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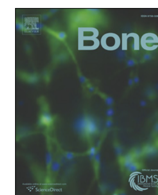

Full Length Article

Phosphoproteome analysis reveals a critical role for hedgehog signalling in osteoblast morphological transitions

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ABSTRACT

The reciprocal and adaptive interactions between cells and substrates governing morphological transitions in the osteoblast compartment remain largely obscure. Here we show that osteoblast cultured in basement membrane matrix (Matrigel™) exhibits significant morphological changes after ten days of culture, and we decided to exploit this situation to investigate the molecular mechanisms responsible for guiding osteoblast morphological transitions. As almost all aspects of cellular physiology are under control of kinases, we generated more or less comprehensive cellular kinome profiles employing PepChip peptide arrays that contain over 1000 consensus substrates of kinase peptide. The results obtained were used to construct interactomes, and these revealed an important role for FoxO in mediating morphological changes of osteoblast, which was validated by Western blot technology when FoxO was significantly up-expressed in response to Matrigel™. As FoxO is a critical protein in canonical hedgehog signalling, we decided to explore the possible involvement of hedgehog signalling during osteoblast morphological changes. It appeared that osteoblast culture in Matrigel™ stimulates release of a substantial amounts Shh while concomitantly inducing upregulation of the expression of the *bona fide* hedgehog target genes Gli-1 and Patched. Functional confirmation of the relevance of these results for osteoblast morphological transitions came from experiments in which Shh hedgehog signalling was inhibited using the well-established pathway inhibitor cyclopamine (Cyc). In the presence of Cyc, culture of osteoblasts in Matrigel™ is not capable of inducing morphological changes but appears to provoke a proliferative response as evident from the upregulation of Cyclin D3 and cdk4. The most straightforward interpretation of our results is that hedgehog signalling is both necessary and sufficient for membrane matrix-based morphological transitions.

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1. Introduction

The extracellular matrix (ECM) is a set of important components essential for tissue integrity with some cell types, such as chondrocytes, osteocytes, and fibroblasts, which are more-or-less completely embedded in it [1–3]. The matrix is highly biologically active and provides ligands that regulate cell-matrix interaction. It also provides important cues with respect to cell plasticity and tissue remodelling [2,4]. The details of the interaction between the ECM and the cells that interact with it are still only partly understood. Our knowledge in this area, however, has been substantially bolstered by the discovery that ECM-based scaffolds are useful tools to analyse both physiology and pathophysiology of cell-matrix interactions [1,4,5]. Increasing insight into the molecular mechanisms involved might revolutionise the pursuit of better biomaterials for combating disease, especially in the quest to improve bone regeneration.

Cellular reaction to the extracellular matrix involves complex signal transduction pathways. Almost all of these pathways involve tyrosine kinases and/or serine/threonine kinases (both receptor type and non-receptor type enzymes), which often operate in intricate signalling cascades that defy analysis using conventional technology. The specific importance of phosphorylation derives from the substantial conformational changes in proteins when the negative phosphate group is introduced into the amino acid chain, which allows fast and reversible regulation of protein function, including enzymatic activity. Classically, the study of different kinases or cellular pathways is typically pursued one protein at the time through Western blotting technology, a fairly

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labour-intensive tool, which hampers the generation of comprehensive description of cellular signalling [6,7]. However, advent of kinome profiling has improved this situation. Multiple technologies exist, but especially peptide arrays allow simultaneous analysis of kinase enzymatic activity of 1000 substrates or more in a single experiment [8–10]. This powerful approach has, however, not been exploited to obtain insight into the biochemical pathways that regulate osteoblast phenotype during its interaction with the ECM.

In this study, we documented that pre-osteoblasts cultured on basement membrane matrix (MATRIGEL™) up to 10 days triggers a morphological transition, then, we explored the global kinase profile associated with this event. The profiles obtained suggest an important role for Shh signalling in these changes, mediating morphological change and exit from the cell cycle, which was confirmed when tested directly. These results open new avenues to understand morphological changes of osteoblast at a molecular level and provide a wealth of data on osteoblast adaptation to organic matrix-based substrates. Thus, this study may be useful for the rational design of therapeutic devices.

2. Material and methods

2.1. Reagents, antibodies, and primers

Cyclopamine, β-glycerophosphate, ascorbate, *p*-nitrophenylphosphate (*p*NPP), and DMSO were purchased from Sigma Chemical Co (St. Louis, MO, USA); A10490 - MEM alpha, nucleosides, with no ascorbic acid, was from Invitrogen; MATRIGEL™ and recombinant-Sonic Hedgehog (rShh) were from R&D (R&D Systems Inc., Minneapolis). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Calbiochem (Calbiochem, San Diego, CA, USA). The general cell culture supplies were purchased from Bio-One (Greiner Bio-One, Germany). The immunoblotting reagents were purchased from Millipore (Millipore Corporate Headquarters, Billerica, MA, USA). Antibodies: CDK6 (DCS83) mouse mAb #3136; CDK6 #3136; $p21^{Waf1/Cip1}$ #2946; phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199) #4228; phospho-Akt (Ser473) #4060; PDK1 #5662; GSK-3β #12456; phospho-PKC (pan) (zeta Thr410) #2060; PKA #3927; p38 MAPK #8690; phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) #4370; phospho-Src Family (Tyr416) #6943; phospho-Paxillin (Tyr118) antibody #2541; Shh antibody #2287; Connexin 43 antibody #3512; FoxO1 #2880; phospho-FoxO3a (Ser253) #9466; phospho-FoxO3a (Ser318) #9465; GADPH #5174; anti-mouse IgG, HRP-linked antibody #7076; and anti-rabbit IgG, HRP-linked antibody #7074 were purchased from Cell Signalling Technology (Boston, MA, USA). Patched (C-20; #6147) and β-Actin (C-4; #sc-47778) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-CyclinD1 (C5588) was from Sigma Chemical Co (St. Louis, MO, USA). Primers were obtained from Invitrogen (Breda, Netherlands) and details are listed in Table 1.

2.2. Cell culture and osteoblast morphological change acquisition protocol

MC3T3-E1 (subclone 4), a calvaria mouse pre-osteoblast cell line, was obtained from the ATCC (Manassas, USA) and grown at 37 °C in α-MEM medium supplemented with 10% Foetal Bovine Serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin under a humidified 5% CO_2 atmosphere. When appropriate, cells were exposed to osteogenic conditions, which involved α-MEM plus β-glycerophosphate (10 mM) plus ascorbate (50 μg/mL). Before experimentation, a gel was prepared by mixing Matrigel™: α-MEM (2:1), as it was used for coating Petri dishes (100 mm diameter) or 6-wells plates, exactly as indicated by the manufacturer. These petri dishes (or plates) were kept at 37 °C for 3 h prior to each experiment in order to allow MATRIGEL™ gelation. Afterwards, cells were seeded (3×10^4 cells/mL) and cultured for 10 days. The medium was freshly replaced every 3 days. In this protocol, 3 experimental conditions were routinely assayed: 1) osteoblasts cultured on polystyrene (conventional cell culture staffs); 2) cells cultured on MATRIGEL™ as ECM-based scaffold; 3) osteoblast cultured on MATRIGEL, but subjected to osteoblast-differentiation condition (as detailed previously).

Table 1
RT-PCR primers.

Primers		
Gene	Forward	Reverse
Alkaline phosphatase (ALP)	5′-aacccagacacaagcattcc-3′	5′-cgaagggtcagtcaggttgt-3′
Osteocalcin (OCN)	5′-gcgctctgtctctctgacct-3′	5′-gccggagtctgttcactacc-3′
Cathepsins B (CtsB)	5′-tgcatcctagcctctccta-3′	5′-ggaagtcctgcagtcaaagc-3′
Cathepsins K (CtsK)	5′-tctctcggcgtttaatttgg-3′	5′-aagtggttcatggccagttc-3′
β-Actin	5′-cctaaggccaaccgtgaaaag-3′	5′-tcttcatggtgctaggagcca-3′

2.3. Treatment with recombinant Shh (rShh) or cyclopamine (Cyc)

In order to confirm whether hedgehog signalling was able to promote osteoblast morphological change, we treated pre-osteoblasts with rShh or cyclopamine (Shh inhibitor) for up to 6 days. Before experimentation, the gel-mix was prepared as described before, but either rShh (10 ng/mL) or cyclopamine (2 μM) was included in the mixture as appropriate, and this gel-mix was used to coat Petri dishes (100 mm) or 6-wells plates. Cyc at the concentration used here did not induce cytotoxicity (data not shown). Coated dishes were incubated at 37 °C for 3 h before seeding 3×10^4 cells/mL and cultured for 6 days. Results were analysed using a Leica microscope/camera and the samples were collected for Western blotting. Medium was replaced every 3 days.

2.4. Cellular viability assay

In order to check the cell viability, MC3T3-E1 cells (3×10^4 cells/mL) were seeded consistent with the appropriate experimental condition in 24-wells plates and maintained for 10 days. Afterwards, the medium was removed and 1 mL (1 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well [11]. After 4 h of incubation at 37 °C, the medium was removed and the reduced formazan was solubilised in 1 mL of DMSO (Sigma-Aldrich, USA). Then, the plate was shaken for 10 min on a plate shaker (Biotek, Winooski, VT, USA) and the absorbance was measured at 570 nm using a micro-plate reader (Biotek, Winooski, VT, USA).

2.5. Determination of alkaline phosphatase (ALP) activity

MC3T3-E1 cells (3×10^4 cells/mL) were seeded on MATRIGEL™ in 24-well plate and grown for 10 days. Afterwards, the cells were rinsed with ice-cold 1× PBS and incubated for 30 min at room temperature in ALP assay buffer (1.5 M of Tris-HCl [pH 9.0], 1 mM $ZnCl_2$, and 1 mM $MgCl_2$) containing 1% (vol/vol) Triton X-100. Cell extracts were collected, centrifuged, and used for an enzyme assay. ALP activity was determined by using 5 mM of *p*-nitrophenyl-phosphate (*p*NPP) as a substrate. The result is expressed as nanomoles of products formed per minute per milligrams of protein. Protein amount was determined by Lowry's method as modified by Hartree [12].

2.6. Western blot

Osteoblasts were lysed using a Lysis-Cocktail [50 mM Tris, tris(hydroxymethyl)aminomethane–HCl (pH 7.4), 1% (vol/vol) Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM O-Vanadate, 1 mM NaF, plus protease inhibitors (1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM 4-(2-aminoethyl)-benzolsulfonyl-fluoride-hydrochloride)] and kept on ice for 2 h, as described previously [13,14]. After clearing the lysate by centrifugation,

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