Substrate Nanotexture and Hypergravity Through Centrifugation Enhance Initial Osteoblastogenesis

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Mimicking the structural nanomolecular extracellular matrix with synthetically designed nanosized materials is a relatively new approach, which can be applied in the field of bone tissue engineering. Likewise, bone tissue-engineered constructs can be aided in their development by the use of several types of mechanical stimuli. In this study, we wanted to combine nanotextured biomaterials and centrifugation in one multifactorial system. Mesenchymal stem cells were isolated from rat bone marrow, and cultured on a nanogrooved polystyrene substrate (200-nm-wide pitch with a depth of 50 nm). Constant centrifugation of 10 g was applied to cells up to 7 days. Results showed that on a nanogrooved substrate osteoblast-like cells align parallel to the groove direction. Centrifugation of 10 g also affected cell morphology on a smooth surface. Moreover, cell alignment was significantly reduced for cells grown on nanogrooved substrates, which were subsequently subjected to centrifugation. Independently, both stimuli increased the number of cells after 7 days of culture. However, when both stimuli were combined, an additive effect on cell number was observed, followed by an enhanced effect on osteocalcin mRNA expression and matrix mineralization. In conclusion, biomaterial surface modification as well as centrifugation are effective means to enhance bone cell behavior, moreover, readily available to many tissue engineers.

Introduction

THE FIELD OF TISSUE ENGINEERING aims to regenerate injured and diseased tissues. Although in the last decade great progress in the development of tissue substitutes is achieved, most of the used tissue constructs have focused on the use of different molecular signals to guide the stem cell differentiation processes.^{1,2} However, after *in vivo* implantation, many of the *in vitro* predesigned load-bearing tissues can fail due to inadequate tissue structure and function.³ Much of this can be attributed to the lack of mechanical stimuli during cell culture. Research has already demonstrated that different mechanical stimuli, such as fluid flow shear stress, hydrostatic pressure, substrate strain deformation,^{4–7} and gravity,⁸ play a critical role in the final cellular morphology, tissue geometry, and function.

Mechanical factors indeed have an important role on life in general. The absence of gravitational force has a negative effect on tissue development and can lead to muscle and bone atrophy, that is, a decreased mineralization and increased mineral resorption, cardiovascular as well as circulatory problems.^{8,9} Exposure to near weightlessness causes changes in focal adhesions and collagen fibrillogenesis,

hampers cell growth, and disturbs osteoblast gene functions.¹⁰ In contrast, short cell culture experiments done in hypergravity conditions via centrifugation have shown to induce a favorable effect on genes involved in osteoblastogenesis, such as osteocalcin, vitamin D receptor, and Runx2.¹¹ Exposure to hypergravity acts on the whole cell mass, and cells exposed to 2 or 3 g reduce 30%–50% in average height,¹² decrease the height of their microtubule network, but also increase the thickness of their actin fibers¹³ without affecting cell viability.¹⁴ Hypergravity can be achieved with different types of centrifuges.^{13–15} Studies examining the response of cells to centrifugation mostly use short and intermittent exposures to increased g levels. The effects on osteoblast morphology and differentiation to continuous increased g levels over longer time periods on osteoblast morphology and differentiation have not been investigated extensively.

Besides mechanical cues, cells are also responsive to the structural elements formed by the surrounding nanomolecular network of the extracellular matrix (ECM), particularly to the most abundant protein collagen.^{16,17} The nanoscale structure of the collagen molecules consists of fibrils with a diameter ranging from 15 to 300 nm¹⁸ and bone mineral

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hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) nanoparticles (20–30 nm) are distributed among the collagen fibrils of bone. Mimicking the structural nanomolecular ECM with synthetically designed nanosized materials has already been demonstrated to cause changes in morphology, as well as to have a positive effect on osteoblastogenesis.^{19,20}

The aim of this study was to combine nanotextured materials and continuous centrifugation of 10 g, and to understand which of these factors guides the cellular response more. Our hypothesis is that centrifugation will have a favorable effect on osteoblast-like cells in terms of differentiation, and that the nanotextured substrate resembling the ECM structure will equally influence this process. In addition, we hypothesize that, when the two stimuli are combined, an additive effect will be observed.

Materials and Methods

Preparation of nanotextured substrate

Nanotextured groove and ridge templates were made on silicon wafers using laser interference lithography and reactive ion etching techniques as described by Lamers *et al.*²⁰ In this study, 120-nm-wide grooves with 200-nm pitch, and a depth of ~50 nm were used. Wafers with a planar (smooth) surface were used as controls.

Using the method of solvent casting, the wafers were used to prepare polystyrene (PS) replicas as described previously by van Delft.²¹ The PS replicas were cut and bonded to PS rings with a diameter of 18 mm, using a PS-chloroform casting solution. Before cell seeding, all experimental substrates received a radio frequency glow discharge treatment (Harrick Scientific Corporation, New York, NY) for 5 min at 100 mTorr Ar, to enhance substrate wettability and cell attachment.

Surface analysis with atomic force microscopy

A multimode atomic force microscopy (AFM) (Nanoscope IIIa; Burker, Santa Barbara, CA) with NanoScope Analysis software (version 1.20, Burker) was used to confirm the surface topography of the nanopatterned replicas. Tapping in ambient air was performed with a high-aspect-ratio NW-AR5T-NHCR cantilever (NanoWorld AG, Wetzlar,

Germany) with average normal spring constants of 30 Nm^{-1} . Height images of each nanopattern were captured in ambient air at 50% humidity at a tapping of ~250 kHz. The analyzed field was scanned at a rate of 80 Hz and with 512 scanning lines.

Cell culture

Rat bone marrow mesenchymal stem cells (MSCs) were isolated from femurs of 40–43-day-old male Wistar rats based on the method described by Maniopoulos *et al.*²² Briefly, MSCs were obtained by flushing out the marrow from two femurs with the α -minimal essential medium (α -MEM) containing 2% of gentamicin (50 mg/mL) (both from Gibco BRL, Life Technologies BV, Breda, the Netherlands). The washout cells were cultured in three T-75 flasks in a humidified atmosphere of 95% air, 5% CO_2 at 37°C with an osteogenic medium containing α -MEM, 10% fetal calf serum (FCS), 10 mM sodium β -glycerophosphate, 10^{-8} M dexamethasone, 50 mg/mL ascorbic acid, and 50 mg/mL gentamycin (all Gibco). The following day, the medium was refreshed to remove all nonadherent cells. After 6 days, the adherent cells were trypsinized using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA) (Sigma-Aldrich, Zwijndrecht, Netherlands) and seeded onto the substrates at 1.0×10^4 cells/cm². Cells were then cultured in the osteogenic medium containing CO_2 -independent α -MEM (Gibco), supplemented with 5% of 200 mM L-glutamine and 0.1% Fungizone (Gibco). Cells were first incubated for 60 min to achieve attachment to the substrates. Then substrates were placed in a custom-made PS holder, which was placed in a tube with a flat silicone rubber bottom (Fig. 1a). The cell culture medium was added to fill the tubes completely.

Hypergravity

Increased mechanical loading in the form of hypergravity was achieved using the Large Diameter Centrifuge (LDC) located at the European Space Agency (ESA) in Noordwijk. (Fig. 1b).²³ The system consists of four large rotating arms with swing gondolas attached at the extremity. The diameter of the centrifuge is 8 meters at full swing-out. The tubes containing the samples were placed inside a 37°C controlled

FIG. 1. General setup. (a) Polystyrene (PS) dishes seeded with cells were placed in a custom-made PS holder in a 50-mL tube equipped with a silicone rubber stopper on the bottom. (b) The Large Diameter Centrifuge (LDC) can provide hypergravity of 10 g. The system comprises of large rotating arms, where a swing-out gondola is attached at the extremity, and a central gondola serves as a rotational control. Color images available online at www.liebertpub.com/tea

incubator in one of the gondolas, accelerated to 10 g (~47 rpm), and cultured continuously for up to 7 days (incubation time was depending on the performed assay). Control samples were placed in the central compartment of the centrifuge, enabling the control samples to undergo the same rotations, yet at 1 g.

Immunofluorescence and image analysis

To observe the cytoskeleton, cells were washed three times with phosphate-buffered saline (PBS), fixed for 10 min in 3% paraformaldehyde (Fluka AG, Buchs, Switzerland), and permeabilized with 1% Triton X-100 (LTD Colebrook, Bucks, England) for 5 min after 24 h and 48 h of centrifugation. Then filamentous actin was stained with Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, Oregon) diluted 1:200 in PBS (Gibco). The cell nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) diluted in PBS 1:2500 for 10 min. Finally, the specimens were examined with an automated fluorescent Zeiss Axio Imager Z1 microscope (Zeiss, Heidelberg, Germany), at magnification of 20 $\times$. For each sample, over 100 microscopic fields were selected randomly, and cells in each field were examined for their overall orientation with respect to the direction of the nanogrooves (~300 cells for each experimental condition). The obtained micrographs were saved as binary images, and the cellular angle, surface area, and elongation index of only mononuclear cells that were attached to the surface, but not in contact with other cells nor with the image perimeter, were measured using ImageJ software (ImageJ, La Jolla, CA).

Scanning electron microscopy

To assess cellular morphology, cells were washed with 1 $\times$ PBS, fixed for 5 min in 2% glutaraldehyde, rinsed for 5 min with 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried in tetramethylsilane to air (all, Acros Organics, Geel, Belgium) after day 1 and 7 of cell culture. The specimens were sputter coated with Pt and examined using scanning electron microscopy (SEM) (JEOL 6330, Tokyo, Japan).

DNA content

After day 1 and 7 of cell culture, total DNA content was determined to obtain information about cellular proliferation, since the DNA content directly correlates to the amount of the cells. The medium was removed and the cell layer washed twice with PBS. 0.5 mL of Milli-Q deionized water was added to each sample and samples were stored at -80°C until further use. To further lyse the cells, sonication of 10 min was used. For analysis, a Pico Green dsDNA Quantitation Kit (Molecular Probes) was used. A standard curve was generated using serial dilutions of lambda DNA ranging from 0 to 2000 ng/ μ L. Briefly, 100 μ L of sample and 100 μ L pico green working solution were added to a 96-well plate. The plate was incubated at room temperature in the dark for 5 min and read at 480–520 nm. The DNA content was determined with the aid of the standard curve.

Mineralized matrix deposition assay

To obtain information about mineralized matrix formation, the calcium (Ca) content was determined after 7 days of

culturing. Ca content was determined using the orthocresolphthalein complexone [OCPC (Sigma)] method. The samples were rinsed twice with PBS followed by the addition of 500 μ L acetic acid (0.5 N) and overnight incubation at room temperature. Samples were frozen at -20°C until further use. The OCPC solution was prepared by the addition of 80 mg of OCPC to 75 mL of demineralized H₂O with 0.5 mL KOH (1 M) and 0.5 mL acetic acid (0.5 N). To prepare the sample solution, 5 mL of OCPC solution was added to 5 mL 14.8 M ethanolamine-boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline (5 g in 100 mL 95% ethanol), and 88 mL of demineralized water. Three-hundred microliters of this sample solution was added to 30 μ L of sample. The plate was incubated at room temperature for 10 min and read at 575 nm using a microplate reader. A standard curve was made from serial dilutions of CaCl₂, ranging from 1–200 μ g/mL.

Isolation of RNA and reverse transcriptase-polymerase chain reaction

Total RNA was extracted after day 1 and day 7 of cell culture using the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA) and performed according to the manufacturer's protocol. After obtaining the mRNA, a first-strand reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Superscript III First-strand Synthesis System for RT-PCR (Invitrogen, Leiden, Netherlands) according to the manufacturer's protocol. Briefly, the cells were lysed in 100 mL of lysis buffer containing 0.7 mL of b-mercaptoethanol. Subsequently, 100 mL EtOH 70% was added, thoroughly mixed, and transferred into an RNA-binding nanospin cup. The sample was centrifuged at 2,000 g for 60 s, the filtrate was discarded, and 300 mL of low-salt wash buffer was added. The sample was centrifuged and the filtrate was removed. 15 mL of DNase-solution (2.5 mL RNase-Free DNase-I mixed with 12.5 mL DNase digestion buffer) was added to the sample and incubated for 15 min at 37°C. 300 mL of high-salt wash buffer was added to, and subsequently, centrifuged at 12,000 g for 1 min. The filtrate was discarded, 300 mL low-salt wash buffer was added to the spin-cup, and centrifuged. 8 mL of elution buffer was added to the sample and incubated for 2 min at room temperature. The sample was collected by centrifugation at 12,000 g for 5 min. After obtaining the mRNA, a first-strand reverse transcriptase PCR was performed using the Superscript III First-strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. For each sample, 150 ng of extracted mRNA was incubated with 1 mL of dNTPs (1 mM end concentration), 0.5 mL random hexamers, and 0.5 mL oligo (dT) 20 primers (both 0.5 mM end concentration) for 5 min at 65°C to anneal the primers to the mRNA. The following components were subsequently added: 2 mL 10 reaction buffer, 4 mL 25 mM MgCl₂, 2 mL 0.1 M DTT, 1 mL RNase-OUT (40 U/mL), and 1 mL superscript III RT (200 U/mL). The reaction mix was incubated for 10 min at 25°C for further primer annealing, 50 min at 50°C for reverse transcription, and 5 min at 85°C to terminate the reaction. Then, 1 mL RNase H was added to the tube and incubated for 20 min at 37°C for RNA digestion. This solution was stored at 20°C until further use.

TABLE 1. GENES FUNCTION, FORWARD AND REVERSE PRIMER SEQUENCES

Gene	Function	Forward (5' → 3')	Reverse (5' → 3')
<i>BCL2</i>	oncogene with antiapoptotic function (opposite of <i>bax</i>)	GGATGACTTCTCTCGTCGCTACCGT	ATCCCTGAAGAGTTCCTCCACCAC
<i>BAX</i>	promotes apoptosis by competing with <i>bcl2</i>	GCTACAGGGTTTCATCCA	CACATCAGCAATCATCCTC
<i>p53</i>	tumor suppressor involved in regulating cell cycle	GATGATATTCTGCCACCAC	GACGGAAGATGACAGAGG
<i>RUNX2</i>	main transcriptional factor involved in osteoblast differentiation	GCCACACTTTCCACACTCTC	CACTTCTGCTTCTTCGTTCTC
osteocalcin (OC)	bone mineralization and calcium ion homeostasis	CGGCCCTGAGTCTGACAAA	GCCGGAGTCTGTTCACTACCTT
β -catenin (β -cat)	anchors the actin cytoskeleton, regulates cell growth, promotes osteoblastogenesis through the wnt/ β -catenin pathway	AGTAGCTGACATTGACGG	GCTTTATTAACGACCACCTG
<i>GAPDH</i>	housekeeping gene	CGATGCTGGCGCTGAGTAC	CGTTCAGCTCAGGGATGACC
<i>HPRT</i>	housekeeping gene	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAAGCTTATAGCC

Gene expression

The gene expression on the RNA level for genes involved in apoptosis (*BCL2*, *BAX*, and *p53*) and osteoblast differentiation [*RUNX2*, osteocalcin (OC), and β -catenin (β -cat)] were evaluated. The expression levels were analyzed versus the average values of the housekeeping genes glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase (*HPRT*). Specific sense and antisense primers for the genes were designed according to published cDNA sequences of GenBank (Table 1). The specificity of the primers was tested separately before the real time PCR reaction. The IQ SYBR Green Supermix PCR kit (BioRad, Hemel Hempstead, United Kingdom) was used for real time measurement. Relative mRNA expression was quantified using the comparative Ct (DCt) method and expressed as $2^{\Delta DCt}$. Each sample was expressed as mean $\pm$ standard deviation. The RNA-negative control consisted of only milli-Q water that is reverse transcribed and amplified in parallel with the cell samples.

Statistical analysis

All experiments were performed three times ($n=3$) with three different rats, and every sample was measured in

duplicate. Since the primary cells were derived from different rats, interanimal differences prohibit the possibility of pooling the results for the DNA content and Ca assay. However, the same trend of results was observed for all three experiments. Therefore, the results from the second experiment are shown and the results from the other two experiments are shown in Table 2 and Table 3. For the gene analysis, since for all samples 150 ng RNA was used, results from all three experiments were analyzed using two-way analysis of variance (ANOVA) with the *post hoc* test. Calculations were performed in InStat (v. 3.05; GraphPad, Inc., San Diego, CA). Statistical analysis for median angles and surface area elongation index were performed using one-way ANOVA with the post-test (Tukey-comparison test).

Results

Substrates

AFM images showed that the PS nanotextured substrates were of constant quality and exhibited uniform nanogrooves. The pitch dimensions of the nanogrooved patterns were 207.3 ± 1.5 -nm wide, and 50.1 ± 5.5 -nm deep as shown on Figure 2. The smooth surfaces had an average roughness (Rq-value) of 3.6 ± 1.6 nm.

TABLE 2. DNA CONTENT FROM ALL THREE EXPERIMENTS

Rat 1	Smooth	nano	smooth 10g	nanotexture 10g
DNA μ g/mL day 7	149.3	725.4	309.5	889.5
SD $\pm$	6.3	2.63	9.1	3.75
Rat 2				
DNA μ g/mL day 7	178.9	388.3	337	698.8
SD $\pm$	15.5	33.3	95.7	159.8
Rat 3				
DNA μ g/mL day 7	379.4	460.5	440	520.4
SD $\pm$	28.4	11.1	120.7	44.6

TABLE 3. CALCIUM ASSAY FROM ALL THREE EXPERIMENTS

Rat 1	smooth	nano	smooth 10g	nanotexture 10g
Ca ng/mL day 7	1.65	2.08	1.84	7.02
SD $\pm$	0.16	0.09	0.14	1.2
Rat 2				
Ca ng/mL day 7	1.29	1.52	1.46	8.96
SD $\pm$	0.13	1.02	0.16	1.8
Rat 3				
Ca ng/mL day 7	1.35	1.17	1.37	1.70
SD $\pm$	0.2	0.2	0.32	0.32

FIG. 2. (a and b) AFM topographies of the nanogrooved PS substrates confirmed that the pattern of grooves and ridges were well reproduced in PS, with an average pitch of 207.3 ± 1.5 nm, and a depth of 50.1 ± 5.5 nm.

Scanning electron microscopy

When comparing the four different experimental conditions, after 1 day of cell culture at normal gravity, SEM showed that cells grown on the smooth substrate spread out in a random fashion with few extensions in multiple directions. When seeded on the nanotextured substrate, most of the cells and their extensions aligned parallel to the groove direction. In contrast, cells grown in hypergravity on the smooth substrate had more extensions, but did not show any obvious shape changes, whereas on the nanotextured substrate, cells had less elongated cell bodies with branched, but also longer cellular extensions in multiple directions as depicted in Figure 3.

Further SEM analysis demonstrated that after 7 days, cells in all experimental conditions became confluent and started to mineralize producing crystal-like particles. Only the particles on the nanotextured substrate in hypergravity were deposited in an aligned mode. In addition, there were also more particles

evident by visual inspection compared to the other three experimental conditions as shown in the inserts of Figure 4.

Image analyses and cell alignment

The fluorescent microscopy corroborated that on the smooth substrate overall cell shape as well as internal actin filaments orientation was as random. On the nanotextured substrates, cells and actin filaments mostly aligned parallel to the direction of the nanogrooves. In hypergravity, on the smooth substrate similar actin orientation to normal gravity was observed. However, on the nanotextured substrate, alignment was far less evident (Fig. 5a).

Cell alignment

The quantified results for the cell alignment are presented as box-whisker plots (Fig. 5b). Such a graph shows the distribution midpoint, the first and third quartile (boxes), and

FIG. 3. Scanning electron micrographs of osteoblast-like cells grown under various conditions for 1 day at $500\times$ magnification. Top left: cells, grown on a smooth substrate at 1 g have few cellular extensions and top right: cells grown on a nanotextured substrate with extensions aligned parallel to the nanogrooves, insert in the corner is $2000\times$ magnification of the nanogrooves beneath the cells. Bottom left: cells grown on a smooth substrate in hypergravity have more extensions and bottom right: cells grown on a nanotextured surface with branched, but also longer cellular extensions in multiple directions. White arrows indicate the direction of the nanogrooves and the letter c is abbreviation for cell.

FIG. 4. Scanning electron microscopy images after 7 days of cell culture at $200\times$ magnification. Note the onset of mineralization. Only the mineral particles on the nanotextured substrate in hypergravity were deposited in an aligned mode. Inserts in the corners show: $2000\times$ magnification the nanogrooves and mineral particles. White arrows indicate the direction of the nanogrooves and the letter c is abbreviation for cell.

the largest and smallest observation (whiskers). The results showed that there was no significant difference in the overall cell alignment for the smooth substrates neither in time, nor in gravity. In contrast, at normal gravity, on the nanotextured substrates, cells aligned parallel to the grooves at all three time points, which was always significant ($p < 0.001$, calculated against the same smooth substrate groups respectively). In addition, after 24 h and 48 h of cell culture under hypergravity conditions, the median angles of the cells grown on the nanotextured substrate changed and increased significantly ($p < 0.001$ and $p < 0.01$) when compared to the median angles of the nanotextured substrates in normal g, respectively, that is, the preferential orientation is less.

Cell elongation

The quantified result for the cell elongation ratio measurements (length/width ratio) showed that cells cultured on the smooth substrate have more rounded bodies with an elongation ratio of ~ 1 . In addition, there was no significant difference for the smooth substrates in time, or in gravity. In contrast, cells grown on the nanogrooved substrate had more elongated bodies and the elongation ratio on the nanogrooved substrates was 1.9 after 24 h and 1.6 after 48 h. In hypergravity, however, the elongation ratio of the cells reduced significantly to 1.3 after 24 h ($p < 0.01$) and to 1.2 after 48 h ($p < 0.01$) (Fig. 5c).

Cell area

Overall, cell area increased significantly over time (24 h vs. 48 h) on the smooth substrate, in both normal and hypergravity conditions ($p < 0.001$). On the smooth substrate, both 24 h and 48 h, cells cultured in hypergravity were significantly smaller than the cells grown at normal conditions ($p < 0.01$). The same significant differences applied for the

nanotextured substrates at both time points. Still, cells grown on the nanotextured substrates had a significantly smaller cell area ($p < 0.001$) than on the respective smooth substrates (Fig. 5d).

DNA content

After day 1 and 7 of cell culture, the total DNA content was determined to obtain information about the cell number. At day 1, no significant difference in the total amount of DNA was observed between the different experimental conditions (Fig. 6a). Cells proliferated under all conditions, as expected more cells were present at day 7 compared to day 1. However, at day 7, a significant increase of cell number ($p < 0.05$) on both nanotextured substrates was observed when compared to the smooth substrates. Furthermore, at this time point there was also a significant increase in cell number in the hypergravity samples compared to smooth ($p < 0.05$) and nanotextured ($p < 0.001$) substrates subjected to 1 g. In addition, there was an additive effect observed when cells were grown on the nanotextured substrate in hypergravity.

Mineralized matrix deposition

At day 1, no mineralization could be detected (data not shown). A low level of mineralization was measured at control conditions, on both smooth and nanotextured substrates as shown in (Fig. 6b). The same amount was observed for cells grown in hypergravity on the smooth substrate at day 7. However, the mineralization was significantly higher only for cells grown on the nanotextured substrate under hypergravity conditions.

Gene expression

The results of gene expression are shown Figure 7. Due to the performed $2^{-\Delta\Delta C_t}$ test, the SD distribution of the error

FIG. 5. Cellular morphological characteristics. **(a)** Fluorescent microscopy graphs of actin staining (red) and nuclei staining (blue) of osteoblast-like cells grown for 48 h. Upper part of the graph: cells grown on a smooth substrate with random cell and actin orientation at 1 g and 10 g, respectively. Lower part of the graph: note that the parallel cell and actin orientation to the nanogrooves in normal gravity is slightly lost in hypergravity. White arrow indicates the direction of the nanogrooves **(b)**. Median angle of $\sim 50^\circ$ is a random cell orientation on the smooth substrates. Note that cells align in parallel direction to the nanogrooves ($p < 0.001$) in normal gravity, and that it changes significantly in hypergravity increasing after 24 h ($p < 0.001$) and 48 h ($p < 0.01$). **(c)** Box-Whisker plot with cell elongation ratio measurements (height/width ratio) showed that cells cultured on the smooth substrate have rounded bodies. Cells grown on the nanogrooved substrate after 24 h had more elongated bodies and in hypergravity the elongation ratio of the cells dropped significantly after 24 h ($p < 0.01$) and 48 h ($p < 0.05$). **(d)** Box-Whisker plot showing the surface area of the cells. Overall cell area increased significantly over time on the smooth substrate in normal gravity and in hypergravity ($p < 0.001$). Note that the median cell area on the nanotextured substrate is significantly smaller in all three time points in both groups when compared with the smooth substrates, respectively. Each box represents ~ 200 cells (* $p < 0.01$, ** $p < 0.001$).

FIG. 6. (a) Cell proliferation graph by a total DNA content measurement (in $\mu\text{g/mL} - 1$). The error bars represent the standard deviations. In normal conditions, at day 1, no significant difference in the total amount of DNA was observed. At day 7 in normal conditions, significant increase of cell number ($p < 0.05$) on the nanotexture substrates was observed when compared to the smooth substrate. The same result was also observed in hypergravity, an additive effect was observed when cells were grown on the nanotextured substrate in hypergravity. (b) The Ca content was significantly higher only for cells grown on the nanotextured substrate under hypergravity conditions. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) $n = 1$. Color images available online at www.liebertpub.com/tea

bars are not even, as analyses are relative (fold) changes with a minimum value of 0 for a decreased expression, while increased expression ranges from 1 to infinite. The relative gene expression for genes involved in cell cycle and apoptosis (*BCL2*, *BAX*) did not show any significant upregula-

tion or down regulation neither at day 1 or day 7, in any of the samples when compared with the smooth substrate. In addition, the ratio of *BAX/BCL2*, which can determine whether or not a cell goes toward apoptosis, showed no significant increase (data not shown). *P53* RNA expression increased significantly at day 7 only for the cells cultured on the smooth substrate in hypergravity (Fig. 7a).

In terms of osteoblast differentiation at day 1, only OC was significantly upregulated on the nanotextured substrate in hypergravity. In contrast, at day 7, a significant increase for *RUNX2* on the nanotextured substrate in normal conditions was also observed, but also on the smooth substrate in hypergravity. β -cat was significantly upregulated only on the smooth substrate exposed to 10 g. Furthermore, an additive effect for OC was observed with a threefold significant increase on the nanotextured substrate in hypergravity ($p < 0.01$) (Fig. 7b).

FIG. 7. Influence of nanotexture, hypergravity, and combination of both on gene expression evaluated after day 1 and day 7 of cell culture. Values were normalized to *GAPDH* and *HPRT* and relative to the smooth substrates (black bar or gray horizontal line). (a) Apoptosis-related genes *BCL2*, *BAX*, and *p53* did not show any significant upregulation or downregulation neither at day 1, or day 7. (b) Osteoblastogenesis genes. At day 1, only OC was significantly upregulated on the nanotextured substrate in hypergravity. Same result was observed at day 7. In contrast, *RUNX2* was significantly upregulated on the nanotextured substrate in normal conditions, but also on the smooth substrate in hypergravity. β -cat was only significantly up regulated only on the nanotextured substrate (* $p < 0.05$, ** $p < 0.01$) $n = 3$. Color images available online at www.liebertpub.com/tea

Discussion

The aim of this study was to combine a nanotextured biomaterial surface with centrifugation in a multifactorial system, and to understand which principle drives the morphological cell response, that is, the cues arising from a specific surface topography or the enhanced physical pull arising from the mechanical environment. MSCs were assessed on morphology, (shape, elongation, and orientation), proliferation, early matrix mineralization, and expression of genes involved in osteoblastogenesis and apoptosis. Overall, the results showed that on a nanogrooved substrate osteoblast-like cells align parallel to the groove direction. Only centrifugation of 10 g does not induce effects on cell alignment on the smooth surface. However, cell alignment is significantly reduced for cells grown on nanogrooved substrates, which are subsequently subjected to centrifugation of 10 g. Thus, the nanotexture demonstrated a stronger effect on cellular morphology, while centrifugation of 10 g played a secondary, but significant role. The nanotextured substrate and the centrifugation, independently increased the number of cells after 7 days of cell culture. Most importantly, when both stimuli were combined, an additive effect on cell

number was observed, combined with an enhanced effect on mineralization, orientation of the deposited mineralized matrix, and increased osteocalcin mRNA expression.

Regarding the study design, mimicking the structural nanomolecular ECM with synthetically designed nanosized materials falls in the range of physiological rationale, but of course for gravity there is no physiological rationale, as on earth gravity is always 1 g. Our previous research had shown that cells can survive and adopt a wide spectrum of gravitational forces (from 10 g to 50 g) without showing apoptotic events.¹⁵ In addition, in the field of hypergravity research, 10 g is commonly used for cell experiments, and therefore we used 10 g in our setup.

Several studies employing centrifugation at various speeds have previously been used and already described the effect of hypergravity.^{24–26} Still, it can be argued that part of the cellular response is caused by indirect effects of the centrifugation. For instance, increased gravity generates hydrostatic pressure due to the column of medium above the cultures. Hydrostatic pressure can be calculated as $P = \rho gh$, where ρ is the density of the medium, g is the acceleration of gravity, and h is the height of the medium in each ring. For our cell culture system, the hydrostatic pressure on the surface of the substrate can thus be calculated to be 1.03 kPa at 10 g ($1.05 \cdot 10^3 \text{ kg m}^{-3} \times (9.81 \text{ m s}^{-2} \times 10) \times 0.01 \text{ m}$). These pressure levels are very low in comparison to hydrostatic pressures as osteoblast-like cells are subjected to, in a common *in vitro* model, which are usually in the range of 0.3 to 5.0 MPa.²⁷ Therefore, the hydrostatic pressure level as produced by centrifugation is neglectable for the current data analysis.

Further, the level of inertial shear from the cell culture medium as experienced by an adherent cell layer is neglectable, since it depends on the radius of the centrifuge and the location of the cells within the sample surface area. This effect is enhanced only in centrifuges with smaller radii, and irrelevant when a LDC is used, like in the current study.²⁴ Thus, the observed effects on the cellular behavior in our setup are due to the nanotextured substrate and the enhanced weight arising from the centrifugation.

Comparison of our data with the available literature indicates that osteoblasts are known to respond to mechanical cues, such as substrate deformation.²⁸ Our initial observations confirmed that cells responded to the substrate topography and centrifugation or a combination of both. Subsequent image analysis showed that cells grown on the smooth substrate under hypergravity had a smaller cellular area. Mammalian cells grown under hypergravity were never assessed and quantified for cellular area. However, this result corroborates with a previous observation of Kato *et al.* on *Paramecium*, where he observed reduced cell size under hypergravity of 20 g.²⁹ The typical elongated cell bodies observed on the nanogrooved substrate decreased significantly after 24 h and 48 h in hypergravity, but not completely to the point of an equal cell elongation index as observed on the smooth substrate. This observation confirms that the effect of substrate topography on the morphological cell response is stronger, and although influential cannot be overruled completely by hypergravity of 10 g.

An intriguing finding in our study was the enhancing effect on proliferation. Based on recent literature, the increased number of cells on the nanotextured substrate can be related to the faster mitotic spindle arrangement. The mitotic spindle

fibers arise from the two poles of the cells toward the nuclei of the mitotic cell and have parallel orientation. The parallel spindle alignment is crucial for the chromosomes to move apart and divide the cell.^{30,31} It is not clear how the arrangement of the spindle arises when cells are cultured on a nanotextured substrate, but using microcontact printing to define patterns of ECM proteins, it has been shown that the mitotic spindle orients to its position faster and results in faster cell division, ergo an increased cell number.³⁰ Short exposure of continuous or intermittent centrifugation was also observed to increase proliferation of MC3T3-E1 cells.³² In addition to these findings, in the current study, an additive effect was found by combining nanotexturing and hypergravity.

Furthermore, a clear and significant increase in matrix mineralization was observed only for cells grown on the nanotextured substrate under hypergravity conditions. Sedimentation of calcium and/or phosphate ions from the culture medium seems unlikely, since sedimentation of proteins as to centrifugation depends on the size, concentration and mass, rotation speed, and viscosity of medium.³³ Sedimentation of even large proteins by centrifugation is only possible when small diameter capillaries are used, in which a high concentration of diluted proteins is placed and subjected to ultra-high-speed rotation. In our study, the ionic components from the medium are infinitely smaller, and thus sedimentation from the cell medium can be excluded. Finally, the increased mineralization was not seen on the smooth substrate at 10 g. Probably, the high amount of mineralization after 7 days of cell culture is due to biological factors, like the higher number of cells. In addition, osteocalcin is a calcium-binding protein involved in bone mineralization and calcium ion homeostasis and expressed with a threefold increase on the mRNA level. It has already been observed that exposure to hypergravity can increase the osteocalcin protein level¹⁴ and higher serum osteocalcin expression levels are relatively well correlated with increases in bone mineral density.³⁴

Analysis of the mRNA expression of genes involved in apoptosis and osteoblast differentiation showed that continuous centrifugation does not affect apoptosis. The ratio of *BAX/BCL-2* determines whether or not the cell goes toward apoptosis, and no significant increase of *BAX/BCL-2* ratio was observed in our samples.^{35,36} The Wnt/ β -catenin-signaling pathway has been identified as one of the signaling pathways that is activated in response to mechanical loading.³⁷ β -catenin anchors to the actin filaments and is involved in regulating cell growth and adhesion, but also enhances osteoblastogenesis.³⁸ After 7 days of cell culture, *β -cat* was highly expressed under hypergravity on the smooth substrate. Further research in the up/downstream pathway is needed to find other molecules involved in this pathway, such as *GSK-3 β* , *APC*, or *TCF*, that might lead to an activation of this pathway under hypergravity. The gene analysis also confirmed that, one of the main transcriptional factors involved in osteoblastogenesis, *RUNX2*, was only significantly upregulated at day 7 on the nanotextured substrate and on the smooth substrate in hypergravity. This showed that independently, or combined together (only for osteocalcin), nanotextured materials and increased gravity by centrifugation have a simulative effect on early osteoblast differentiation.

Conclusion

Up to date, the present study is the longest continuous cell culture experiment ever carried out in a centrifuge generating 10 g, demonstrating that cells maintained viable, proliferated faster, and differentiated over 7 days toward osteoblastogenesis. Our results showed that independently, nanotextured substrates and centrifugation, increased the number of cells. When both stimuli were combined, an additive effect on cell number, followed by an enhanced effect in osteocalcin mRNA expression and matrix mineralization were observed. Thus, the use of new materials with nanoscale features can improve bone tissue development on the surface of the implant *in situ*. Further, centrifugation can be exploited to enhance osteoblastogenesis in other similar tissue-engineered materials seeded with stem cells, and thus speed-up the bone tissue development before clinical trials. The specificity of cellular responses to externally applied mechanical stress and the interaction between various environmental cues requires more in depth research using new multifactorial models. These results can be used to improve our fundamental understanding on how physical pull by centrifugation affects cell function and bone tissue engineering. Still, in conclusion, biomaterial surface modification as well as centrifugation are effective means to enhance bone cell behavior, moreover, readily available to many tissue engineers.

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Disclosure Statement

No competing financial interests exist.

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