Cell Interactions at the Nanoscale: Piezoelectric Stimulation

Adam S. G. Curtis*, Stuart Reid, Iain Martin, Ramanathan Vaidyanathan, Carol-Anne Smith, Habib Nikukar, and Matthew J. Dalby

***Abstract*—Nanometric movements of the substrate on which endothelial cells are growing, driven by periodic sinusoidal vibration from 1 Hz to 50 Hz applied by piezo actuators, upregulate endothelin-1 and Kruppel-like factor 2 expression, and increase cell adhesion. These movements are in the z (vertical) axis and ranges from 5 to 50 nm and are similar in vertical extent to protrusions from the cells themselves already reported in the literature. White noise vibrations do not to produce these effects. Vibrational sweeps, if suitably confined within a narrow frequency range, produce similar stimulatory effects but not at wider sweeps. These effects suggest that coherent vibration is crucial for driving these cellular responses. In addition to this, the applied stimulations are observed to be close to or below the random seismic noise of the surroundings, which may suggest stochastic resonance is being employed. The stimulations also interact with the effects of nanometric patterning of the substrates on cell adhesion and Kruppel-like factor 2 and endothelin-1 expression thus linking cell reactions to nanotopographically patterned surfaces with those to mechanical stimulation.**

***Index Terms*—Cell adhesion, endothelial cd 133+ cells, gene expression, nanoscale mechanical stimulation, stochastic resonance.**

I. Introduction

CELLS LIVE IN environments where there are often nanometrically sized features, arising from the presence of other cells, intercellular materials, parasites, and even some prosthetic devices. There is also often mechanical stimulation which can be repetitive, small in magnitude for example fluid circulating systems (e.g., blood flow) or acoustical sound. This paper addresses experimental systems where both mechanical stimulation and nanofeatures are brought into interaction at the nanoscale.

There have been many reports that surfaces that bear nanotopographic patterns modify cell adhesion [1]–[5] thereby reducing it in several situations but increasing it in the interesting case where the regularity of the pattern is deliberately degraded (controlled disorder rather than randomness). The processes involved are unclear, possibly in part because few experimental methods of modifying such reactions have been reported. We chose to investigate the possible effects of mechanical vibration on cells grown on nanotopographic patterns because considerable similarities have been reported in the effects of mechanical stimulation of cells grown on planar unpatterned surfaces [6]–[12] to that of growth of cells on various nanotopographic patterns [3]–[5]. Mechanical vibration has been reported to change adhesion and gene expression [13], [14] at 100–700 nm amplitude. These similarities include features of cell adhesion, cell cycle duration, cell shape, cytoskeletal assembly and gene expression, etc. Recently we [15] have shown that nanoscale low frequency vibration will lead to changes in the expression of gene products and differentiation in human mesenchymal stem cells. In this paper we test whether the findings of [15] can be duplicated in another cell type (endothelia) examining different expression factors (Krüppel-like factor 2 (KLF-2) and endothelin 1) and testing still lower signal amplitudes.

Another related reason for choosing this topic lies in the work of Pierres *et al.* [16], [17] who showed that as cells settle and adhere to a planar surface the cells protrude and retract very small "toes" towards and away from the surface. Such moving protrusions up to about 50 nm length could be expected to have local nanomechanical effects. Such work relates to earlier reports [18], [19] on nanotopography and its effects on cells.

Thus, we chose to examine the effects of imposing very small-scale movements on cells in the 5 to 50 nm range. The need for accurate, fast, and reproducible (and perhaps repetitive) movements on this scale suggests the use of piezo actuators, because they can meet these requirements. The use of piezoactuators allows questions about the importance, if any, of frequency and of temporal stimulation pattern to be considered as well. Because mechanical stimulation is known to alter gene expression and cell adhesion we considered whether the nanoscale piezo stimulation we used would have similar effects. Investigations in this area may throw light on the linkage, if any, between mechanotransduction and the reaction of cells to the nanoenvironment and the ways in which biomaterial properties may affect this. The mechanical displacements applied to cell cultures are shown to be far away from any resonant condition of the setup, suggesting that no unintended amplification was present above the stated amplitudes.

II. Materials and Methods

A. Substrata

Tissue culture-grade polystyrene culture vessels were used as the substrata for the culture of cells.

Manuscript received October 26, 2012; revised March 20, 2013; accepted April 08, 2013. Paper submitted on 18th March 2013. This work was supported by the BBSRC, EPSRC, STFC and the Royal Society of Edinburgh. *Asterisk indicates corresponding author.*

*A. S. G. Curtis is with the Centre for Cell Engineering, University of Glasgow, Glasgow G12 8QQ, U.K. (e-mail: adam.curtis@glasgow.ac.uk).

S. Reid is with the Thin Film Centre, University of West of Scotland, Paisley PA1 2EB, U.K. (e-mail: stuart.reid@uws.ac.uk).

I. Martin is with the Institute for Gravitational Research, University of Glasgow, Glasgow G12 8QQ, U.K. (e-mail: iain.martin@glasgow.ac.uk).

R. Vaidyanathan is with AIBN, University of Queensland, St Lucia, QLD 4072 Australia (e-mail: r.pudhukodevaidyanath@uq.edu.au).

C.-A. Smith, H. Nikukar, and M. J. Dalby are with the Centre for Cell Engineering, University of Glasgow, Glasgow G12 8QQ, U.K. (e-mail: carol-anne.smith@glasgow.ac.uk; h.nikukar.1@research.gla.ac.uk; Matthew.Dalby@glasgow.ac.uk).

Color versions of one or more of the figures in this paper are available online at http://ieeexplore.ieee.org.

Digital Object Identifier 10.1109/TNB.2013.2257837

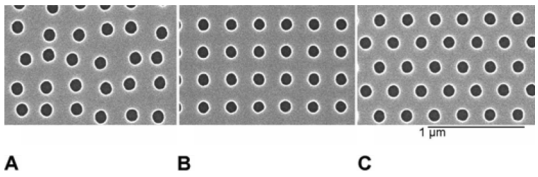


Fig. 1. Examples of nanopit topography generated from electron-beam lithography. A. shows a Near-Square (NSQ pattern, B. a Square pattern (FSQ pattern) and C. a Hexagonal array (not used in this work).

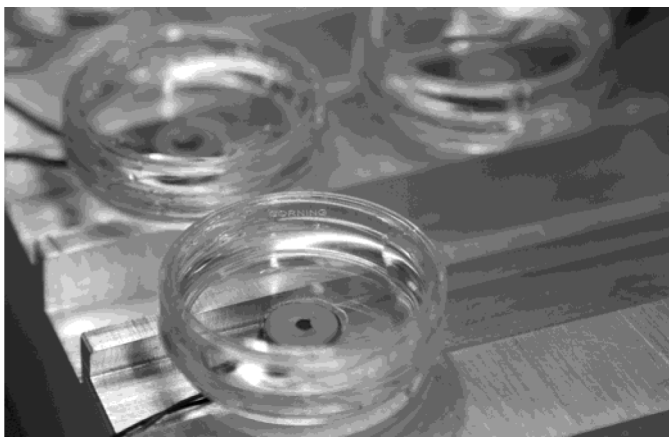


Fig. 2. To illustrate general method of mounting a piezoactuator on the base of a petri dish.

Nanotopographically patterned surfaces in polycarbonate were kind gifts from N. Gadegaard and R. Petersen (School of Engineering, University of Glasgow) fabricated by techniques [20] which yielded the same surface patterns as those described before [1]–[5]. Each surface carried control unpatterned areas and arrays of nanopits in square array, and the near-square (controlled disorder) array, where each category of pattern covered 1 cm^2 each and where the pits were 120 nm diameter 77 and 100 nm deep, as shown in Fig. 1.

For the square arrays there was an absolute center-to-center spacing of 300 nm. For the disordered arrays the center-center spacing was an average of 300 nm in a square arrangement but each pit had up to 50 nm center offset in both dimensions. For the random patterns (not illustrated) a 1 mm^2 array was written with a random center-center placement and then tiled to fill 1 cm^2. These patterned surfaces were attached to the culture dishes with epoxy cements. The use of nanopits in square or hexagonal pattern is known to reduce cell adhesion [6] and the near-square array increases cell adhesion [1], [4].

B. Mechanical Actuation

Piezoelectric transducers model no. 010–05H from Physik Instrumente, Karlsruhe/Palmbach, Germany were used to generate the required mechanical stimulation. The underneath sides of the culture flasks/dishes (52 mm diameter) or nanostructured surfaces were attached to the piezostacks by applying solvent-free (PVA) adhesive or epoxy adhesive from Bostik, Stafford, U.K., as shown in Fig. 2.

Immediately thereafter the structures were tested for fiducial replication of pattern and that the impedance between the culture region and the piezoelement was high [$> 1\ \mathrm{M}\omega$].

The culture regions were sterilized with 70% ethanol in a sterile airflow and then residual ethanol was washed out with

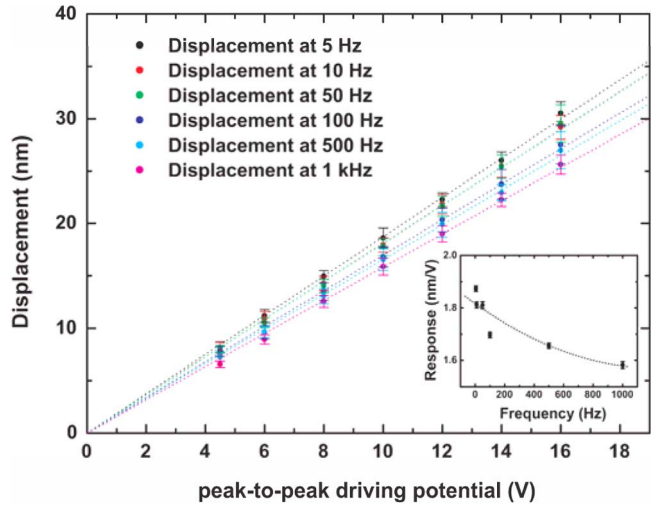


Fig. 3. Vertical displacements of the substratum under various drive conditions for the piezo actuator.

sterile Hanks' saline. The devices were then stored sterilely before use.

The piezostacks were driven by a waveform generator [Marconi Instruments, St. Albans, U.K., type TF 2120] to produce a sine wave 10V peak-to-peak output or an Agilent 33210A function generator (Agilent, Santa Clara, CA, USA) driving PI 010–05H ring-type piezo stacks to produce 5–10 V peak-to-peak sine wave potentials. In addition, one-to-one transformers were used to center bias the voltages in order to maintain positive-only potentials to the piezo devices. The Agilent generator was also used to provide the white noise and sweep signals.

The small substrate displacements suggested to us that the actual forces applied to the cells might be so small as to lie close to the random thermal noise limit. Therefore if stimulation at this level were effective it would imply that coherence may be important. However, a white noise signal would decohere the signal. Thus, if using a white noise signal decoheres the signal and then subsequently removes cellular effects, this would provide evidence that coherent vibration was important in the reaction of cells to the substrata at low signal levels.

The piezoactuators move the culture surface (polystyrene cell culture grade or polycarbonate when nanotopography was being studied) in a direction normal to the cells, i.e., z-axis. Since the cells are already adherent this means that during the part of the movement of the cell toward the substratum the actuator the cell may be compressed or indented by the movement of its substrate and during the opposite movement stretched.

C. Piezoelectric Transducer Calibration

The piezo devices used for inducing the mechanical stimulation were all calibrated between 1 Hz and 1 kHz over a range of sine wave driving potentials. For displacements below 100 nm calibration curves typically started at 5 Hz due to seismic vibration (background noise) tending to saturate the readout at lower frequencies. The overall response of all the piezo devices was observed to be broadly linear with respect to the driving potential at constant frequency and to the frequency at constant driving potential as can be seen Fig. 3.

Displacements were sensed using a commercial SIOS interferometer model SP-S120, SIOS Meßtechnik gmbH, D-98693 Ilmenau, Germany, mounted on an air table with an average of

8 measurements per data point and the corresponding standard error.

D. Stimulation Regime for Cultures

Stimulation at 1 Hz, 2 Hz, 37 Hz, and 50 Hz at 5 or 10 V peak-to-peak drive was carried out for 16 to 24 hours. The cultures were then fixed and stained, and images of the cells adherent on the surfaces acquired over areas of 480 000 $\mu\mathrm{m}^2$. The number of adherent cells per counting area was measured.

E. Cell Culture

Le-2 endothelial cells, a line of mouse lung capillary endothelia, [21] (from B10D2 congenic mice, cd 133+) were plated out after suspension by trypsinisation into polystyrene culture flasks. For experiments, the plating numbers of cells were 1×10^4 cells ml^{-1} diluted into 5 ml of culture medium in flasks or petri dishes of surface area approx 25 or 38 cm^2. The endothelial cells were grown in Ham's F10 medium with 3% fetal bovine and insulin-selenite-transferrin supplement for 30 to 48 hours. At the completion of the experiments the cultures were fixed in 4% formol saline followed by permeabilisation with Triton X 100 in buffer (tris pH 7.6) for 15 minutes if immunofluorescent staining was to be carried out. Otherwise, cells were stained in 1% aqueous methylene blue or fixed and then stained in Coomassie Blue stain in acetic acid/methanol.

F. Cell Viability

Cell viability was measured by counting the proportion of cells that fluoresced green with 488 nm stimulation after incubation in calcein AM at 37 °C for 30 minutes as compared to those that incorporated ethidium bromide.

G. Detecting Changes in Gene Expression

Adhesion was measured as the percentage of cells adhering to a specified substrate specified from a cell suspension in four hours. A cell was judged to be in adhesion if the cell was not rounded in plan view and was resistant to detachment in a shear flow of approximately $4\,\mathrm{sec}^{-1}$. Such cells did not detach spontaneously and nearly all proceeded to spread more extensively over the next 24 hours.

H. Detecting Changes in Gene Expression

1) Immunocytochemical Methods: After periods of stimulation, the test and control cells were fixed in 4% formol-saline for 5 minutes, permeabilized, and then stained with rabbit anti-KLF2 antibody H60 for 3 hours (sc-28675, Santa Cruz Biotechnology, CA, USA) followed by sulforhodamine-conjugated Goat anti-rabbit IgG antibody (Vector Laboratories, Peterborough, U.K.) for 2 hours. A similar protocol was used to test for Endothelin-1 production using Rabbit polyclonal anti-Endothelin reagent Abbiotec (San Diego, CA, USA, Lot 11100305). The fixed cells were washed repeatedly with PBS between and after each treatment with antibody reagents. Finally, the cells were examined and imaged using a Leitz fluorescence microscope operating at 546 nm excitation.

2) RT-PCR Methods: After piezo stimulation at constant frequency the cells were trypsinised to make suspensions and then centrifuged. RNA was extracted from cell pellets using the RNeasy Micro Kit (Qiagen, Crawley, U.K.) following the manufacturer's instructions.

cDNA synthesis was performed on the extracted RNA using QuantiTect Reverse Transcription Kit (Qiagen, Crawley, U.K.). 1 μl of extracted RNA was incubated with 2 μl gDNA Wipeout buffer and 1 μl RNase-free water for 2 minutes at 42 °C. This was then added to a reaction mix containing 1 μl QuantiTect Reverse Transcriptase, 4 μl QuantiTect buffer, and 1 μl primer mix, Primers used were RT-PCR primers and was incubated for 15 minutes at 42 °C, then 3 minutes at 95 (°C).

Primer Sequence Product Tm (°C):
NFkB Forward
CAGCTGGCTGAAGATGTGAA 81
NFkB Reverse
GTGTTTTGGAAGGAGCAGGA 81
Endothelin 1 Forward
GGAGAAACCCACTCCCAGTC 86
Endothelin 1 Reverse
GATGTCCAGGTGGCAGAAGT 86

Real Time PCR [22], [23] was carried out on the 7500 Sequence Detection System (Life Technologies UK). 2 μl cDNA was added to a reaction mixture $(20\mu\mathrm{l})$ containing 10 μl TaqMan Gene Expression Mastermix (Life Technologies, UK), and 1 μl of TaqMan Gene Expression Assay (Life Technologies, UK). The reaction mix was incubated a 95 °C for 5 minutes followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Confirmation of product was from melting curve analysis.

Each assay and controls were repeated three times with three samplings of each separate one so nine estimates of standard errors of the repetitions were obtained for each treatment. The significance of differences between control and experimental RT-PCR ratios was calculated by ANOVA.

3) Statistical Methods: 2-way ANOVA was applied to examine the interaction of nanotopography and vibration of nanostructures for both cell adhesion and KLF2 expression.

III. Modeling of Forces Applied to Cells

A. Average Force on Entire Cell

It is of interest to estimate the mechanical forces being applied to individual cells as a result of mechanical stimulation. During each experiment cells become adherent to the substrate surface and will be supporting a column of aqueous solution above, of height 3 mm to 5 mm. Any force being applied to this column of solution by the piezoelectric transducer must therefore be transmitted through the cell. Taking the typical surface area of the adhered cells to be 50 $\mu\mathrm{m}$ by 20 $\mu\mathrm{m}$, the force transmitted through each cell is simply the accelerative force, being the product of the mass of the column of solution and the acceleration, as described in Newton's second law. The peak accelerative force is therefore estimated to be in the range 0.03 pN to 0.05 pN at 5 Hz and 20 nm peak-to-peak displacement (Fig. 4).

The maximum peak force applied at 50 Hz would be in the range 3 pN to 5 pN. It should be noted that viscous forces may be a contributing factor in the cell responses reported here. In order to help evaluate this, the motion of the top surface of the water solution with respect to the petri dish was recorded using the interferometric instruments described previously. The solution surface and piezo followed each other within the measurement accuracy achievable (a few nm) and therefore viscous forces will be assumed to be negligible. It can also be demonstrated that the stimulation frequencies used here are well below any resonant condition of the cells, solution and/or enclosure. For

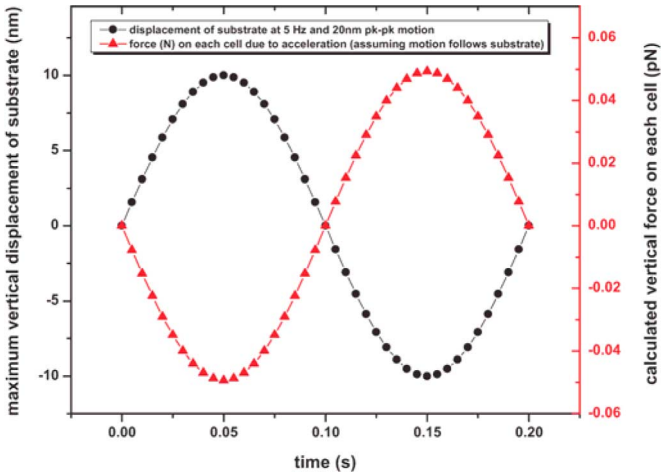


Fig. 4. Substrate displacement as a function of time compared to the calculated vertical accelerative force on each cell, assuming the cell and substrate move as a rigid body and a solution (culture medium depth/height of 5 mm).

this reason, the setup is expected to directly follow the piezo displacement and behave as a rigid body.

Though it would have been desirable to use techniques such as AFM to measure these forces our experimental system is not amenable to force measurement methods such as AFM since both the substratum and the AFM probe would be moving independently.

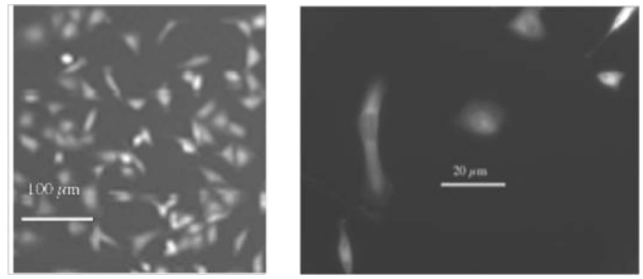
Another question is whether the stimulation causes changes in the separation of cell and substratum. The acceleration of the cells due stimulation can be estimated to be around $4 \times 10^{-7}\ \mathrm{m/s^2}$ at 1 Hz and $1 \times 10^{-3}\ \mathrm{m/s^2}$ at 50 Hz, assuming both have 20 nm peak-to-peak motion. The gravitational force on the cell will be the product of gravity (9.81) and the difference in density of the cell verses the water solution. Knowing that the density of endothelial cells to be in the range 1.03 to 1.05 $\mathrm{g\,cm^{-3}}$, then the static force of gravity remains at least 20 times larger than the stimulation at 50 Hz. For all intents and purposes, the cell sees the same effect as if gravity is being modulated by 1 part in 20 at most. Therefore, this appears as a negligible effect and cannot explain how the cells would change their separation with the substrate during stimulation. In addition, the evidence that cell adhesion is required before stimulation for changes in the cells to result suggests that cell and substrate move as one.

B. Other Mechanisms of Mechanotransduction

There could be other possible mechanisms for the cell responses reported here, which cannot be excluded based on basis of the experimental evidence. Further experiments would be required in order to identify the specific mechanism(s) that are responsible within this area of mechanotransduction. The role of other cellular components, such as the microfilament scaffold and the cell membrane, may also be critical in these responses.

IV. Results

Since mechanical stimulation of cells can lead to increased gene expression especially of transcription factors [8], [9] we looked for this but shifted the mechano-stimulation to very low intensity (nanoscale) repetitive stimulation which to the best of our knowledge has not been examined previously. KLF2 and endothelin are two gene products which have already been shown to be stimulated by more intense (x:y axes stimulation). Results are shown in Figs. 5(a), (b), 6, and Table I.



(a) (b)

Fig. 5. KLF2 expression, stained with antiKLF 2antibody. Fig. 5(b) Endothelim-1 expression, stained with anti-endothelin-1. In each case, the second antibody was sulforhodamine labeled anti-mouse antibody. Piezo-stimulation 5V pk to pk.

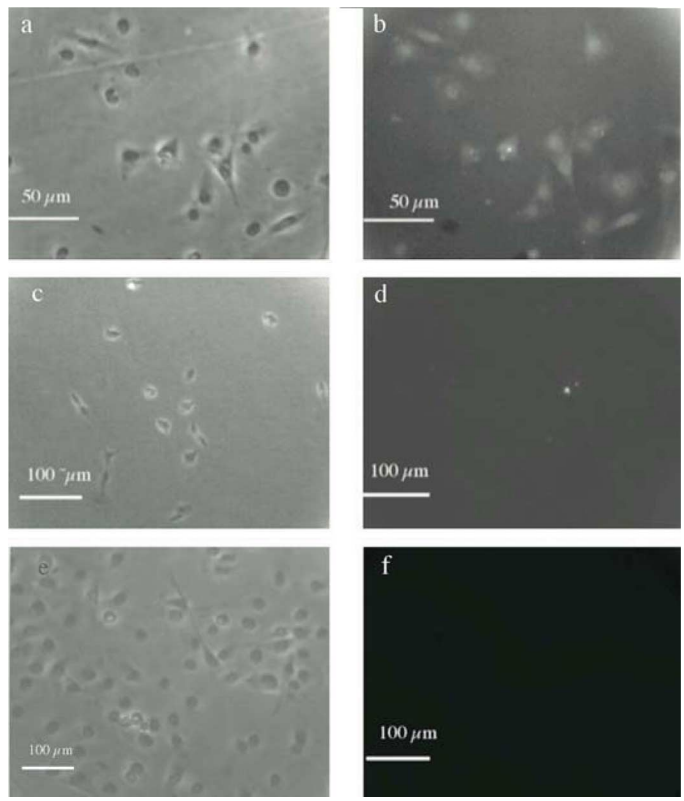


Fig. 6. KLF2 expression in Le2 cells. LH column: phase contrast images of the same fields viewed by fluorescence microscopy in the RH column. a and b: 24 hours piezo-stimulation 5 V peak-to-peak. At this voltage level stimulation is weak and piezo movement low at ca. 5 nm. For results of more intense stimulation, see Fig. 5(a), (c), and (d): white noise stimulation and f: no stimulation.

A. KLF2 and Endothelin-1 Expression

The repeated, continuous stimulation of the cultures by the piezoactuators over periods of at least 24 hours (after 2–4 hours non-stimulation to allow cell settlement) does not appear to damage or kill the cells. The regular substrate excursions in the range 5–20 nm lead to increased expression of genes KLF2 [9] KLF2 and endothelin-1 [10] as evidenced by immunocytochemical results (Figs. 5(b) and 6) and also by RT-PCR data (Fig. 7).

The immunocytochemical detection of KLF2 shows that cells treated with white noise [Fig. 6(d)] and those in static cultures [Fig. 6(f)] showed little or no KLF2 expression.

TABLE I
EFFECT OF NANOSCALE STIMULATION ON CELL ADHESION ON VARIOUS NANOTOPOGRAPHIES

Surface	0 Hz	1 Hz	50 Hz	Test of significance
Square FSQ	Mean 10.6 SD 2.25 n=7	Mean 25.5 SD 5.26 n=4	Mean 13.3 SD 4.71 n=6	ANOVA F=38.4 (2.14) P<0.001
Near-square NSQ	Mean 45.2 SD 1.7 n=5	Mean 43.3 SD 3.03 n=5	Mean 43.25 SD 1.8 n=4	

mean attachment % of cells at frequencies 0 Hz to 50 Hz on three different nanotopographies. Numbers represent cell counts per area (count area) 480,000 μm2. 5V peak-to-peak.

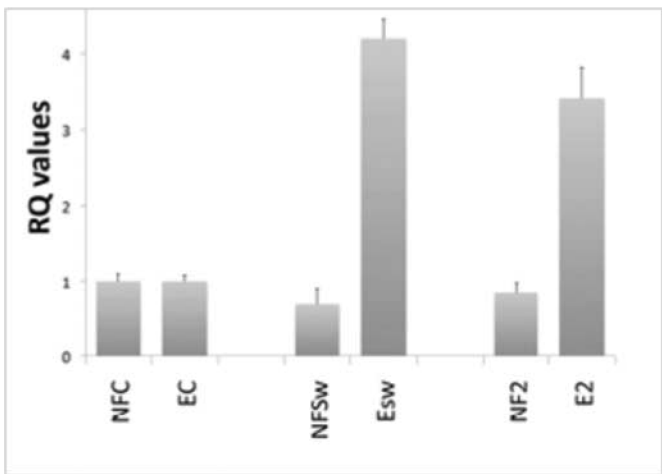


Fig. 7. Real-time PCR results with mean and standard deviation for three expression factors as RNA for endothelin-1.NF is NF-kappa beta, E is Endothelin 1, C denotes control series, Sw refers to expressions from Sweep type stimulation 0.8 Hz to 1.2 Hz sine wave stimulation. Groups all denoted by 2 received constant frequency stimulation at 1Hz 10V pk-to-pk stimulation. This experiment was repeated three times and controls (EC) and experimental (E2) were tested for significance of difference in mean RQ values EC2 v EC, $t = 5.6.\mathrm{df} = 16, P < 0.005$ demonstrating that constant frequency stimulation significantly enhances $\mathrm{Endothelin} = 1\ \mathrm{RNA}$ expression over this Sweep range.

These two genes are already known to be upregulated by relatively large mechanostimulation of endothelia [9], [10] and it is claimed that fluid flow around the endothelial cells is required for this to happen. However, the mechanical stimuli required for this are likely to be much less specific than flow alone because any mechanical stimulus is likely to spread from the point of application to nearby tissues producing a variety of mechanical effects (e.g., tension, compression, flow). Cell viability is vey high both before and after stimulation.

Piezo stimulation for up to at least 16 hours at 7 V and 1 Hz after a 2 hours delay for the cells to adhere, see Table I, did not kill the cells.

Low intensity stimulation (5 V peak-to-peak) at 1 or 2 Hz lead to expression of KLF2 but not in all cells but higher intensities such as 10 or 15 V peak-to-peak leads to all cells expressing this factor.

B. Effects of Modifying the Signal

1) Frequency Effects: Table I shows the effects on cell adhesion of using a small range of low frequencies to stimulate the cells and the results suggest that the system is not highly tuned to a particular frequency but further investigation see below suggests that the response system may be not of a simple type.

TABLE II
WHITE NOISE AND KLF2 EXPRESSION

Treatment	Result KLF2 by antibody	RT-PCR detection
control 2 Hz	positive KLF2 expression	positive endothelin-1 expression
white noise	no KLF2 expression	not carried out

TABLE III
WHITE NOISE AND CELL ATTACHMENT

Mode of stimulation	% Cells adhering	Statistics
2 Hz sine wave 10V for 24 hrs	mean 46.7	SD=4.9, n=8
white noise 10V for 24 hrs	mean 9.6	SD=3.38, n=23
static control no stimulation	mean 10.6	SD=4.71, n=6

C. White Noise Stimulation

The possible effects of white noise stimulation were assessed by examining cells for KLF2 expression and by RT-PCR methods after 24 hours stimulation and also for changes in adhesion over the same period (results presented in Tables II and III). The results shown in Fig. 6 show that white noise stimulation does not lead to KLF2 expression by the cells, and this was investigated further using sweep stimulation.

D. Sweep Stimulation

Sweep stimulation was carried out at frequencies between 0.7 and 10 Hz and at rates of frequency change between 0.92 $\mathrm{Hz\,s^{-1}}$ and 0.06 $\mathrm{Hz\ s^{-1}}$. Stimulation was measured as the percentage of cells showing KLF2 expression after 24 hours. These sweeps were only run in the increasing frequency mode, as shown in Fig. 8.

Fig. 8 reveals that the observed KLF2 expression drops markedly as the sweep frequency range of the stimulation widens. This result is consistent with the concept that a lack of coherence in the stimulation waveform (with the most extreme case of incoherence being that of white noise) will reduce or prevent KLF2 expression, in agreement with the PCR data in Fig. 7. For sweeps over a small frequency range, the observed cell response remains high, however these responses markedly decrease as the frequency range of stimulation broadens.

Fig. 9 illustrates this concept further by indicating that as sweep duration rises, KLF2 expression drops, perhaps since the most effective regime (e.g., a specific frequency) for stimulation is revisited less and less frequently as the sweep duration rises.

E. Effects of Mechanical Stimulation on Adhesion of Le2 to Nanotopographically Patterned Surfaces

These experiments were designed to discover whether adhesion of Le2 cells and expression of KLF2 to nanopatterned

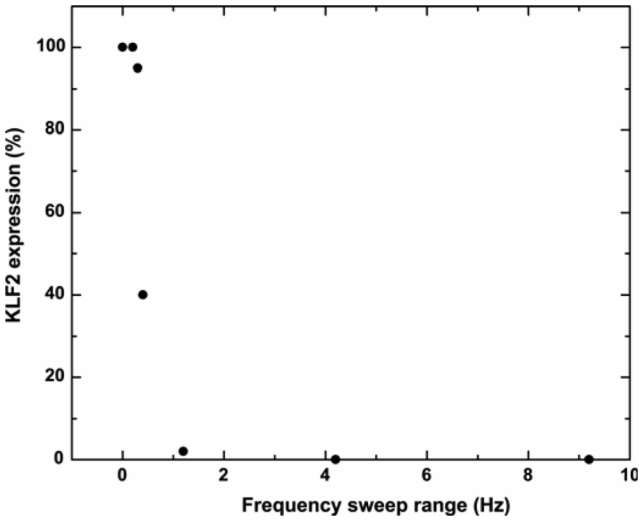


Fig. 8. KLF2 expression (% of cell population) in relation to bandwidth of sine wave stimulation.

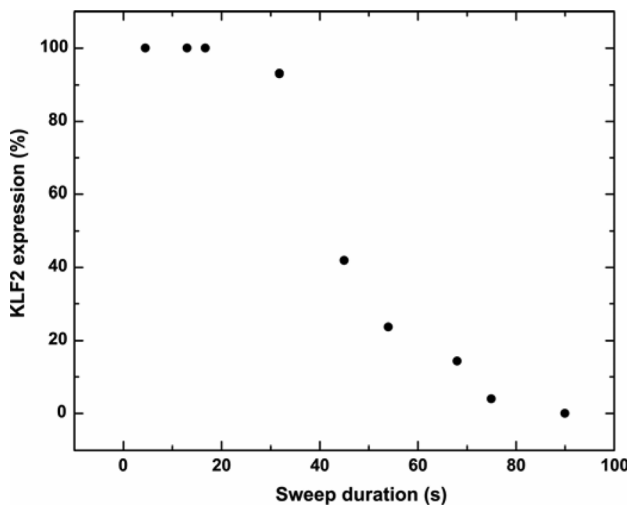


Fig. 9. KLF2 expression [% of cell population] in relation to duration in seconds of each sweep.

surfaces is modified when the surfaces are piezostimulated for 24 hours after initial settlement. It had already been shown that some nanotopographical patterns can increase or decrease cell adhesion under static conditions compared with controls attaching to a planar surface or with each other [8]. Therefore it is of interest to test whether piezostimulation changes the effects of nanotopography.

Table IV shows that the proportion of cells displaying KLF2 expression increases as vibration or nanotopography is added to the stimuli the cells are exposed to and even more when both factors are applied simultaneously. The significance of the results was examined using ANOVA with interaction methods to look for interaction between mechanical stimulation (vibration) and type of nanotopography. The raw data on frequency of KLF expression was used to calculate the F ratio for data grouped by substratum type. An F value of 18.65 df 2/58 $P < 0.001$ was obtained.

The data on cell adhesion Table IV shows that there is a rise in cell adhesion with type of topography on mechanostimulation from none (planar) to square (FSQ) to near-square (NSQ).

TABLE IV
OF CELLS EXPRESSING KLF2 ON THREE TYPES OF NANOTOPOGRAPHY

Surface	Static (S) or vibration stimulated (V)	n	mean	SD
1. planar	S	20	0.23	0.17
2. planar	V	20	0.25	0.22
3. nanotopography FSQ	S	20	0.35	0.28
4. nanotopography FSQ	V	20	0.40	0.26
5. nanotopography NSQ	S	20	0.59	0.29
6. nanotopography NSQ	V	20	0.78	0.21

V. DISCUSSION

Sections II–IV establish that our experimental system produces nanoscale z-axis excursions of the culture surface. The second section shows that the nanoscale biological effects of these excursions are very similar to those described in the literature for other larger scale mechanical stimulations, e.g., general effects of flow on endothelial cells at the microscale are well reviewed for instance in [8]–[10] The third section suggests that our stimulations must have temporal coherence to be effective at modifying cell behaviour. This is a novel finding and may suggest that a mechanism such as stochastic resonance might operate in these systems, allowing the cells to respond to very small (and coherent) mechanical signals at or below the thermal noise and/or seismic background. Spatial coherence was maintained due to the geometrical construction of the piezoactuator. The fourth section indicates that the cells may respond to the stimulation by self-tuning and suggests future experiments that need to be done.

The main results of this study show that these endothelial cells of capillary origin respond to nanoscale stimulation where the movements produced by the stimulatory system are seen in the range 5–20 nm and the force below 10 pN and that effects are clearly seen on gene expression. Low intensity stimulation, e.g., 5 V pk-to-pk at low frequency leads to only a proportion of cells expressing KLF2 factor and forces are below 1 pN but higher intensity (10 to 15V pk-to-pk) and higher frequency results in all cells expressing this factor. Higher forces also lead to KLF2 expression but resonance may set in under such conditions.

The movements produced by the piezostimulation correspond well with the movements observed by Pierres [15] and the force range lies in the regions calculated or observed for *Drosophila* mechanosensory cells [24]. Uzer *et al.* [25] obtained an increase in COX2 gene expression in osteoblast-like cells using rather similar though more intense mechanostimulation but could not entirely exclude shear forces. In our case the fact that the cells need to be attached to the substrate to redifferentiate suggests that shear forces are unimportant.

A. The Processing of the Mechanical Signal by the Cell

Our findings that white noise stimulation abolishes the response of the cells found with single frequency stimulation suggests strongly that coherent stimulation is essential for stimulation of specific changes in transcription. The sweep experiments show that reaction to mechanical stimulus is lost as the bandwidth of the sweep is increased and coherence lost. The loss

of reaction with frequency switch is also an indication that coherence is important. It should be pointed out that the piezo devices are coherent in the time domain when a single frequency is used for stimulation and also likely to be spatially coherent due to their construction. Stochastic resonance [26] may act in the system we are using. Tanaka and colleagues [27] suggested that stochastic resonance could account for the stimulation of osteopontin synthesis by osteoblasts exposed to mixed low frequency and broadband strains of fairly high intensity. These could be the events with which the piezo-derived oscillations interact and we postulate that such interactions could drive cell surface activity.

The data shown in Table III show that stimulation frequencies in the broad range of 1 to 50 Hz produce responses from the cells while the evidence from the sweep experiments (Fig. 8) suggests fine tuning with narrow bandwidths. This seeming contradiction could be explained by the concept of "self-tuning" which has been observed in biological systems as well as in others [28] The experiments on altering the stimulation frequency upwards or downwards after a period of stimulation are also consistent with self-tuning. The large number of cycles of stimulation needed to produce changes in gene expression are again consistent with this concept. Random protrusions by the cell could provide the random element required for stochastic resonance. We suggest that for many animals the heartbeat could also provide a regular time coherence reference when experimental ones of the type we use are absent. Stochastic resonance might interact with the contraction cycle of protrusions from the cell. Mixing stimulation frequencies might provide a further experimental approach for future use.

It is interesting to note that the results presented within Figs. 7 and 8 appear to be broadly consistent with the expected response of a simple resonant system. Using the analogy of a resonant electrical circuit, e.g., LCR, it can be shown that driving the circuit with an input waveform that is suitably close to the resonant frequency will allow the output (or response) of the circuit to remain high, as was observed in the KLF2 expression in Fig. 7. Likewise the response of the resonant electrical circuit will fall as the range of input frequencies broadens from the central resonant frequency. Fig. 8 is consistent with a model in which the energy supplied by the excitation signal is stored in a resonant system, with an associated decay time over which the energy is dissipated. If the resonant frequency is visited suitably often in the input waveform, then there would be insufficient time for all of the energy in the system to fully dissipate before additional energy is introduced. Likewise, if the resonant frequency is visited less often than the associated decay time, then there would be significant periods during which the resonant system has no response, perhaps leading to an overall null response, consistent with the observed cell responses in Fig. 8. Although the comparison to resonant systems is speculative at this stage, it may be important to use this observation to help plan future experiments where mechanical stimulation is employed to drive particular cell responses, and further modelling of the observed KLF2 expression as a resonant process may lead to clearer insights into the underlying processes responsible for mechanotransduction.

One matter of practical importance is will it be possible to establish long-term or permanent changes in differentiation by applying nanoscale forces? A full test of this is needed before nanoscale stimulation can be applied practically.

If stochastic resonance or any other resonance acts there would be a certain element of persistence in the effects of the stimulus frequency assisting the frequency stability. Stochastic resonance would also explain how very small repeated signals could have biological effect such as those seen at the lower intensities and frequencies we used.

B. Cell Interactions

Tables I and IV show that nanotopography, mechanotransduction, and adhesion interact closely. These are three major areas of research and it seems likely that they reflect parts of a general cellular mechanism. Thus, we suggest that the cell responds to mechanical stimuli primarily in the vicinity of the cell surface which is very close to the surface nanotopography of the substratum. Pierres *et al.* [16] showed that the cell makes protrusions "plucking" at the substratum surface. Piezostimulation will stimulate a related process-the cell being forced into the "arms" of the substratum. It can be argued that very small scale mechanical stimulations which are effective are most likely to be located close to the structures or systems involved in transduction. On that argument the very small piezoactuation effects are likely to be acting directly on the final transducer.

These findings are compatible with explanations applied to results obtained by others using larger scale movements, usually in the x:y plane, where mechanoreceptors in or near the cell surface may act. The experiments we describe differ from others because they were run at appreciably lower amplitude than probably all other experiments and with movements in the z plane rather than the x/y. There is evidence that we only obtained responses to coherent signals and that loss of coherence leads to a failure of response.

VI. Conclusions

We show from our experimental results that repeated mechanical stimulation of the Le2 line of mouse endothelial capillary cells with frequencies in the range 1 to 50 Hz with movements in the range 5–20nm and calculated force per cell of 0.05 to 5pN leads to:

1) Appearance of KLF2 and endothelin-1 expression factors and increases in cell adhesion and that with sufficiently intense stimulation all cells in the cultures express these factors.
2) Effective stimulation in this mode has to be coherent in time and space and if this is altered the gene expression of KLF2 and endothelin-1 is lost.

Both nanotopography and vibration stimulation can produce these effects and there is evidence for stochastic resonance in the system (cell-piezostimulator).

Acknowledgment

The authors thank Dr. N. Gadegaard and Dr. R. Pedersen for providing the nanopatterned materials we used. The authors also thank BBSRC and EPSRC for support. The authors thank Andrew Hart for technical assistance. Colin Craig is thanked for technical assistance in constructing the apparatus for mechanical stimulation.

References

[1] A. S. G. Curtis, B. Casey, J. D. Gallagher, D. Pasqui, M. A. Wood, and C. D. W. Wilkinson, "Substratum nanotopography and the adhesion of biological cells. Are symmetry or regularity of nanotopography important?," *Biophys. Chem.*, vol. 94, pp. 275–283, 2001.

[2] M. J. P. Biggs, R. G. Richards, N. Gadegaard, C. D. W. Wilkinson, and M. J. Dalby, "The effects of nanoscale pits on primary human osteoblast adhesion formation and cellular spreading," *J. Mater. Sci: Mater. Med*, vol. 18, no. 2, pp. 399–404, 2007.
[3] M. A. Wood, C. D. W. Wilkinson, and A. S. G. Curtis, "The effects of colloidal nanotopography on initial fibroblast adhesion and morphology," *IEEE Trans. NanoBiosci.*, vol. 5, no. 1, pp. 20–31, 2008.
[4] M. J. Dalby *et al.*, "Increasing fibroblast response to materials using nanotopography. Morphological and genetic measurements of cell response to 13 nm high polymer demixed islands," *Exp. Cell Res.*, vol. 276, pp. 1–9, 2002.
[5] L. E. McNamara *et al.*, "The role of microtopography in cellular mechanotransduction," *Biomaterials*, vol. 33, no. 10, pp. 2835–2847, 2012.
[6] M. Eastwood, D. A. McGrouther, and R. A. Brown, "A culture force monitor for measurement of contraction forces generated in human dermal fibroblast-cultures-evidence for cell-matrix mechanical signaling," *Biochem. Biophys. Acta.*, vol. 1201, no. 2, pp. 186–192, 1994.
[7] R. A. Brown, R. Prajapati, D. A. McGrouther, I. V. Yannas, and M. Eastwood, "Tensional homeostasis in dermal fibroblasts: Mechanical responses to mechanical loading in three-dimensional substrates," *J. Cell. Physiol.*, vol. 175, no. 3, pp. 323–332, 1998.
[8] S. Lehoux and A. Tedgui, "Cellular mechanics and gene expression in blood vessels," *J. Biomech.*, vol. 36, no. 5, pp. 631–643, 2003.
[9] R. J. Dekker *et al.*, "Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2)," *Blood*, vol. 100, no. 5, pp. 1689–1698, 2002.
[10] A. Ishibazawa, T. Nagaoka, A. Yofa, T. Takahshi, and Tabano, I, "Effect of shear stress on the gene expression of endothelial nitric oxide synthase. Endothelin 1 and thrombomodulin in human retinal microvascular endothelial cells," *Invest. Opthalmol. Vis. Sci.*, vol. 52, pp. 8504–8946, 2011.
[11] A. Mammoto *et al.*, "A mechanosensitive transcriptional mechanism that controls angiogenesis," *Nature.*, vol. 457, no. 7233, pp. 1103–1108, 2009.
[12] L. H. Ting *et al.*, "Flow mechanotransduction regulates traction forces, intercellular forces, and adherens junctions," *Amer. J. Physiol.—Heart Circ. Physiol.*, vol. 302, no. 11, pp. H2220–H2229, 2012.
[13] Y. Ito *et al.*, "Nano-vibration effect on cell adhesion and its shape," *Bio-Med. Mater. Eng.*, vol. 21, pp. 149–158, 2011.
[14] Y. Ito, T. Kimura, K. Nam, A. Katoh, T. Masuzawa, and A. Kishida, "Effects of vibration on differentiation of cultured Pc12 cells," *Biotechnol. Bioeng.*, vol. 108, no. 3, pp. 592–599, 2011.
[15] H. Nikukar *et al.*, "Osteogenesis of mesenchymal stem cells by nanoscale mechanotransduction," *ACS Nano*, vol. 7, no. 3, pp. 2758–2767, 2013.
[16] A. Pierres, A.-M. Benoliel, D. Touchard, and P. Bongrand, "How cells tiptoe on adhesive surfaces before sticking," *Biophys. J.*, vol. 94, pp. 4114–4122, 2008.
[17] A. Pierres, V. Monnet-Corti, A.-M. Benoliel, and P. Bongrand, "Do membrane undulations help cells probe the world?," *Trends Cell Biol.*, vol. 19, no. 8, pp. 428–433, 2009.
[18] Y. L. Lim and H. J. Donahue, "Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning," *Tissue Eng.*, vol. 11, no. 8, pp. 1879–1891, 2007.
[19] A. M. Rajnicek, S. Britland, and C. D. McCaig, "Contact guidance of cns neurites on grooved quartz: Influence of groove dimensions, neuronal age and cell type," *J. Cell Sci.*, vol. 110, pp. 2905–2913, 1997.
[20] N. Gadegaard *et al.*, "3D fabrication methods for producing tissue engineering scaffolds," *IEEE Int. Microprocesses Nanotechnol. Conf. Dig. Papers*, pp. 410–411, 2007.
[21] H. Kitamura *et al.*, "Induction of src-suppressed C kinase substrate (SSeCKS) in vascular endothelial cells by bacterial lipopolysaccharide," *J. Histochem. Cytochem.*, vol. 50, no. 2, pp. 245–255, 2002.
[22] R. Higuchi, C. Fockler, G. Dollinger, and R. Watson, "Kinetic PCR analysis-real time monitoring of DNA amplification reactions," *Nat. Biotechnol.*, vol. 11, no. 2, pp. 1026–1030, 1993.
[23] T. W. Woudenberg and J. Stevens, "Quantitative PCR by real-time detection," in *Ultrasensitive Biochemical Diagnostics*, G. E. Cohn, S. A. Soper, and C. H. W. Chen, Eds. Bellingham, WA, USA: SPIE, 1996, vol. 2680, pp. 306–315.
[24] B. J. Nadrowski, T. Albert, and M. C. Göpfert, "Transducer-based force generation explains active process in Drosophila hearing," *Curr. Biol.*, vol. 18, no. 18, pp. 1365–1372, 2008.
[25] G. Uzer *et al.*, "Separating fluid shear stress from acceleration during vibrations in vitro: Identification of mechanical signals modulating the cellular response," *Cell. Mol. Bioeng.*, vol. 5, pp. 266–276, 2012.
[26] F. Moss, "Stochastic resonance at the molecular level: The Poisson wave model," *Biophys. J.*, vol. 73, no. 5, pp. 2249–2250, 1997.
[27] S. M. Tanaka *et al.*, "Effects of broad frequency vibration on cultured osteoblasts," *J. Biomech.*, vol. 36, no. 1, pp. 73–80, 2003.
[28] G. G. Turrigiano, "The self-tuning neuron: Synaptic scaling of excitatory synapses," *Cell.*, vol. 35, no. 3, pp. 422–435, 2008.

Adam S. G. Curtis is Emeritus Professor of Cell Biology at the University of Glasgow, U.K., and has published more than 140 papers especially on the reactions of cells and the nanoscale.

Stuart Reid is a lecturer at the University of the West of Scotland, U.K. (UWS), having gained his Ph.D. from the University of Glasgow, U.K., in 2006 on the development of novel optical and suspension technology for gravitational wave detectors. He is a member of various international research collaborations and invited member of international advisory panels, and currently holds a prestigious research fellowship from the Royal Society of Edinburgh.

Iain Martin is a Royal Society Research fellow in the School of Physics and Astronomy at the University of Glasgow, U.K., having received his Ph.D. in gravitational wave physics in 2009. His research interests include the properties of optical coatings, thermal noise in precision measurement, and the detection of gravitational waves.

Ramanathan Vaidyanathan is a Ph.D. student at the Australian Institute for Bioengineering and Nanotechnology (AIBN), University of Queensland, Australia. He is currently working on developing novel technologies for the detection of cancer cells and protein biomarkers. He was awarded the International Postgraduate Research Scholarship (IPRS) and Australian Postgraduate Award (APA) in 2011. Prior to undertaking his Ph.D. at the AIBN, he gained his M.S. in biomedical sciences (M.Res) from the University of Glasgow, U.K., in 2010. He has published six peer-reviewed articles in various multidisciplinary journals.

Carol-Anne Smith After graduating with a B.Sc. (Hons.) in medical biochemistry in 1999, she started work as a research technician in the Division of Virology, University of Glasgow, U.K., where she worked for several years on numerous clinical projects performing real time PCR. She then transferred to the Centre for Cell Engineering, University of Glasgow, where she used her expertise to look at gene expression levels in response to different cell interactions at the nanoscale.

Habib Nikukar studied medicine and then a pediatric subspecialty in the Shiraz University of Medical Sciences, Shiraz, Iran, from 1986 to 1997. He then worked as a pediatrician (1997–2009) in Yazd, Iran, as a member of Shahid Sadoughi University of Medical Sciences. His clinical duties were prevention, diagnosis, and treatment of internal medical diseases in the pediatric age group. At the same time he worked for the University as a researcher and trainer of medical and paramedical staff and students. Due to his interest in the basic sciences especially molecular and cellular biology, he moved to the CC to undertake a Ph.D. course on nanobiotechnology (since 2010). His research focuses on finding the possible effects of nanoscale mechanical stimulation on the behaviors of mesenchymal stem cell fate. His interests are regenerative therapy, stem cell therapy, and uses of nanosciences in medicine.

Matthew J. Dalby is a Reader in Cell Engineering in the Institute of Molecular, Cell and Systems Biology at the University of Glasgow, U.K. After completing a Ph.D. in Biomedical Materials at the Interdisciplinary Research Centre in Biomedical Materials, London, U.K., he moved to the Centre for Cell Engineering as a PDRA on EU framework 5 grant Nanomed. In 2003 he was awarded a BBSRC David Phillips Fellowship and then a Lectureship in 2008. He has established the Glasgow Orthopaedic Research Initiative with consultant surgeon Mr. Dominic Meek, and his research focuses on understanding the interaction of cells with nanoscale features.