Growth Hormone & IGF Research 24 (2014) 245–250

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

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

Nanotopology potentiates growth hormone signalling and osteogenesis of mesenchymal stem cells

Jessie R. Wang [a], S. Faisal Ahmed [a], Nikolaj Gadegaard [b], R.M. Dominic Meek [d], Matthew J. Dalby [c], Stephen J. Yarwood [c,*]

[a] Developmental Endocrinology Research Group, Royal Hospital for Sick Children, University of Glasgow, Yorkhill, Glasgow G3 8SJ, UK
[b] School of Engineering, University of Glasgow, Glasgow G12 8LT, UK
[c] Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, G12 8QQ, UK
[d] Department of Orthopaedics, Southern General Hospital, 1345 Govan Road, Glasgow G514TF, UK

ARTICLE INFO

Article history:
Received 20 August 2014
Received in revised form 3 October 2014
Accepted 20 October 2014
Available online 28 October 2014

Keywords:
Stem cells
Growth hormone
Nanotopology
Osteogenesis

ABSTRACT

Custom engineered materials can influence the differentiation of human mesenchymal stem cells (MSCs) towards osteoblasts, chondrocytes and adipocytes, through the control of chemistry, stiffness and nanoscale topography. Here we demonstrate that polycaprolactone growth surfaces engineered with disordered (but controlled) 120 nm diameter dots (NSQ50), but not flat surfaces, promote osteogenic conversion of MSCs in the absence of other osteogenic stimuli. Differentiating MSCs on NSQ50 were found to express growth hormone receptors (GH) and stimulation with recombinant human GH (rhGH) further enhanced NSQ50-driven osteogenic conversion of MSCs. This increased osteogenesis coincided with an enhanced ability of GH to activate ERK MAP kinase on NSQ50, but not on flat topology. The importance of ERK for MSC differentiation was demonstrated by using the inhibitor of ERK activation, U0126, which completely suppressed osteogenesis of GH-stimulated MSCs on NSQ50. The ability of GH to activate ERK in MSCs may therefore be a central control mechanism underlying bone development and growth.

1. Introduction

The growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis is closely linked to chondrogenesis and osteogenesis [17]. This is evidenced by GH deficiency and deletion of GHRs in mice and humans leading to a decrease in bone mass and mineral density [6,16]. Moreover, in rodent models, GH enhances osteoblast precursor pool size and increases osteoblast differentiation [11]. These osteogenic actions of GH appear to occur independently of the classical route of transcriptional control by GH, via activation of the transcription factor STAT5 [16]. GHRs have been detected on murine bone marrow mesenchymal stem cells (MSCs), suggesting a role in osteogenesis [8] and osteogenesis of MSCs appears to be dependent on signalling pathways downstream of GH [3] and involves the activity of the MAP kinase, ERK [5].

MSCs are readily found within the human bone marrow where they undergo self-renewal and differentiate into osteoblasts (bone), chondrocytes (cartilage), adipocytes (fat), fibroblasts (connective tissue) and myoblasts (muscle) and their multi-potency can be demonstrated using defined culture medium, which leads to simultaneous formation of bone, cartilage, fat and reticular tissues through the formation of a range of developmental stages [12,14]. Currently, the major problem for detailed MSC studies is their tendency for spontaneous differentiation, resulting in a heterogeneous population of mainly fibroblastic cells when grown on plastic surfaces in vitro. However, in recent years, advances in high-resolution electron beam lithography (EBL) means that nanoscale topographies can be precisely fabricated and then embossed into biocompatible polymers. These nanometric growth surfaces have been shown to promote osteogenesis from stem and progenitor mesenchymal populations in the absence of osteogenic supplements [4]. Whilst this osteogenic property can be seen as a potential superior implant material in orthopaedics [4], it also provides a better control substrate for MSC growth and bone differentiation without recourse to complex media formulations. In the current study we use a nanometric growth surface to explore the effects of GH on the osteogenic conversion of human MSCs in vitro. This revealed a novel, regulatory interplay between nanoscale topography and GH signalling through the ERK MAP kinase cascade in the control of the osteogenic conversion of human MSCs. For the first time we demonstrate how nanometric topology can influence signalling from hormone receptors to direct the differentiation of MSCs.

* Corresponding author at: Rm239, Davidson Building, Institute of Molecular, Cell and Systems Biology, University of Glasgow, G12 8QQ, UK. Tel.: +44 141 330 3908; fax: +44 141 330 4620.

http://dx.doi.org/10.1016/j.ghir.2014.10.003
1096-6374/

2. Materials and methods

2.1. Materials

Anti-recombinant bovine GH antiserum was a generous gift from Professor David Flint (University of Strathclyde).

2.2. Preparation of cell growth surfaces

The cell culture growth surfaces used throughout this study were nanoscale topography near square 50 (NSQ50) or flat surfaces embossed onto the biodegradable, polyester polycaprolactone (PCL). The nanoscale growth substrate was fabricated by electron beam lithography to form arrays of 120 nm diameter pits of 100 nm depth and average 300 nm pitch in a square arrangement. The EBL tool was programmed to introduce random displacements of up to ±50 nm in X and Y, maintaining an average 300 nm pitch, as previously described [4]. After fabrication, nickel dies were made directly from the patterned resist samples. A thin (50 nm) layer of Ni–V was then sputter-coated on the samples. This layer acted as an electrode in the subsequent electroplating process. The dies were plated to a thickness of about 300 μm. Polymeric replicas were made in PCL by hot embossing. Beads of PCL were melted on a hot plate and the nickel die was pressed by a thumb into it for about 10 s and then transferred to an ice box to allow the sample to cool. All the subsequent replicas possessed a nano-imprinted area of 1 cm^2. As a control surface, flat PCL with similar area was used, which was made by hot-embossing over a glass slide (Ra = 1.174 nm over 10 μm).

2.3. Extraction of bone marrow MSCs

Stromal progenitors containing MSCs were extracted from bone marrow samples obtained from haematologically normal adults

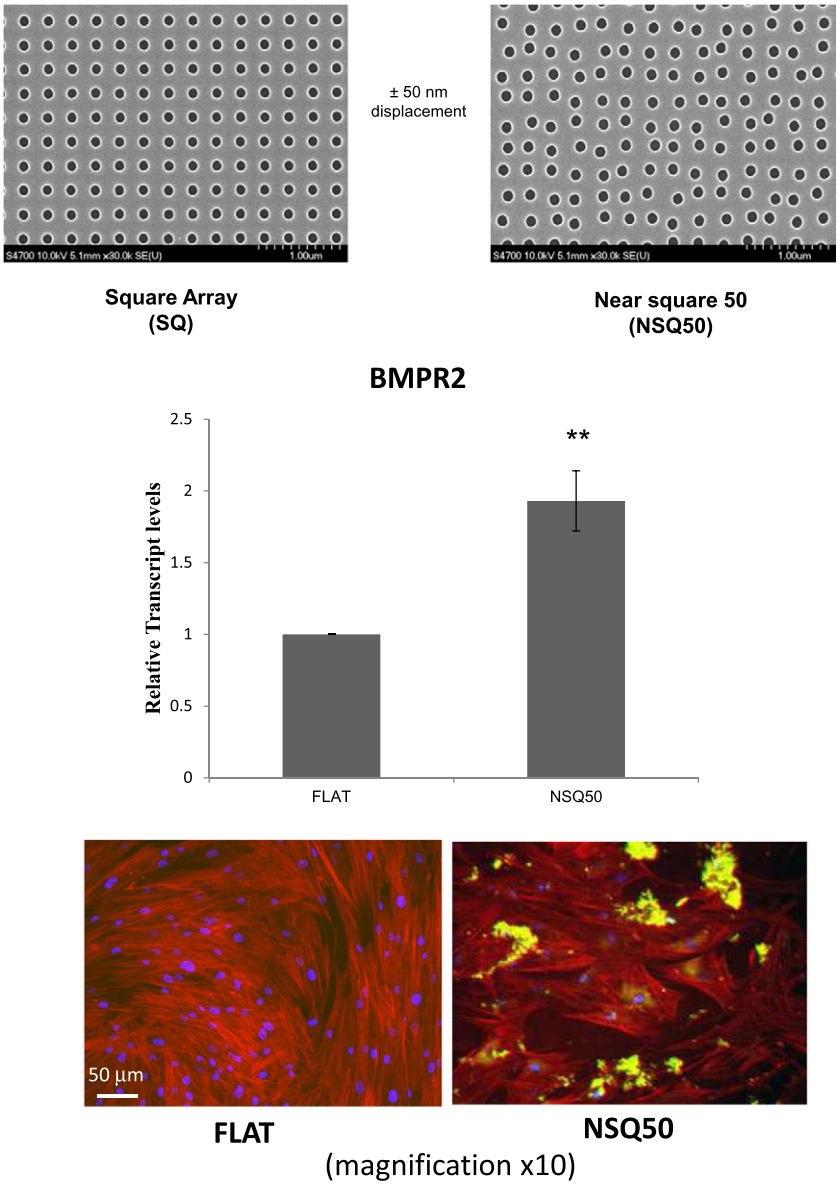

Fig. 1. In the *upper panel* are nanotopographies fabricated by electron beam lithography. Both contain 120 nm diameter pits (100 nm deep). The left image shows a square array with an even average centre–centre spacing of 300 nm. The image on the right is a disordered square (NSQ50) array with dots displaced randomly at ±50 nm on both axes from their position in a true square. NB. In the *lower panel* are qRT-PCR results measuring transcript levels for BMPR2 in MSCs cultured on flat or NSQ surfaces. The relative transcript levels were normalised to cells cultured on the flat substrates and are means ± SD for three separate experiments. Statistical differences are indicated, $^{**}p < 0.01$. Osteopontin (OPN) staining of MSCs after 28 days of culture with the indicated treatments is also shown in the *lower panel*. OPN is stained green, actin is stained red and nuclei are stained blue.

undergoing routine hip-replacement surgery at the Southern General Hospital, Glasgow. The methods used (Ficol gradient and selection through adhesion to tissue culture plastic) have been previously described [9]. Extracted cells were maintained in a humidified atmosphere at 37 °C and 5% (*v*/*v*) CO_2 in 87.7% Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Poole, UK), 8.77% foetal calf serum (FCS; Life Technologies, UK), 1.75% antibiotics (Penicillin–Streptomycin, P0781, Sigma), 0.877% 100 mM sodium pyruvate (Life Technologies), and 0.877% non-essential amino acids (Life Technologies). Cells were used at passages 1–2 throughout the study. Cells used for individual experiments were from different donors.

2.4. Cell culture

All nanotopography substrate replicas used in the experiments (NSQ50 and flat) were briefly (20 s) air plasma-treated (Harrick plasma cleaner) to increase hydrophilicity of the polymer and hence enhance cell binding, and then washed in 70% (*v*/*v*) ethanol, HEPES-buffered saline and cell growth medium (DMEM with 10% FBS, 1% (*v*/*v*) 200 mM l-glutamine, 1% (*v*/*v*) sodium pyruvate and antibiotics (6.74 U/ml Penicillin–Streptomycin, 0.2 μg/ml Fungizone)) for 5 min each. The substrates were then placed in a 24-well plate and seeded with MSCs at a concentration of 1×10^4 cells/ml of growth medium per well. The growth medium was changed twice weekly until the end of culture period.

2.5. Immunohistochemistry

At the end of cell culture periods (e.g. 3, 14 and 28 days), the cells were washed in phosphate-buffered saline (PBS) and then fixed in 4% (*v*/*v*) formaldehyde in PBS containing 1% (*w*/*v*) sucrose at room temperature for 15 min. After fixing the cells, samples were washed with PBS three times and then permeabilising buffer (10.3 g sucrose, 0.292 g sodium chloride, 0.06 g magnesium chloride, 0.476 g HEPES buffer, 0.5 ml Triton X, in 100 ml PBS, pH 7.2) was added for 5 min at 4 °C. Cells were then incubated at 37 °C for 5 min in 1% (*w*/*v*) BSA/PBS. This was followed by the addition of either anti-osteopontin (OCN) or anti-growth hormone receptor (GHR) primary antibody for 1 h (37 °C) [1:50 in 1% (*w*/*v*) BSA/PBS; anti-osteopontin: mouse monoclonal antibodies (IgG_1), Autogen Bioclear; GHR ab (ab134078): rabbit monoclonal antibodies, Abcam]. To visualise actin, rhodamine phalloidin was also added for the duration of this incubation (1:500 in 1% (*w*/*v*) BSA/PBS, Molecular probes, Life Technologies UK). The

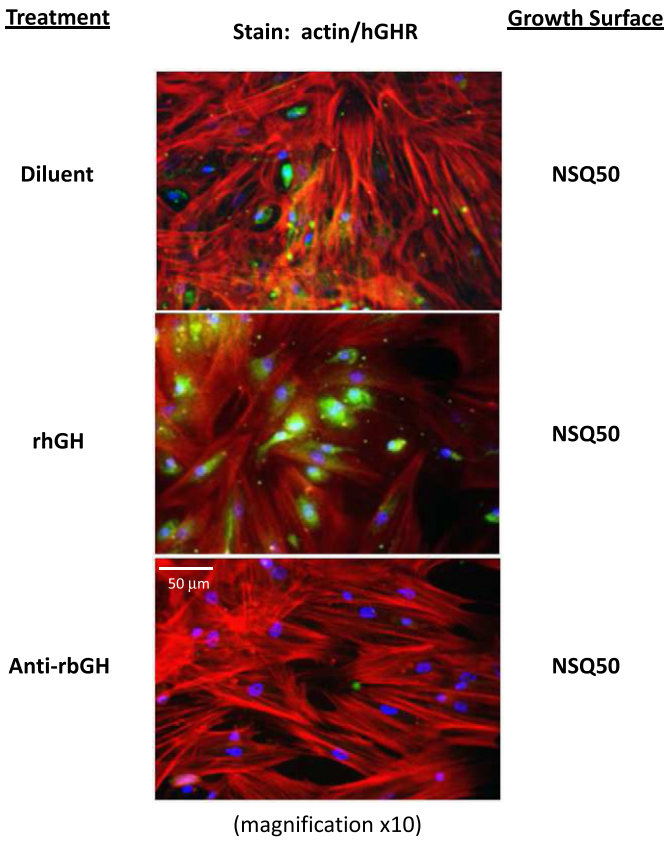

Fig. 2. Immunofluorescent staining of GH receptors (green) in human MSCs grown on NSQ50 topography. Osteoprogenitors were cultured for three days in growth media (top), growth media plus human recombinant GH (middle) or growth media plus anti-bovine GH antiserum (bottom). Actin is stained red and nuclei are stained blue.

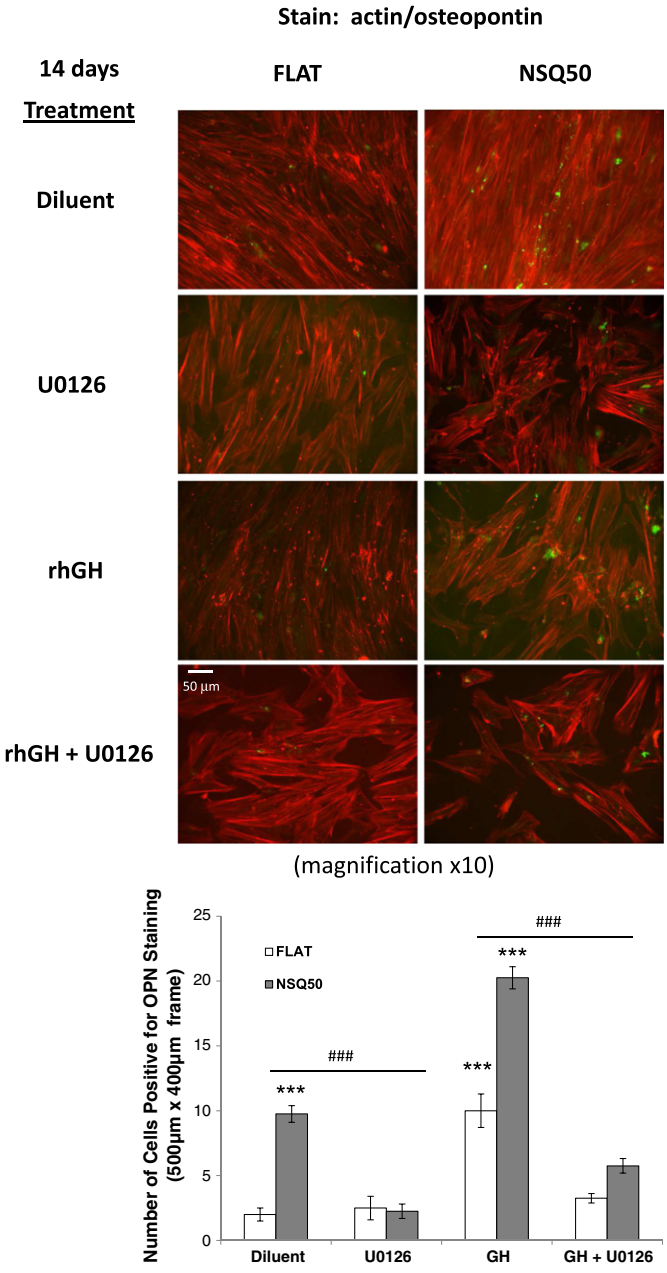

Fig. 3. Osteopontin (OPN) staining of MSCs after 14 days of culture with the indicated treatments. OPN is stained green, actin is stained red and nuclei are stained blue. Quantification of the number of OPN-positive cells is presented as a histogram in the *lower panel*. Significant increase in OPN staining relative to diluent-treated cells is indicated, ***, $p < 0.001$, as are significant decreases in cells treated with U0126, ###, $p < 0.001$.

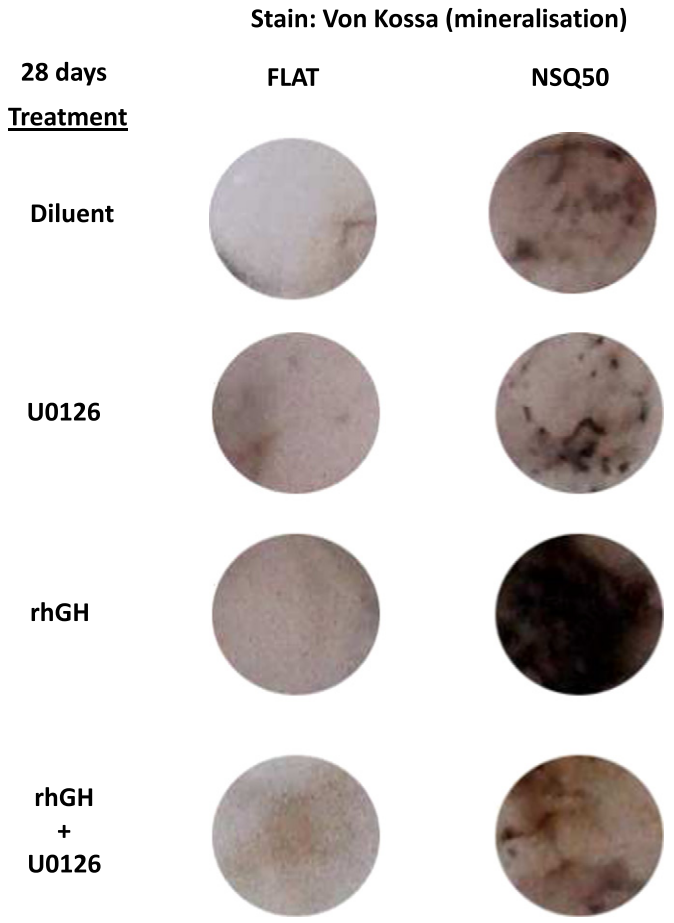

Fig. 4. Von Kossa staining of MSCs after 28 days of culture with the indicated treatments. Mineralisation is indicated by a black colouration whereas individual cells are stained brown.

samples were next washed in 0.5% (*v*/*v*) Tween 20/PBS (3 × 5 min). A secondary biotin conjugated antibody, either anti-mouse or anti-rabbit antibody was added for 1 h (37 °C) followed by washing (1:50 in 1% (*w*/*v*) BSA/PBS, monoclonal horse anti-mouse/rat IgG, Vector laboratories). A FITC-conjugated streptavidin third layer was added (1:50 in 1% BSA/PBS, Vector Laboratories) at 4 °C for 30 min. Finally, samples were mounted with DAPI nuclear stain (Vector Laboratories), and slides were viewed by fluorescence microscopy (Zeiss Axiophot, 200 M or 100 M).

2.6. Real-time quantitative PCR

At the end of the 7-day culture period (i.e. experiment 2), cells were lysed and total RNA was extracted using a Qiagen RNeasy kit (three replicates for each condition). The RNA samples were treated with RNAse and reverse-transcribed using the SuperScript first-strand synthesis system for real-time quantitative polymerase chain reaction (RT qPCR, Invitrogen). Real-time qPCR was carried out using the 7500 Real Time PCR system from Applied Biosystems for analysing expression of bone morphogenetic protein receptor type II (BMPR2) mRNA with a SYBR Green PCR kit. GAPDH gene served as the house-keeping gene, and expression of all the genes of interest was normalised to GAPDH expression. Primer sequences were BMPR2 (fwd), 5′-GATGGCAAATCA GGATCAGG-3′, BMPR2 (rev), 5′-CTTCACAGTCCAGCAGTTCA-3′, GAPDH (fwd), 5′-GTCAGTGGTGGACCTGACCT-3′ and GAPDH (rev), 5′-ACCTGG TGCTCAGTGTAGCC-3′. The comparative cycle-threshold method was used for quantification of gene expression.

2.7. Von Kossa stain for mineralisation

Silver nitrate (5% *w*/*v*) was used to cover the substrates, which were then exposed to UV light for 20 min and rinsed in deionised water. Following this 5% (*w*/*v*) sodium thiosulphate was added to the substrates for 10 min and then rinsed with tepid water for 5 min followed by deionised water. The substrates were then counter stained with nuclear fast red for 5 min and then rinsed three times with deionised water followed by 70% ethanol. The substrates were allowed to dry and observed using an Axiophot-Zeiss microscope under white light.

2.8. In cell western analysis of ERK phosphorylation

Isolated MSCs grown on either NSQ50 or flat surfaces were stimulated and then fixed with 10% (*v*/*v*) formaldehyde in PBS, containing 2% (*w*/*v*) sucrose. Cells were then permeabilised in 0.5% (*v*/*v*) Triton, 10% (*w*/*v*) sucrose, 0.3% (*w*/*v*) NaCl, 0.06% (*w*/*v*) MgCl2 in HEPES-buffered PBS, pH7.2. Following permeabilisation, cells were blocked for 1.5 h at room temperature with 1% (*w*/*v*) milk powder in PBS. Antibodies to detect both total (raised in rabbit) and phosphorylated (Thr202/Tyr204) forms of ERK (raised in mouse) were then added to a concentration of 1:500 in blocking solution and left overnight at 4 °C. Cells were then washed 5 times with 0.1% (*v*/*v*) Tween20 in PBS and then incubated for 1 h at 37 °C with a second antibody mix containing near infra-red, fluorescently labelled anti-mouse and anti-rabbit antibodies (1:1000; Licor Biosciences) in 0.2% (*v*/*v*) Tween20 in PBS. Cell were then washed 5 times with 0.1% (*v*/*v*) Tween20 in PBS and visualised using an ODYSSEY® Sa Infrared Imaging System (Licor Biosciences, Nebraska, USA).

2.9. Statistics

Statistical analysis was carried out using the Tukey–Kramer multiple-comparisons post-test analysis of variance (ANOVA) and Prism Software (GraphPad Software).

3. Results

3.1. Osteogenesis

To establish a reproducible osteogenic cell model, human MSCs were isolated form human bone marrow and grown on either flat or nanometric, near square 50 (NSQ50) growth surfaces (Fig. 1; *upper panel*), since the NSQ50 topology has been previously shown to promote the osteogenic conversion of MSCs in the presence of normal serum [4]. After 7 days of culture on flat or NSQ50 surfaces, mRNA was extracted from cells and quantitative RT-PCR was carried out to determine whether cells expressed the early marker for osteogenesis, BMPR2. Results demonstrated that cells grown on NSQ50 demonstrated significantly higher levels of BMPR2 mRNA than cells grown on flat surfaces (Fig. 1; *lower panel*), which is in agreement with previous studies demonstrating that NSQ50 topology can promote the expression of late-markers of osteogenesis [4]. In addition to early marker expression immunostaining cells following 28 days of culture showed a marked increase in the expression of the late osteogenic markers, osteopontin and minerilisation, in NSQ50, but not flat, cells (Fig. 1, *lower panel* and Fig. 4, respectively). These results demonstrate the efficacy of NSQ50 in promoting osteogenic conversion of human MSCs.

3.2. GH and osteogenesis

Having established a reproducible system for MSC differentiation, we next examined the potential role of growth hormone (GH) in the control of osteogenesis from MSCs, since GH has been shown to induce

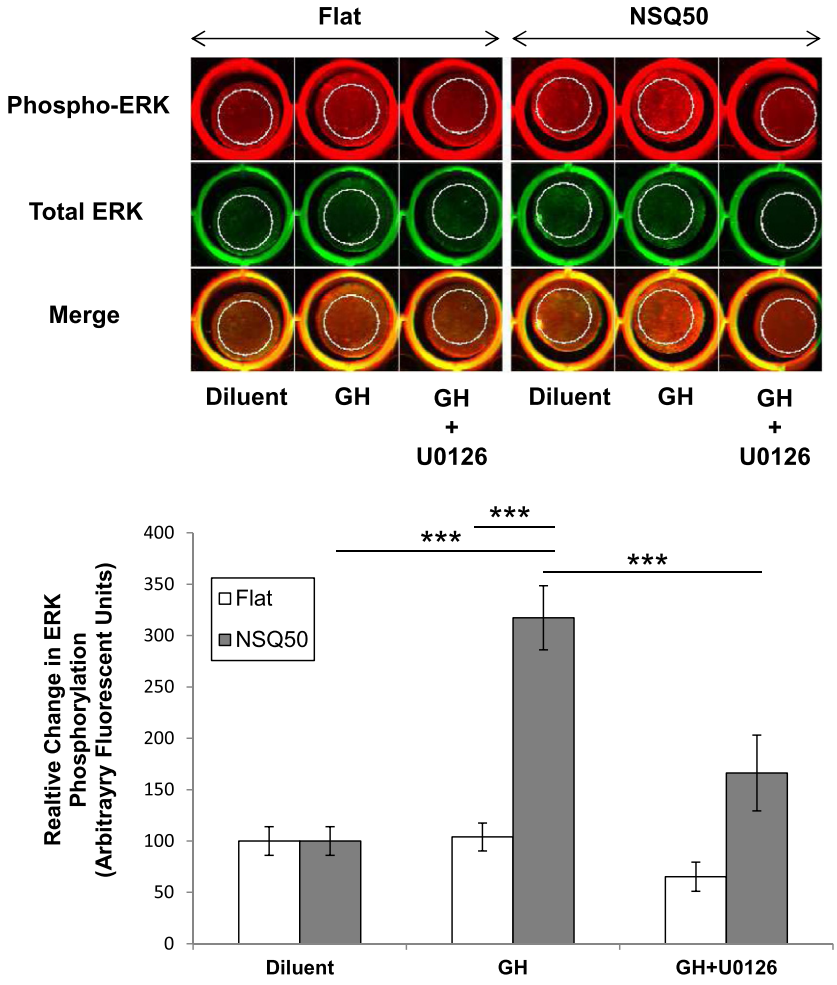

Fig. 5. MSCs were plated onto flat or NSQ50 surfaces and then stimulated for 1 h in the presence or absence of rhGH and the inhibitor of ERK activation, UO126. Cells were then fixed and fluorescently stained with antibodies (*upper panel*) that recognise the phosphorylated, active form of ERK (red) and total ERK protein (green), respectively. Relative fluorescent intensities were then determined. The histogram in the *lower panel* represents the relative change of phosphorylated ERK to total ERK and is means ± SEM for three separate observations. Significant changes in ERK phosphorylation are indicated ***, $p < 0.001$.

expression of BMPRs and osteopontin in osteoblasts [7]. We first used anti-GH receptor antibodies to detect the expression of GHRs on MSCs grown on NSQ50 topology. Immunofluorescent staining confirmed the presence of GHRs on MSCs cultured in growth media on NSQ50 surfaces (Fig. 2) and found that stimulation of cells with recombinant human GH (rhGH) enhanced the expression of GHRs (Fig. 2), indicating that GHR expression may be regulated by GH present in the growth medium. Since the cell culture medium normally contains bovine GH, we added anti-recombinant bovine GH (anti-rbGH) antiserum to neutralise this and determine the effect GHR expression. We found that on addition of the anti-rbGH antibody to the growth medium, there was a noticeable reduction in GHR immunofluorescent staining in MSCs, indicating that the level of GHR expression in MSCS grown on NSQ50 is dependent on the extracellular concentration of GH in the growth medium.

3.3. GH and MSC differentiation

We next examined whether stimulation of MSCs with GH affects the differentiation of MSCs on NSQ50 topography. In preliminary experiments, cells were cultured for 14 days on flat or NSQ50 topology or NSQ50 plus recombinant human growth hormone (rhGH). Cells were then stained for the late marker of osteogenic differentiation, osteopontin. Immunofluorescence staining for osteopontin indicated significant osteogenesis on NSQ50, but not on flat topography (Fig. 3). Furthermore, the expression of osteopontin on NSQ50 topography was enhanced by the addition of rhGH to the medium (Fig. 3).

To test for a possible mechanism by which GH potentiates MSC differentiation, cells were co-incubated with the ERK MAP kinase inhibitor, UO126 (Fig. 3). Addition of UO126 (10 μM) to the medium was found to completely inhibit osteogenic conversion of GH-stimulated MSCs on NSQ50 topography, as determined by staining for osteopontin expression (Fig. 3), indicating that ERK activation by GH may be required for osteogenesis.

Growth of cells on NSQ50 topology was also found to increase mineralisation of cells, as determined by Von Kossa staining, following 28 days of culture (Fig. 4). Moreover, stimulation of cells with rhGH was found to dramatically increase the extent of cell mineralisation on NSQ50, but not flat surfaces, demonstrating that NSQ50 enhances the osteogenic action of GH in MSCs. In addition, U0126 also effectively inhibited the enhanced osteogenic differentiation of MSCs in response to rhGH, cultured for 28 days on NSQ50 morphology (Fig. 4), demonstrating the importance of ERK activity for the osteogenic programme. Indeed, using an "in-cell" Western blotting approach to measure the phosphorylation/activation state of ERK MAP kinase in MSCs, we found that rhGH promoted activation of ERK on NSQ, but not on flat topography. ERK activation in response to GH was also significantly inhibited in the presence of UO126 (Fig. 5). Together these results suggest a novel mode of regulation whereby NSQ topology enhances the ability of GHRs to couple to the activation of the ERK MAP kinase

signalling pathway in MSCs. This leads to a potentiation of MSC differentiation towards a bone-forming phenotype.

4. Discussion

Here we have developed a system of nanometric topology to trigger the spontaneous osteogenesis of human MSCs, as previously described [4]. This system is an important development because it allows the in vitro differentiation of MSCs in the absence of complex, hormonally defined differentiation media [4]. Moreover, because nanotopographic differentiation is largely dependent on cell–substrate interaction, this provides a highly amenable system to investigate how interactions with external topographic cues control the responsiveness of MSCs to other differentiative factors, including hormones and growth factors. In the long term studies like this may lead towards a full understanding of the factors controlling stem cell differentiation and lead towards the development of an in vitro model of the stem cell niche [9].

In the current study we have examined how interactions between osteogenic nanotopography and the GH receptor affect the osteogenic differentiation of human MSCs. Our results show that MSCs grown on NSQ50 topology express growth hormone receptors (GHRs), the expression levels of which were dependent on the concentration of GH in the growth medium. To our knowledge this is the first time that GHR levels have been shown to be modulated by GH in MSCs. In other tissue and cell types, the data regarding the regulation of GHR expression is conflicting and may be dependent on timing and what epitope on the GHR is used for detection [15]. Moreover, differential effects of GH on the expression of GHR can be observed in the same tissue. For example, and consistent with our results, physiological levels of GH promote GHR up-regulation in human glomerular mesangial cells, whereas supra-physiologic levels of GH promote GHR down-regulation and use of the GHR antagonist, pegvisomant, had no effect in the same cells [10]. Clearly the ability of GH to regulate GHR levels in MSCs requires future investigation and may cast new light on the link between bone density and the response of MSCs to GH in disease syndromes, such as GH resistance and GH deficiency.

We also found that stimulation of NSQ50 cells with rhGH leads to an enhancement of osteogenesis, as determined by the expression of the late osteogenic marker, osteopontin, and Von Kossa staining of mineralisation. This enhancement coincided with an enhanced ability of GH to activate the ERK MAP kinases cascade on NSQ50. This is the first time that NSQ50 topology has been shown to affect GH signalling. Thus, GH can potentiate ERK signal transduction on NSQ50 but not on flat surfaces. Inhibition of ERK activation severely blunted osteogenesis promoted by NSQ50 topology and GH treatment, indicating that ERK activity may be important for the actions of both GH and NSQ50 on MSC differentiation. While it has been previously shown that ERK may be important for MSC osteogenesis in response to differentiation supplements and NSQ50 topology [5,9], this is the first time that ERK activity has been shown to be so critical for GH-driven differentiation of human MSCs.

With respect to the action of NSQ50 on MSC differentiation it is clear that this engineered surface is altering the balance between osteogenesis, adipogenesis and chondrogenesis [4]; however how this is achieved remains to be determined. Interaction with NSQ50 topology may mimic the effect of hormonal or extracellular matrix (ECM) stimuli otherwise found in the stem cell niche that facilitates GH signalling towards osteogenesis. In this respect, it is known that topographic sensing and interaction with the ECM are both governed by the integrin family of adhesion receptors [1]. Integrin engagement has been shown to modulate signalling from growth factor receptors in general [18] and, particularly, a key element for signalling to the ERK cascade in mechanically induced MSCs [13]. The role of integrin engagement in the sensitisation of GHR signalling by NSQ50 topology in MSCs also remains to be determined as is whether NSQ50 is affecting elements up-stream or down-stream of GHR receptor activation. For example, the GH/IGF-1 axis becomes impaired in transgenic mice lacking specific β1-integrins, which is associated with a lack of hypothalamic GHR expression and antagonism of GH signalling [2]. Further work will therefore be necessary to determine the role of integrins and ERK signalling in controlling the response of MSCs to GH and nanometric topology. Overall this new work advances the field of biomaterials since it demonstrates that NSQ50 topology can be used to study the individual actions of isolated hormones, such as GH, on the differentiation of MSCs. This will pave the way for future studies on the interaction between growth factors, hormones and cell adhesion molecules in the control of stem cell function.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgements

This work was supported by a grant from the Bioscience and Biotechnology Research Council awarded to MJD (grant number BB/K006908/1). The authors acknowledge the technical assistance of Carol-Anne Smith.

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