Biomaterials 34 (2013) 2177–2184

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A genomics approach in determining nanotopographical effects on MSC phenotype

Penelope M. Tsimbouri [a,*], Kate Murawski [d], Graham Hamilton [c], Pawel Herzyk [c], Richard O.C. Oreffo [d,e], Nikolaj Gadegaard [b], Matthew J. Dalby [a]

[a] Centre for Cell Engineering, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK
[b] Division of Biomedical Engineering, School of Engineering, University of Glasgow, Glasgow G12 8LT, Scotland, UK
[c] Sir Henry Wellcome Functional Genomics Facility, College of Medical, Veterinary and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK
[d] Bone & Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton SO16 6YD, UK
[e] Stem Cell Unit, Department of Anatomy, College of Medicine, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 10 September 2012
Accepted 15 December 2012
Available online 9 January 2013

Keywords:
Mesenchymal stem cells (MSCs)
Nanotopography
Mechanotransduction
Gene expression
Cell signalling
Molecular biology

ABSTRACT

Topography and its effects on cell adhesion, morphology, growth and differentiation are well documented. Thus, current advances with the use of nanotopographies offer promising results in the field of regenerative medicine. Studies have also shown nanotopographies to have strong effects on stem cell self-renewal and differentiation. What is less clear however is what mechanotransductive mechanisms are employed by the cells to facilitate such changes. In fastidious cell types, it has been suggested that direct mechanotransduction producing morphological changes in the nucleus, nucleoskeleton and chromosomes themselves may be central to cell responses to topography. In this report we move these studies into human skeletal or mesenchymal stem cells and propose that direct (mechanical) signalling is important in the early stages of tuning stem cell fate to nanotopography. Using fluorescence *in situ* hybridization (FISH) and Affymetrix arrays we have evidence that nanotopography stimulates changes in nuclear organisation that can be linked to spatially regulated genes expression with a particular focus on phenotypical genes. For example, chromosome 1 was seen to display the largest numbers of gene deregulations and also a concomitant change in nuclear positioning in response to nanotopography. Plotting of deregulated genes in reference to band positioning showed that topographically related changes tend to happen towards the telomeric ends of the chromosomes, where bone related genes are generally clustered. Such an approach offers a better understanding of cell–surface interaction and, critically, provides new insights of how to control stem cell differentiation with future applications in areas including regenerative medicine.

1. Introduction

Current developments in the application of nanotopography have provided us with promising results in the field of regenerative medicine. Major results with mesenchymal stem cells, a key regenerative cell target given their indicated immune-privilege and availability as autologous cells, have included the ability to target osteogenesis using controlled disorder, NSQ50 (pits of 120 nm diameter, 100 nm deep with a near square arrangement – average 300 nm centre–centre with up to ±50 nm offset in X and Y) indicating that implant modifications may be possible to improve clinical outcome [1–4]. More recently, it was shown that nanostructured surfaces with tightly controlled arrangement, SQ (similar to NSQ50 but with no offset) can retain stem cell phenotype and maintain stem cell growth with implications therein for provision of high quality stem cells to clinic [5]. Furthermore, recent literature has also highlighted the potential for modifying embryonic stem cell response with nanotopography [6,7].

Understanding the mechanism of the physiological processes that control cell–biomaterial interactions and the influence of nanotopography on cell adhesion and phenotype is fundamental to understanding stem cell differentiation. In this study isolated multipotential bone marrow skeletal stem cells also known as mesenchymal stem cells (MSCs), with the potential to differentiate along the stromal lineages, were examined. MSCs can give rise to

* Corresponding author. Tel.: +44 (0) 7801434679.
E-mail addresses: penelope.tsimbouri@glasgow.ac.uk, p.tsimbouri@btinternet.com (P.M. Tsimbouri).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.12.019

different lineages including fibroblastic, chondrogenic, myoblastic, adipogenic, and osteoblastic cell types [8–11]. Recent studies have highlighted that MSC function follows form, with alterations in cell adhesion and subsequent cytoskeletal tension modulating lineage commitment [9,12]. There is evidence demonstrating the importance of intracellular tension in MSCs with a high-tension state inducing osteogenic differentiation, whilst a low-tension state inducing adipogenic differentiation [12–14]. Recent advances are indicative of the requirement of an intermediate level of cellular tension for MSC self-renewal [5,15,16].

Interactions between stem cells and the ECM can have indirect or direct effects on cells, otherwise known as mechanotransduction, to elicit changes in gene expression.

Indirect mechanotransduction includes the canonical biochemical signalling cascades which result from integrin binding and focal adhesion formation [17]. The second form of mechanotransduction, direct, occurs as a consequence of conformational changes in the cell cytoskeleton, which forms a direct link between the extracellular matrix and the nucleus of the cell via the nucleoskeletal lamins (the intermediate filaments of the nucleus) and potentially further to the chromosomes via telomeric chromatin/lamin interactions [18,19].

The nucleus itself is supported by the nucleoskeleton consisting of a network of proteins comprising lamins A and C (derived by differential splicing of the same gene) [20], B1 [21] and B2 [22] (products of two genes) in most somatic cell types. The lamina provides structural support to the nucleus, forming part of the link to the cytoskeleton ensuring the correct nuclear and centrosomal organization. The lamina is also associated with DNA replication; lamin B foci are associated with proliferating cell nuclear antigen (PCNA), a protein acting as a processivity factor for DNA polymerase δ in eukaryotic cells creating topological links to the genome during DNA replication [23,24]. Nanotopography produces tension related changes in-line with the literature for stiffness and chemistry. For example, high-tension for MSCs on NSQ50 nanotopographies resulting in osteogenesis and intermediate tension for MSCs on SQ nanotopographies supports self-renewal [5,16]. We also understand that these tension states drive indirect cascades such as extracellular-signal-regulated kinases, (ERK1/2), c-Jun N-terminal kinase (Jnk) and low density lipoprotein (LDL) signalling [16]. However, what is less clear is whether these tension states drive direct changes in nuclear architecture and if there is a possible link to phenotype arising from such changes.

To address the role of direct mechanotransduction on MSC differentiation, the SQ (self-renewal promoting) and NSQ50 (osteogenesis promoting) nanotopographies were employed in these studies. We have examined the nucleus and have used fluorescence *in situ* hybridisation (FISH) to study movement of chromosomes in the MSCs on the defined nanotopographies. Chromosome choice was informed by microarray analysis implicating the chromosomes with the greatest expression profile change. In addition, using the gene expression data, spatial activity along the chromosomal arms was examined and gene and protein level data on key transcription factors for differentiation and phenotypical markers for MSC phenotype were linked to these spatial 'bins'. The experiments were performed after three days of culture in order to capture morphological changes in the early stages of cell decision making in maintaining self-renewal or starting to express early differentiation-related transcription factors.

2. Materials and methods

2.1. Nanopatterning and mastering

The substrates were made in a three-step process of electron beam lithography [25] nickel die fabrication and polymer replication using injection moulding. Briefly, the master substrates were fabricated to form an array of 120 nm diameter pits of 100 nm depth and 300 nm pitch in a square (SQ) arrangement with the near square (NSQ50) substrate has a random displacement of ±50 nm, and maintaining an average 300 nm pitch. Nickel dies were made directly from the patterned resist samples and a thin (50 nm) layer of Ni–V was sputter coated on the samples, acting as an electrode in the subsequent electroplating process. The dies were plated to a thickness of approximately 300 μm. The nickel shims were cleaned by stripping the protective polyurethane coating using chloroform in an ultrasound bath for 15 min. An injection moulder was used to make polymer replicas in polycarbonate.

2.2. Cell extraction and culture

MSCs or skeletal stem cells were enriched from human bone marrow using the STRO1 antibody and magnetic activated cell sorting (MACS) as previously described [2]. MSCs were maintained in basal media (αMEM (PAA)) supplemented with 10% FBS (PAA), 1% (v/v) 200 mM L-glutamine (Gibco) and antibiotics (6.74 U/ml Penicillin–Streptomycin, 0.2 μg/ml Fungizone, Gibco) at 37 °C with 5% CO_2 in a humidified incubator. MSCs were seeded onto the materials at 1×10^4 cells/ml and allowed to grow for 3 days. Cells were used at passages P1–P2 throughout the study. Cells were isolated from a large number of patients (>10) and were used over the course of the studies to help show robustness of the data.

2.3. Chromosome territory staining: fluorescence in situ hybridisation (FISH)

MSCs were fixed in 3:1 methanol:acetic acid for 30 min at room temperature and rinsed in 2× SSC (saline sodium citrate; diluted from 20× stock of 3 M NaCl, 0.3 M *tri*-sodium citrate, pH7.4) for 3 h at 37 °C. The appropriate chromosome probe (biotinylated human chromosome 1 paint; Cambio, Cambridge, UK) was brought to 37 °C, vortexed, and pelleted by centrifugation for ~3 s at 11,000 × *g*. The probe was denatured at 65 °C for 10 min, followed by a 30 min incubation at 37 °C. The samples were rinsed in H_2O for 30 s and then dehydrated through a 70%, 90%, 90% (v/v) ethanol series, with a 2 min incubation at each step, followed by a 5 min dehydration step in 100% ethanol. The samples were then air dried for 1 min and incubated in denaturation solution (7:1 formamide: 2× SSC buffer) at 65 °C for 2 min. The samples were quenched using an ice-cold ethanol series as above and air-dried for 1 min. The denatured probe (8–15 μl) was added to each sample, the samples were covered with coverslips and incubated for 44 h at 37 °C in a humidified chamber. Following hybridization, the samples were rinsed in 45 °C pre-warmed 1× SSC buffer for 5 min followed by 2 × 5 min washes in stringency wash solution (1:1 formamide: 1× SSC). The probe was detected using the Biotin Painting Kit (Cambio), according to the manufacturer's protocol. Three replicates of each topography (NSQ50, SQ, FLAT) were used in each experiment.

2.4. Territory analysis

The distances from the nearest edge of the nuclei to the centres of the chromosomal territories and the interterritory distances were measured using Image J (version 1.34s; Rasband, W.S., Image J, U.S. National Institutes of Health – http://rsb.info.nih.gov/ij/). Statistics were generated using Prism (GraphPad at www.graphpad.com/prism) the Tukey–Kramer multiple comparisons post-test analysis of variance (ANOVA).

2.5. Affymetrix arrays

MSCs were cultured on the topographies (4 material replicates/biological replica/topography) for 3 days. At this point, the cells were lysed and total RNA was extracted using a Qiagen RNeasy micro kit (Qiagen, UK). Gene expression changes were detected by hybridization of mRNA to Affymetrix HuGene 1.0 ST human arrays according to the manufacturers instructions. Initial bioinformatic analysis was based on rank product. A false discovery rate of 20% was used to upload selected genes changes to the Ingenuity Pathway Analysis (IPA) server to identify canonical signalling pathways, functional pathways and to produce networks. Statistics for functional analysis were carried out by Fischer's exact test (automatically performed by the software). For the chromosomal band identification a custom script was written to add annotations to the ANOVA results file. The script generated a transcript cluster ID to the chromosome lookup table from the HuGene-1_0-st-v1.na32.hg19.probeset.csv file obtained from Affymetrix (www.affymetrix.com). Then the results file was parsed and the chromosomal location details, from the lookup table, were appended to the corresponding transcript.

2.6. Quantitative real time (q)PCR

MSCs were cultured on topographies for 3 days (4 biological replicas (each consisting of 3 replicas each pooled) for each NSQ50, SQ and FLAT) at a density of 1×10^4 cells/ml. Total RNA was extracted using a Qiagen RNeasy micro kit. Real-time qPCR was carried out and analysed as previously described to assess the expression of Runx2, HOP26, ALCAM, SOX9 and PParγ (Tables 1 and 2). RNA samples were reverse transcribed using the Omniscript First Strand System (Qiagen). Real-time qPCR was carried out using the 7500 Real Time PCR system from Applied Biosystems. GapDH