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Functional differences between healthy and diabetic endothelial cells on topographical cues

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Abstract

The endothelial lining of blood vessels is severely affected in type II diabetes. Yet, there is still a paucity on the use of diabetic endothelial cells for study and assessment of implantable devices targeting vascular disease. This critically impairs our ability to determine appropriate topographical cues to be included in implantable devices that can be used to maintain or improve endothelial cell function *in vivo*. Here, the functional responses of healthy and diabetic human coronary arterial endothelial cells were studied and observed to differ depending on topography. Gratings (2µm) maintained normal endothelial functions such as adhesiveness, angiogenic capacity and cell-cell junction formation, and reduced immunogenicity of healthy cells. However, a significant and consistent effect was not observed in diabetic cells. Instead, diabetic endothelial cells cultured on the perpendicularly aligned multi-scale hierarchical gratings (250nm gratings on 2µm gratings) drastically reduced the uptake of oxidized low-density lipoprotein, decreased immune activation, and accelerated cell migration. Concave microlens (1.8µm diameter) topography were additionally observed to overwhelmingly deteriorate diabetic endothelial cell function. The results of this study support a new paradigm and approach in the design and testing of implantable devices and biomedical interventions for diabetic patients.

Graphical Abstract

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Author Contributions Statement

MFAC and EKFY designed experiments. MFAC, BMXC, DJHN and MR carried out experiments. MFAC, BMXC and EKFY performed data analysis. MFAC, BMXC and EKFY wrote the manuscript. MFAC, MR and EKFY edited the manuscript. All authors have read and agreed to the final version of this manuscript.

Introduction

Type II diabetes is a worldwide epidemic that is characterized by the lack of response to insulin and the consequent disability in storing glucose [1,2]. Type II diabetes (henceforth referred to as simply diabetes) is a complex metabolic disease that systemically affects organ systems, including the cardiovascular system. Prolonged availability of excess glucose leads to the production of excessive amounts of reactive oxygen species, causing oxidative stress in the endothelial cells (ECs) through activation of damaging metabolic and biochemical pathways including the protein kinase C and the polyol pathway [3,4]. The endothelial defense system is also aberrantly activated in response to a hyperglycemic environment, leading to increased attachment of inflammatory cells on the vascular wall. Further exposure to the peculiar metabolic environment in type II diabetes, which includes hypertension, hyperlipidemia, and hypercoagulability, leads to EC senescence, death and detachment without a normal mechanism for repair [5]. Such a dysfunctional endothelium is the critical first step in the development of atherosclerosis and cardiovascular disease [6]. Due to the diabetic milieu, structural and functional damage to the vascular system is accelerated, leading to a two- to four-fold increase in the risk of cardiovascular disease, such as peripheral arterial disease and coronary heart disease [7]. Thus, cardiovascular disease is the main cause of morbidity, mortality and economic burden for patients with diabetes [8,9].

Exploration of strategies for better treatment of cardiovascular disease remains a relevant field of study today. For instance, biomaterials that can stimulate tissue regeneration in situ has been growing to address the need for implantable devices, such as vascular grafts and stents, that can have better functionality than the current state of the art materials. Research is now geared towards the use of synthetic substrates with nano- to micron-sized topographies that redirect the various cell behaviors through mechanotransduction. Mimicking the normal endothelial milieu, comprised of fibers, pits and bumps [10], to direct EC behavior is being investigated to improve vascular graft endothelialization, without which thrombosis and intimal hyperplasia occurs with few restraint. The problem of graft restenosis and occlusion is even more urgent in diabetic patients, who have an accelerated rate of coronary artery bypass occlusion [11], leading to more adverse long-term outcomes [12,13]. Animal studies have shown that the introduction of micron-sized gratings topographies into the luminal surfaces of stents [14–17], and gratings and lens structures on vascular grafts [18–21], have shown potential over conventional treatments. Improvement in EC behaviours such as adhesion, proliferation [18,22,23], migration [22,24], gene expression [25], inflammatory capability [26], and secretion of vascular remodelling cytokines [27], induced by gratings topographies plausibly stimulate in situ

endothelialisation of implanted vascular devices. In addition, EC alignment induced by micron-sized gratings recapitulates the characteristic EC morphology under physiological, laminar shear blood flow. In vitro, laminar shear stress can decrease immunogenicity [28,29], increase EC retention [22], and improve atheroprotection [30].

The literature is replete with studies on the efficacy of topographical cues to influence EC behavior using normal endothelial cells. The inherent phenotypic, genotypic [31], and microenvironment differences between healthy and diabetic ECs precludes the application of this knowledge on diabetic patients. Therefore, it is necessary to precisely assess topography-induced functional responses of diabetic ECs and to compare those effects with healthy ECs. In this manner, biomimetic, topography-based strategies may be more accurately employed for personalized cardiovascular treatment of diabetic patients.

Materials and methods

Cell culture

Healthy human coronary artery ECs (Healthy; Lonza) and type II diabetic human coronary artery ECs (Diabetic; Lonza) were used from passages 5 to 7 in this study. The characteristics of donors for both cell types are listed in Table 1. All cells were expanded in endothelial growth medium, supplemented with the 2MV bullet kit (EGM-2MV; Lonza) on standard tissue culture polystyrene plates. Cells were washed in HEPES buffered saline solution and trypsinized with 0.05% trypsin for harvest and seeding on topographies. Human monocytes U937 (ATCC) were cultured as suspension cells. Complete U937 media, made of RPMI media (Life Technologies), supplemented with 10% fetal bovine serum (HyClone), 1% penicillin streptomycin (GE Healthcare) and 1% 100mM Sodium Pyruvate (Sigma), was added to the monocyte cell suspension every 3 days. All cells were maintained in standard cell culture conditions of 37°C and 5% CO₂ until 90–95% confluency. Initial characterization of *in vitro* behavior of healthy and diabetic EC on Unpatterned PDMS were performed (Figure S1–S3).

Soft lithography

Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning), used at a ratio of 10:1 base agent:curing agent, was used throughout this study due to robust and reproducible nano- and micro-patterning attainable on this material. The mechanical similarity with native tissue, and hemocompatible and biostable properties of PDMS have made it ideal as an implant or surface coating for vascular applications [32–34]. Patterning of PDMS was performed through standard soft lithography methods on patterned polycarbonate molds. The PDMS was allowed to age at room temperature for at least 1 week at 60°C before use in experiments. Soft lithography of the multi-architectural chip (MARC) [35,36] was also performed to generate an array for screening topographies that elicit differential responses between the healthy and diabetic ECs (Figure S4). From the screening results, six patterns were selected for most of the functional response assays. The patterns are listed as follows: 2µm gratings (Gratings), hierarchical 2µm gratings with 250nm lines parallel to the micro-gratings (Parallel), hierarchical 2µm gratings with 250nm lines perpendicular to the micro-gratings (Perpendicular), 1.8µm convex microlens (Convex) and, 1.8µm concave microlens

(Concave). Dimensions of the topographies used are given in Table 2, and the scanning electron microscopy (SEM) or atomic force microscopy (AFM) characterization is given in Figure S5. An additional nano-pattern with 250nm gratings (250nm in ridge, gap-size and height) was also studied for the cell morphology and proliferation.

EC seeding

PDMS substrates were prepared for cell seeding by cutting them into circular pieces in order to fit into the wells of specific culture plates. The substrates were also washed in absolute ethanol. Immediately before cell seeding, all PDMS substrates were plasma treated at 85 watts and 8.5 L/min of oxygen gas (FEMTO Science Cute), and subsequently sterilized with UV light. PDMS substrates were coated with bovine collagen I ($2.5\mu\text{g}/\text{cm}^2$; Gibco) in 0.5N acetic acid for 1 hour at room temperature. PDMS substrates were then washed with phosphate buffer solution (PBS) three times before seeding with 10000 cells/ cm^2 , unless otherwise specified below.

Proliferation assay

After 4 days on topographies, the ECs were incubated with 5-ethynyl-2'-deoxyuridine for 16 hours (EdU; LifeTechnologies). All cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) and subsequently fixed with 4% paraformaldehyde. EC proliferation was reported as number of EdU-labelled cells normalized in comparison to total cell number. Proliferation assay was performed across 2 biological and 3 technical replicates.

Cell morphometry

ECs on various topographies were fixed with 4% paraformaldehyde and stained using Alexa Fluor-conjugated phalloidin after 4 days of culture. The images of the stained actin cytoskeleton were used to manually trace individual cell boundaries and measure cell area and cell alignment using Image J (1.49v, National Institutes of Health), as described previously [18]. Multiple cells measured were obtained across 2 biological and 2 technical replicates.

Gene expression quantification

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify changes in gene expression levels. RNA was extracted from ECs using the RNeasy mini kit (Qiagen), following the manufacturer's protocol, after 4 days of culture. cDNA conversion of total mRNA was performed (RevertAid First Strand cDNA synthesis kit, ThermoFisher) before gene expression quantification using the One-step Evagreen qRT-PCR Low Rox kit (ABM). The qPCR reactions were run in the ABI 7500 Fast Real Time PCR system according to manufacturer's protocol. Expression of various endothelial markers were measured and primers against targeted genes are listed in Table S1. Cycle threshold values were collected, normalized to 18s ribosomal RNA, and subsequently used to calculate fold change against healthy ECs on Unpatterned. One primary cell source for each EC type was measured in one biological experiment with three technical replicates (n=3).

Matrigel assay

After 4 days of culture, healthy and diabetic ECs were trypsinised and seeded onto growth factor-reduced Matrigel (BD Biosciences) at a seeding density of 15000 cells/cm². Matrigel was incubated at standard culture conditions (5% CO₂ at 37° C) for 24 hours before images were taken using an optical microscope (Leica DMIRB). Percentage of area covered by tubes, tube length, number of branching points, and number of loops formed in each sample was assessed using WimTube (ibidi GmbH) [36,37]. Matrigel assay was performed with 3 biological replicates and 6 technical replicates.

Permeability assay

Permeability assay was adapted from Rizwan et al [38]. After formation of a monolayer on PDMS, EC were trypsinized and seeded on transwell inserts (Greiner ThinCert, 2 µm pore size). Thereafter, the medium inside the top chamber was supplemented with FITC-dextran (40 kDa) at a final concentration of 100 µg/ml. The movement of FITC-dextran from the top chamber to the bottom chamber was monitored by sampling with replacement 100 µl of media from the bottom chamber at specific intervals. The fluorescence signal from each sample was measured using a microplate reader (Tecan; excitation: 485nm, emission 535 nm). The concentration of FITC-dextran was interpolated using a standard curve.

Oxidized low density lipid (oxLDL) uptake assay

The oxLDL uptake assay was adapted from Teupser et al. [39], and Stephan and Yuracheck [40]. Briefly, ECs were grown for 3 days on various patterned substrates. After 3 days of culture, ECs were serum deprived by replacing full media with endothelial basal media with 0.5% fetal bovine serum. After 12 hours, the cells were replaced with EGM-2MV supplemented with 5µg/ml oxLDL (Kalen Biomedical). ECs were incubated with oxLDL for 2, 4, and 6 hours. At the end of each incubation period, the ECs were lysed with 0.1N NaOH + 0.1% Triton X-100. Fluorescence of the cell lysate was measured at 546 nm against a standard curve of oxLDL with known concentrations. Total protein content of lysates was measured using a microBCA assay (ThermoFisher). OxLDL uptake was reported as oxLDL mass to total protein mass, as previously described [39]. Rate of oxLDL uptake was performed by fitting a hyperbolic curve onto oxLDL uptake across time [41]. OxLDL uptake was measured across 2 biological and 3 technical experiments.

VE-cadherin and ZO-1 protein measurement

ECs were seeded on selected topographies (Unpatterned, Gratings, Parallel and Perpendicular) until formation of a confluent monolayer. Cells were fixed with 4% paraformaldehyde and stained using anti-vascular endothelial-cadherin (VE-cadherin; Cell Signaling Technology, 1:200) and anti-zonula occludens-1 (ZO-1; BD Biosciences; 1:50) antibodies. Alexa Fluor conjugated secondary antibodies (LifeTechnologies; 1:500) were used to detect primary antibodies. Nuclei were stained using DAPI. Images of each fluorescent marker were captured using an epifluorescence microscope (Leica DMIRB). Integrated intensity of VE-cadherin and ZO-1 was calculated by subtracting total image integrated intensity with cytoplasmic integrated intensity and normalizing to image cell

number [18,42]. VE-cadherin and ZO-1 measurement was performed on 2 biological experiments across 3 technical replicates.

Monocyte adhesion assay

Monocyte adhesion assay was adapted from Huang et al. [23]. ECs were grown to confluency on selected topographies after seeding with 20000 cells/cm². EC monolayers were then activated with 2.5 ng/ml tumor necrosis factor-alpha in EGM-2MV media for 5 hours. Meanwhile, monocytes were labelled with CellTracker DiO dye (LifeTechnologies) according to manufacturer's protocol. After activation, EC monolayers were gently washed with 300 µl of serum-free monocyte media thrice. Labeled monocytes (150000 cells/cm²) were added to activate EC monolayers and incubated at standard culture conditions under rolling conditions (100 rpm) for 45 minutes. Afterwards, non-adherent monocytes were vigorously washed off with PBS and the substrates were fixed with 4% paraformaldehyde. The samples were then stained against fluorescently-conjugated PECAM1 (Miltyi Biosciences; 1:100) to mark EC boundaries and DAPI to mark the cell nuclei. Samples were imaged using an epifluorescence microscope (Leica DMIRB). The results were presented as number of monocytes attached per EC monolayer area. A total of 3 biological experiments and 3 technical replicates were obtained, with at least 20 images captured for each technical replicate.

Migration assay

Migration assay was adapted from Rizwan et al. [42]. Before EC seeding, equally sized strips of Unpatterned PDMS were temporarily stuck onto the center of topography substrates to create a barrier for EC growth. ECs were grown on selected topographies until the formation of a monolayer. Afterwards, the strip of PDMS was removed from each substrate to effectively create uniformly sized wounds aligned orthogonally to the micron-sized gratings axis with reduced distortion of the underlying topography. Media was immediately replaced with fresh EGM-2MV medium supplemented with 15.83 µg/ml of collagen I. Cells were immediately viewed using phase contrast live microscopy (Olympus EZ) at regular intervals. MetaMorph image analysis software (Molecular Devices) was used for individual cell tracking and analysis of cell speed and cell directionality. Percentage of wound healing was analyzed using ImageJ and was calculated as the ratio of the remaining wound area to the original wound area. Wound healing and individual cell tracking was measured across 2 biological and 3 technical replicates, from which multiple cells were obtained for individual cell tracking.

Statistical analysis

All data is presented as mean ± standard deviation. The sample size (n) reported is a combination of biological and technical replicates. Key comparisons were made between cell types (comparing healthy with diabetic ECs when cultured on the same pattern) and between topographies (comparing topography to Unpatterned within the same cell type). Two-way ANOVA with Tukey's multiple comparison post-hoc test (without adjustment for multiple correction) was used for all statistical analyses (GraphPad Prism v6.0). Differences were considered significant at p<0.05.

Results

Diabetic EC proliferation and morphometry are affected by Perpendicular and Concave topographies

In this study, ECs from coronary arteries of healthy and diabetic patients were used to study topography-induced EC responses (Table 1). Initial characterization revealed weaker expression of EC markers (Figures S1–S2) and lower stability of tubes formed (Figure S3) in diabetic EC compared with healthy EC. A strong association has been specifically established for coronary artery heart disease and type 2 diabetes [43,44]. Healthy and diabetic ECs were cultured on the multi-architectural chip, containing an array of topographies with various geometries and dimensions, to investigate the VE-cadherin expression (Figure S4). Topographies that were observed to significantly increase VE-cadherin expression of diabetic ECs were used for further analysis in this study (Table 2, Figure S5). A preliminary analysis of protein expression by healthy and diabetic EC markers on different topographies was performed through Western blot (Figure S6) and flow cytometry (Figure S7). Flow cytometry and western blot data show some differences in expression of various EC markers between healthy and diabetic ECs.

To assess the most basic changes in ECs after exposure to various topographies, both cell proliferation and morphometry were measured (Figure 1). EC proliferation at sites near injury have been identified as an important mechanism to repair the vessel wall, while increase in EC area correlated with enhanced cell attachment through integrin engagement [45]. Culture of healthy ECs on anisotropic topographies (Gratings, Parallel, Perpendicular) after 4 days showed significant decrease in EC area when compared to those grown on Unpatterned (Figure 1A–1B). Comparing both healthy and diabetic ECs on Unpatterned, no significant differences in cell area were observed. On the other hand, diabetic EC area increased on Gratings and decreased on Perpendicular and Concave in comparison to Unpatterned. The Gratings and the Concave structures significantly changed the area of healthy and diabetic ECs: Gratings increased the cell area of diabetic cells, whereas Concave decreased it in comparison to the healthy ECs.

In contrast to cell area, healthy ECs showed statistically equivalent cell proliferation rates across all topographies (Figure 1C). It is notable that the two different cell types only showed significant difference in cell proliferation when grown on Parallel, on which diabetic ECs showed slower proliferation. Meanwhile, cell proliferation rates of diabetic ECs on Perpendicular and Concave topography were significantly reduced when compared to the Unpatterned control. Convex topography significantly increased diabetic EC proliferation compared to Unpatterned.

Healthy and diabetic EC alignment along the micron-sized gratings axis was measured for the anisotropic topographies (Figure 1D–1G). The alignment of ECs along the micron-sized gratings axis was previously shown to mimic the endothelial arrangement in linear blood vessels [15,18,46]. While the alignment of healthy and diabetic ECs were mostly similar on the Gratings and Parallel topographies (Figure 1D–1E), larger differences in frequency and angle against the micron-sized gratings axis were observed between the two cell types on Perpendicular and 250 nm Gratings topographies (Figure 1F–1G)

Cell area is used to indicate cell adhesion, which highly determines cell viability and proliferation. Here, we observed that the proliferative capacity of healthy ECs were unchanged, while the cell area was decreased on anisotropic topographies. The diabetic EC characteristics of area and proliferation were consistently decreased after culture on Perpendicular and Concave topographies.

Angiogenic capability of diabetic ECs is severely impaired on Concave topography

Angiogenic capability of both healthy and diabetic ECs were assessed (Figure 2). Angiogenesis, or the process of forming new blood vessels, is important for collateral vessel formation and revascularization. An *in vitro* Matrigel assay was used to assess the capability of the endothelial cells to form tubes after culture on selected topographies (Figure 2A). Qualitative analyses show that healthy ECs grown on all topographies showed tube formation. Notably, healthy ECs grown on the Gratings, Parallel, and Perpendicular topographies for 4 days induced better network formation on healthy ECs compared with Unpatterned from visual inspection. Diabetic ECs show non-uniform and poor formation of endothelial tubes after growth on Unpatterned surfaces; this was improved when diabetic ECs were grown on Gratings and Parallel topographies. Quantitative assessment of tube formation at day 4 reiterates these observations (Figure 2B–2F). Network area remained similar regardless of cell type or topography type (Figure 2B). Total tube length (Figure 2C) and total branching points (Figure 2D) improved after healthy ECs were cultured on all Parallel and Perpendicular (hierarchical structures), and Convex and Concave (microlens structures) compared to healthy ECs on Unpatterned. Measurement of total loops reiterated that Perpendicular and Convex improved diabetic EC tube formation in Matrigel (Figure 2E). Diabetic ECs notably showed consistent and significant reduction tube formation metrics after culture on Concave compared with Unpatterned (Figure 2B–2E). In addition, diabetic ECs on Concave were also found to create worse networks than that of healthy ECs on the same topography (Figure 2B–2E). Prolonged 9-day culture of diabetic ECs on topographies retained the improved network formation on Gratings and Parallel topographies compared to the Unpatterned control (Figure S8).

Expression of *FLT1* and *KDR1*, which encode for vascular endothelial growth factor receptors (VEGFR) 1 and 2 respectively, were measured (Figure 2G–2H). All gene expression values were presented relative to the gene expression of healthy ECs cultured on Unpatterned. *FLT1* was significantly downregulated in all healthy ECs cultured on topographies compared with Unpatterned (Figure 2G), while only Parallel and Perpendicular downregulated *KDR1* expression on healthy ECs (Figure 2H). Both genes were significantly downregulated on diabetic ECs compared with healthy ECs on Unpatterned, indicative of the dysfunctionality of the diabetic ECs. Aside from the significant decrease in *FLT1* expression in diabetic ECs on Concave compared with Unpatterned, there was generally a lack of changes in *FLT1* and *KDR1* expression in diabetic ECs in response to topography. *FLT1* and *KDR1* expression were consistently different between healthy and diabetic ECs on Convex and Concave.

Both quantitative and qualitative measures showed an improvement in healthy EC angiogenic capacity after culture on Parallel, Perpendicular, Convex and Concave. On the

other hand, diabetic ECs on Concave consistently decreased tube formation capacity and angiogenic marker expression compared with Unpatterned.

Oxidized low density lipoprotein (OxLDL) uptake and metabolism is unchanged in healthy ECs and is reduced in diabetic ECs when using hierarchical topographies

OxLDL is a key component in atherosclerotic plaque formation and receptors that are used for its uptake are predominantly presented in dysfunctional ECs [47]. Thus, oxLDL metabolism of healthy and diabetic ECs after growth on topography was interrogated over time (Figure 3). No drastic changes in oxLDL uptake were observed in healthy ECs in response to topography (Figure 3A–3F). Only the extrapolated oxLDL uptake rate of healthy ECs on Perpendicular showed significant reduction compared with healthy ECs on Unpatterned (Figure 3H). OxLDL uptake by diabetic ECs was markedly decreased in Parallel (Figure 3C) and Perpendicular (Figure 3D) at 6 hours when compared to Unpatterned, but cumulative uptake of oxLDL was markedly higher on diabetic ECs cultured on Convex compared to Unpatterned (Figure 3G). The oxLDL uptake rate in diabetic ECs was significantly decreased on Gratings compared with Unpatterned (Figure 3H). OxLDL uptake rate was additionally reduced on diabetic ECs compared with healthy ECs on Gratings and Convex (Figure 3H).

Changes in gene expression of receptors for specific uptake of oxLDL were also measured in response to topography. *CD36* (Figure 3I), encoding for fatty acid translocase, remained unchanged in healthy ECs on different topographies compared with Unpatterned. *CD36* was significantly upregulated in diabetic ECs compared with healthy ECs on Unpatterned, and further downregulated in diabetic ECs grown on Parallel, Perpendicular, Convex and Concave topographies compared to Unpatterned. Concave exhibited significant difference in *CD36* expression between healthy and diabetic ECs. *OLR1* (Figure 3J), which encodes for oxidized low-density lipoprotein receptor-1, showed significant increase in expression in healthy ECs across all topographies compared with Unpatterned. *SRECI*, encoding for scavenger receptor expressed by endothelial cells 1, only showed increased expression in healthy ECs on Concave compared with Unpatterned, and on diabetic ECs compared with healthy ECs on Unpatterned (Figure 3K). Meanwhile, *SRECI* expression in diabetic ECs on Parallel and Concave were significantly decreased compared to Unpatterned.

The expression of the gene for endothelial lipase (*LIPG*, Figure 3I), which is involved in lipoprotein metabolism, was significantly downregulated in all healthy ECs cultured on topography compared with Unpatterned. *LIPG* was also significantly downregulated in diabetic ECs compared with healthy ECs on Unpatterned. Only diabetic ECs grown on Concave showed a significant decrease in *LIPG* expression compared with Unpatterned.

Dysfunctionality in lipid metabolism is a hallmark in diabetic ECs. Generally, there was a lack of significant changes in oxLDL uptake and lipid metabolism in healthy ECs in response to topography. In contrast, diabetic EC oxLDL uptake was reduced after culture on Parallel, Perpendicular and Concave topographies.

Monolayer marker expression in healthy ECs is decreased in response to topography but remains poor in diabetic ECs

Pertinent endothelial function relies heavily on the capability of ECs to form an intact monolayer. Here, it was observed that healthy and diabetic human coronary arterial ECs were unable to form a monolayer on isotropic microlens topographies (Figure S9). Therefore, only Unpatterned, Gratings, Parallel and Perpendicular were used for further examination.

EC monolayer formation and integrity depends on the extent of formation of tight junctions, which is implicated by cell-cell junction proteins such as VE-cadherin and ZO1.

Representative images of VE-cadherin and ZO1 on healthy and diabetic EC monolayers after culture on different topographies qualitatively show differences in monolayer marker expression (Figure 4A–4B). Semi-quantitative analysis of immunofluorescence images of VE-cadherin and ZO1 at the cell boundary were also performed. Significant decrease in membrane localization of VE-cadherin occurred in healthy ECs cultured on topographies compared with Unpatterned (Figure 4C). Healthy ECs exhibited drastically higher expression of VE-cadherin at the membrane compared with diabetic ECs on Unpatterned. Meanwhile, diabetic ECs on different topographies showed no significant changes in VE-cadherin expression in response to topography. In contrast, an increase in ZO1 membrane localization only occurred in healthy ECs on Perpendicular (Figure 4D). Similarly, ZO1 membrane intensity remained unchanged in diabetic ECs in response to topography. Comparing the ZO1 level on each topography between healthy and diabetic ECs, a significantly lower ZO1 expression was observed for diabetic ECs on Perpendicular.

Expression of genes *CDH5* (Figure 4E), encoding for VE-cadherin, and *TJP1* (Figure 4F), encoding for ZO1, was also downregulated in healthy ECs on Parallel and Perpendicular compared to Unpatterned. Both cell-cell junction genes were significantly reduced in diabetic ECs compared with healthy ECs on Unpatterned. Notably, expression of both genes was significantly downregulated on diabetic ECs on compared with healthy ECs on Gratings.

Corneal EC [48], mesenchymal stem cells [36,49] and human embryonic stem cells [50] have been shown to retain information and cellular responses from previous exposure to topographical cues. Thus, EC monolayers that had been exposed to topographies were recultivated into transwells to test functional integrity and permeability (Figure 4G–I). Healthy and diabetic EC from Unpatterned showed similar rates of FITC-dextran movement (Figure G). Healthy EC on Parallel and Perpendicular retained similar FITC-dextran permeabilities compared with Unpatterned (Figure 4H–4I). Diabetic EC from Parallel and Perpendicular topographies compared with diabetic EC on Unpatterned significantly increased FITC-dextran permeability. Moreover, diabetic EC from Perpendicular exhibited an increased FITC-dextran permeability compared with healthy EC on the same topography.

To summarize, the VE-cadherin cell-cell adhesion on EC monolayer was negatively affected in healthy ECs by Parallel and Perpendicular topographies. On the other hand, the poor capability for cell-cell junction assembly of diabetic ECs on Unpatterned remained unimproved after culture of these cells on various topographies.

Hierarchical and Gratings topographies influence immunogenicity and adhesiveness of both healthy and diabetic ECs

Low level inflammation of ECs is an important event in endothelial dysfunction and consequent plaque formation. Monocyte adhesion to the EC monolayer and consequent intravasation is highly involved in the generation of lipid-laden macrophages (foam cells) that are an important step in the generation of atherosclerotic plaque [51]. Hence, a monocyte adhesion assay on healthy and diabetic EC monolayers was carried out (Figure 5A). Healthy ECs exhibited an increase in monocyte adhesion on Gratings compared with Unpatterned. Similarly, diabetic ECs on Gratings and Perpendicular showed higher monocyte adhesion compared with Unpatterned. When comparing healthy and diabetic ECs on the same topographies, no statistically significant differences in monocyte adhesion were observed (Figure 5B).

Several genes that contribute to leukocyte-endothelium interaction and immunogenicity were examined to explain the changes in monocyte adhesiveness of healthy and diabetic ECs in response to topography. Soluble factors such as nitric oxide are involved in the leukocyte adhesion process. Nitric oxide, produced by nitric oxide synthase, is a pleiotropic factor significantly involved in numerous EC functions and also functions as a potent anti-adhesion molecule [52]. The expression of *NOS3* in healthy ECs was downregulated on all topographies compared to healthy EC on Unpatterned, (Figure 5C). The production of nitric oxide in diabetic ECs is known to be severely impaired [53,54], and this was strongly indicated in the drastic downregulation of *NOS3* (nitric oxide synthase) on diabetic ECs compared with healthy ECs on Unpatterned. In diabetic ECs, *NOS3* expression was upregulated on all topographies compared to diabetic ECs on Unpatterned. Gratings also exhibited consistent and significant upregulation of *NOS3* in diabetic ECs compared with healthy ECs (Figure 5C). The opposing effect of Gratings was clear in *NOS3* expression, which decreased in healthy ECs and increased in diabetic ECs, when compared to the corresponding Unpatterned control.

Gene expression of EC surface receptors involved in the EC immune activation and leukocyte adhesion cascade were also examined. Both *VCAM1* (vascular cell adhesion molecule 1, Figure 5D) and *ICAM1* (intercellular cell adhesion molecule 1, Figure 5E) were significantly reduced in healthy ECs after culture on hierarchical topographies when compared to Unpatterned. However, no changes in diabetic EC expression of *VCAM1* and *ICAM1* compared with Unpatterned were observable. Yet both *VCAM1* and *ICAM1* were significantly reduced in diabetic ECs compared with healthy ECs on all topographies. *PECAM1* (platelet and endothelial cell adhesion molecule 1) expression in healthy ECs was not altered by topographies compared with Unpatterned (Figure 5F). *PECAM1* expression on diabetic ECs compared with healthy ECs was significantly downregulated on Unpatterned. Additionally, *PECAM1* expression in diabetic ECs cultured on Parallel decreased significantly compared with Unpatterned. *SELE* gene that encodes for endothelial selectin showed significant downregulation in diabetic ECs compared with healthy ECs on Unpatterned, similar to *NOS3*, *VCAM1* and *ICAM1* (Figure 5G). No significant change in *SELE* expression was observed in healthy ECs in response to topography, but diabetic ECs showed a significant upregulation in *SELE* expression after culture on Parallel and

Perpendicular compared to Unpatterned. Similar to *VCAMI* and *ICAMI*, expression of *SELE* was significantly downregulated in diabetic ECs compared with healthy ECs on all topographies. *VWF* gene expression was significantly reduced in healthy ECs on all topographies compared to Unpatterned, while diabetic ECs were only changed on Parallel compared to Unpatterned (Figure 5H). Significant upregulation in *VWF* expression was observed in diabetic ECs compared with healthy ECs on Gratings and Perpendicular. The low expression of immunogenic genes in diabetic ECs was unexpected but could be explained by the prolonged *in vitro* culture of diabetic ECs. A previous study has observed the increasing equivalence in *VCAMI*, *ICAMI* and *SELE* expression between nondiabetic and diabetic saphenous ECs with prolonged growth on tissue culture plastic [55]. Possibly, prolonged EC culture on topographies have accelerated the decline of immunogenic marker expression, leading to significantly lower levels of gene expression in diabetic ECs compared with healthy ECs.

In general, Gratings, Parallel and Perpendicular topographies decreased expression in *NOS3* and immunogenicity genes in healthy ECs in response to topography. Diabetic ECs showed less changes in immunogenic gene expression in response to topography, despite showing increased monocyte adhesion on Gratings and Perpendicular. Nevertheless, the positive outcome of *NOS3* upregulation in diabetic ECs after culture on topographies may imply a restoration of normal EC functionality.

Perpendicular topography improves migration of both healthy and diabetic ECs

A wound healing study was performed to assess the differences in healthy and diabetic EC migration when cultured on different topographies (Figure 6A–6D). Wound healing and EC migration are critical in the repair of the injured endothelium and necessary for re-endothelialization of implanted vascular devices. While healthy ECs showed a significant improvement in the rate of wound healing after culture on Gratings compared with Unpatterned (Figure 6B). On the other hand, healthy ECs grown on Parallel performed worse in wound healing than healthy ECs on Unpatterned (Figure 6C). There was no significant change in wound healing of diabetic ECs in response to topography. Cell migration at the wound leading edge was also measured (Figure 6E–6H). Healthy EC velocity was increased on Gratings and Perpendicular topographies compared with Unpatterned (Figure 6E), but accumulated distance (Figure 6F) and Euclidean distance (Figure 6G) was only improved on healthy ECs cultured on Perpendicular compared with Unpatterned. Similarly, Perpendicular improved diabetic EC velocity compared with Unpatterned (Figure 6E). Accumulated distance (Figure 6F) and Euclidean distance (Figure 6G) were both increased in diabetic ECs on Parallel and Perpendicular compared with Unpatterned. Directness of diabetic EC migration was improved on Gratings, Parallel and Perpendicular compared with Unpatterned (Figure 6H). Significant difference between healthy and diabetic EC velocity (Figure 6E), accumulated distance (Figure 6F) and Euclidean distance (Figure 6G) were observed on Unpatterned. The significantly lower velocity of diabetic ECs compared with healthy ECs on Gratings also matches the observations in accumulated distance (Figure 6F) and directness (Figure 6H). Meanwhile, the difference between healthy and diabetic EC velocity on Perpendicular (Figure 6C) was mirrored in accumulated distance (Figure 6F). The consistent and significant increase in

healthy and diabetic EC migration on Perpendicular have enormous impact on improving the repopulation of implanted vascular devices with a new endothelial population.

Discussion

It is imperative that the efficacy of topographies for potential biomedical applications be assessed using a relevant model that can fully recapitulate *in vivo* physiology. The prevalence of type II diabetes as a systemic metabolic disease needs to be accounted for when interrogating the benefit of topographical cues for altering EC behavior. Here, we have systematically compared several functions of healthy and diabetic ECs on various isotropic and anisotropic topographies. The results support the hypothesis that healthy and diabetic ECs respond to similar topographies in different ways (Table 3). In some instances, it was observed that topography did not affect the EC function in question. Moreover, the results have further implications on the use of topographical cues for specific biomedical applications.

Endothelial cell morphometry, proliferation and tube formation assay are well-established, standard assays in most laboratories that can quickly provide quantitative and robust output. For this purpose, cell proliferation, cell area and angiogenic capacity are cell responses that are most often used to assess EC functionality. While healthy EC proliferation was unaffected by all topographies, hierarchical topographies were observed to significantly reduce cell area and tube formation capacity of healthy ECs. The minimal changes in healthy EC proliferation, area, and angiogenic capacity after exposure to topography is unsurprising as resistance to change or improvement may be a mechanism to maintain homeostasis. Consistent and significant reduction in initial EC markers was observed in diabetic ECs on Concave topography. On Concave microlens, diabetic EC responses overwhelmingly deteriorated compared with Unpatterned and even prevented the formation of a confluent monolayer. In addition, Concave was demonstrated to induce drastic differences in cell area and angiogenic capability between the two cell types. The results indicate a negative effect on diabetic EC functionality related to adhesion on the Concave topographies. Nevertheless, our results strongly preclude the use of the tested Concave microlens for vascular applications, both *in vitro* and *in vivo*. Further study on microlens topographies of other dimensions should be performed, especially in light of ultrastructural data showing that the basement membrane of the aorta in rhesus macaque are defined by pore-like structures around 60 nm in diameter [56].

The implications of the results in this study are mostly relevant for device-based vascular surgical interventions. The vast differences between diabetic and nondiabetic ECs are also manifested in significantly variable rates of healing and surgical success (e.g. patency rates) between the two [54]. For instance, patency rates and survival rates after stenting or vascular bypass are significantly lower in diabetic patients compared with nondiabetic patients [57,58]. In addition, studies of glucose management have shown that the positive effects of restoring normoglycemia in diabetic patients on the microvasculature (e.g. of the eyes and the kidneys) does not extend to the macrovasculature (e.g. the coronary, femoral and saphenous arteries) [59,60].

Endothelialization of stents and grafts is key to improving success rates of vascular surgical interventions. By mimicking the natural tendency of ECs to be aligned along the direction of shear stress, it is postulated that transanastomotic migration, proliferation, and capture of ECs from the adjoining native artery or the circulation will improve. Similar to proliferation, adhesion, and angiogenic capacity, minimal effects of Gratings on critical monolayer responses in healthy ECs such as cell-cell junction formation and monocyte adhesion were observed. Previous studies have already shown positive influence of 400 nm to 4 μm [61], 2 μm to 5 μm [46] or 100 μm gratings [26] topographies on healthy EC migration, alignment and cell-cell marker expression, yet have no additional reports on its effects in other EC functions pertinent to endothelialization. Indeed, this indicates a need for rigorous testing of cell functions in response to topography. To our knowledge this is the first study that has extensively measured various aspects of behavior in response to topography of primary human ECs, regardless of patient source. Only Lehle et al. has studied proliferation and production of immunogenic markers between non-diabetic and type II diabetic human primary saphenous vein endothelial cells cultured on regular tissue culture polystyrene [55].

Importantly, the same minimal changes were induced by Gratings on diabetic EC behavior, thereby challenging the paradigm of incorporating simple gratings structures into luminal surfaces of vascular grafts and stents. Indeed, the use of multi-scale hierarchical structures is argued to more naturally mimic the cell niche compared with traditional micro- and nano-topographical substrates [62–64]. One notable finding of this study is the significant and consistent increase in both healthy and diabetic EC migration on Perpendicular hierarchical topographies. Migration of ECs across the anastomosis (connection between native vessel and synthetic vascular graft or stent) is a crucial step in biomaterial endothelialization. The effect of increased migration rates and directionality can have a significant impact on vascular device endothelialisation, which was shown to reach only 1–2 mm beyond the anastomosis site after 2 weeks in a rabbit model [65,66]. In contrast, spontaneous endothelialisation of implanted vascular devices is a rare occurrence in humans and transanastomotic migration is severely limited to the perianastomotic regions even after prolonged implantation [67,68]. In addition to what we have demonstrated, it would be interesting to study if Perpendicular topography preferentially induces increased migration of ECs over smooth muscle cells, as others have observed [15,69].

The endothelium formed on the surface of stents and grafts also need to be able to carry out its function in maintaining selective permeability, low lipid uptake, and preventing thrombosis, inflammation, and cellular adhesion. Again, the reduction in oxLDL uptake and improvement in *NOS3* gene expression in diabetic ECs on Perpendicular are indeed important findings that may reduce the likelihood of stent or graft restenosis due to the loss of control in vessel tone [70,71] and deterioration in wound healing in diabetic patients [53,72]. It is possible that for dysfunctional diabetic ECs, the mechanical stimuli provided by Perpendicular topographies may aid to mimic the natural cell niche that shows two orthogonal directional axes: the direction of laminar flow and the direction of circumferential blood vessel stretch. This may be particularly important to restore the *in vivo* state since diabetic ECs will unlikely receive the appropriate mechanical stimulation from the hypertensive microenvironment. Numerous studies have demonstrated the importance of both directional mechanical cues in maintaining endothelial homeostasis [73,74].

Meanwhile, healthy ECs exposed to normotensive environment may interpret Perpendicular topography as an abnormal mechanical state, as evidenced by decreased angiogenesis, monolayer formation and immunogenicity of healthy ECs on Perpendicular topography.

Diabetic ECs were generally less responsive than healthy ECs across the cell functions interrogated in this study, as shown by the comparison of responses between healthy and diabetic EC on the same topographies (Table 3). Observed differences in topographical response were notably in the absence of hyperglycemic conditions, which until now have been the prevailing mechanism of mimicking diabetic conditions [55,75]. It may be speculated that mechanical changes to the diabetic ECs, through changes in membrane stiffness, decrease in capacity for cell contractility, or even impairment of the integrin-cytoskeleton-nucleus mechanotransduction axis, has caused these cells to be less responsive to certain types of topographies. In fact, EC membrane stiffness has been reported to increase in response to artificially induced hyperglycemic environment *in vitro* [76]. Additionally, abnormal activation of the RhoA/ROCK pathway has been shown to be present in dysfunctional endothelium, leading to increased vasoconstriction and decreased vasodilation due to an imbalance of EC-derived effectors [77]. The effect of a broken RhoA/ROCK axis, a critical pathway in mechanotransduction, may also cause the impaired response to topography and indeed merits further study. It is also likely metabolic memory of the diabetic ECs, likely involving epigenetic changes, may contribute to some dysfunctionality in lack of diabetic EC responses to topography [78].

While a number of EC functional assays have been interrogated here, it is not an exhaustive list that definitively assesses EC function. Further study on other EC functions, such as selective permeability using porous and patterned PDMS membranes and anti-thrombogenicity, can be performed. Additional testing of a wider topography library may be useful in screening for topographies that can be used to holistically improve diabetic EC function. Yet, at the least, this study certainly reiterates the fundamental differences in non-diabetic and diabetic ECs, and is the first to demonstrate their variable sensitivities and responses towards topographical cues. This is best exemplified by the opposite functional responses of either cell type on Concave, Gratings and Perpendicular topographies. Indeed, our findings provide more evidence for the management of comorbidities of diabetic patients differently from non-diabetic patients and the quasi-personalization of treatment based on biomedical devices with topographical cues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Effect of endothelial cell dysfunction in type 2 diabetes needs studying
- Diabetic endothelial cells are less responsive to substrate topography
- Hierarchical 250nm gratings on 2µm gratings improve diabetic endothelial functions
- Concave microlens deteriorates diabetic endothelial cell functions
- New implant design needed for cardiovascular devices used in diabetics

Figure 1.

Morphometry of healthy and diabetic endothelial cells (ECs) on different topographies. (a) Representative images of healthy and diabetic ECs cultured on various topographies for 4 days. Single EC were stained against the actin cytoskeleton (red) and the nucleus (blue). White arrows denote the direction of the micron-sized gratings axis. (b) Average area of healthy and diabetic single EC on different topographies. (c) Proliferation rate of healthy and diabetic cells on different topographies (n=6). Alignment of healthy and diabetic cells on (d) Gratings, (e) 250nm Gratings, (f) Parallel and (g) Perpendicular topographies. Cell alignment was measured against the micron-sized gratings axis. Total number of ECs measured for area and alignment: Unpatterned (Healthy: n=37; Diabetic: n=42), Gratings (Healthy: n=50; Diabetic: n=32), Parallel (Healthy: n=64; Diabetic: n=44), Perpendicular (Healthy: n=37; Diabetic: n=51), Convex (Healthy: n=44; Diabetic: n=44), Concave (Healthy: n=44; Diabetic: n=46). Black lines indicate significant differences between healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant differences between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate the significant differences between healthy and diabetic ECs when cultured on the same topography. Two-way ANOVA with level of significance denoted by * (P<0.05), ** (P<0.01), *** (P<0.001), or **** (P<0.0001).

Figure 2.

In vitro angiogenic capability of healthy and diabetic ECs after culture on different topographies. (a) Representative images of tube formation of healthy and diabetic ECs after culture on different topographies. Extent of angiogenic function, measured by (b) percentage of area covered by tubes, (c) total tube length in pixels, (d) total branching points, (e) total loops formed, and (f) total nets (n=18). Tube formation assays were performed on all 4 EC donor types. Relative gene expression of (g) FLT1 (vascular endothelial growth factor receptor [VEGFR] 1) and (h) KDR1 (VEGFR2) genes in healthy and diabetic ECs (n=3).

Black lines indicate significant differences between healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant differences between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate the significant differences between healthy and diabetic ECs when cultured on the same topography. Two-way ANOVA with level of significance denoted by * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), or **** ($P < 0.0001$).

Figure 3.

Uptake of oxidized low density lipoprotein (oxLDL) by healthy and diabetic ECs cultured on different topographies. Measurement of oxLDL uptake by healthy (n=6) and diabetic (n=7) ECs cultured on (a) Unpatterned, (b) Gratings, (c) Parallel, (d) Perpendicular, (e) Convex, and (f) Concave topographies across time (n=6). Gray asterisks indicate the significantly different oxLDL uptake of diabetic ECs on the indicated topography against Unpatterned. OxLDL uptake was normalized to total protein content. Cumulative oxLDL uptake after 6 hours is also presented in (g). The oxLDL uptake rate is presented in (h) and

was calculated by fitting a hyperbolic curve into oxLDL uptake across time. Measurements of oxLDL uptake were made on two types of healthy and diabetic ECs. Relative gene expression of lipid receptors (i) CD36 (fatty acid translocase), (j) SREC1 (scavenger receptor expressed by endothelial cells 1) and (k) OLR (like oxidized low-density lipoprotein (LDL) receptor-1), and (l) LIPG (endothelial lipase) in healthy and diabetic ECs (n=3). Black lines indicate the significant differences between healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant differences between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate the significant differences between healthy and diabetic ECs when cultured on the same topography. Two-way ANOVA with level of significance denoted by * (P<0.05), ** (P<0.01), *** (P<0.001), or **** (P<0.0001).

Figure 4.

Monolayer formation of healthy and diabetic ECs cultured on different topographies. Representative images of (a) vascular endothelial cadherin (VE-cadherin, red) and (b) zona occludens 1 (ZO1, green) expressed in healthy and diabetic ECs after culture on different topographies. All cells were stained against the nuclei (blue). Semi-quantitative measurement of integrated fluorescence intensity of (c) VE-cadherin and (d) ZO1 at the cell boundary (n=6). Relative gene expression of (e) CDH5 (VE-cadherin) and (f) TJP1 (ZO1) genes in healthy and diabetic ECs (n=3). Black lines indicate significant differences between

healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant differences between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate the significant differences between healthy and diabetic ECs when cultured on the same topography. Permeability of FITC-dextran (40kDa) through healthy and diabetic EC after culture on (g) Unpatterned, (h) Parallel, and (i) Perpendicular topographies (n=2). The presence of FITC-dextran in the bottom chamber was measured over time. Gray asterisks indicate significantly different FITC-dextran permeability of diabetic ECs on the indicated topography against Unpatterned. Red asterisks indicate the significant differences in FITC-dextran permeability between healthy and diabetic ECs on the same topography. Two-way ANOVA level of significance denoted by * (P<0.05), ** (P<0.01), *** (P<0.001), or **** (P<0.0001).

Figure 5.

Monocyte adhesion on EC monolayers formed by healthy and diabetic ECs on selected topographies. (a) Representative images of monocyte adhesion on monolayers of healthy and diabetic ECs formed on different topographies. For clarity, a separate panel of images showing only monocytes are included. A merged image of the ECs are stained against the EC marker PECAM1 (denoted by red) and nuclei (denoted in blue), while monocytes are depicted in green. (b) Quantification of monocyte adhesion on healthy and diabetic EC monolayers (n=9). Measurements of monocyte adhesion were made on both types of healthy

and diabetic ECs. Relative expression of various immunogenicity genes involved in the leukocyte adhesion process (n=3): (c) NOS3 (endothelial nitric oxide synthase [eNOS]), (d) VCAM1 (vascular cell adhesion molecule 1 [VCAM1]), (e) ICAM1 (intercellular adhesion molecule 1 [ICAM1]), (f) PECAM1 (platelet endothelial cell adhesion molecule 1 [PECAM1]), (g) SELE (endothelial selectin), and (h) VWF (von Willebrand factor [VWF]) transcripts in healthy and diabetic ECs (n=3). Black lines indicate the significant differences between healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant differences between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate the significant differences between healthy and diabetic ECs when cultured on the same topography. Two-way ANOVA level of significance denoted by * (P<0.05), ** (P<0.01), *** (P<0.001), or **** (P<0.0001).

Figure 6.

Migration and wound healing capability of healthy and diabetic ECs on topographies. Wound closure capacity of healthy and diabetic ECs grown in a monolayer on (a) Unpatterned, (b) Gratings, (c) Parallel and (d) Perpendicular topographies. Percent wound length is denoted as a percentage relative to the original wound length at time 0 (n=6). All wounds were orthogonal to the direction of the micron-sized Gratings. Black asterisks indicate significantly different percent wound length of healthy ECs on the indicated topography against Unpatterned, while gray asterisks indicate the same for diabetic ECs

against Unpatterned. Red asterisks indicate the significant differences in wound length between healthy and diabetic ECs on the same topography. (e) Single EC velocity of healthy and diabetic ECs on various topographies. (f) Accumulated distance travelled by healthy and diabetic ECs on different topographies. (g) Euclidean distance travelled by healthy and diabetic ECs on different topographies. Euclidean distance is the straight line distance between two points. (h) Directness of healthy and diabetic EC migration on different topographies. Directness was calculated as a ratio of Euclidean distance to accumulated distance. Total number of ECs tracked to measure velocity and distances: Unpatterned (Healthy: n=81; Diabetic: n=86), Gratings (Healthy: n=75; Diabetic: n=78), Parallel (Healthy: n=42; Diabetic: n=64), Perpendicular (Healthy: n=53; Diabetic: n=75). Black lines indicate the significant differences between healthy ECs grown on topography compared to healthy ECs grown on Unpatterned. Grey lines indicate the significant difference between diabetic ECs grown on topographies compared to diabetic ECs grown on Unpatterned. Red lines indicate significant differences between healthy and diabetic ECs when cultured on the same topography. Two-way ANOVA with level of significance denoted by * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), or **** ($P<0.0001$).

Table 1

Characteristics of diabetic HCAEC compared with normal HCAEC

Characteristic	Normal EC 1	Normal EC 2	Diabetic EC 1	Diabetic EC 2
Phenotype	Normal	Normal	Type II diabetes	Type II diabetes
Gender	Male	Male	Male	Female
Age	30	35	60	56
Race	Caucasian	Hispanic	Caucasian	Hispanic
Tobacco use?	No	Yes	No	No
Alcohol use?	No	Yes	Yes	No

Table 2
Topographies used for studying differential responses of healthy and diabetic ECs

	Hierarchical topographies			Lens topographies	
	Unpatterned	Gratings	Parallel	Perpendicular	
Dimensions of topography	None	2µm ridge × 2 µm gap × 2 µm height	2µm ridge × 2 µm gap × 2 µm height with 250nm ridge × 250 nm gap × 250 nm height nanogratings aligned parallel to gratings axis on top	2µm ridge × 2 µm gap × 2 µm height with 250nm ridge × 250 nm gap × 250 nm height nanogratings aligned perpendicularly to gratings axis on top	<div>Convex</div> <div>Concave</div>
					<div>1.8 µm diameter, 2 µm pitch, 0.7 µm height spherical protrusion</div> <div>1.8 µm diameter, 2 µm pitch, 0.7 µm depth spherical depression</div>

Table 3
Summary of significant changes in healthy and diabetic EC function after culture on various topographies

Arrow pointing upwards indicates improvement of function, and arrow pointing down indicates deterioration of function on the specified topography.

Cell function	Comparison towards Unpatterned control		Comparison between the same topography
	Healthy	Diabetic	Healthy vs Diabetic
1. Cell area	Gratings ↓ Parallel ↓ Perpendicular ↓	Gratings ↑ Concave ↓ Perpendicular ↓	Gratings, Concave
2. Cell proliferation	No significant changes	Convex ↑ Concave ↓ Perpendicular ↓	Parallel
3. Angiogenic capacity	Parallel ↑ Perpendicular ↑ Convex ↑ Concave ↑	Concave ↓	Unpatterned, Concave, Convex
4. Oxidized LDL uptake	No significant changes	Parallel ↓ Perpendicular ↓ Concave ↓	Unpatterned, Gratings, Concave
5. Monolayer formation and integrity	Parallel ↓ Perpendicular ↓	Perpendicular ↓	Unpatterned, Gratings, Parallel, Perpendicular
6. Monocyte adhesion	Gratings ↑	Gratings ↑ Perpendicular ↑	No significant changes
7. Immunogenicity	Gratings ↓ Parallel ↓ Perpendicular ↓	Parallel ↓ Perpendicular ↓	Unpatterned, Gratings, Parallel, Perpendicular
8. Wound healing	Gratings ↑ Parallel ↓	No significant changes	Gratings
9. Migration	Perpendicular ↑	Perpendicular ↑	Unpatterned, Gratings, Perpendicular