiPS-CMs have been found to lack certain physiological properties, which include short sarcomeres with structural disorganization as a result of small contractile forces, lack of T-tubules, and decreased ion channel expression. Further differences include the absence of a positive force-frequency relation and the absence of calcium signaling in hiPS-CMs (1, 3).

Uosaki *et al.* confirmed that the development of hiPS-CMs remains at the late embryonic stage, even when maturation is promoted by long-term culture (4). However, since hiPS-CMs transplanted into neonatal rat hearts develop into cells that exhibit equivalent morphology and function to adult cells (8), cell maturation of hiPS-CMs may be possible if they are placed in an environment that is structurally and physiologically similar to the *in vivo* environment.

In order to increase the cell maturity of hiPS-CMs and reproduce the morphological and physiological characteristics of *in vivo* cells, there have been various attempts to develop methods to induce differentiation by changing the culture medium and culture substrate. Examples include long-term culturing, three-dimensional (3-D) tissue engineering, mechanical loading, electrical stimulation, modulation of substrate stiffness, and treatment with neurohormonal factors (1). In particular, there have been many attempts to improve the physiological maturity of cells by aligning hiPS-CMs to induce rod-shaped morphology (3, 9–10).

Various culture media substrates that produce anisotropic orientations in cardiomyocytes have been reported. These include the highly aligned PLGA nanofiber scaffold, in which biodegradable, electrospun poly(lactic-co-glycolic acid) (PLGA) fibers are aligned into a scaffold to create a substrate for producing 3-D cardiac tissue-like constructs (11, 12); a scaffold formed by aligning electrospun polycaprolactone (PL) fibers (13); substrates with a structure of aligned micro-grooves (18–19); substrates using textile structures of polyethylene terephthalate (14); and substrates in which fibronectin is applied in a linear pattern by means of microcontact printing in order to align cells by limiting the cell adhesion area (9).

It has been confirmed that cell culture of cardiomyocytes using these substrates aligns the cells and promotes morphological and functional maturation. However, based on the results of calcium flow analysis and expression analysis of the genes related in maturation, Han found that the degree of cell maturation was limited when hiPS-CMs were aligned by cell culture on aligned fibers and concluded that cell alignment needs to be combined with other methods to promote maturation (3, 13).

Using our original nanostructure fabrication technology, we have developed a cell culture substrate that has regions of nanopillars with a pitch of several hundred nm and planar regions with no nanopillars, with the two types of region arranged in alternate stripes of width 10–30 μm (line/dot structure) on the surface of the cell culture vessel. An important feature of this substrate is that the tips of the nanopillars in the pillar regions are at the same level with the planar regions, so that cells can adhere to both the nanopillar regions and the planar regions. When myoblasts, cardiomyocytes, or fibroblasts are cultured on this substrate, the long axes of the cells extend along the stripe direction, forming cell sheets with the long axes of the cells aligned in one direction. In the present study, hiPS-CMs were cultured using the

line/dot structure, and morphological observations, orientation analysis, and gene expression analysis were performed to evaluate changes in the maturity of hiPS-CMs cultured on this structure.

2. Materials And Method

2 – 1. Cell Culture Substrates

A master mold of the line/dot structure was fabricated at the surface of a 6-inch-diameter Si wafer by combining conventional colloidal lithography and photolithography methods to fabricate a nanostructure consisting of multiple nanopillar and planar regions alternately arranged in a striped pattern (15, 16).

A Ni stamper for a replication mold with the inverse line/dot structure was fabricated by Ni electroforming on the Si wafer master mold. A cell culture substrate with this nanostructure was then manufactured by transferring the line/dot configuration to the surface of a polystyrene sheet (0.15 mm, $T_g = 99\text{ }^\circ\text{C}$) by means of thermal nanoimprinting using the Ni stamper (168 $^\circ\text{C}$, 6 MPa, 5 min). The surface of the resin sheet was then examined by means of a scanning electron microscope (SEM) to confirm that the nanostructure had been replicated successfully (Fig. 2).

A dish-type cell culture substrate was prepared by means of injection molding forming the line/dot structure on the base of a 35-mm-diameter culture dish (ND Cell Aligner Dish, Oji Holdings). A 35-mm dish (3000-035 Tissue Culture Dish, Iwaki) was used as a planar substrate for comparison. There were three types of line/dot structure, as shown in Table 1.

Table 1

The line/dot structures formed on the polystyrene surface. Line refers to the planar region, space is the nanopillar region, and pitch is the distance between the vertices of adjacent nanopillars.

Structural parameters	Structure \boxtimes	Structure \boxtimes	Structure \boxtimes
Line width (planar region)	10 μm	10 μm	30 μm
Space width (nano pillar region)	10 μm	10 μm	30 μm
Pitch of nano pillars	300 nm	600 nm	600nm
Shape of pillars	cone	cone	cone

2–2. Cell culture (human iPS cell-derived cardiomyocytes)

The cell culture substrates and dishes with the transferred line/dot structure were treated by UV-ozone (1 min) to make the surfaces hydrophilic and to sterilize them. Surface coating with human-derived fibronectin was then carried out to enhance cell adhesion. For this, fibronectin solution (Wako, 063-

05591) was diluted with phosphate buffered saline (PBS without Ca^{2+} , Mg^{2+}) to a concentration of 50 $\mu\text{g}/\text{ml}$, and this was added to the dishes at a volume of 0.1 ml/cm^2 and allowed to stand for 2 hours at room temperature. The dishes were washed with sterile water and then used for cell culture.

The human iPS cell-derived cardiomyocytes used were MiraCell Cardiomyocytes (Takara Bio, MiraCell Cardiomyocytes v2 [from ChiPSC12] Kit, Y50025). Cultures were performed according to the protocol provided.

MiraCell Cardiomyocytes were thawed using MiraCell CM Thawing Medium, and cells in a vial were precultured. The culture medium was changed every 2 days for 5 days until the cells reached confluency. The cells were then collected by trypsin treatment and suspended in MiraCell CM Culture Medium.

The cells thus collected were then seeded at a cell concentration of 2×10^5 cells/well on a 48-well plate with line/dot structure substrates, cut to small pieces of 8.8 mm in diameter and placed at the base of each well and at a cell concentration of 8×10^5 cells/dish on a 35-mm-diameter culture dish with a line/dot structure (ND Cell Aligner, Oji Holdings). After seeding, the cells in the well plate and the dish were cultured in an incubator at 37 °C and an atmosphere of 5% CO_2 for 14 days, with the culture medium changed every 2 days until day 6 and then changed every day.

For the control, 8.8-mm-diameter polystyrene substrates placed in a 48-well plate and a 35-mm-diameter dish (3000-035 Tissue Culture Dish, Iwaki) were used.

2–3. Immunocytochemistry

Immunostaining was performed on samples cultured for 14 days on line/dot structure substrate (Structure I, Structure III) and on planar substrate. The samples were stained for α -actinin to observe the sarcomeres in the myocardium. The samples were fixed using 4% paraformaldehyde phosphate buffer solution, and they were then permeabilized (1% BSA and 0.2% Triton-X100 PBS[-/-]) and blocked. Primary antibody treatment was carried out using anti-sarcomeric alpha actinin antibody (mouse) (Abcam, ab9465) overnight at 4 °C, after which the samples were washed 3 times with wash buffer. Secondary antibody treatment was carried out using goat anti-mouse IgG Alexa Fluor 488 antibody (Abcam, ab150117) for 1 hour at room temperature. The immunostained samples were observed using a fluorescence microscope (Keyence, BZ-X800).

2–4. Analysis of cell orientation and sarcomere orientation

For evaluation of the orientation of the sarcomeres (units) of cardiomyocytes cultured on the substrate with the line/dot structure, A-zou Kun image analysis software (Asahi Kasei Engineering) was used to measure and analyze the orientation distribution of rod-like materials.

The substrates used for this analysis were Structure I, Structure III, and planar substrate. Cardiomyocytes were cultured on these three types of substrate for 14 days and then stained for α -actinin in the

sarcomeres. Images from three fields of view were taken of each substrate type. The orientation of the rod-like structures within the sarcomeres stained for α -actinin was measured at 10-degree intervals using the directional distribution measurement and analysis function of the A-zou Kun software. The greatest number of rod-like structures in the interval of one direction was set as 1, and the number of rod-like structures in the other intervals was expressed as a value relative to this. The relative value for the rod-like structures in each interval was calculated for the three fields of view, and the mean, standard deviation, and CV value of each interval were determined.

2–5. Quantitative reverse transcription

After 14 days of culture, cells were collected from the culture with line/dot structure (Structure II) and the planar surface dishes, and total RNA was extracted and used for expression analysis.

Total RNA extraction was carried out using NucleoSpin RNA (Takara Bio, U0955B) according to the protocol provided. Reverse transcription of total RNA was carried out using the PrimeScript RT reagent Kit (Takara Bio, RR037A) to synthesize cDNA. Real-time PCR was carried out using a LightCycler 480 II (Roche). The reaction was carried out using TB Green Premix Ex Taq II (Takara Bio, RR820A), and for the gene-specific primers, the target gene primer set was purchased from Perfect Real Time Primer (Takara Bio). Three separate tests were performed for all samples, and the expression levels were analyzed using the $\Delta\Delta C_t$ method for relative comparison. The expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to correct the expression levels of the target genes. A list of the target genes is given in Table 2.

Table 2

Gene groups from hiPS-CMs cultured on the line/dot structure for which mRNA expression analysis was performed.

Gene	Description	Function
RYR2	ryanodine receptor 2	ER-Ca ²⁺ + function
PLN	phospholamban	ER-Ca ²⁺ + function
ATP2A2	ATPase, Ca + + transporting	Calcium ATPase
CACNA1C	calcium voltage-gated channel subunit alpha1 C	Calcium channel
SCN5A	sodium voltage-gated channel alpha subunit 5	Sodium channel
KCNE1	potassium voltage-gated channel subfamily E regulatory subunit 1	Potassium channel
HCN1	hyperpolarization activated cyclic nucleotide gated potassium channel 1	Potassium channel
KCNJ2	potassium voltage-gated channel subfamily J member 2	Potassium channel
KCNA4	potassium voltage-gated channel subfamily A member 4	Potassium channel
CASQ2	calsequestrin 2	Calcium-binding protein
TNNT2	troponin T2, cardiac type	Sarcomere structures
TNNI3	troponin I3, cardiac type	Sarcomere structures
MYL2	myosin light chain 2	Ventricular structures
MYL7	myosin light chain 3	Sarcomere structures
MYH6	myosin heavy chain 6	Sarcomere structures
MYH7	myosin heavy chain 7	Sarcomere structures
ACTN2	actinin alpha 2	Sarcomere structures
TTN	titin	Z-disc and M-line anchor protein
HAND2	heart and neural crest derivatives expressed 2	Ventricular structures
GJA1	gap junction protein alpha1	Component of gap junctions
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Endogenous control gene

2–6. Statistical analysis

Gene expression analysis was performed using the Mann-Whitney U test.

3. Results

3 – 1. Nanofabrication

At the surface of the fabricated Si nanostructure master mold, parallel stripes of equal width of nanopillar regions and planar regions were formed, and each region was 10 μm or 30 μm wide. In the nanopillar region, cone-shaped nanopillars with pitch of 300 nm or 600 nm were arranged in a quasi-closest packing (triangular lattice) geometry (Fig. 1 shows SEM images of the surface nanostructure of the Si master mold). Important features of the nanopillar region are that the aspect ratio of the nanopillars was approximately 1, and the tips of the nanopillars in the pillar regions were at the same level with the planar regions. For this study, three types of line/dot master molds with line/dot structures of differing stripe width and nanopillar pitch were prepared (Table 1).

Figure 2 (b) shows an SEM image of the surface of a polystyrene sheet with the line/dot structure fabricated by nanoimprinting using the inverse structure Ni stamper. The structure transferred satisfactorily, with the nanostructure of the Si master mold accurately reproduced on the surface of the polystyrene sheet.

3 – 2. Morphology and sarcomere orientation of hiPS-CMs

Human iPS cell-derived cardiomyocytes were cultured on the substrates with the line/dot structure, and they were also cultured on a planar substrate for comparison. Figure 3 shows sarcomere observations of cells that were cultured for 14 days and immunostained with α -actinin antibody.

Cardiomyocytes cultured on the cell culture substrate with line/dot structure were observed to be attached to the substrate and to be elongated, with the long axis of the cells and the direction of the stripe structure at a slight angle to each other. The cell morphology was rod-shaped. When the sarcomeres were visualized by α -actinin staining, it was confirmed that they were oriented orthogonally to the long axis of the cells.

Cardiomyocytes cultured in the planar dish were elongated in random directions. Observation of fluorescence-stained sarcomeres also showed that the cells had various orientations, and that the cell morphology was a mixture of cells elongated in a straight line and cells with a curved shape.

3–3. Distribution analysis of sarcomere orientations

Cardiomyocytes cultured on the substrate with the line/dot structure were α -actinin-stained, and A-zou Kun image analysis software was used to identify the sarcomeres and determine the distribution of their angles of orientation. The substrates used were Structure I (10 μm /10 μm , nanopillar pitch 600 nm), Structure III (30 μm /30 μm , nanopillar pitch 600 nm), and the planar structure. Three fields of view were taken for a single substrate, and the distribution of the angles of orientation for each substrate are shown in Fig. 4.

Since the sarcomeres in cardiomyocytes appear in an orthogonal direction to the long axis of the cell, if the long axis of the cell is oriented along the stripe structure, the sarcomeres will be at an angle of 90° to the stripes.

Cardiomyocytes cultured on substrate with line/dot structure were oriented with the long axis of the cell at a slight angle to the direction of the stripe structure. With Structure I, the peak distribution of sarcomere structure orientations was in the range 70–100° to the direction of the stripe structure, and in Structure III, it was 70–90° to the direction of the stripe structure. These results indicate that the sarcomeres of cardiomyocytes cultured on the line/dot structure were at an orientation of roughly 90° to the stripes.

When the distribution angles of orientation of sarcomere structures in cardiomyocytes cultured on the control planar substrate were determined, the histogram of mean values obtained from the three fields of view showed a shape with no peaks, with no distribution found in any specific direction.

The mean, standard deviation, and CV value of each interval of the histogram of means from the three fields of view were Structure I: 0.819 ± 0.080 (CV value 0.097), Structure III: 0.861 ± 0.080 (CV value 0.093), and control (planar substrate): 0.898 ± 0.014 (CV value 0.016).

3–4. Expression of cardiac-specific genes

Relative quantification of cardiac-specific gene expression was performed by quantitative PCR. Figure 5 shows the results from cardiomyocytes in dishes with the line/dot structure (Structure II) compared to cardiomyocytes in dishes with the planar structure as a control, both cultured for 14 days. *GAPDH* was used as an endogenous gene for correction.

Overall, the gene expression level was higher in cardiomyocytes cultured on the substrate with line/dot structure than in cardiomyocytes cultured on the planar substrate. Significantly greater expression of the *TNNT2*, *MYL7*, *MYH7*, *ACTN2*, and *TTN* gene groups, which are related to myocardial contraction, was seen in the cardiomyocytes cultured on the substrate with line/dot structure than in the cardiomyocytes cultured on the planar substrate ($p < 0.01$). However, expression of the *MYL2* gene group was significantly lower in the line/dot substrate cardiomyocytes than in the planar substrate cardiomyocytes ($p < 0.01$).

With the genes relating to myocardial ion channels as well, cardiomyocytes cultured on the substrate with line/dot structure showed significantly higher expression of the *RYR2*, *PLN*, *ATP2A2*, *CACNA1C*, *KCNE1*, *KCNJ2*, *KCNA4*, and *CASQ2* ($p < 0.01$, Fig. 5) gene groups and the *SCN5A* and *HCN1* ($p < 0.05$, Fig. 5) gene groups than the planar substrate cardiomyocytes.

GJA1, which encodes the gap junction structural protein connexin 43 (Cx43), showed significantly higher expression in cardiomyocytes cultured on the substrate with line/dot structure ($p < 0.01$).

4. Discussion

For hiPS-CMs to be used in drug discovery or regenerative medicine, they need to be made to reach maturation. For this reason, there have been various attempts using different methods to bring about maturation of hiPS-CMs (1). In particular, these have included numerous attempts to improve the level of physiological maturity of hiPS-CMs by orienting them to induce rod-shaped cell morphology (3, 9–10). In the present study, nanostructure fabrication technology was applied to develop a line/dot structure capable of culturing hiPS-CMs with a specific orientation. By introducing a microstructure of planar stripes of width 10–30 μm into a whole surface structure of nanopillars of pitch 300–600 nm fabricated by colloidal lithography, it was shown that it is possible to culture cells in a particular orientation. Specifically, it was confirmed that, as a result of culture on a substrate with this line/dot structure, compared to the planar substrate, cardiomyocytes became oriented along the stripes, and their morphology changed to a rod shape. It was therefore shown that, through the use of this line/dot structure as a cell culture substrate, immature hiPS-CMs were induced to take on a more mature morphology.

In addition, mRNA expression analysis showed that the expression of gene groups related to myocardial structural proteins and to ion channels was generally enhanced, which showed that maturation of cardiomyocytes from iPS cells was promoted at the level of mRNA expression as a result of anisotropic culture of cells by means of the line/dot structure.

The important features of the line/dot structure are that the tips of the nanopillars are at the same level with the planar regions, and that cells adhere to and elongate along not just the planar regions, but the nanopillar regions as well. As a result of these features, when cells are cultured on the line/dot structure, they become oriented along the stripe structure, and, at the same time, neighboring cells are on the same plane as each other, so that a flat cell sheet is formed. Parker *et al.* (17) stated that the cell-cell junction between neighboring cells is necessary not just for contractile function at the tissue level, but also for optimal mechanical and electrical coupling. It appears that the flatness of the planar and nanopillar regions of the line/dot structure is effective for functional expression between neighboring cells.

It is also important to note that, because the tips of the nanopillars are at the same level with the planar region as described above, the cells are therefore free to choose where to adhere. This is different from methods where cells are physically aligned by fitting them into the grooves of the stripe structure (18–19). Therefore, the cell orientation achieved with the line/dot structure is thus not forced by spatial constraints, but it is the result of recognition and subjective selection of the adhesion surface by the cells themselves.

Prior studies have used various methods to induce cell orientation and promote morphological and physiological maturation, as a result of which elongated, rod-shaped cell morphology and more developed sarcomeres have been observed. However, the degree to which maturation is promoted in terms of mRNA expression has varied depending on the substrate.

One method of forming two-dimensional (2-D) tissue with aligned hiPS-CMs is to culture the cells on a nanofiber scaffold of PGLA produced by electrospinning. It has been shown that using an aligned

nanofiber scaffold improves the anisotropic mechanical properties of the tissue, induces myocardial morphology similar to normal myocardium, and improves mechanical function (20). However, it has also been shown that there was no notable difference in the mRNA levels of alpha-actinin and troponin-I in tissue from aligned nanofiber scaffold and tissue from a planar cell culture substrate (12). Han *et al.* (13) investigated the maturity of cells produced by anisotropic culture on an aligned nanofiber scaffold made of polycaprolactone (PCL), and they found that, compared to a randomly ordered nanofiber scaffold, only two genes (*CASQ2* and *KCNJ2*) were significantly upregulated at the level of mRNA expression, and they concluded that there was no improvement in the maturity of hiPS-CMs. In the culture of hiPS-CMs on a 3-D nanofiber scaffold, cell sheets have been produced ranging in thickness from 0.3 μm to 4 μm , depending on the nanofiber scaffold and the number of cells, and they showed increased mRNA levels in addition to improved mechanical and physiological functions (11). The idea has therefore been put forward that, in order to achieve maturation of hiPS-CMs at the gene expression level, a combination of cell orientation with some other method (3-D culturing, long-term culturing, electrical stimulation, induction with drugs, etc.) is needed (3, 13).

Gene expression in hiPS-CMs cultured on substrates with the line/dot structure was increased in gene groups relating to myocardium structural proteins, cell-cell junction Cx43, and ion channels. The increased mRNA expression of gene groups related to myocardial structural proteins is associated with a well-organized sarcomere structure, and the increased gene expression of *GJA1* is associated with the Cx43 cell-cell junction protein. Increased gene expression of *GJA1* indicates evidence of electrical properties (12), suggesting that there is also promotion of maturation of physiological functions (electrical properties) in hiPS-CMs cultured on substrates with the line/dot structure. A detailed investigation of whether culture on substrates with the line/dot structure can promote maturation in terms of physiological function in hiPS-CMs is needed.

Several methods have been developed to culture cardiomyocytes with an elongated morphology by means of the nano-micro structural approach. These include the use of photolithography techniques to create cell adhesion regions, the use of microcontact printing to apply extracellular matrix (ECM) components such as fibronectin to limit the cell adhesion regions, the use of a substrate with grooves of nano-micrometric width, and functionalization of the culture substrate using a chimeric peptide containing the Arg-Gly-Asp (RGD) cell adhesion motif (18, 19, 21–23).

It has been reported from the results of culture of neonatal rat ventricular myocytes on a substrate with nanosized uniform ridges and grooves that the nanotopographic pattern of the substrate affects cell size and the amount and distribution of Cx43, and that the structure and function of cardiomyocytes respond to the nanoscale organization and structure of the ECM. There are also reports that, with ridges and grooves of optimal size, adhesion of cells to grooves increases, cell elongation and intercellular connectivity are promoted, and physiological function is increased (higher action potential propagation velocity) (18–19).

An important feature of the line/dot structure is that the tips of the nanopillars and the planar regions are at the same level, unlike substrates made by conventional techniques in which walls or grooves are formed on the flat region, and there are thus differences in height. Cardiomyocytes cultured on the line/dot structure recognize the shape characteristics of the nanostructure through the sites by which they adhere to the substrate surface, and because they are stimulated by the nanostructure, they spontaneously choose to orient themselves and elongate along the direction of the stripes. It may be surmised that, as a result of this, the cardiomyocytes in the formed cell sheets take the optimal shape and size (similar to *in vivo* myocardium), and they form cell-cell junctions, undergo cell contraction, and transmit electrical stimuli. This morphology cannot be achieved by passive cell orientation using pre-designed scaffolds such as grooves or fibers, and it is likely to be effective in promoting the functional and physiological maturation of cardiomyocytes.

Two-dimensional culture substrates have the advantages that drug evaluations can be carried out with fewer cells than 3-D cultures, and the evaluation cycle is shorter than with 3-D cultures. In addition, as an application for regenerative medicine, 3-D layered bodies with blood vessels can be fabricated from 2-D substrates, because the method of fabricating and layering cell sheets allows blood vessels to be arranged between the cell sheets. This can solve the problems of oxygen supply and nutrient supply that are challenges with 3-D tissues (24).

The line/dot structure has a substrate surface in which the top of the nanopillar region and the planar region are at the same height, and it is effective as a fabrication substrate not just for drug evaluation, but also for cell sheets for regenerative medicine. This is because cell sheets made with scaffolds such as grooves and fibers are difficult to detach and collect as individual cell sheets, and, therefore, cannot be used directly for regenerative medicine. We plan to conduct further studies to determine whether it is possible to promote the maturation of physiological functions of hiPS-CMs using only 2-D substrates, without combining this with any other method of cardiomyocyte maturation.

5. Conclusions

The hiPS-CMs were cultured on a substrate with a line/dot structure comprising nanopillar structures and planar regions arranged in alternating stripes and showed a clear cell orientation with their long axis aligned in one direction, inducing a rod-shaped cell morphology. In addition, analysis of the expression of cardiac-specific genes confirmed a significant increment of mRNA of myocardial structural proteins, ion channels, and Cx43, suggesting that the line/dot structure promoted maturation of hiPS-CMs. Culturing hiPS-CMs on the line/dot structure is thought to be a useful and effective method for drug discovery and regenerative medicine applications.

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Figures

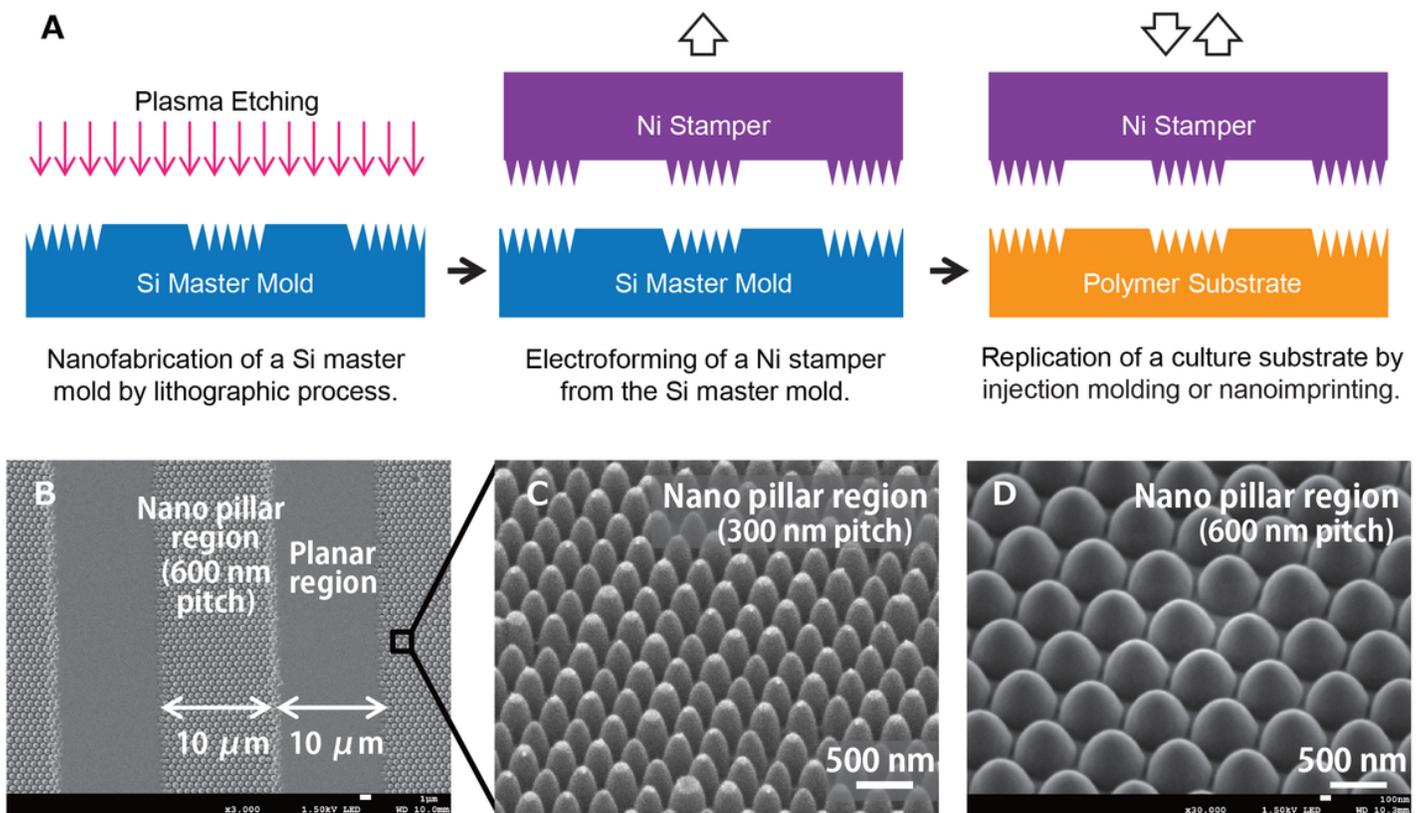


Fig.1 Fabrication process and Si master mold of the culture substrate formed with a line/dot structure of regions of nanopillars with pitch of several hundred nm and planar regions arranged in alternating stripes.

(A) The process of forming the culture substrate from the silicon master mold with the line/dot structure.

(B)–(D) SEM images of the nanostructure of the silicon master mold.

(B) Nanopillar region and planar region of Structure II. The pitch is 600 nm.

(C) Magnified view of the nanopillar region of Structure I. The pitch is 300 nm.

(D) Magnified view of the nanopillar region of Structure II. The pitch is 600 nm.

Figure 1

See image above for figure legend

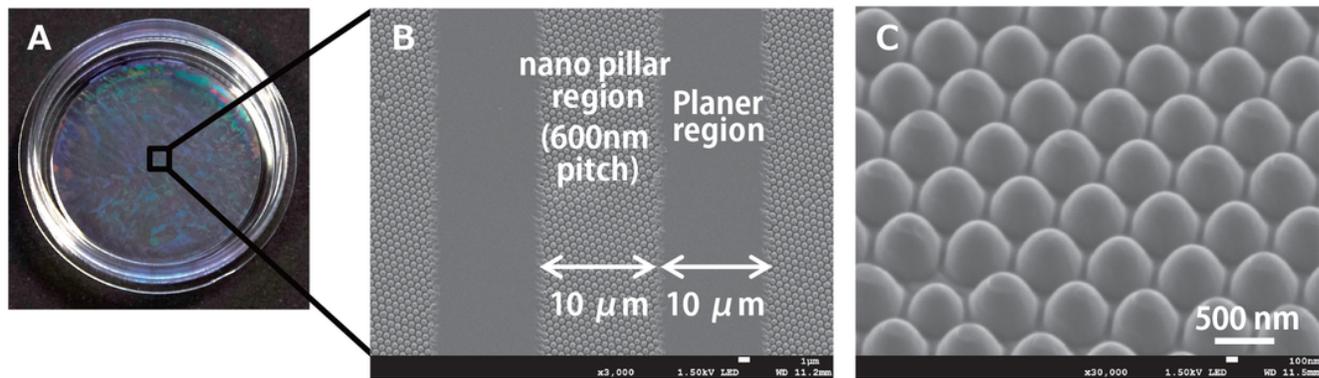


Fig.2 Cell culture substrate with the line/dot structure formed at the surface of the polystyrene resin by injection molding.

(A) 35-mm-diameter dish with the microstructure formed at the surface.

(B), (C) SEM images of the nanostructure of the cell culture substrate.

(B) Nanopillar region and planer region of Structure II.

(C) Magnified view of the nanopillar region of Structure II. The pitch is 600 nm.

Figure 2

See image above for figure legend

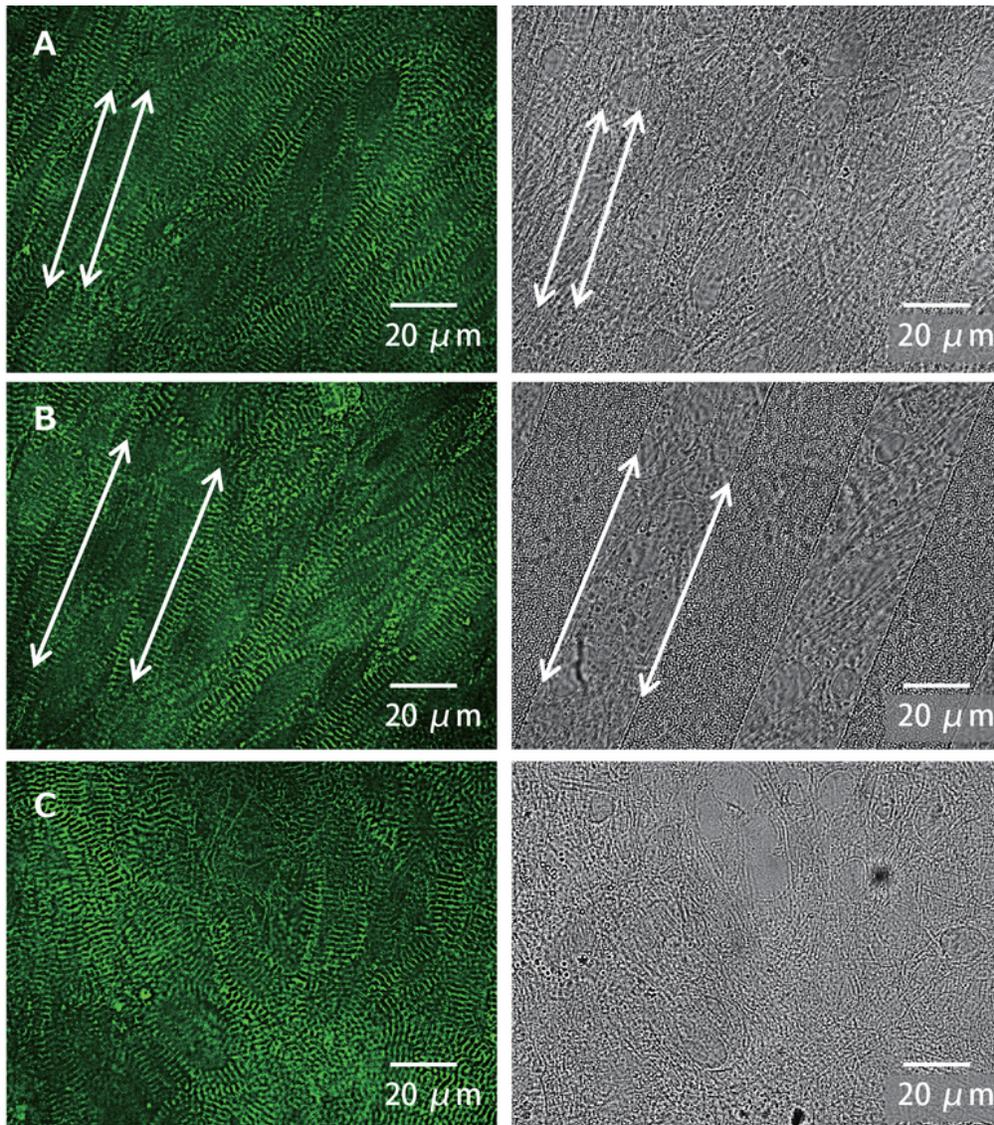


Fig. 3 Microscope image of hiPS-CMs cultured for 14 days on the line/dot structure substrate and the planar substrate. Left are immunofluorescent images of sarcomeric α -actinin (green), right are phase-contrast images. Adjacent left and right images are from the identical field of view.

The white arrows show the direction and the interval of the stripes.

(A) Structure I: Line/dot width = $10 \mu\text{m}/10 \mu\text{m}$, nanopillar pitch = 300 nm .

(B) Structure III: Line/dot width = $30 \mu\text{m}/30 \mu\text{m}$, nanopillar pitch = 600 nm .

(C) Planar substrate

Figure 3

See image above for figure legend

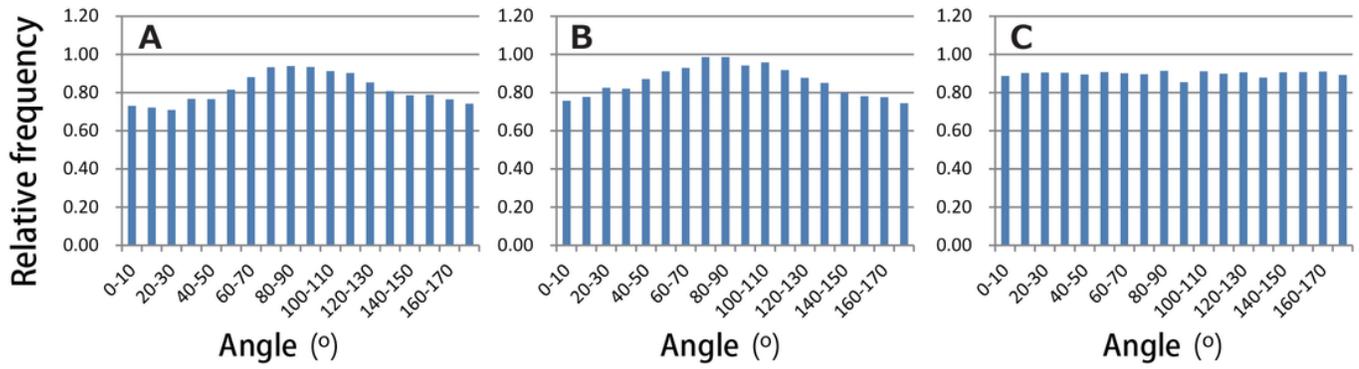


Fig.4 Orientation analysis of sarcomeres of hiPS-CMs cultured for 14 days on the line/dot structure substrate and the planar substrate. Angles were determined with the orientation of the stripes taken as 0°.

(A) Structure I: Line/dot width = 10 μm /10 μm , nanopillar pitch = 300 nm.

(B) Structure III: Line/dot width = 30 μm /30 μm , nanopillar pitch = 600 nm.

(C) Planar substrate

Figure 4

See image above for figure legend

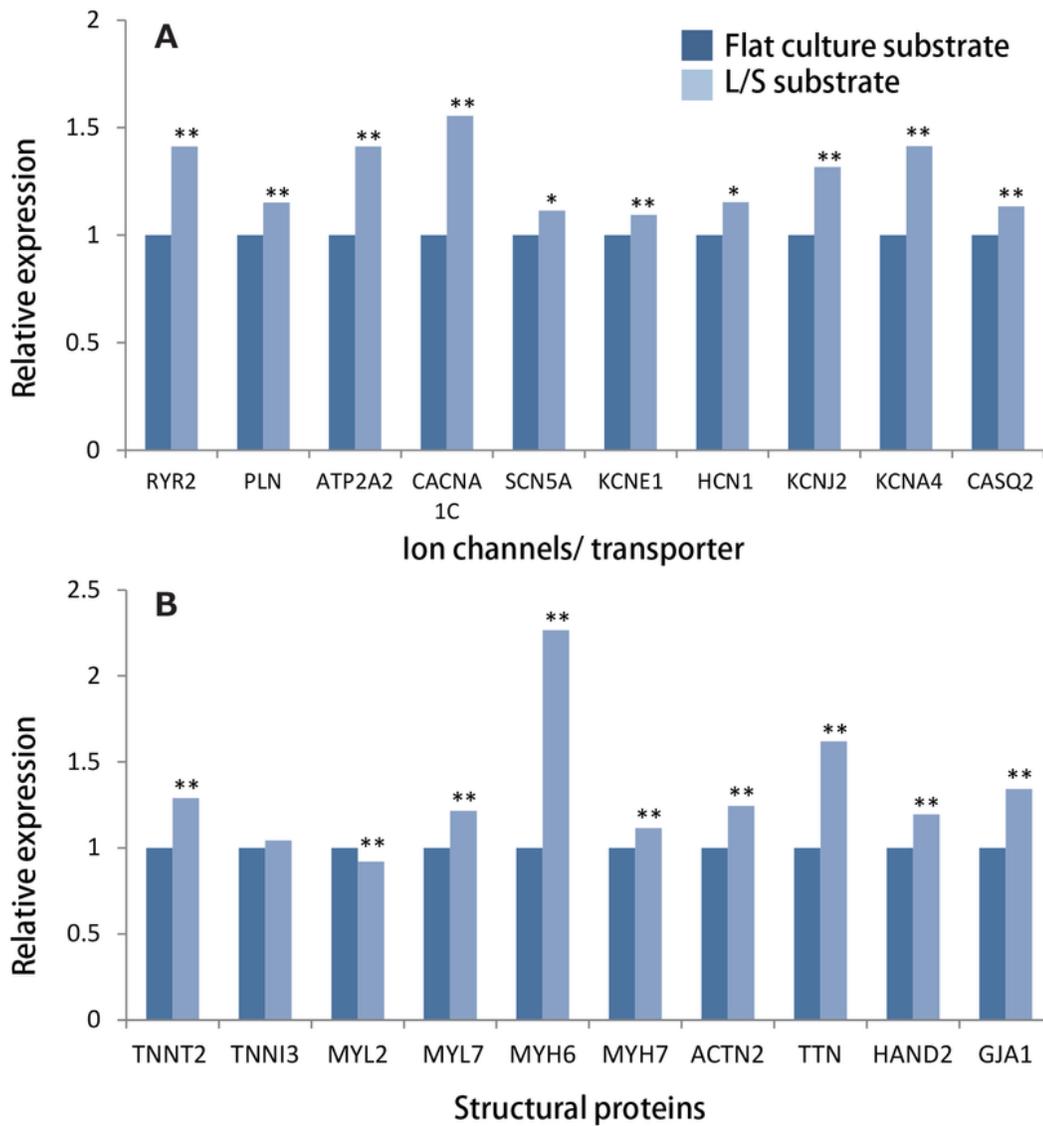


Fig.5 Relative expression of cardiomyocyte specific genes. hiPS-CMs cultured for 14 days on the planar substrate and on the Line/ dot structure substrate (line/dot width = 10 μ m/10 μ m, nanopillar pitch = 600 nm).

(A) Relative expression of Ion channels and transporter genes.

(B) Relative expression of cardiomyocyte structural genes.

The experiment was performed with three independent trials.

Mann-Whitney U test **: $P < 0.01$, *: $0.01 < P < 0.05$

Figure 5

See image above for figure legend