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Osteogenic lineage restriction by osteoprogenitors cultured on nanometric grooved surfaces: The role of focal adhesion maturation ☆

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ABSTRACT

The differentiation of progenitor cells is dependent on more than biochemical signalling. Topographical cues in natural bone extracellular matrix guide cellular differentiation through the formation of focal adhesions, contact guidance, cytoskeletal rearrangement and ultimately gene expression. Osteoarthritis and a number of bone disorders present as growing challenges for our society. Hence, there is a need for next generation implantable devices to substitute for, or guide, bone repair in vivo. Cellular responses to nanometric topographical cues need to be better understood in vitro in order to ensure the effective and efficient integration and performance of these orthopedic devices. In this study, the FDA-approved plastic polycaprolactone was embossed with nanometric grooves and the response of primary and immortalized osteoprogenitor cells observed. Nanometric groove dimensions were 240 nm or 540 nm deep and 12.5 μm wide. Cells cultured on test surfaces followed contact guidance along the length of groove edges, elongated along their major axis and showed nuclear distortion; they formed more focal complexes and lower proportions of mature adhesions relative to planar controls. Down-regulation of the osteoblast marker genes RUNX2 and BMPR2 in primary and immortalized cells was observed on grooved substrates. Down-regulation appeared to directly correlate with focal adhesion maturation, indicating the involvement of ERK 1/2 negative feedback pathways following integrin-mediated FAK activation.

1. Introduction

By 2020 it is expected that the current number of patients suffering from bone diseases will double, with 9.3% of US adults predicted to suffer from osteoarthritis alone [1,2]. Hence, there is a need to maximize the life expectancy of primary joint implants through understanding cell–material interactions.

It is becoming increasingly evident that the interface between host and implant is far more than a simple boundary of definition. Rather, host–implant interfaces provide a context for cellular adhesion and cell-specific orientation through first stage protein interactions and subsequent support of tissue neogenesis and cellular differentiation [3,4]. The range of orthopedic biomaterials currently in use clinically typically lack in biofunctionality, resulting in micromotion of prosthesis after implantation and an increased risk of revision surgery. Aside from the clear morbidity and socio-economic implications, secondary surgery is accompanied by a twofold increased risk of further medical complications [5]. It is essential that future orthopedic devices are able to withstand micromotion by directing differentiation of locally derived mesenchymal (specifically skeletal) stem cells (MSCs) and osteoprogenitors (OPGs) into bone-matrix-secreting osteoblasts.

Contact guidance on grooved topographies is a well-documented cellular behaviour in vitro [6] and has biological relevance in vivo. The natural bone extracellular matrix provides an environment rich in both chemical and topographical cues capable of influencing and modulating cell behaviours. This macromolecular network of proteins, glycoproteins and polysaccharides is known to influence the differentiation of skeletal progenitor cells through focal adhesion formation, contact guidance, cytoskeletal rearrangement and ultimately gene expression [7,8]. These are effects that

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we, and others, have replicated across a variety of cell types [7–11]. It is now recognized that capitalizing on topographical cues, alongside traditional chemical and biological signalling, is necessary to facilitate the successful integration and performance of a device in vivo.

A critical mechanism for cellular interaction with the extracellular matrix (ECM), and with biomaterials, is the process of cell adhesion. In addition to facilitating cellular tethering to substrata, focal adhesions form the basis of filopodia exploration of the ECM, subsequent lamellipodia ruffling and cellular spreading [12] in response to the extracellular landscape. As a cell spreads across a substrate, transient focal complexes (FX; adhesions of less than 1 μm) form along the cell's leading edge, which may precede formation of larger focal adhesions (FA; 1–5 μm adhesions) [13].

Intrinsically linked to cellular spreading is the phenomenon of contact guidance, i.e. cellular tracing along the edge of a physical feature or obstacle. Contact guidance is associated with regular grooved substrata in Ref. [14] and drives processes such as the migration of immature neurons along scaffolds of glial fibres during development [15]. The use of grooved substrates provides a reproducible model to study the effects of focal adhesion formation in osteogenesis in vitro [17].

The fundamental component of the focal adhesion plaque and the receptor through which MSCs, osteoprogenitors and osteoblasts (amongst others) physically interact with the ECM is the integrin [16]. Integrins function in adhesion plaques by binding and mechanically coupling the cell's cytoskeleton to the ECM and translating mechanical into biochemical signals within the cell; a process termed mechanotransduction [18]. In osteogenesis, focal adhesion-mediated mechanotransduction and integrin-dependent signalling pathways are dependent on non-receptor tyrosine kinases such as focal adhesion kinase (FAK) [19]. In addition to directly activating survival pathways (inhibiting anoikis and inactivating caspase 9 [20]), FAK promotes osteogenesis through extracellular signalling-related kinase 1 and 2 (ERK 1/2) pathways [21]. Through the integrin-dependent translocation of ERK, FAK mediates phosphorylation of the Runt-related transcription factor RUNX2 [22], the expression of which is highly restricted to skeletal tissue. RUNX2 acts as a master regulator in progenitor commitment to the osteoblast lineage and in transcription of osteoblast-specific genes essential for bone homeostasis (osteocalcin, osteopontin and matrix metalloproteinase 13 [7]). Through attachment to ECM elements, integrin clustering and the formation of actin stress fibres, focal adhesion formation and maturation are directly linked to FAK signalling, RUNX2 transcription and the induction of osteogenesis.

Here, we draw on the observation that as cellular spreading increases (either uniformly or as part of a polarized cellular morphology), tension placed on actin stress fibres anchored at focal adhesion plaques will result in a proportional and marked elongation of the adhesion itself through dynamic reinforcement [23]. We hypothesize that increased cellular motility caused by contact guidance on grooved substrates will inhibit osteogenesis through a reduction in adhesion maturation and reduced RUNX2 transcription. To investigate this, we have examined focal adhesion formation in response to 240 nm and 540 nm deep-grooved (12.5 μm wide) polycaprolactone (PCL) substrate, a biodegradable polyester approved by the US Food and Drugs Administration (FDA) for use in vivo. The osteoblast-like MG63 cell line and primary human osteoprogenitors cell populations (OPGs) were used in experiments. Comparisons were drawn between cells cultured on grooved and planar substrates and significance tested by statistical analysis of focal adhesion classification (FX, FA, SMA), proportions (%FX, %FA, %SMA) and total adhesion numbers. Focal adhesion orientation and nuclear distortion were quantified and polarized cellular morphologies were noted. Quantitative PCR was used to determine expression of osteoblast master transcription factor RUNX2 and related gene BMPR2 after 3 days of culture on planar control and groove substrates.

2. Materials and methods

2.1. Generation of grooved and planar growth surfaces

Grooved and planar slides were produced as previously described [24]. Planar slides were blanket-etched to ensure comparable chemistry to the grooved slides. Slide topographies were confirmed after original fabrication to be 12.5 μm × 240 nm and 12.5 μm × 540 nm. Quartz slides were used to create multiple polymer replicas by manual hot (80 °C) embossing polycaprolactone beads ($(C_6H_{10}O_2)_n$; PCL, washed in methanol for 1 week, Sigma Aldrich). Substrates were cooled on ice and trimmed to sizes suitable for cell culture in 24 well culture dishes: typically discs were 13 mm in diameter.

Wettability of PCL substrates was quantified by water contact angle analysis (WCA) with a KSV CAM 100 contact angle meter (KSV Instruments) using the sessile drop technique. Measurements were carried out in triplicate on three replicates of each sample. PCL substrates were treated for 30, 60, 90 or 120 s at MHz-range radio-frequency (RF) (10.15 W, 720 V DC, 15 mA DC) in a plasma cleaner (PDC-002, Harrick Plasma) to remove organic contaminants and improve cell surface attachment. Surfaces were sterilized in 70% ethanol for 1 h immediately following plasma treatment, followed by three sequential 5 s immersions in 70% ethanol, two sequential 5 s immersions in HEPES saline solution and a final 10 immersion in cell culture media prior to cell seeding.

2.2. Atomic force microscopy

Surfaces were analysed by atomic force microscopy (AFM) (scanning mode) after plasma treatment. The tip used was a non-conducting silicon nitride (MSCT, Veeco) with dimensions of 140 μm length by 18 μm width (resonance frequency 38 kHz, spring constant (K) 0.1 N m^{-1}). Planar control substrates showed an average roughness of 18.5 nm over 100 μm^2 (RMS 23.0 nm). Groove depths of 240 nm-grooved substrates were 210, 220 and 235 nm with an approximate width of 12.8 μm. 540 nm depths were 510, 530 and 535 nm and width ~13.1 μm.

2.3. Cell isolation and culture

OPGs were isolated from haematologically normal patients undergoing routine surgery as previously described [25]. Only tissues that would have been discarded were used and only with the approval of the Southampton and South West Hants Local Research Ethics Committee. OPGs were cultured in growth media containing 88% Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 8.8% fetal bovine serum (FBS; Lonza), 1.5% penicillin streptomycin, 0.85% 100 × non-essential amino acids (Invitrogen) and 0.85% 100 mM sodium pyruvate (Life Technologies). The medium was changed every 3 days and cells were passaged as required by trypsinization. MG63 human osteoblast-like cells were cultured in media containing 71% DMEM, 17.5% Medium 199 (Sigma), 10% FBS, 1% penicillin streptomycin and 0.5% 100 mM sodium pyruvate. Cells were incubated at 37 °C with a 5% CO_2 atmosphere and serum starved for 24 h prior to seeding to encourage populations with synchronized cell cycles. Cells were seeded at 1 × 10^4 cells ml^{-1} in assay media containing 1% FBS.