CHAPTER 2

MECHANORESPONSIVENESS OF HUMAN ADIPOSE STEM CELLS ON NANOCOMPOSITE AND MICRO-HYBRID COMPOSITE

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Submitted
Chapter 2

ABSTRACT
Resin-based composites are used for bone repair applications and comprise resin matrix and different sized filler particles. Nanometer-sized filler particles improve composite’s mechanical properties compared to micrometer-sized filler particles, but whether differences exist in the biological response to these composites is unknown. Natural bone comprises a nanocomposite structure, and nanoscale interactions with extracellular matrix components influence stem cell differentiation. Therefore we hypothesized that nanometer-sized filler particles in resin-based composites enhance osteogenic differentiation of stem cells showing a more bone cell-like response to mechanical loading compared to micrometer-sized filler particles. Pulsating fluid flow (PFF; 5 Hz, mean shear stress: 0.7 Pa; 1 h) rapidly, within 5 minutes, increased nitric oxide production in human adipose stem cells (hASCs) on nanocomposite, but not on micro-hybrid composite. PFF increased RUNX2 expression in hASCs on micro-hybrid composite, but not on nanocomposite after 2 h post-incubation. PFF did not affect mean cell orientation and shape index of hASCs on both composites. In conclusion, the PFF-increased nitric oxide production in hASCs on nanocomposite, and increased osteogenic differentiation of hASCs on micro-hybrid composite suggest different responses to mechanical loading of hASCs on composite with nanometer-sized and micrometer-sized filler particles. This might have important implications for bone tissue engineering.

Key words: Mechanoresponsiveness, Adipose stem cells, Nanocomposite, Micro-hybrid composite, Pulsating fluid flow
INTRODUCTION
Composite scaffolds are used for tissue engineering. They are composed of two or more materials, and when combined, they have superior properties. Resin-based composites are widely used as dental restorative materials, but also for bone repair applications.1-4 These composites comprise an organic resin matrix and inorganic filler particles of different size, e.g. nanometer-sized (nanocomposite) and micrometer-sized (micro-hybrid composite). Nanometer-sized filler particles enable mechanical strengthening of resin-based composite.5 Reduction of filler particle size increases surface area-to-volume ratio and filler volume, which improves the mechanical properties including flexural strength.6

Natural bone comprises a complex organic-inorganic nanocomposite structure. The organic phase is mainly composed of collagen type I nanofibers, and the inorganic phase consists of hydroxyapatite nanometer-sized crystals. Within the stem cell niche, nanoscale interactions with extracellular matrix components influence stem cell behavior and differentiation.7,8 To engineer bone tissue, it is important that stem cells differentiate into bone cells and display a bone cell-like response to mechanical loading.5 Adipose stem cells (ASCs) constitute an interesting target for bone tissue engineering. Clinically relevant stem cell numbers with high proliferative capacity can be easily extracted from adipose tissue.10,11 ASCs have multilineage potential including the osteogenic lineage.12 Osteogenically stimulated ASCs display a bone cell-like response to mechanical loading by pulsating fluid flow (PFF).9,13 Moreover, bone cell mechanosensitivity increases during bone cell differentiation.14 Osteocytes, the mechanosensors in bone, align in the direction of mechanical loads.14 Bone cells with a round morphology are more mechanosensitive than elongated cells.15-17

Understanding biological responses to resin-based composites is important for bone tissue engineering. MC3T3-E1 osteoblasts seeded on micro-hybrid composite show similar attachment, spreading, and proliferation compared to cells on polystyrene culture dishes.18 C2C12 pluripotent mesenchymal precursor cells seeded on micro-hybrid composite show similar attachment, proliferation, and alkaline phosphatase activity compared to cells on resin-based cements.19 Human peripheral blood leukocytes exposed to 5 day eluates from nanocomposite do not exhibit cell genotoxicity.20 Polyethylene tubes filled with nanocomposite and empty tubes implanted in the subcutaneous connective tissue of isogenic mice evoke a similar inflammatory response.21 Thus the biological response of cells to nanocomposite and micro-hybrid composite seems quite similar, but the mechanoresponse of cells on both materials is unknown.

Human ASCs (hASCs) on polylysine-coated glass slides display a bone cell-like response to mechanical loading by PFF13, but whether hASCs on nanocomposite or micro-hybrid composite respond similarly to mechanical loading by PFF is unclear. We investigated whether differences exist in the response to PFF of stem cells on nanocomposite or micro-
hybrid composite. We hypothesized that nanocomposite enhance mechanosensitivity of hASCs. hASCs were cultured on nanocomposite or micro-hybrid composite for 1 day, subjected to 1 h PFF (pulse: 5 Hz; mean shear stress: 0.7 Pa), representing a physiological loading regime. Static cultures were used as control. Medium samples were assayed for nitric oxide (NO) production. At 2 h post-PFF, gene expression of osteogenic differentiation markers RUNX2, ALP, OPN, ON, DMP1, COL1, nitric oxide synthase marker NOS3, mechanosensitivity marker COX2, and vasculogenic differentiation markers VEGF165 and VEGF189 was assessed, as well as cell alignment in the flow direction and cell shape index by using image-processing algorithms.

MATERIALS AND METHODS

Nanocomposite and micro-hybrid composite
A nanocomposite (Filtek™ Supreme XT flowable restorative; 3M ESPE, St Paul, MN, USA), and a micro-hybrid composite (Clearfil Majesty™ Flow; Kuraray Medical Inc., Okayama, Japan) were used (Table 1).

Table 1. Dental composite resins used in this study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Type</th>
<th>Filler</th>
<th>Monomer</th>
<th>FS (MPa)</th>
<th>CS (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtek™ Supreme XT</td>
<td>nanocomposite</td>
<td>SiO&lt;sub&gt;2&lt;/sub&gt; (75 nm)</td>
<td>Bis-GMA</td>
<td>125&lt;sup&gt;3&lt;/sup&gt;</td>
<td>375&lt;sup&gt;39&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>zirconia (15-20 nm)</td>
<td>Bis-EMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>zirconia/SiO&lt;sub&gt;2&lt;/sub&gt; (0.6-1.4 μm)</td>
<td>TEGDMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 vol%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearfil Majesty™ Flow</td>
<td>micro-hybrid composite</td>
<td>Ba glass (0.02-19 μm)</td>
<td>TEGDMA</td>
<td>145&lt;sup&gt;+&lt;/sup&gt;</td>
<td>329*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SiO&lt;sub&gt;2&lt;/sub&gt; (0.02-19 μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 vol%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* According to the manufacturer.

FS: flexural strength; CS: compressive strength; Bis-GMA: Bis-phenol A diglycidylmethacrylate; Bis-EMA: ethoxylated bisphenol A dimethacrylate; TEGDMA: Triethyleneglycol dimethacrylate; SiO<sub>2</sub>: silica; Ba: barium.

Donors
Subcutaneous adipose tissue was harvested from residues of abdominal wall resections of five healthy female donors (aged 33, 40, 47, 50, and 54), who underwent elective abdominal wall correction at the Tergooi Hospital Hilversum and a clinic in Bilthoven, The Netherlands.
The Ethical Review Board of the VU Medical Center, Amsterdam, The Netherlands, approved the protocol. Informed consent was obtained from all patients.

**Isolation and culture of hASCs**

hASCs were isolated from the resection material from above mentioned donors as described earlier with minor modifications. In brief, adipose tissue was cut into small pieces, and enzymatically digested with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 45 min at 37°C in phosphate-buffered saline containing 1% bovine serum albumin (Roche Diagnostics GmbH) under continuous stirring. Ficoll® density-centrifugation step (Lymphoprep™, Axis-Shield, Oslo, Norway; 1000xg for 20 min, ρ=1.077 g/ml Ficoll®, osmolarity 280±15 mOsm) was performed to remove remaining erythrocytes from the stromal vascular fraction. The resulting stromal vascular fraction pellet containing hASCs was resuspended in Dulbecco’s modified Eagle’s medium (Life Technologies™ Europe BV, Bleiswijk, The Netherlands), counted, frozen, and stored in liquid nitrogen until further use. Heterogeneity studies including cell characterization and multipotent differentiation potential of these cells have been reported previously by our group.

Cryopreserved stromal vascular fraction-containing cell suspensions were pooled and cultured in α-Minimum Essential Medium (α-MEM; Life Technologies™, Waltham, MA, USA) with 5% platelet lysate, 100 U/ml penicillin (Sigma-Aldrich, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Sigma-Aldrich), and 10 IU/ml heparin (LEO Pharma A/S, Ballerup, Denmark) to prevent coagulation at 37°C in a humidified atmosphere with 5% CO₂. The medium was refreshed three times/week. After reaching confluency, cells were harvested with 0.25% trypsin (Life Technologies™) and 0.1% ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) in phosphate-buffered saline at 37°C, replated, cultured until passage 2 (P2), and stored in liquid nitrogen until further use. Cryopreserved pooled hASCs-containing cell suspensions were thawed and seeded at 0.5x10⁶ cells per T-225 culture flask (Greiner Bio-One, Kremsmuenster, Austria) in α-MEM with 2% platelet lysate, antibiotics, and 10 IU/ml heparin. Cells were cultured until P3-P4, and used for PFF experiments.

**Nanocomposites and micro-hybrid composites preparation**

For PFF experiments, microscopic slides (size 3.6x7.6x0.1 cm; Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) were coated as described. In brief, microscopic slides were grinded with 400 grits Carbimet paper discs (Buehler, Lake Bluff, IL, USA) to provide a rough bonding surface, and covered on two parallel edges with two layers of adhesive tape (height 80 µm) to control the thickness of the composite layer. A thin film of Clearfil SE bond (Kuraray) was applied and light-cured to provide a chemical bond between the microscopic slide and the composite. Nanocomposite or micro-hybrid composite was applied to one of the free edges of a microscopic slide, distributed with a glass rod sliding over the tape-covered edges, and
light-cured (DentaColor XS, Heraeus Kulzer, Wehrheim, Germany) for 180 s. An oxygen inhibition layer was removed by brushing with ethanol, and subsequently with demineralized water. Nanocomposites had a roughness average ($R_a$) of 0.6±0.1 µm (mean±SEM) and micro-hybrid composites a $R_a$ of 1.5±0.1 µm. Composite-coated slides were sterilized with 70% ethanol for 5 min, rinsed with Milli-Q water, and radiated with ultraviolet light.

**Pulsating fluid flow**

One day before PFF-treatment, 1.0x10$^5$ or 2.0x10$^5$ hASCs were cultured on nanocomposite or micro-hybrid composite in a petri dish with 13.5 ml α-MEM with 2% platelet lysate, antibiotics, and 10 IU/ml heparin overnight at 37°C in a humidified atmosphere with 5% CO$_2$. For PFF treatment, medium was replaced by α-MEM containing 2% fetal clone serum and antibiotics. Cells were subjected to 1 h PFF as described earlier.$^{14}$ Briefly, PFF (pulse: 5 Hz, mean shear stress: 0.7 Pa; pulse amplitude: 0.7 Pa; peak shear stress: 22.7 Pa/s) was generated by pumping 13.0 ml PFF medium through a parallel-plate flow chamber containing hASCs on nanocomposite or micro-hybrid composite. Static cultures were kept in a petri dish under similar conditions as the experimental cultures. Medium samples of 500 µl were taken at 5, 15, 30, and 60 min of static or PFF treatment, and assayed for NO production. After 60 min of static or PFF treatment, cells were post-incubated in 2 ml fresh medium α-MEM with 2% fetal clone serum and antibiotics for 2 h without mechanical loading, and conditioned medium was collected for NO determination. Composites with 1.0x10$^5$ hASCs were used for scanning electron microscopy (SEM). Composites with 2.0x10$^5$ hASCs were lysed in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) for total DNA content and RNA isolation for quantitative real-time PCR (qPCR).

**Nitric oxide**

NO production was measured as nitrite (NO$_2^-$) accumulation in conditioned medium using Griess reagent containing 2% sulfanilamide, 0.2% naphtylethelene-diamine-dihydrochloride, and 5% H$_3$PO$_4$. Serial dilutions of 0.1 M NaNO$_2$ in α-MEM were used as a standard curve. The absorbance was measured at 540 nm with a Synergy HT® spectrophotometer.

**DNA content**

DNA was isolated by conventional phenol-chloroform extraction to quantify the total DNA content of hASCs on nanocomposite or micro-hybrid composite. Total DNA concentration was measured at 260 nm with a Synergy HT® spectrophotometer.

**Analysis of gene expression**

Total RNA was isolated and stored at -80ºC prior to further use. Complementary DNA (cDNA) synthesis was performed according to the First Strand cDNA Synthesis kit (Thermo Fisher
Mechanoresponsiveness of hASCs on composites

Scientific, Vilnius, Lithuania) in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA, USA). cDNA was stored at -20°C prior to qPCR analysis, and diluted 5x for gene expression analysis. qPCR reactions were performed using 1 µl cDNA per reaction and LightCycler® 480 SYBR® Green I Mastermix (Roche Diagnostics GmbH) in a LightCycler® 480 (Roche Diagnostics GmbH). qPCR conditions for all genes were as follows: 10 min pre-incubation at 95°C, followed by 45 cycles of amplification at 95°C for 10 s, 56°C for 5 s, and 78°C for 5 s, after which melting curve analysis was performed. With Light Cycler® software (version 1.2), crossing points were assessed and plotted versus the serial dilution of known concentrations of the internal standard. PCR efficiency (E) was obtained by using the formula E=10\(^{-1/\text{slope}}\). Data were used only if E=1.85-2.00. For gene expression analysis, the values of target gene expression were normalized using a factor derived from the equation \((\text{YWHAZ} \times \text{HPRT1})^{1/2}\) to obtain relative gene expression. qPCR was used to assess expression of the following genes: runt-related transcription factor-2 (RUNX2), alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON), dentin matrix acidic phosphoprotein-1 (DMP1), vascular endothelial growth factor-165 (VEGF165), vascular endothelial growth factor-189 (VEGF189), collagen-1 (COL1), nitric oxide synthase-3 (NOS3), and cyclooxygenase-2 (COX2). Primer sequences used for qPCR are listed in Table 2.

hASC alignment and shape index on nanocomposite and micro-hybrid composite

hASCs on nanocomposite and micro-hybrid composite were fixed with 4% paraformaldehyde (Merck), 1% gluteraldehyde (Merck), and 0.1 M natriumcacodylate (pH 7.4) (Merck) for 2.5 h. To visualize hASCs using SEM, samples were dehydrated in a graded series of ethanol (35%, 50%, 70%, 80%, 90%, 96%, 100%), and overnight air dried in hexamethyldisilazane (Sigma-Aldrich, St. Louis, MO, USA). To evaluate cell alignment and cell shape index, the specimens were sputter-coated with gold and examined using a scanning electron microscope XL20 (FEI Europe B.V., Eindhoven, The Netherlands) at an accelerating voltage of 15 kV. Cell alignment and cell shape index were quantified using an image-processing algorithm developed in MATLAB (Mathworks, Natick, MA, USA). SEM images were first imported in MATLAB for further analysis (Fig. 1A). After the images were converted to a grayscale format and a noise-reduction filter was used, a gradient detection algorithm was applied to the images. A binary threshold was applied on the gradient image by setting the mean gray value as the threshold value (Fig. 1B). Moreover, a geometrical filter (Fig. 1C) was applied to remove remaining small artifacts. The cell orientation angle was considered as the angle between the longest diameter of the cells with the horizontal (x) axis. Finally, a histogram containing all cell orientation data in each image was obtained (Fig. 1D). The mean angle and dispersity of the cell distribution were calculated for each image by applying a density function to the histogram. Shape index (SI), as a measure of cell elongation, was calculated as
[SI = \((4\pi \times \text{area})/\text{perimeter}^2\)]. A circle has a shape index of 1 and a line has a shape index of zero.\textsuperscript{27,28}

Table 2. Primer sequences for determination of gene expression of osteogenic, vasculogenic, and mechanosensitive markers through PCR. Tyrosine 3-monoxygenase / tryptophan 5-monoxygenase activation protein, zeta (YWHAZ); hypoxanthine phosphoribosyltransferase (HPRT1); runt-related transcription factor-2 (RUNX2), alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON), dentin matrix acidic phosphoprotein-1 (DMP1), vascular endothelial growth factor-165 (VEGF165), vascular endothelial growth factor-189 (VEGF189) collagen-1 (COL1), nitric oxide synthase-3 (NOS3), and cyclooxygenase-2 (COX2); bp: base pairs.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>YHWAZ</td>
<td>Forward 5’ gATgAAGCCATTgCTGAACCTTg 3’</td>
<td>229</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CTTTTgTgggACAgCATggA 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>Forward 5’ gCTgACCTgCTggATTACAT 3’</td>
<td>260</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CTTgCgACCTTgACCATCT 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>Forward 5’ ATgCTTCATTcGcCTCACC 3’</td>
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<td>56</td>
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<td></td>
<td>Reverse 5’ ACTgCTTgCAgCCTTAAT 3’</td>
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<td></td>
</tr>
<tr>
<td>ALP</td>
<td>Forward 5’ AgggACATTgACgTgATCAT 3’</td>
<td>242</td>
<td>56</td>
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<tr>
<td></td>
<td>Reverse 5’ CCTggCTCgAAgAgACC 3’</td>
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<td></td>
</tr>
<tr>
<td>OPN</td>
<td>Forward 5’ TTCCAAGTAAGTCCAACgAAAg 3’</td>
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<td>56</td>
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<tr>
<td></td>
<td>Reverse 5’ gTgACCAgTTCATCAgATTCAT 3’</td>
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<tr>
<td>ON</td>
<td>Forward 5’ CtgTCCAggTggAAgTAgg 3’</td>
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<td>56</td>
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<tr>
<td></td>
<td>Reverse 5’ GTggCAggAAgAgTcgAag 3’</td>
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<td></td>
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<tr>
<td>DMP1</td>
<td>Forward 5’ TAggCTAgCTggCTTCT 3’</td>
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<td>56</td>
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<td></td>
<td>Reverse 5’ AACTCggAgCCgTCTCCCAT 3’</td>
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<tr>
<td>VEGF165</td>
<td>Forward 5’ ATCTTCAAgCCATCTCTgTgAC 3’</td>
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<td></td>
<td>Reverse 5’ CAAgCCgCACgAGggATTITT 3’</td>
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<tr>
<td>VEGF189</td>
<td>Forward 5’ ATCTTCAAgCCATCTCTgTgAC 3’</td>
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<td></td>
<td>Reverse 5’ CACAgggAAgCCgCTCCAggAC 3’</td>
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<tr>
<td>COL1</td>
<td>Forward 5’ TCCggCTCCTgCTCCCTT 3’</td>
<td>336</td>
<td>56</td>
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<td></td>
<td>Reverse 5’ ggCCgAgTgTCTCCCTTg 3’</td>
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<td></td>
</tr>
<tr>
<td>NOS3</td>
<td>Forward 5’ AAgCCgCATAgCACCACgAgAg 3’</td>
<td>346</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ TgggTACCgCTgCTggAgg 3’</td>
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<td></td>
</tr>
<tr>
<td>COX2</td>
<td>Forward 5’ gCATTCTTtgCCAgACCTT 3’</td>
<td>299</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ AgACCgAgCACCACgACCACgA 3’</td>
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</table>
Mechanoresponsiveness of hASCs on composites

**Figure 1.** Image-processing procedure used to determine cell alignment and cell shape of hASCs cultured on nanocomposite and micro-hybrid composite. (A) Representative scanning electron microscopic image of 1 h PFF-treated hASCs on a micro-hybrid composite after 2 h of post-incubation, (B) Corresponding binary image before removal of small artifacts, (C) Corresponding binary image after removal of small artifacts, (D) Histogram containing cell orientation data. White arrow: direction of fluid flow. SD, standard deviation. Magnification: 100x.

**Statistical analysis**

Data were obtained from four independent experiments for hASCs on nanocomposite or micro-hybrid composite. Data are mean±SEM. Data were tested with an independent-samples t-test. Differences were considered significant if p<0.05. Statistical analysis was performed using IBM® SPSS® Statistics version 21 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism® 5.0 (GraphPad Software Inc., La Jolla, CA, USA).
RESULTS
NO production in response to PFF was increased in hASCs on nanocomposite, but not on micro-hybrid composite, compared to static control conditions after 5 min (1.4-fold), 30 min (3.3-fold); and 60 min (4.2-fold; Fig. 2A-C). NO production was similar in hASCs on micro-hybrid composite with or without PFF treatment at all time points (Fig. 2D-F).

**Figure 2.** Effect of 1 h PFF on NO production by hASCs on nanocomposite and micro-hybrid composite after 2 h of post-incubation. (A) PFF rapidly, within 5 min, increased NO production by hASCs on nanocomposite. (B) PFF did not affect NO production in hASCs on nanocomposite after 2 h of post-incubation. (C) PFF increased NO production by hASCs on nanocomposite after 30 min. (D-F) PFF did not affect NO production by hASCs on micro-hybrid composite. (C-F) Values are mean±SEM of PFF over static control ratios (PFF/static, n=7-10), dashed line, PFF/static=1 (no effect). *Significantly different from static control cultures, p<0.05. NO, nitric oxide; PFF, pulsating fluid flow.
Gene expression of the early osteogenic differentiation marker *RUNX2* was increased in hASCs on micro-hybrid composite, but not on nanocomposite, after 1 h PFF treatment (2.3-fold) compared to static controls (Fig. 3A). Gene expression of osteogenic markers *ALP, OPN, ON, DMP1, COL1*, nitric oxide synthase marker *NOS3*, mechanosensitivity marker *COX2*, vasculogenic markers *VEGF165*, and *VEGF189* were not affected by PFF treatment for 1 h in hASCs on both composites (Fig. 3B-J). The expression level of *ALP, ON, COL1*, and *VEGF165* was low, but high for *OPN, VEGF189, COX2*, and *NOS3* was high (Fig. 3B-J).

![Figure 3](image_url)

**Figure 3.** Effect of 1 h PFF on (A) *RUNX2*, (B) *ALP*, (C) *OPN*, (D) *ON*, (E) *DMP1*, (F) *VEGF165*, (G) *VEGF189*, (H) *COL1*, (I) *NOS3*, and (J) *COX2* gene expression in hASCs on nanocomposite and micro-hybrid composite after 2 h of post-incubation. (A) PFF increased *RUNX2* expression in hASCs on micro-hybrid composite. (B-K) PFF did not affect *ALP, OPN, ON, DMP1, VEGF165, VEGF189, COL1, NOS3*, and *COX2* expression in hASCs on nanocomposite and micro-hybrid composite. Values are mean±SEM (n=6-9). PFF, pulsating fluid flow; nano-comp, nanocomposite; micro-hybrid comp, micro-hybrid composite; *RUNX2*, runt-related transcription factor-2; *ALP*, alkaline phosphatase; *OPN*, osteopontin; *ON*, osteonectin; *DMP1*, dentin matrix acidic phosphoprotein-1; *VEGF165*, vascular endothelial growth factor-165; *VEGF189*, vascular endothelial growth factor-189; *COL1*, collagen-1; *NOS3*, nitric oxide synthase-3; *COX2*, cyclooxygenase-2.
The mean angle of hASCs on nanocomposite under static condition was not significantly different from PFF condition (static: 12.5±3.5 (mean±SEM); PFF: 11.7±4.0) as well as for hASCs on micro-hybrid composite (static: 9.8±1.4 (mean±SEM); PFF: 14.8±3.3) under both conditions (Fig. 4A). There were no differences in dispersity between static and PFF conditions for hASCs on nanocomposite (static: 78.1±0.0 (mean±SEM); PFF: 78.1±0.0) as well as for hASCs on micro-hybrid composite (static: 79.0±0.9 (mean±SEM); PFF: 78.1±0.0; Fig. 4B).

After 2 h of post-incubation, 1 h PFF treatment did not change the shape index of hASCs on nanocomposite (static: 0.60±0.01 (mean±SEM); PFF: 0.60±0.06), nor on micro-hybrid composite (static: 0.60±0.04 (mean±SEM); PFF: 0.60±0.01; Fig. 4C). hASCs on nanocomposite as well as on micro-hybrid composite showed a spindle-shaped morphology under static and PFF conditions (Fig. 4D).

**Figure 4.** Effect of 1 h PFF on mean angle and dispersity of cell distribution, and shape index of hASCs on nanocomposite and micro-hybrid composite after 2 h of post-incubation. (A, B) PFF treatment did not affect the mean angle and dispersity of cell distribution of hASCs on nanocomposite nor on micro-hybrid composite. (C) PFF did not change the shape index of hASCs on nanocomposite nor on micro-hybrid composite. (D) Spindle-shaped morphology of hASCs on nanocomposite or on micro-hybrid composite under static and PFF conditions. PFF, pulsating fluid flow; deg, degrees. Magnification: 100x.
DISCUSSION

We investigated whether differences exist in the response to PFF of stem cells on nanocomposite or micro-hybrid composite. We found that (i) PFF rapidly, within 5 minutes, increased NO production by hASCs on nanocomposite, but not on micro-hybrid composite; (ii) PFF increased \textit{RUNX2} expression in hASCs on micro-hybrid composite, but not on nanocomposite after 2 h post-incubation; (iii) PFF did not affect mean cell orientation and shape index of hASCs on both composites after 2 h post-incubation. Therefore, the PFF-increased NO production by hASCs on nanocomposite, and increased \textit{RUNX2} gene expression in hASCs on micro-hybrid composite, suggest different responses to mechanical loading by PFF of hASCs on composite with filler particles of different size, and chemical composition.

Mechanosensitivity of bone cells increases during bone cell differentiation.\textsuperscript{14,29-31} PFF increases NO synthesis by bone cells and osteogenically stimulated ASCs.\textsuperscript{13,14} Therefore, the rapidly PFF-increased NO production by hASCs on nanocomposite, but not on micro-hybrid composite, might indicate a more bone-cell like response to mechanical loading for hASCs on nanocomposite than micro-hybrid composite.

PFF increased expression of the early osteogenic differentiation marker \textit{RUNX2} in hASCs on micro-hybrid composite, but not on nanocomposite, indicating an early stage of osteogenic differentiation of hASCs on micro-hybrid composite. hASCs on both composites did not upregulate gene expression of late osteogenic differentiation markers \textit{ALP}, \textit{OPN}, \textit{ON}, \textit{DMP1}, and \textit{COL1} after mechanical loading by PFF. This suggests that hASCs have not yet reached the differentiation state needed to respond to PFF with change in gene expression of late osteogenic markers. Possibly, the parameters of our physiological mechanical loading regime used in this study,\textsuperscript{9,13,22,23} e.g. duration of stimulation and/or post-incubation, may not have been optimal for ASCs to evoke a mechanical response. Polycaprolactone with surface roughness $R_a \sim 2.1$-$3.1 \mu m$ promotes faster osteogenic differentiation of human bone marrow derived mesenchymal stem cells than polycaprolactone with surface roughness $R_a \sim 0.64$-$0.80 \mu m$.\textsuperscript{32} Therefore, another explanation for the PFF-increased \textit{RUNX2} expression in hASCs on micro-hybrid composite might be a rougher surface ($R_a = 1.5 \mu m$) than nanocomposite ($R_a = 0.6 \mu m$). Osteogenically differentiated hASCs exhibit longer cilia than undifferentiated hASCs.\textsuperscript{33} Therefore, it might be possible that the primary cilium of hASCs on micro-hybrid composite, implicated as a mechanosensor in a variety of cell types, is longer than on nanocomposite.\textsuperscript{34,35} Further studies are needed to confirm the length of the primary cilium of hASCs on both composites.

Mechanical loading of ASCs increases NO production and gene expression of \textit{COX2},\textsuperscript{9,13} which are implicated in mechanical adaptation of bone, and expression of \textit{RUNX2}, which is a transcription factor expressed in early osteogenic differentiation.\textsuperscript{13} PFF increased NO production in hASCs on nanocomposite, but not on micro-hybrid composite, and did not
upregulate expression of COX2. Mechanical loading by PFF increased RUNX2 expression in hASCs on micro-hybrid composite, but not on nanocomposite. Besides these contradictions with our previous findings, possibly due to the use of different coating materials, ASCs on both composite showed a bone-cell like response to PFF. The PFF-increased NO response by hASCs on nanocomposite, and increased RUNX2 expression in hASCs on micro-hybrid composite can not be explained by cell mean orientation or shape since we did not find any difference. Flow-induced cell alignment is highly dependent on the magnitude and exposure time of the flow applied as well as attachment time prior to flow. Cell attachment to biomaterial surfaces depends on adhesion time and adhesion force which are dependent on e.g. cell type and cell substrate. Human mesenchymal stem cells are more mechanosensitive after a longer time to attach prior to PFF.\textsuperscript{36} Round shaped osteocytes are more mechanosensitive than flat shaped.\textsuperscript{17} hASCs on both composites were spindle-shaped, suggesting similar mechanosensitivity based on cell shape. Therefore, mechanosensitivity of hASCs on both composites might also be greater after cells have longer time to attach prior to PFF. Much research has been done on the response of ASCs to mechanical forces and the underlying molecular mechanisms by which mechanical forces are transduced into physiological responses, but the exact mechanisms are still unknown. ASCs show an osteogenic response to our physiological regime used in this study,\textsuperscript{9,13,22,23} but possibly the pulse and mean shear stress may not have been optimal for ASCs on both composites to evoke a mechanical response. Future studies will address whether different mechanical loading regimes including higher magnitude and/or frequency of PFF will affect the mechanoresponsiveness of hASCs on both composites.

PFF increased NO production in hASCs on nanocomposite and osteogenic differentiation potential of hASCs on micro-hybrid composite, but did not affect cell mean orientation and shape index of hASCs on both composites. Therefore, we conclude that differences exist in the response to PFF of stem cells on nanocomposite and micro-hybrid composite, suggesting different responses to mechanical loading of hASCs on composite with nanometer-sized and micrometer-sized filler particles which might have important implications for bone tissue engineering.
REFERENCES


Mechanoresponsiveness of hASCs on composites


