Scalable alignment of three-dimensional cellular constructs in a microfluidic chip†

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There have been considerable efforts to engineer three-dimensional (3D) microfluidic environments to enhance cellular function over conventional two-dimensional (2D) cultures in microfluidic chips, but few involve topographical features, such as micro/nano-grooves, which are beneficial for cell types of cardiac, skeletal and neuronal lineages. Here we have developed a cost-effective and scalable method to incorporate micro-topographical cues into microfluidic chips to induce cell alignment. Using commercially available optical media as molds for replica molding, we produced large surface areas of polydimethylsiloxane (PDMS) micro-grooved substrates and plasma-bonded them to multiple microfluidic chips. Besides aligning a 2D monolayer of cells, the micro-grooved substrate can align 3D cellular constructs on chip. C2C12 mouse myoblasts were cultured three-dimensionally in a microfluidic chip with incorporated PDMS micro-grooved substrate remodeled into an aligned 3D cellular construct, where the actin cytoskeleton and nuclei were preferentially oriented along the micro-grooves. Cells within the 3D cellular constructs can align without being in direct contact with the micro-grooves due to synergism between topography and fluid shear stress. Aligned C2C12 3D cellular constructs showed enhanced differentiation into skeletal muscles as compared to randomly aligned ones. This novel method enables the routine inclusion of micro-topographical cues into 2D or 3D microfluidic cultures to generate relevant physiological models for studying tissue morphogenesis and drug screening applications.

Introduction

Microfluidic technologies have been increasingly used for studies such as stem cell differentiation, cell selection, disease modeling and drug screening applications.\textsuperscript{1,2} This is because culturing cells in microfluidic systems permit the use of a small quantity of cells and reagents, and can be readily adapted to multiplexed and high throughput assays.\textsuperscript{2} In addition, they allow for more precise experimental manipulation of cellular systems, such as spatial patterning of molecules, control over mass transfer regimes, and application of shear stresses.\textsuperscript{1,3} To fully realize the utility of microfluidic cell-based models in biomedical applications, it is important to create a microfluidic cell microenvironment which is conducive for maintaining physiological cellular phenotypes. Earlier developments have focused on the surface modification of microfluidic substrates to facilitate cell attachment.\textsuperscript{4} More recently, there has been much emphasis to incorporate 3D cellular microenvironments into microfluidics since 3D cell cultures better mimic the in vivo environment for more physiological function, differentiation and drug responses.\textsuperscript{5–7} Besides 3D cell morphology, cell alignment imposed by the geometry and topography of the cell culture substrate e.g., the extracellular matrix (ECM) is important for maintaining physiological phenotypes and functions, especially in anisotropic cardiac, neuronal and skeletal muscular tissues.\textsuperscript{8} Therefore, the incorporation of cell alignment cues in microfluidic platforms is of interest in the customization of microfluidic cell models to better support these specific tissues.\textsuperscript{9}

Despite the advantages of aligning cells in cultures, there have been relatively few attempts to incorporate engineered features for aligning cells in microfluidic systems.\textsuperscript{10} Some
Researchers achieved an aligned monolayer of cells in microfluidic devices by patterning ECM molecules on glass substrates. The alignment was produced by geometrically constraining cell attachment and spreading to only the desired area. The technique can only create an aligned monolayer of cells but not 3D cellular constructs in microfluidic chips. The mechanism of eliciting alignment by ECM patterning is different from that of cell alignment in vivo, which has been shown to be due to groove-like topographical features of the ECM. Therefore, inducing cell alignment via engineered topographical cues may elicit a cellular phenotype similar to aligned tissues in vivo.

A major challenge of making topographical cues, such as micro-grooves, a routine feature of microfluidic platforms is the high cost of fabrication of these micro- or nano-topographies. Yang et al. proposed a stitching method to generate large surface areas of micro-grooved substrate. This consists of pre-fabricating micro-grooved substrates using photolithography or electron beam lithography and then stitching them together. Wallin et al. used electrospun polymers to create aligned and random patterns in polydimethylsiloxane (PDMS) microfluidic chips. However, both methods require costly or specialized photolithography or electrospinning equipment. Alternative methods of producing micro-grooved substrates have been developed, such as micropatterned surfaces ground with abrasives or using metal wrinkles. Although these techniques help to reduce the cost and specialized equipment required, their adaptation into microfluidic cell culture systems has not yet been demonstrated.

Methods for incorporating topographical features into a microfluidic system should also be compatible with the implementation of microfluidic 3D cell culture. To date, cell alignment with micro-patterned ECM or topographical features in microfluidics has only been demonstrated for 2D monolayer cultures. While the generation of aligned 3D cellular constructs have been developed in static bulk cultures, which include stacking of aligned cell sheets, encapsulation of cells in hydrogels or culture in a scaffold, these methods are not readily implemented in microfluidic systems. The ability to create an aligned 3D culture of cells from anisotropic tissues, which require polarity or alignment for proper functioning, will enable one to customize microfluidic cell models of these specific tissues and fully realize their potential in desired applications, such as drug screening.

Here we report a cost-effective and scalable method to incorporate micro-topographies into microfluidic chips. We utilized commercially available optical media, which contain micro-grooves of various dimensions that support alignment of cardiac, skeletal and neuronal cell lines, as molds to transfer the topographical features to a PDMS sheet. These optical media offer a simple and cost effective method to generate a large surface area (up to 15 cm × 30 cm) of micro-grooved PDMS substrates, which can be easily incorporated into PDMS microfluidic devices. When coupled with a 3D microfluidic cell culture system, we show that the micro-grooved substrate can align 3D cellular constructs along the orientation of the grooves; and the aligned 3D cellular constructs exhibited improved differentiation of C2C12 myoblast cells into skeletal muscle cells in the microfluidic device.

**Experimental**

**Design, fabrication and assembly of the microfluidic chip**

The top layer of the microfluidic chip containing the microfluidic network was adapted from a previously developed microfluidic 3D cell culture chip. Briefly, microfluidic channels with a micropillar array were designed using AutoCAD (Autodesk, USA) and L-Edit v10.20 (Tanner Research, USA). The dimensions of the microfluidic channel were 1 cm (length) × 600 μm (width) × 100 μm (height) and had one inlet for the cell culture medium, one inlet/cell reservoir and one outlet. An array of 30 μm × 50 μm micropillars with a gap size of 20 μm was situated in the middle of the microfluidic channel, bounding a cell residence volume that was 1 cm (length) × 200 μm (width) × 100 μm (height). Silicon templates were fabricated by a standard deep reactive ion etching (DRIE) process (Oxford Instruments Plc, UK). The microfluidic channels were then obtained by replica molding polydimethylsiloxane (PDMS) (Dow Corning, USA) on the silicon templates.

The top layer was bonded to a PDMS bottom layer containing micro-grooves instead of glass substrates. To generate a large surface area for the bottom layer, optical media (Vivoalign™ licensed to Bio-Byblos, Taiwan ROC, 8 x 8 cm) were placed into 150 mm cell culture dishes (CORNING, Singapore). The optical media were glued to the bottom of the dish with double-sided tape, exposing the grooved surface. Subsequently, a mixture of PDMS with curing agent at a ratio of 10 : 1 was poured on the surface of the optical media. The mixture was degassed and left to cure in 80 °C for 2 h.

Both the top layer and bottom layer were subsequently treated with oxygen plasma-bonded and put into an oven at 80 °C for another 2 h. This was to aid the bonding between both PDMS layers. Three different configurations of the bottom layer were used: 1) grooves were arranged to be parallel to the fluidic flow; 2) grooves were perpendicular to the flow; and 3) no grooves on the bottom layer. The microfluidic chips were sterilized by autoclaving and connected to a perfusion circuit as described previously.

**Surface characterization of PDMS micro-groove substrates by Atomic Force Microscopy (AFM)**

AFM samples were prepared by cutting appropriate sizes of the PDMS micro-groove substrates. An atomic force microscope, DI Nanoscope Dimension 3100 (Digital Instruments, USA), was used in tapping mode to identify the groove features on the PDMS substrate.

**Microfluidic cell culture**

C2C12 is a sub-clone of a murine myoblast and consists of a pure population of myogenic cells that proliferate and differentiate in culture. The cells were obtained from ATCC.
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(USA) and were used at a passage number below 20 for this study. Prior to cell seeding, the microfluidic chip was primed by perfusing the following solutions in order: 70% ethanol, DMEM (Gibco®, 31600, Invitrogen, Singapore), PBS and again with DMEM. Each solution was left to run for 20 min at 0.5 ml h⁻¹. After the priming step, the bottom layer of the chip was coated with a mixture of 10 μg ml⁻¹ fibronectin (F1141, Sigma-Aldrich, Singapore) and 1 mg ml⁻¹ gelatin (G1890, Sigma-Aldrich, Singapore). The solution was added into the cell reservoir and the outlet connected to a syringe pump in withdrawal mode to allow for the solution to flow into the chip. The chips were incubated with the fibronectin-gelatin solution for 2 h before washing with DMEM. A suspension of C2C12 cells (6 million cells ml⁻¹) was introduced into the chip using a syringe pump to withdraw cells from a cell reservoir. Cells were immobilized three-dimensionally by the micropillar array within the microfluidic chip. Cells were allowed 4 h to attach before perfusion was initiated at a flow rate of 0.05 ml h⁻¹.

To study the effect of the groove orientation on the cell alignment in the microfluidic chip, cells were cultured for three days in a proliferation medium, containing DMEM with 10% Fetal Bovine Serum (FBS) (Gibco®, 16000, Invitrogen, Singapore) and 1% Penicillin-Streptomycin (P4333, Sigma-Aldrich, Singapore). The three different configurations mentioned above were used. To study the differentiation of C2C12 cells into skeletal muscle lineage, the cells were cultured for 3 days in proliferation medium, followed by another 3 days in differentiation medium, which consisted of DMEM, 1% FBS, 1% Penicillin-Streptomycin and 1% insulin transferrin-sodium selenite (Gibco®, 41400, Invitrogen, Singapore). At the end of the 3 or 6 days culture period, samples were either fixed for immunofluorescence staining or cells were lysed for qPCR analysis. For the differentiation experiment, two configurations were compared: a) no grooves and b) grooves parallel to the direction of the fluidic flow.

A 2D monolayer culture in the microfluidic chip was established by using a low-density cell suspension (1 million/ml) so that the cells cannot be efficiently trapped by the micropillar array. After the cells were introduced into the chip, they were incubated for 4 h before perfusion culture was initiated.

**Total RNA isolation and gene expression analysis**

Total RNA was harvested and purified with the RNeasy Plus Microkit (74034, Qiagen, Singapore). Cells lysis was performed by perfusing the chip with RLT Plus buffer (supplied with the RNeasy Plus Micro Kit), supplemented with 10 μl ml⁻¹ of β-mercaptoethanol (M3148, Sigma-Aldrich, Singapore) to inhibit RNases, at a flow rate of 0.5 ml h⁻¹ until a volume of 350 μl was collected. Total RNA was isolated and purified from the cell lysate according to the manufacturer’s protocol, and reverse transcribed to cDNA using high capacity RNA to cDNA kit (4387406, Invitrogen, Singapore). A total of 300–600 ng μl⁻¹ of RNA was obtained from each sample. Previously published primers for C2C12 cells were used for the quantitative PCR and the reactions were performed using the LightCycler® FastStart DNA MasterPLUS SYBR Green I (03515869001, Roche, Singapore) in a Roche LightCycler® 1.5 carousel based system (Roche, Singapore). Analysis of each gene was performed using the relatively quantitative ΔACT method. Transcript levels were first normalized to the housekeeping gene GAPDH and expressed as relative level to that of day 0.

**Immunofluorescence staining**

Cells were fixed, permeabilized and blocked as described previously. Alexa fluor phalloidin 488 (A12379, Invitrogen, Singapore) was used for F-actin visualization and DAPI (D9542, Sigma, Singapore) at 1 μg ml⁻¹ for nuclei staining. The samples were imaged with a confocal microscope (Olympus FV1000, Japan).

**Quantification of nuclei and cell orientation**

The nuclei alignment angle and elongation ratio (E.R.) was calculated using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The nuclei alignment is the orientation of the major elliptic axis of the nucleus with respect to the horizontal axis along which the micro-grooves were oriented, while the E.R. is the ratio of the long to short axis of the nucleus. For each sample, images were acquired from three different imaging planes along the height of the microfluidic chip: the bottom plane, mid plane and top plane, with each plane being 8–12 μm higher than the previous plane. For this analysis, the nuclei alignment angles were normalized to the mean nuclei angle for each sample and the percentage of nuclei within 20° of the mean was calculated.

**3D image rendering**

3D image rendering of the z-stack confocal images was performed using the Imaris software (Bitplane, Switzerland).

**Statistical analysis**

Graphs and statistical differences for nuclei alignment, elongation ratio (E.R.) and gene expression were performed using GraphPad Prism 5 (GraphPad Software Inc. California, USA). A one-way analysis of variance followed by Bonferroni’s multiple comparison tests was employed to compare the nuclei alignment and E.R. across the three configurations of groove alignment. For the gene expression studies, paired t-test studies were performed comparing flat and grooved for each gene, p < 0.05 was considered significant.

**Results and discussion**

**Fabrication and incorporation of PDMS micro-grooved substrate into microfluidic chips**

We report a simple and scalable method of fabricating a microfluidic chip incorporating topographical features that enabled the alignment of 3D cellular constructs. The chip comprised of a top layer with a microfluidic network optimized for supporting 3D culture of mammalian cells, and a bottom PDMS substrate containing micro-grooves to align cells (Fig. 1A). The top layer consisted of a microfluidic channel which was segregated by a micropillar array into a central cell culture compartment and two flanking perfusion compartments (Fig. 1A). The micropillars permitted 3D trapping of cells during seeding and after perfusion culture,
the trapped cells formed a 3D cellular construct with about 3 layers of cells. The bottom PDMS layer was fabricated by simply molding PDMS on a commercial optical media, (Fig. 1A) which was pre-imprinted with micro-grooves. These commercial optical media offer a readily available and affordable source of micro-grooved substrates and come in different topographical dimensions, ranging from 500 nm–2 μm. We have previously demonstrated that the micro-grooves on these commercial optical media can efficiently align multiple cell types, including cardiac, muscular and neuronal cells. The microfluidic chip was assembled by plasma bonding the two layers together (Fig. 1A). Due to the large surface area of the optical media used, we can achieve a 8 cm x 8 cm PDMS micro-grooved substrate, which can simultaneously be bonded to up to 12 microfluidic chips (Fig. 1B). After bonding both layers by oxygen plasma treatment and heating in an oven, we checked for leakages by setting up the chip and perfusing a colored dye through the channels. There was no evident leakage from any of the chips (Fig. 1B), which indicated the robustness of the method to incorporate micro-grooved substrates into multiple microfluidic chips. This method can produce micro-grooved substrates of considerably larger area than the stitching method proposed by Yang et al.

Larger scale fabrication can be achieved due to the availability of optical media with an even larger footprint.

To determine the fidelity of transferring the topographical features from the optical media to PDMS, AFM images of the PDMS substrate were obtained to assess the grooves. The AFM image showed that the grooves were successfully transferred from the optical media to the PDMS sheet (Fig. 2A). The depth of the PDMS micro-grooves was 225 nm ± 51 nm and the pitch was 2.080 μm ± 0.06 μm. The optical media used as mold had a depth of 282 nm ± 80 nm while the pitch was 2.100 μm ± 0.05 μm. This represented a 80% fidelity in the transfer of the micro-grooves from the optical media to the PDMS.

To achieve different configurations of the microfluidic chip, the bottom layer was bonded to the top layer such that the grooves run either parallel (Fig. 2B) or perpendicular to the direction of the flow (Fig. 2C). As a control, we also had chips consisting of a flat PDMS substrate without any grooves (Fig. 2D). We validated that the PDMS micro-grooved substrate incorporated into the microfluidic chip could support the attachment and alignment of a 2D monolayer of C2C12 cells (Fig. S1, ESI†). This method provides a simple and robust means to routinely incorporate topographical cues into microfluidic cell culture systems.

**Alignment of 3D myoblast cellular construct in a microfluidic chip**

The alignment of 3D cellular constructs formed from C2C12 mouse myoblasts was examined to determine the topographical effects of the PDMS micro-grooves in the microfluidic chip. During cell seeding, the C2C12 cells were packed three-dimensionally at a high density due to the sieving mechanism of the micropillar array. We have previously validated that various three-dimensionally immobilized cells in this microfluidic chip can maintain high viability for up to 5 days. The C2C12 cells were cultured in proliferation medium for 3 days to allow remodeling into a 3D cellular construct. Confocal images of fluorescently labeled F-actin revealed that the cells cultured on the flat surface without grooves showed a random orientation of the actin cytoskeleton after 3 days (Fig. 3A and B); however the cells cultured on the grooved substrate were aligned along the direction of the grooves irrespective of their orientation to the flow direction (Fig. 3C–F). An analysis of a 3D confocal optical stack showed that the cell alignment in both the parallel and perpendicular-oriented chips was consistently observed throughout the 15–25 μm thick 3D cellular construct, formed with about 2–3 layers of cells (Fig. 3C and 3E). The multilayer cell alignment was likely to be a result of layer-by-layer cytoskeletal rearrangement.
due to the cadherin mediated cell–cell coupling of the C2C12 cells. The bottom layer of the cells, which came in direct contact with the PDMS micro-grooves, could sense the topography and rearranged their cytoskeleton to align along the direction of the groove; the other layers subsequently align on top of the bottom layers. This is in agreement with published literature on the generation of multiple layers of aligned cells in static bulk culture, in which a monolayer of cells was first cultured on micro-patterned polyacrylamide gel and after 2 days, another layer of cells was overlaid on the already existing monolayer. The second layer of the cells aligned along the direction of the first layer of cells, thus forming a double layer of aligned cells.

Cell alignment not only alters the actin cytoskeleton arrangement, it also produces anisotropy in the cell nuclei morphology along the direction of alignment. Hence, we also assessed for cell nuclei morphology and orientation using quantitative image analysis. For each configuration of the microfluidic chip (i.e., parallel, perpendicular or no grooves), nuclear measurements were made at 3 different layers of the 3D cellular construct: the bottom layer in contact with the micro-grooves, the mid-layer and the top layer furthest from the micro-grooves. The overall alignment of the 3D cellular construct was taken to be the averaged nuclear measurements from the 3 cell layers.

We calculated the nuclear elongation ratio (E.R.) to study the deformation of the nucleus. This index is a quotient of the long and short axis of the nucleus and is commonly used to assess for nuclear deformation. Nuclear deformation has been shown to alter gene and protein expression, and has been linked to differentiation of progenitor cells and commitment into a particular lineage. An E.R close to one shows a more rounded nuclear morphology while values higher than 1 tend towards an anisotropic ellipse. The average nuclear E.R. of C2C12 3D cellular constructs cultured in different topographical configurations is shown in Fig. 4A. It was much higher for the cells cultured on the parallel grooves, followed by cells on the perpendicular grooves and lowest on cells cultured without grooves. We found that these differences were a result of variations in the proportion of nuclei exhibiting different extents of deformation across the 3 configurations. A breakdown of the overall nuclear E.R. in the C2C12 3D cellular constructs was performed by binning the E.R. of approximately 700 nuclei from each configuration into 3 groups, representative of (1) round nuclei (1.0 < E.R. < 1.5), (2) moderately elongated nuclei (1.5 < E.R. < 2.5) and (3) very elongated nuclei (E.R. > 2.5) (Fig. 4B). The results show that the proportion of cells with moderately elongated nuclei in all the 3 configurations was approximately the same, with 48% (no grooves), 55% (perpendicular grooves) and 57% (parallel grooves) falling into this category. However, there was
a significant difference in the proportion of cells with round or very elongated nuclei across the 3 configurations. ~50% of the cells cultured on substrates without grooves had round nuclei (group 1), while only 35% and 10% of the cells on the perpendicular and parallel grooves respectively were in this group. The reverse was the case for the proportion of cells with very elongated nuclei (group 3), with about 2% (no grooves), 10% (perpendicular grooves) and 33% (parallel grooves) of the cells falling into this group. To determine if these observed differences were due to variability in the transmission of alignment cues across the thickness of the 3D cellular construct, the degree of elongation was investigated in three planes. The results showed that ~38% and 22% of the nuclei in the bottom layer of the parallel and perpendicular chips respectively had a ratio of >2.5 (group 3). This percentage was reduced in the top layer, with the reduction being more evident in the perpendicular chips (from 22% to 6%) than in the parallel chip (from 38% to 33%) (Fig. S2, ESI†). This suggests that transmission of alignment cue was dependent on the distance of a cell in the 3D cellular construct from the micro-grooves.

To quantitatively measure the extent to which nuclear elongation within the C2C12 3D cellular construct was directed by the PDMS micro-grooves, we determined the direction of cell nuclei orientation. For the chips with micro-grooves oriented parallel to the flow direction, 78% of the nuclei were correctly aligned to within 20° from the orientation of the micro-grooves. For 3D cellular constructs cultured on the perpendicular grooves, 53% of the cells were correctly aligned; while correct alignment for cells on a flat substrate was about 20% (Fig. 4C). This suggests that the direction along which nuclear deformation occurs was indeed dependent on micro-grooves since nuclear alignment in both parallel and perpendicular configurations were significantly higher than that of the flat substrate configuration. A breakdown of the top, mid and bottom planes for each configuration again revealed that the percentage of nuclei alignment along the micro-grooves reduced from the bottom layer (which was in direct contact with the micro-grooves) to the top layer for both the parallel and perpendicular grooves. However, there was no significant change for the chips with a flat surface (Fig. 4D). For the parallel and perpendicular grooves, the strongest alignment was at the bottom plane, which came in direct

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Fig. 3 Alignment of actin cytoskeleton in C2C12 3D myoblast cellular constructs. C2C12 cells were cultured three-dimensionally for 3 days in proliferation medium with different configurations of the bottom micro-grooved substrates in the microfluidic chip: (A and B) flat surface, (C and D) micro-grooves parallel to flow direction and (E and F) micro-grooves perpendicular to the flow direction. (A,C,E) are the 3D orthogonal views of F-actin and DAPI staining of C2C12 cells. (B,D,F) shows a corresponding selection of individual z-stack images of the C2C12 3D cellular constructs from the bottom to the top of the microfluidic chip. Scale bar = 50 µm.
contact with the PDMS micro-grooves (82% for parallel and 60% for perpendicular) while the top layer had the weakest alignment (70% for parallel and 42% for perpendicular) (Fig. 4D). This further buttresses the fact that the grooves were the key determinant of the alignment.

Both the actin cytoskeleton and nuclear deformation results collectively demonstrated that alignment of 3D myoblast cellular constructs could be achieved by incorporating micro-grooves into the 3D microfluidic chip. Cell alignment was the most efficient in the parallel configuration, where we observed the highest extent of nuclear deformation and alignment along the micro-grooves (Fig. 4A and C). In the perpendicular configuration, even though the actin cytoskeleton was aligned along the micro-grooves, the transmission of the cytoskeletal alignment to nuclear deformation was significantly lower than that in the parallel configuration (Fig. 4A and C), which suggests that other factors in addition to topography can also modulate cell alignment in the microfluidic chip. One of such factors is fluid shear stress. Using computational fluid simulation, we estimated that cells at the periphery of the 3D cellular construct experienced shear stress ranging from 0.3 to 15 dynes cm$^{-2}$ depending on how far they were away from the micro-pillar array (Fig. S3, ESI). This range of shear has been reported to produce cell alignment. Therefore, in

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**Fig. 4** Quantification of nuclear elongation and alignment in C2C12 3D myoblast cellular constructs. C2C12 cells were cultured three-dimensionally for 3 days in proliferation medium with different configurations of the bottom micro-grooved substrates before assessing for nuclei morphology and orientation. (A and B) show the nuclear elongation ratio (E.R.). (A) is the average E.R. of 3D cellular constructs cultured in the three configurations of the micro-grooved substrate. A total of >700 nuclei were analyzed for each configuration. * indicates p < 0.05 when compared to the chip with parallel grooves, # indicates p < 0.05 when compared to the chip with perpendicular grooves. (B) The E.R. of all nuclei in each configuration was binned into 3 groups: (i) 1.0 < E.R. < 1.5 represents nuclei that are rounded, (ii) 1.5 < E.R. < 2.5 represents moderately elongated nuclei and (iii) E.R. > 2.5 represents very elongated nuclei. (C and D) show the percentage of nuclei that align to within 20° of the groove direction. (C) shows the averaged nuclear angle alignment in the 3D cellular constructs. * indicates p < 0.05 when compared to the chip with parallel grooves, # indicates p < 0.05 when compared to the chip with perpendicular grooves. (D) shows the breakdown of the nuclear alignment by imaging planes at different depths of the 3D cellular construct. Three different planes were analyzed (top, middle and bottom) with each plane being about 8–12 μm higher than the previous plane. *indicates p < 0.05 when compared to the bottom plane.
the parallel configuration, in addition to alignment cues provided from the micro-grooves, the shear stress provided a synergistic cue to align the entire 3D cellular construct. However, in the perpendicular configuration, this shear stress could act antagonistically to the alignment cues from the micro-grooves, causing cytoplasm and nuclear spreading, thereby reducing both the nuclear E.R. and the percentage of cells aligned along the micro-groove orientation. Another possible explanation is the geometry of the microfluidic channel. Aubin et al. showed that even in the absence of flow, when cells with an innate tendency to align (such as C2C12 and fibroblasts) are encapsulated in a gel with high aspect ratio (50 μm wide × 150 μm long), the cells align along the direction of the long axis. This alignment reduces as the width of the channel increases, thereby decreasing the aspect ratio of the gel strip. Similarly, when the C2C12 cells were trapped within the 200 μm × 1 cm 3D microfluidic compartment, they may experience a similar geometrical aspect ratio and tend to align along the long axis. The presence of the perpendicular grooves therefore has to compete against the flow direction and the shape of the cell compartment in the chip in aligning the cells.

Enhancement of differentiation in aligned 3D myoblast cellular constructs

3D culture has been shown to better mimic the in vivo environment and hence represents a more physiological model for in vitro studies. To determine the importance of alignment to a 3D myoblast cellular construct in the microfluidic chip, we further investigated the cells' ability to differentiate into skeletal muscle. C2C12 cells were seeded into 2 configurations (i.e., flat substrate and parallel grooves) of the microfluidic chip and cultured in proliferation medium for 3 days to allow cells to remodel into 3D myoblast cellular constructs. Differentiation into skeletal muscle was initiated by perfusing with differentiation medium for 3 days.

We first used immunocytochemistry to analyze myotube formation in the 3D cellular construct. Myotube formation is a good indicator of skeletal myocyte's maturation and is enhanced by cessation of proliferation and end-to-end contacts between myoblast. Immunostaining results showed that both the cells on the grooved and flat substrates had started fusing (Fig. 5A–D) with the alignment facilitating a better end-to-end contact of the myoblasts (Fig. 5C and D).

We also examined the expression levels of 4 skeletal muscle genes, which are key components of the sarcomere in the skeletal muscle and are used as indicators of myoblast maturation into skeletal muscle. Gene expression results indicated that the 3D myoblast cellular construct cultured on the micro-grooved substrate had a significantly higher expression of the skeletal genes, especially the myosin heavy chains 1, 2 and 4 (Fig. 6). These genes are important for the skeletal muscle development and form part of the contractile apparatus. This result is in agreement with a large number of published literatures that show that alignment enhances myotube formation and maturation of C2C12 cells in both 2D and 3D cultures.

C2C12 cells have been used widely as a model to study the development of skeletal muscle and cell alignment since they possess intrinsic potential to align both in vivo and in vitro. They are also valuable as cell models to study effects of drugs on skeletal muscle contraction, insulin induced glucose metabolism of muscle cells, and muscle regeneration. Engineering 3D cellular constructs of skeletal muscle can

![Fig. 5 Morphology of nascent myotubes after differentiation of C2C12 3D myoblast cellular constructs. C2C12 cells in the microfluidic chips were first cultured for 3 days in proliferation medium to form the 3D myoblast cellular construct, before switching to differentiation medium for 3 days. (A,C) show the 3D orthogonal view of F-actin and DAPI staining of C2C12 cells in the chips after 6 days of perfusion culture using two configurations of the micro-grooved substrate in the microfluidic chips. The configurations were: (A) chips with no grooves, and (C) chips with parallel grooves. (B,D) show a corresponding selection of individual z-stack images of the C2C12 nascent myotubes. They belong to (B) chips with no grooves and (D) chips with parallel grooves. Scale bars = 50 μm.](image-url)
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