Surface chemistry, substrate, and topography guide the behavior of human articular chondrocytes cultured *in vitro*

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Abstract

Understanding the behavior of chondrocytes in contact with artificial culture surfaces is becoming increasingly important in attaining appropriate *ex vivo* culture conditions of chondrocytes in cartilage regeneration. Chondrocyte transplantation-based cartilage repair requires efficiently expanded chondrocytes and the culture surface plays an important role in guiding the behavior of the cell. Micro- and nanoengineered surfaces make it possible to modulate cell behavior. We hypothesized that the combined influence of topography, substrate, and surface chemistry may affect the chondrocyte culturing in terms of proliferation and phenotypic means. Human chondrocytes were cultured on polystyrene fabricated microstructures, flat polydimethylsiloxane (PDMS) or polystyrene treated with fibronectin or oxygen plasma, and cultured for 1, 4, 7, and 10 days. The behavior of chondrocytes was evaluated by proliferation, viability, chondrogenic gene expression, and cell morphology. Contrary to our hypothesis, microstructures in polystyrene did not significantly influence the behavior of chondrocytes neither under normoxic- nor hypoxic conditions. However, changes in the substrate stiffness and surface chemistry were found to influence cell viability, gene expression and morphology of human chondrocytes. Oxygen plasma treatment was the most important parameter followed by the softer substrate type PDMS. The findings indicate the culture of human chondrocytes on softer substratum and surface activation by oxygen plasma may prevent dedifferentiation and may improve chondrocyte transplantation-based cartilage repair.

Keywords: Human chondrocytes, topography, substrate stiffness, surface chemistry, PDMS

INTRODUCTION

Autologous chondrocyte implantation (ACI) is a preferred and recommended treatment for larger focal articular cartilage defects in the knee[1]–[4]. The ACI procedure is among the U.S. Food and Drug Administration (FDA) approved cell-based treatment of focal cartilage repair[5]. Short and long-term studies have shown promising outcomes with this two-step procedure harvesting cartilage from a non-weight bearing area and expanding the chondrocytes *ex vivo* prior to surgical implantation in the defect[6]–[8]. The efficiency and cost-determining step of this treatment are associated with the chondrocyte culturing. A few studies have been addressing the expansion and growth of human chondrocytes in monolayer *in vitro* at flat surfaces[9]–[11]. In the native environment, chondrocytes are located in lacunae sensing the physical interface between the cytoplasmatic membrane proteins and the pericellular matrix (PCM) consisting of a meshwork of proteoglycans, collagens, hyaluronan etc.[12]. Native resident chondrocytes do not proliferate in contrast to *in vitro* cultured chondrocytes demonstrating considerable proliferation[9]. As chondrocytes attach and spread on the flat surface they start dedifferentiating by expressing a fibroblast-like morphology, which is accompanied by considerable proliferation. During this phenotypic switch synthesis of cartilage-specific extracellular matrix components such as collagen type II and proteoglycan expression decrease while the expression of collagen I increases consistent with fibrogenesis[13]–[17]. While the chondrocytes may partially or fully regain their chondrogenic phenotype over time following transplantation, the dedifferentiated chondrocytes also pose a risk of developing fibrous or fibrocartilaginous tissues. Hence, the properties of the *ex vivo* surface play an important role in cell adhesion, proliferation, and differentiation and subsequently for the clinical outcome of ACI.

Advancements in the engineering of biophysical surface properties such as topography, stiffness, and chemistry have allowed for modulation and guidance of specific cellular responses. In

chondrocytes, nano- and microscale topographical features such as pillars, grooves, and pits have been found to influence cellular responses[11],[18]–[22]. The proliferative capacity was enhanced when cultured on microstructured surfaces compared with flat surfaces of the same material[11],[20],[23]. While minimizing the tendency of dedifferentiation and maintaining the chondrocytes phenotype, hyaluronan micro-structured surfaces resulted in the alignment of cells inducing chondrogenic differentiation as suggested by increased collagen II and aggrecan expression and synthesis[18]. Apart from guiding mature chondrocytes topography also controls chondrogenesis in mesenchymal stem cells (MSCs) as indicated in different morphology, proliferation, and expression of chondrogenic markers[24]. Besides topography, substrate elasticity also induces lineage specification for MSCs and especially softer substrates induce the neurogenic phenotype, while stiffer substrates favor the osteogenic phenotype[25]. A study combining nanotopography and substrate elasticity, including polydimethylsiloxane (PDMS) and polystyrene (PS) with Young's modulus of 4.8 MPa and 2.9 GPa, respectively, fostered the dominance of topography over substrate stiffness on cytoskeletal organization and focal adhesion for MSCs[26]. Studies have shown that chondrocytes respond differently to substrate stiffness in the range 2 to 20 kPa and the response to matrix elasticity depending on the 3D versus 2D culture environment[27],[28]. But the chemical composition of a surface is also known to influence cellular responses. MSC differentiation on silane-modified surfaces with a range of chemical end groups was reported to control the phenotype of the attached MSCs. Negatively charged -COOH surfaces gave rise to a rounded morphology and an increase in collagen type II expression indicative of chondrogenic differentiation, while positively charged -NH2 surfaces led to an increase in osteogenic differentiation[29]. Also, the base material, as well as the type of procedure for protein adsorption may influence chondrocyte behavior[30]. In this study, we aimed at investigating the effect of surface topography, substrate, and surface chemistry on primary human chondrocyte culture. Earlier studies have shown the combined microstructure and

soft substrate polydimethylsiloxane (PDMS) influence chondrocytes where specific microstructures promoted proliferation and rate of differentiation of muscle cells[11],[31]. However, polystyrene is preferred owing to its common use *in vitro* and in clinical applications. We hypothesize that combined biophysical features may affect the chondrocyte behavior during *in vitro* culture.

MATERIALS AND METHODS

Fabrication of microstructured polystyrene microtiter plates

Seven different microstructures and a flat structure (control) were manufactured by injection molding of polystyrene plates. Each of the eight structures was produced in triplicate giving 24 test areas on each slide. The master substrate was fabricated to contain the microstructures pattern A and B visualized in Fig. 1. with different pillar size and inter-pillar gap size (X,Y) in μm. Culture plate numbering is as followed the smooth control 1=(SM), pattern A; 2=(4,1), 3=(4,2), 4=(4,6), and pattern B; 5=(2,1), 6=(4,1), 7=(4,2), 8=(4,6) as seen in Fig. 1[11],[32]. The pillar height was 2.4 μm and culture area per well was 28 mm^2 for each of the test fields on the microstructured polystyrene microtiter plates. In brief, the fabrication was in four steps[33]: (1) the original pattern was exposed by electron beam lithography. Silicon substrates were coated with a bilayer of PMMA 2010 and 2041. After exposure the substrate was developed in 1:1 (IPA:MIBK). Prior to reactive ion etching, 80 nm of NiCr was deposited by e-beam evaporation and lift-off carried out. The metal pattern acted as a hard mask during an ICP etch (SF_6/C_4F_8) of the silicon substrate. After dry etching, the NiCr layer was removed by chrome etchant. (2) From the etched silicon master, an electroplated nickel shim was produced. (3) The shim was inserted in an injection molding tool for the manufacture of microscope slide sized (75x25x1.2 mm^3) polymer slides. Manufacturing was conducted using a Victory Tech 28 (Engel GmbH). (4) Finally, the slides were ultrasonically welded (Rinco Ultrasonic, Switzerland) to an upper structure forming a microtiter plate.

Fabrication of flat PDMS surfaces

The material used for the PDMS substrates was SYLGARD® 184 (Dow Corning, USA)[11]. Prior to casting the PDMS substrates, 10% (w/w) curing agent was added to the elastomer followed by thorough mixing. Subsequently, the mixture was degassed in three cycles using a vacuum desiccator. The degassed PDMS mixture was added to 6-well dishes and cured by heating at a temperature of 70 ºC for 24 hours. The cured samples were rinsed with sonication in ethanol and subsequently in water for 10 min each followed by drying under a stream of nitrogen. The mechanical properties of the terminally cured substrates were evaluated by tensile mechanical testing (Alwetron TCT5, Lorentzen & Wettre, Sweden) and found to have an elastic modulus of 4.8 ± 0.2 MPa. Inserts of 28 mm^2 were punched out and placed in a 96-culture wells plate (Sarsted 83.1835). Prior to using the 10% PDMS surfaces were functionalized with gas oxygen plasma treated vacuum (Advanced Vacuum Vision 320 RIE) or 0.5 $\mu g/cm^2$ fibronectin (Sigma-Aldrich). This surface functionalization will be referred to as surface chemistry: Oxygen plasma (PL) and fibronectin (FN).

Cell culture procedures

Articular cartilage biopsies and chondrocytes were processed and cultured as described earlier[34],[35]. The ethical approval was obtained through patients' written consent and the protocol was approved by the local ethical committee under the Danish National Committee on Research Ethics (#M-2082453). In brief, chondrocytes were seeded at a density of 2,500 cells/cm^2 on the surfaces in Dulbecco's Modified Eagle Medium/F12, GlutaMAX (DMEM/F12) (Gibco, Life Technologies, Europe) with 10% FBS and 1:100 penicillin-streptomycin, and cultured at 37°C in a humidified atmosphere in normoxia (21% oxygen) or hypoxia (5% oxygen). For hypoxia cultures, the cell-seeded surfaces were transferred to a designated hypoxia chamber workstation (Xvivo

System, BioSpherix, NY) that was pre-balanced for the desired culture environment. Medium was replaced twice a week and equilibrated to 21% and 5% oxygen before use.

Relative cell number – proliferation indices

The relative cell number was analyzed for chondrocytes in passage 1 (P1) at day 1, 4, 7 and 10 using staining on a Victor Multilable counter (Perkin Elmer, Waltham, MA). Briefly, cells were fixated in 70% ethanol and washed in PBS before staining with 0.1% (w/v) methylene blue for 30 minutes. Excess dye was removed with 0.01 M borate buffer and images obtained with Motic AE21 inverted microscopy. Elution of dye was performed using 1:1 ethanol and 0.1 M HCl. The methylene blue dye was transferred to a 96-well ELISA plate for the measuring of absorbance at 650 nm using a Victro Multilable counter[36].

Cell viability

Cell viability was analyzed at day 4, 7, and 10 using an XTT assay (11465015001, Roche) according to manufacturer's recommendation and with the proportion of methylene blue measured at day 4, 7, and 10. Briefly, 50 µL of XTT labeling mixture was added to the well, which contained cells and 100 µL DMEM/F12 supplemented with 10% FCS (final XTT concentration 0.3 mg/mL). A Victor^3 Multilabel counter at 450/650 nm was used to measure the generated orange formazan dye. The absorbance was read after incubation at 37°C and 5 % CO_2.

Total RNA extraction and RT-qPCR

Total RNA was extracted using TaqMan® Fast Cells-to-CT™, (Ambion, Life Technologies) according to the manufacturer's instructions. In brief, each sample was lysed and reverse transcriptase polymerase chain reaction (PCR) was performed on cell lysate, which was transcribed

into cDNA using TaqMan® Fast Cells-to-Ct™ (Ambion). Quantitative real-time RT-PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using TagMan® Fast Universal PCR Master Mix (Applied Biosystems) and TagMan® Gene Expression Assays (Applied Biosystems) with the following assay: sex-determining region Y box 9 (*SOX9*) Hs00165814_m1, Collagen type 2 alpha 1 (Collagen II); Hs00264051_m1, aggrecan (*Aggrecan*); Hs00153936_m1, and Collagen type 1 alpha 1 (Collagen I); Hs00164000_m1. Standard enzyme and cycling conditions for the 7500 Fast System were used. Each biological sample was run in technical duplicates for each gene. Data analysis was performed using 7500 Fast System Sequence Detection Software version 3.1 (Applied Biosystems). Target gene expression was normalized to the housekeeping genes (HKG) Beta-2-microglobulin (B2M) Hs99999907_m1 and ribosomal protein L13a (RPL13a) Hs03043885_g1 based on BestKeeper values[34],[37].

KI67 staining

Chondrocytes cultured for 4 days were rinsed with PBS, fixed in 70% ethanol, and rehydrated in dH_2O before permeabilized with 0.1% triton X-100 in PBS, blocked with 10% bovine serum albumin (BSA) (Sigma-Aldrich), and incubated with KI67 (SP6) Rabbit monoclonal antibody at 1:300, Sigma-Aldrich, in 1% BSA overnight at 4°C. The next day chondrocytes were incubated with biotinylated goat anti-rabbit 1:300, (Dako, Denmark) and HRP-conjugated streptavidin tertiary antibody (Dako, Denmark) 1:300 for 1 hour for each antibody at RT. Images were captured using a Zeiss AX10 microscope equipped with a digital color camera (Axiocam 506 color Zeiss). Cells with nuclear staining were considered as positive.

Actin staining

Chondrocytes cultured for 4 days were fixed in 4% formaldehyde (VWR), permeabilized with 0.5% triton X-100 in PBS, blocked with 1% BSA, and incubated with Alexa Fluor 488® phalloidin at 1:100 (Invitrogen) in 1% BSA for 1 hour at room temperature. Chondrocytes were rinsed in PBS and counterstained with 4',6-diamidino-2-phenylinolde (DAPI) at 1:1000 (Invitrogen) in 1% BSA. To visualize and quantify the average cell size corresponding to actin fluorescence a single in-focus plane image was required using a 10x objective lens and Olympus BX50 fluorescence microscope (Olympus Denmark A/S). The regions of interest were drawn around each cell and cellular fluorescence was automatic quantified using image analysis newCAST™ software (Visopharm, Hørsholm, Denmark).

Statistics

For comparison of data, normality was checked using histogram and Q-Q plots and statistical analyses were performed using two-ANOVA on cell viability, cell proliferation, gene expression containing (microstructure*time) and relative number of cells (substrate*chemistry) information. Multivariate ANOVA was performed on hypoxia data containing (microstructure*oxygen-tension*time). All data that passed ANOVA was either analyzed with pairwise comparisons of means using tukey post-hoc test or one-way ANOVA. Student's t-test was performed on KI67 positive cells. Data are presented as mean ± SD of three independent experiments in triplicates (n=3). Stata Statistical Software: release 13.1 (StataCorp LP) was used. P-values <0.05 were considered significant.

RESULTS

Cell proliferation and viability on microstructures of polystyrene

Equal seeding and random distribution of chondrocytes onto the flat smooth surface (no. 1) and 7 microstructures (nos. 2-8) of polystyrene were found, which were visualized and measured using methylene blue staining on culture day 1 (Fig. 2A and C-D). Validation of chondrocyte expansion was based on a proliferation index showing the significant increase in proliferation within 1, 4, 7, and 10 days of culture. Thus, the seeding and proliferative findings revealed no significant preferences with regard to the microstructures. The morphological appearance of the chondrocytes on microstructures and smooth surfaces are found in Fig. 2C-D. The effect of microstructures on morphology was not different from the smooth surface representing a random orientation of cells and islet based outgrowth on the surfaces. Cell viability decreased significantly over time (Fig. 2B). At day 10 of culture, the cell viability was at its lowest. There were, however, no significant differences in cell viability between the smooth surface and the 7 microstructures.

Chondrogenic gene expression on microstructures of polystyrene

To investigate the chondrogenic state of the proliferating chondrocyte on microstructures of polystyrene relative quantitative gene expression measurements of transcription factor *SOX9*, *collagen II*, and *aggrecan* were performed at day 4 and 7 of culture. The expression of *SOX9* and *aggrecan* were significantly different when compared between day 4 and 7 (Fig. 3). The smooth surface and microstructures did not influence any of the examined genes.

Hypoxia and microstructures of polystyrene

Hypoxia was used to mimic the cartilaginous environment when studying the effect of topography as microstructures. There was a significant difference in proliferation when studying the oxygen concentration 21% compared with 5% (Fig. 4A). At day 4, chondrocytes in hypoxia (5% oxygen) had increased proliferation. This effect was, however, reversed at day 7 and 10 where chondrocytes

in 21% oxygen expressed increased proliferation. No observations were found in cell viability between the microstructures and oxygen tension. Time influenced the cell viability and was significantly reduced at day 10 (Fig. 4B). All genes showed a difference in expression when comparing 21% with 5% at the different days. Furthermore, the relative gene expression of *SOX9*, *collagen II and aggrecan* were significantly increased between day 4 and 7 (Fig. 4C). There was no influence of the microstructures under hypoxia on proliferation, cell viability, and expression of the chondrogenic genes examined. The effects observed were solely owing to hypoxia and time.

Characterization of cellular features on flat substrates and surface chemistries

Proliferation increased over time on both substrates (polystyrene and PDMS) and chemistries PL and FN (Table 1, Proliferation index). Viability was significantly higher on the polystyrene compared with PDMS at all time points (Table 1, Cell viability). The largest difference in viability was observed on day 4. Both substrates had decreased viability over time when correlated with the increased proliferation. Significantly more KI67 positive chondrocytes were found on PDMS compared with polystyrene on day 4, corresponding to cells being proliferative (Fig. 5A-B and Table 1, KI67 index).

Viability was significantly higher for FN compared with PL (Table 1, Cell viability). However, chondrocytes cultured on surfaces with chemistry of FN were associated with a significantly lower number of cells with a cell size < 1000 μm^2 and a larger number of cells with a cell size > 2000 μm^2 (Fig. 6A and Table 1, Cytoskeleton) compared with surface chemistry PL. The reverse observation was seen with PL (Fig. 6A). Figure 6B shows representative images illustrating the cytoskeletal morphology of chondrocytes cultured on PDMS with PL and FN visualized with green fluorescence actin filament and blue nucleus staining .

Chondrogenic differentiation of substrate and surface chemistry

To investigate the chondrogenic differentiation of cultured chondrocyte on polystyrene and PDMS with different surface chemistry gene expression analysis was performed (Table 2). Chondrocytes cultured on PDMS had significantly higher *SOX9* and *collagen II* expressions at both time points (Table 2C). The differentiation index "ratios of mRNA expression" also indicated a higher COLII/COLI expression in chondrocytes cultured on PDMS compared with polystyrene (Table 2D). Chondrocytes cultured on PL treated surfaces had a significantly higher expression of *SOX9*, *collagen II*, *aggrecan*, COLII/COLI and AGG/COLI compared with FN.

DISCUSSION

The understanding of the chondrocyte behavior in contact with artificial surfaces is essential in attaining adequate *ex vivo* culture conditions of chondrocytes in cartilage regeneration. Low native proliferative capacity and a high tendency of dedifferentiation when cultured remain a huge challenge to overcome in chondrocyte-based articular cartilage. In the present study, we show that human chondrocytes can be guided using the biophysical featured microstructure, substrate, and surface chemistry *in vitro*. We found that selected cell-culture substrates tended to be more efficient than microstructures in influencing cell viability and differentiation of human chondrocytes. Specifically, we showed that microstructures fabricated with 1, 2, and 6 μm inter-pillar distance in polystyrene did not facilitate variations in the cell-culture interactions regarding proliferation, cell viability, and chondrogenic expression compared to the flat polystyrene. A previous study has shown an effect of those microstructures in PDMS[11]. Advances had led to increased interest in the investigations of how cells react to topographical modulated surfaces and studies have reported the effect of topography on cellular mechanisms in cells[11],[38],[39]. In chondrocytes, the dimensional feature size range investigated has been more than 80 nm and less than 80 μm[18],[22],[23]. We used

polystyrene because of its ubiquity in most *in vitro* cell culture materials. Polystyrene has also previously been used for the investigation of the effect of topographical modulations[40],[41]. The inability of the microstructures to augment proliferation, cell viability, or gene expression could be due to the high rigidity of the material.

We used low oxygen tension for mimicking the physiological conditions of the environment of cells in articular cartilage exploring any increased chondro-inductive effect of the microstructures under low oxygen tension. However, the assisted chondrogenic conditions through hypoxia, 5% oxygen tension, did not induce a specific response. In agreement with findings in earlier publications, hypoxia decreased chondrocyte proliferation and increased the *SOX9* and *collagen II* expressions[42],[43]. We did not investigate other hypoxic conditions but chose to use 5% oxygen where we observed the same cell viability as in normoxia. Interestingly in chondrocyte respect, was the reversible phenotype-switch between proliferation and differentiation when observing the influence of oxygen.

Chondro-inductive properties of the substrate stiffness and/or surface chemistry were examined. Since soft materials have shown, in combination with topographies or oxygen plasma, to influence cellular responses, which could change the mechanical properties of the outer nanometers at the surface[44]–[46]. As such, PDMS has garnered great attention as a material used for soft lithography fabrication owing to its low elasticity[47]–[50]. The culture of human chondrocytes onto soft PDMS surfaces generated remarkable differences in cell viability, KI67 positive cells, and chondrogenic gene expression in comparison with polystyrene. Most notably were the differences in the viability of chondrocytes on the substrates. Cell viability, in this case, is correlated with mitochondria activity suggesting that the surface substrate influences the guidance of the metabolic activity. Chondrocytes operate under low oxygen consumption and with energy requirement primarily

coming from anaerobic glycolysis allowing chondrocytes to have only a few mitochondria[51]–[53]. Gene expressions of the chondrogenic markers *SOX9*, *collagen II* and *COLII/COLI* on PDMS showed an increase compared with polystyrene indicating a correlation between low cell viability and more differentiated chondrocytes. To this extent, it should be noted that the findings were independent of surface chemistry. However, including the surface chemistry oxygen plasma is a simple and widely used technique for introducing oxygen-containing groups on the surface[46],[54]. Oxygen plasma has previously been shown to have pronounced effect on the cell adherence and phenotype[46]. In this study, the cells cultured on the oxygen plasma treated surfaces resulted in the smallest cytoskeleton, lowest cell viability, and highest expression of chondrogenic genes. Dedifferentiation is associated with rapidly proliferating fibroblast-like cells. Fibronectin is a RGD-containing glycoprotein often used for surface coating, but fibronectin matched the general trend as indicated by spread adherence and reduced chondrogenic differentiation[55]. We cannot directly convert the information observed in this study to this process, but a behavior reflecting a loss of phenotypic traits when cultured on polystyrene and fibronectin compared with PDMS and oxygen plasma. It is important to note that the present results were obtained in monolayer culture and therefore cannot be extrapolated to the appearance of chondrocytes in 3D-cultures. A variety of different approaches enhancing proliferation and/or preventing dedifferentiation of chondrocyte cultures have been proposed. Our findings indicate that surface chemistry > substrate > topography guides the behavior of human chondrocytes cultured *in vitro* and that these results may foster the potential use of softer substrate and oxygen plasma treatment for the culture of chondrocytes.

CONCLUSION

In this study, surface functionalization as changes in substrate stiffness and surface chemistry were found to influence the behavior of human chondrocytes: oxygen plasma treatment was the most

important parameter followed by substrate type PDMS. Microstructures in polystyrene were also introduced. However, no significant influence was observed either under normoxic- or hypoxic conditions. Given the substantial use of different functionalizations for cell culture experiments, this study highlights the importance of understanding how human chondrocytes are guided by their surroundings and especially cell-surface chemistry and cell-substrate interactions. These findings indicate the culture of human chondrocytes on softer substratum and surface activation by oxygen plasma may prevent dedifferentiation and may improve chondrocyte transplantation-based cartilage repair.

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Disclosure statement

No competing financial interests exist.

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FIG 1. Microstructure design. (A) The topographical design consisting of pattern A and B organized in different pillar sizes (X) and inter-pillar gap sizes (Y) visualized in the combination of (X,Y) [8]. Pattern A represents circles and pattern B a combination of circles and squares both arranged in the same structure combination. (B) Microstructures are arranged in the nos. 2-8 and no. 1 is the control (SM). (C) Microstructured polystyrene microtiter plate design.

FIG 2. Human chondrocyte expansion on microstructures of polystyrene. **(A)** Proliferation of chondrocytes over time. Vertical axis represents the methylene blue optical density data normalized to the smooth surface at day 1. Horizontal axis represents the smooth surface (no. 1) and 7 combinations of microstructures (nos. 2-8). Asterisks indicate significant between days of culture * $p<0.05$. Data are expressed as mean ± SD (n=3). **(B)** Cell viability of chondrocytes over time. Vertical axis represents cell viability based on XTT/relative cell number data normalized to the smooth surface at day 4 (% of control). Horizontal axis represents the smooth surface (no. 1) and 7 combinations of microstructures (nos. 2-8). Asterisks indicate significant difference between days of culture * $p<0.05$. Data are expressed as mean ± SD (n=3). **(C)** Representative methylene blue images illustrating the morphology of chondrocytes seeded on different microstructures over time. Magnification was 4x. (D) Selected enlargement of representative methylene blue images with morphology of chondrocytes seeded on smooth surface (1) and topography microstructured surface (4).

FIG 3. The relative gene expression levels of *SOX9*, *collagen II*, and *aggrecan* of chondrocytes culture on microstructures of polystyrene for 4 and 7 days. Vertical axes represent the normalized to control relative gene expression level and horizontal axes represent chondrocytes cultured on the 8 different microstructures at day 4 and 7. Asterisks indicate significant difference between days of culture* $p<0.05$. Data are expressed as mean ± SD (n=3).

FIG 4. Chondrocytes cultured on microstructures of polystyrene under normoxia and hypoxia. **(A)** Proliferation of chondrocytes ± hypoxia over time. Vertical axis represents the methylene blue optical density data normalized to the smooth surface at day 1 under normoxia (21% oxygen). Horizontal axis represents the microstructures under normoxia (21% oxygen) and hypoxia (5% oxygen) at day 1, 4, 7 and 10, respectively. Asterisks indicate significant difference between oxygen tension * $p<0.05$ and days of culture ** $p<0.05$. Data are expressed as mean ± SD (n=3). **(B)** Cell viability of chondrocytes ± hypoxia over time. Vertical axis represents the XTT/relative cell number data normalized to the smooth surface at day 4 under normoxia. Horizontal axis represents the microstructures under normoxia (21% oxygen) and hypoxia (5% oxygen) at day 4, 7 and 10, respectively. Asterisks indicate significant difference between days of culture ** $p<0.05$. Data are expressed as mean ± SD (n=3). **(C)** Vertical axis represents the relative gene expression level normalized to control at day 4 under normoxia and horizontal axis represents microstructures normoxia (21% oxygen) and hypoxia (5% oxygen) at day 4 and 7. Asterisks indicate significant difference between oxygen tension * $p<0.05$ and days of culture ** $p<0.05$. Data are expressed as mean ± SD (n=3).

FIG 5. Human chondrocytes cultured on polystyrene or PDMS with different surface chemistry PL and FN. Human chondrocytes were seeded with 2,500 cells/cm^2 and cultured for 4 days before KI67 staining. (A) KI67 positive chondrocytes. Vertical axis

represents the percentage of positive KI67 chondrocytes. Horizontal axis represents the average level of pooled data for substrates and surface chemistry. Significant difference between substrates * $p<0.05$. Data are expressed as mean ± SD (n=3). (B) KI67 positive (red nucleus) and negative chondrocytes cultured on PDMS for 4 days. Scale bar 50 µm.

FIG 6. Human chondrocytes cultured on polystyrene or PDMS with surface chemistry of PL or FN. Human chondrocytes were seeded with 2,500 cells/cm^2 and cultured for 4 days before cytoskeleton staining. (A) Relative cell number of the cell size distribution. Vertical axis represents relative cell number. Horizontal axis represents cell size: 1000 µm^2 < 1000-2000 µm^2 < 2000 µm^2. Significant difference between the surface chemistries* $p<0.05$. Data are expressed as mean ± SD (n=3). (B) Actin (green) and DAPI (blue) staining of the cytoskeleton and nucleus, respectively, of chondrocytes cultured on PDMS with PL or FN. Scale bar 50 µm.

TABLE 1. Proliferation index, cell viability, cytoskeleton, and KI67 index. Human chondrocytes were seeded with 2,500 cells/cm^2 and culture for 1, 4, 7, or 10 days (D1, D4, D7, and D10). Proliferation index: methylene blue optical density. Cell viability: XTT/relative cell number. Cytoskeleton: Cell size in µm^2. KI67 index: ratio of KI67 positive chondrocytes. Significant difference between substrates* and surface chemistry** $p<0.05$. Data are expressed as mean ± SD (n=3).

TABLE 2. Relative gene expression of chondrogenic genes. Human chondrocytes were seeded with 2,500 cells/cm^2 and culture for 4 and 7 days (D4 and D7). Genes of interest *SOX9, collagen II, aggrecan* and the ratios of collagen II/collagen I (COLII/COLI) and aggrecan/collagen I (AGG/COLI). Significant difference between substrates* and surface chemistry** $p<0.05$. Data are expressed as mean ± SD (n=3).

A		B								
Substrate/Chemistry		Proliferation index				Cell viability			Cytoskeleton	KI67 index
		D1	D4	D7	D10	D4	D7	D10	D4	D4
Polystyrene	O_2 plasma	0.064 ±0.02	0.083 ±0.02	0.127 ±0.04	0.157 ±0.04	5.330 ±2.2	6.022 ±1.9	3.219 ±0.6	1391 ±662	0.461 ±0.10
	Fibronectin	0.027 ±0.01	0.099 ±0.04	0.203 ±0.05	0.259 ±0.05	8.661 ±3.3	6.480 ±1.5	4.098 ±0.3	1658 ±729	0.473 ±0.04
PDMS	O_2 plasma	0.130 ±0.05	0.075 ±0.02	0.113 ±0.05	0.156 ±0.05	1.166 ±0.2	1.580 ±0.7	0.786 ±0.2	1365 ±691	0.488 ±0.08
	Fibronectin	0.051 ±0.02	0.082 ±0.04	0.158 ±0.08	0.211 ±0.05	2.878 ±2.0	3.883 ±2.1	2.050 ±0.8	1840 ±707	0.534 ±0.05
P value*	Substrate	0.818				0.00001			0.135	0.0016
P value**	Chemistry	0.064				0.00001			0.00001	0.0532

Mean values with standard deviation (SD) are reported for each substrate/chemistry, along with the statistical P-values * and **

A = Classification of groups

B = Cellular features

**P*-value for the difference between the two substrates

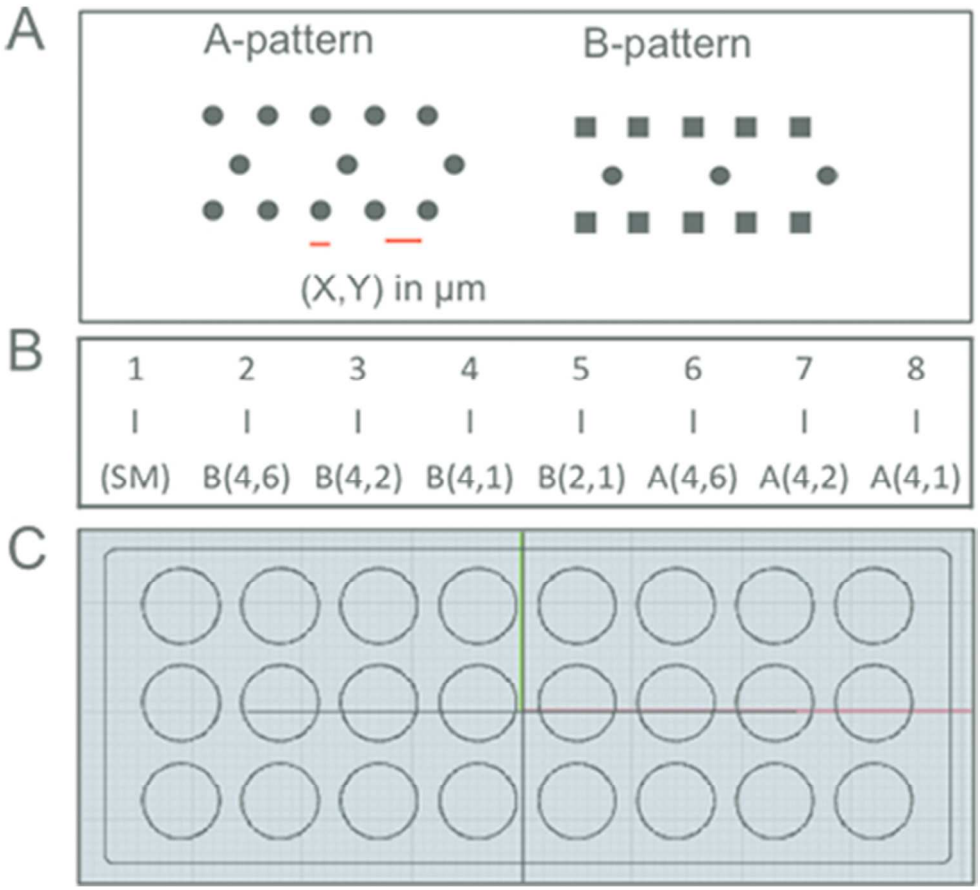
***P*-value for the difference between the two surface chemistry

A		C						D			
Substrate/Chemistry		SOX9		COLLAGEN II		AGGRECAN		COLII/COLI		AGG/COLI	
		D4	D7	D4	D7	D4	D7	D4	D7	D4	D7
Polystyrene	O_2 plasma	7.077 ±0.8	6.364 ±1.0	10.734 ±1.0	8.632 ±1.4	12.005 ±0.5	10.599 ±0.3	0.687 ±0.1	0.597 ±0.1	0.768 ±0.02	0.734 ±0.03
	Fibronectin	5.558 ±0.7	5.595 ±0.9	6.623 ±1.4	3.299 ±1.8	10.315 ±0.5	9.458 ±0.3	0.418 ±0.1	0.244 ±0.1	0.712 ±0.1	0.717 ±0.04
PDMS	O_2 plasma	9.320 ±0.9	7.758 ±0.9	15.201 ±1.0	10.596 ±1.9	12.839 ±1.2	10.343 ±0.8	0.892 ±0.1	0.699 ±0.1	0.754 ±0.1	0.686 ±0.1
	Fibronectin	6.811 ±0.5	6.752 ±1.2	10.172 ±0.7	7.089 ±1.3	10.114 ±0.5	9.423 ±0.6	0.614 ±0.1	0.481 ±0.1	0.641 ±0.04	0.638 ±0.03
P value*	Substrate	0.00001		0.00001		0.1988		0.00001		0.00001	
P value**	Chemistry	0.00001		0.00001		0.00001		0.00001		0.00001	

Mean values with standard deviation (SD) are reported for each substrate/chemistry, along with the statistical P-values * and **

A = Classification of groups
C = mRNA expression
D = Ratios of mRNA expression

**P*-value for the difference between the two substrates
***P*-value for the difference between the chemistry



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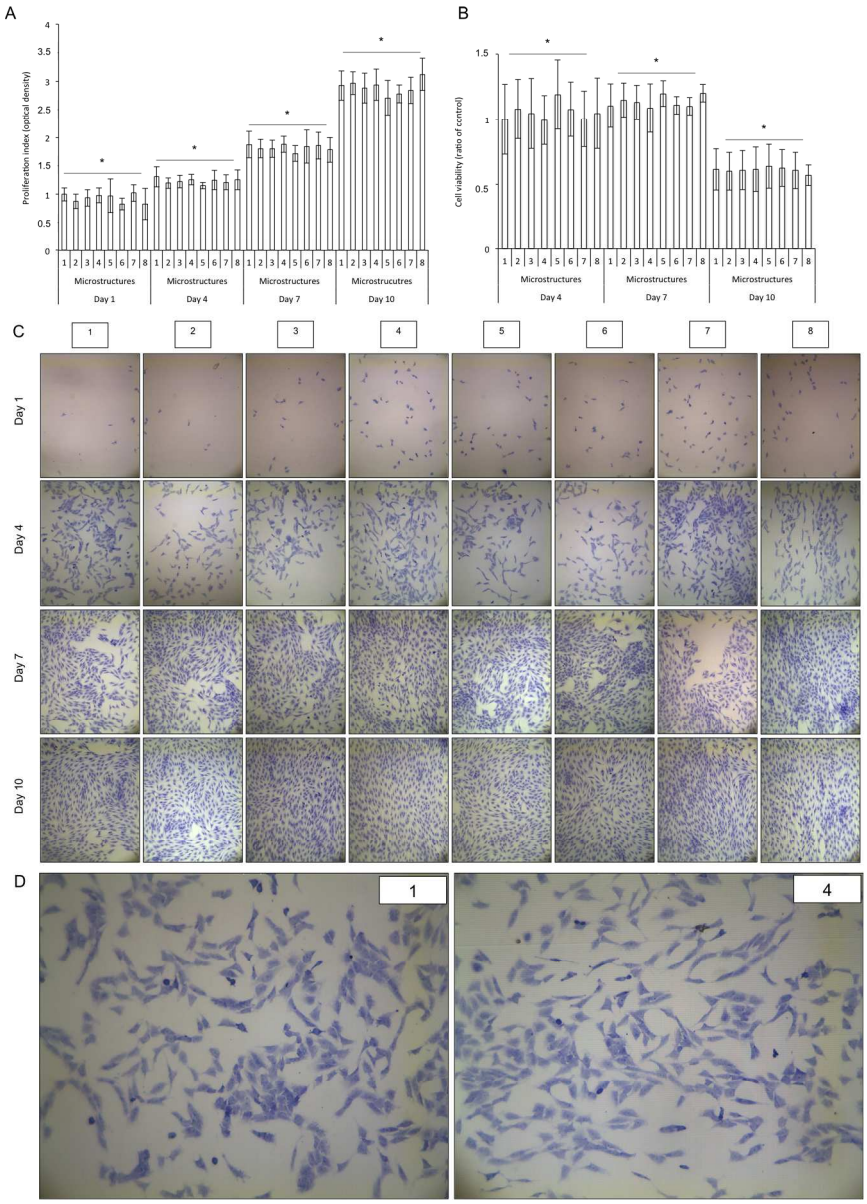

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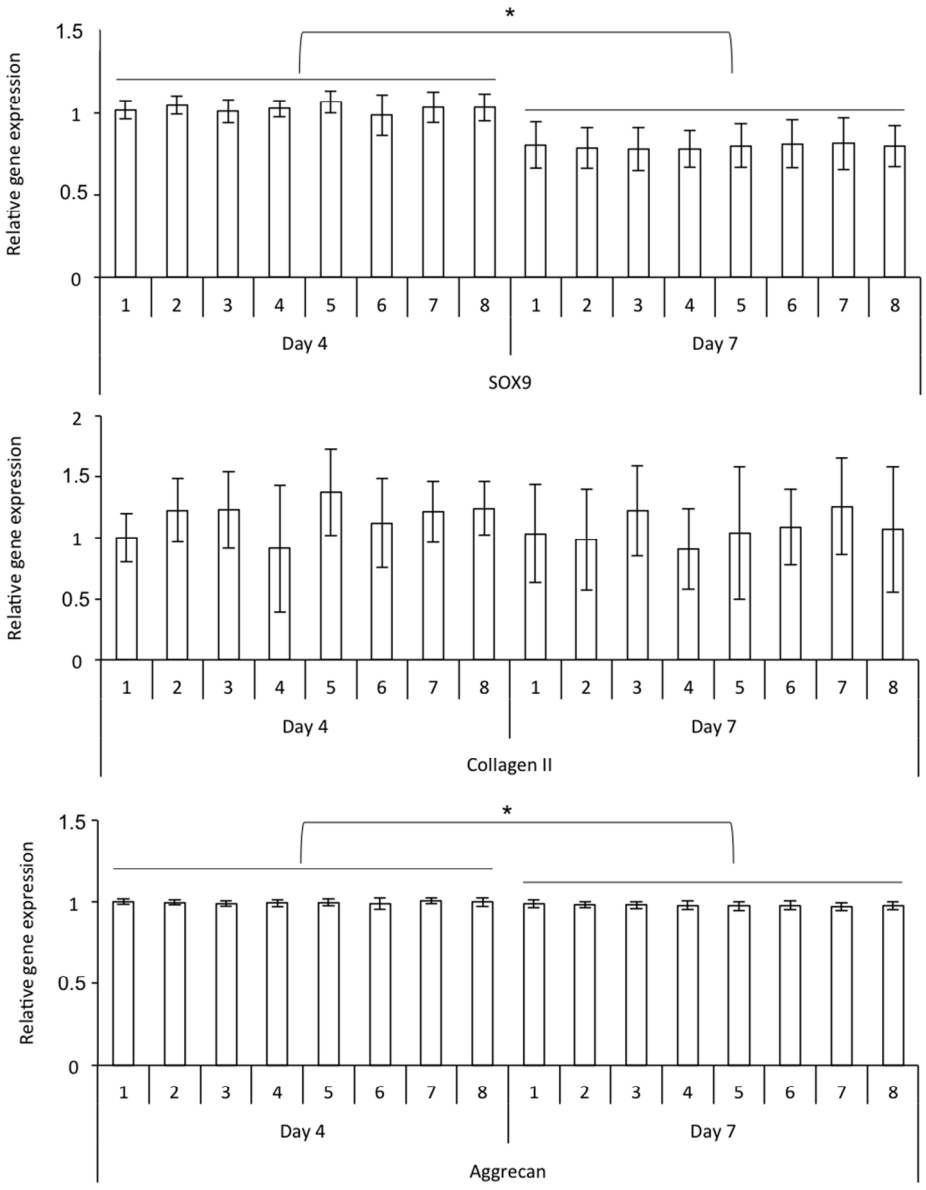

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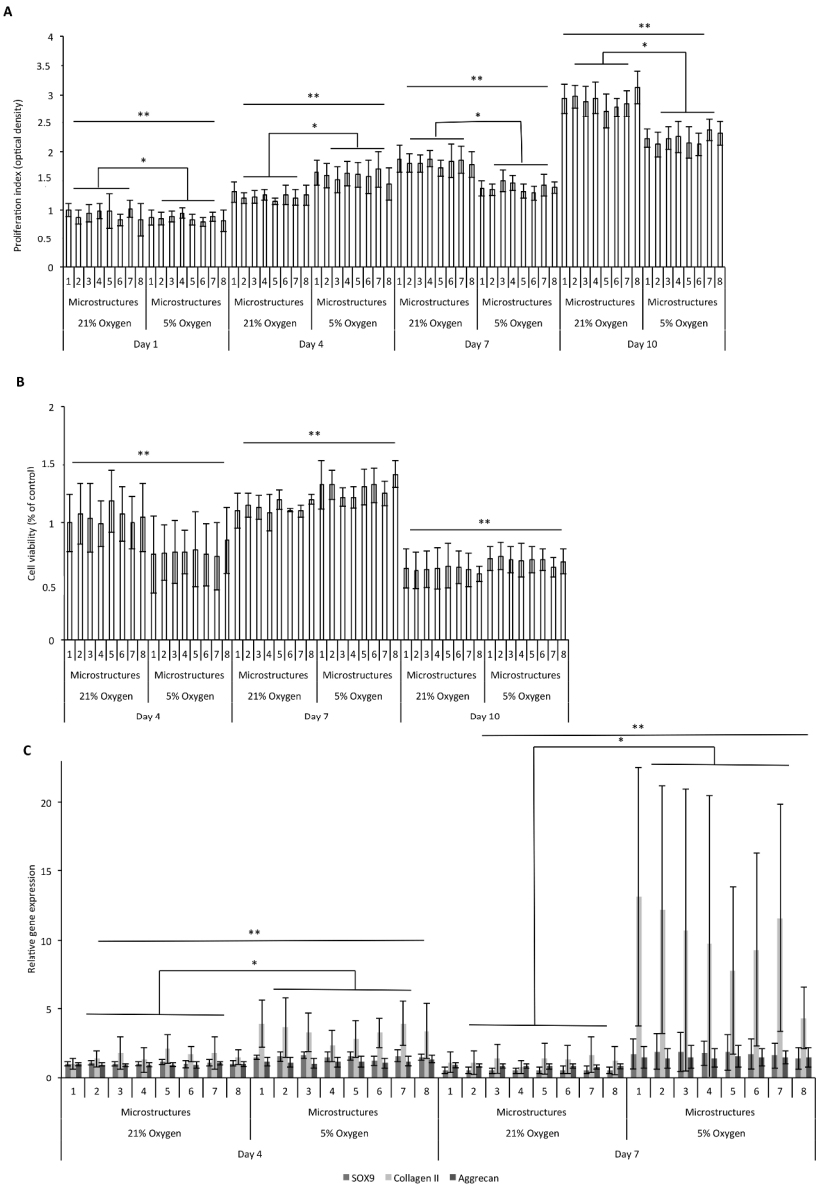

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as mean ± SD (n=3).

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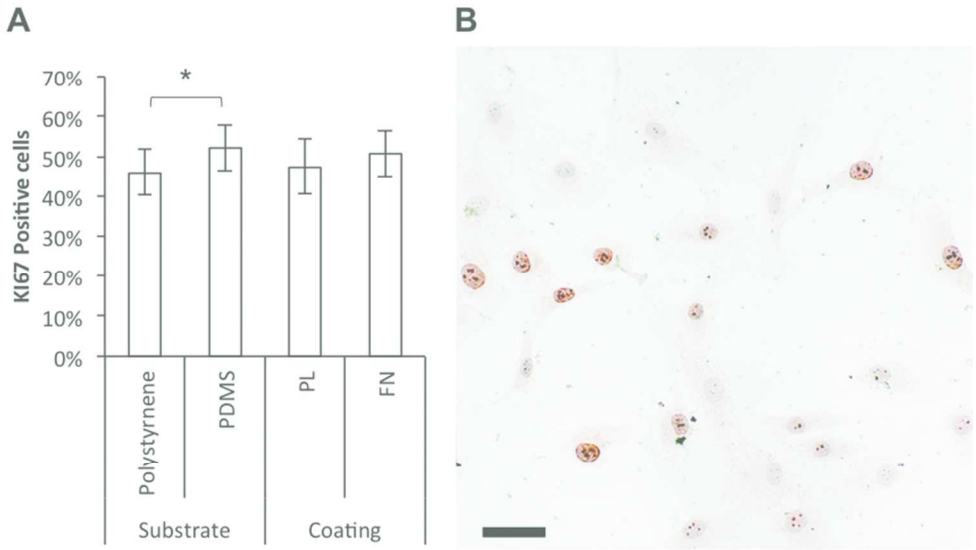

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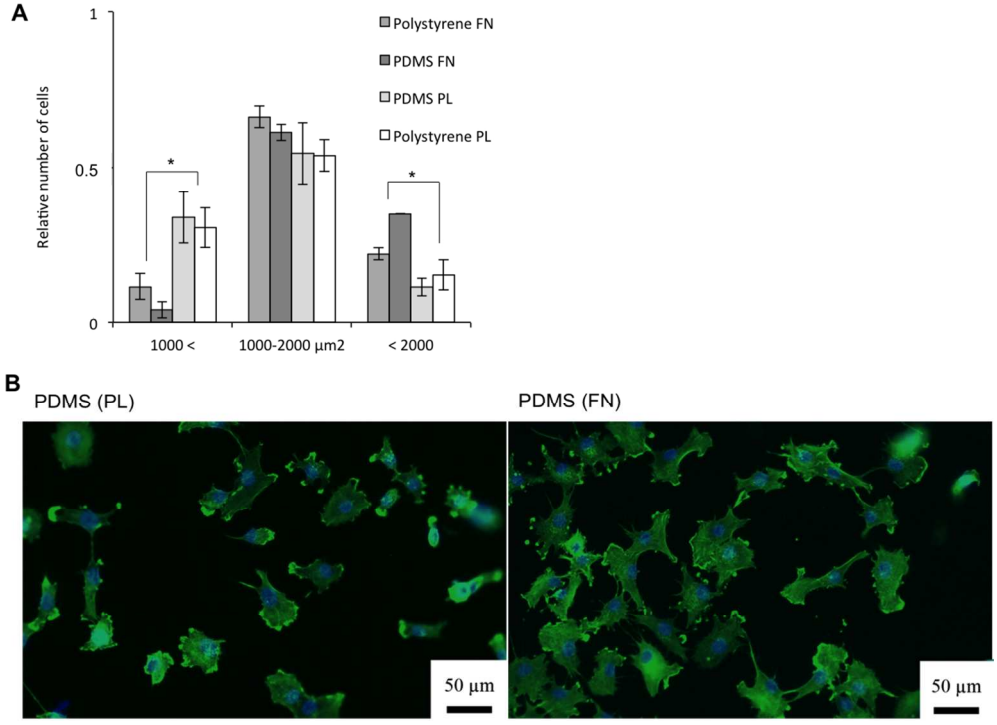

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213x157mm (300 x 300 DPI)