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Paxillin localisation in osteocytes—Is it determined by the direction of loading? ☆

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Abstract

External mechanical loading of cells aligns cytoskeletal stress fibres in the direction of principle strains and localises paxillin to the mechanosensing region. If the osteocyte cell body can indeed directly sense matrix strains, then cytoskeletal alignment and distribution of paxillin in osteocytes *in situ* will bear alignment to the different mechanical loading patterns in fibulae and calvariae. We used confocal microscopy to visualise the immunofluorescence-labelled actin cytoskeleton in viable osteocytes and paxillin distribution in fixated osteocytes *in situ*. In fibular osteocyte cell bodies, actin cytoskeleton and nuclei were elongated and aligned parallel to the principal (longitudinal) mechanical loading direction. Paxillin was localised to the 'poles' of elongated osteocyte cell bodies. In calvarial osteocyte cell bodies, actin cytoskeleton and nuclei were relatively more round. Paxillin was distributed evenly in the osteocyte cell bodies. Thus in osteocyte cell bodies *in situ*, the external mechanical loading pattern likely determines the orientation of the actin cytoskeleton, and focal adhesions mediate direct mechanosensation of matrix strains.

Keywords: Osteocyte; Bone cells; Cytoskeleton; Actin stress fibres; Mechanosensing; Paxillin; Focal adhesions; Fibula; Calvaria; Confocal laser scanning microscopy

Bone is a dynamic tissue, which remodels in accordance with the external mechanical loading. Osteocytes are widely acknowledged as the pivotal cells orchestrating the biomechanical regulation of bone mass and structure [1–5]. It is believed that mechanosensation of external loads by osteocytes initiates the production of signalling molecules, which modulate the activity of bone resorbing cells, the osteoclasts, and bone forming cells, the osteoblasts. The stellate-shaped osteocytes reside within the bone matrix, with their cell bodies occupying the lacunae and their cell processes occupying the canaliculi [6,7]. According to the lacuno-canalicular flow hypothesis, mechanosensing by osteocytes occurs predominantly through the cell processes [8]. The strain-derived flow of fluid through the lacuno-canalicular network imparts shearing forces on the cell surface, which are amplified via the interaction of pericellular matrix and the cell process cytoskeleton [5,9,10]. The osteocyte cell body has not been implicated in mechanosensation as the fluid flow-mediated shear forces on the osteocyte cell bodies are too small to be sensed [11]. However, our recent *in vitro* studies have shown that both cell bodies and cell processes are mechanosensitive to localised loading [12], which suggests that cell bodies might also be involved in mechanosensing *in vivo* albeit via some different mechanism than fluid flow.

External mechanical loading on the mammalian cells results in surface integrin-mediated elongation of stress fibres in the direction of principle strains [13,14]. The integrins mechanically couple the cellular cytoskeletal network to the extracellular matrix via focal adhesions, which

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comprise of multiple actin-associated proteins such as paxillin, vinculin, talin, and zyxin. It has been suggested that the application of tension on surface integrins causes elongation of stress fibres along the direction of tension [14], and that the cells in general orient in the direction of the principle strain [13]. Moreover, stresses applied to integrins can also regulate gene expression [15,16]. These unique features of the adhesion complexes have led to the suggestion that they can function as mechanosensors in cells [17].

In vitro studies have shown that focal adhesions play a role in mechanosensation in bone cells [18]. However, little is known about the presence, distribution, and physiological relevance of focal adhesions in osteocytes in bone. Recently, we demonstrated differences in 3D morphology and the alignment of osteocytes in unidirectionally loaded mouse fibulae and multidirectionally loaded calvariae [19]. We proposed a possible role of osteocytes in direct mechanosensing of matrix strains via their cell body cytoskeleton. Paxillin is an integral component of focal adhesion complexes and is widely used as a parameter to study the distribution of focal adhesions [20,21]. Moreover, paxillin localises to the cell region, which is actively involved in mechanosensing [22], thus making paxillin an ideal candidate to investigate the presence and distribution of focal adhesions and their possible physiological relevance in direct mechanosensing of matrix strains by osteocytes.

The location of osteocytes in hard bone matrix has been an impediment in the study of their fine structural details at the subcellular level *in situ*. Nevertheless, high resolution imaging by confocal microscopy allows us to visualise osteocytes in their natural environment of bone matrix up to a depth of almost 60 μm. Moreover, combination of recently developed live-cell staining with fluorescent markers [23] and visualisation of osteocytes *in situ* [19] allowed us to investigate fine structural details of osteocyte cytoskeleton in their natural milieu of bone matrix.

In the present study we applied the above-mentioned techniques, i.e. the live-cell staining with fluorescent markers and visualisation of the osteocytes *in situ* to demonstrate the differences in alignment of actin cytoskeleton and distribution of paxillin protein in osteocytes *in situ*, in unidirectionally loaded mouse fibulae and multidirectionally loaded calvariae. This will help us understand better the plausible role of the cytoskeleton of osteocyte cell bodies in direct mechanosensing of matrix strains via their focal adhesions.

Materials and methods

Bone explant preparation

Whole calvariae and fibulae were aseptically isolated from 3–6 months old adult C57 B1/6 wild type mice as described earlier (permission of the Animal Review Committee was obtained) [19]. Briefly, the periosteal surfaces of the bone explants were gently scraped to remove muscular attachments and periosteum for better imaging of the deeper embedded osteocytes. In fibular explants, bone marrow was flushed out thrice with 1 ml phosphate-buffered saline (PBS). The bone was then dissected transversly to obtain pieces of approximately 3×1 mm. In calvarial explants, the suture edges were trimmed and pieces of approximately 3×1 mm were prepared. The bone explants were then prepared for either live actin staining or were fixated and stained for paxillin visualisation as described below.

Osteocyte staining

Live actin staining. For *in situ* live-cell imaging, bone explants were incubated and shaken at 350 rotations per minute for 7–24 h at room temperature with Hank's Balanced Salt Solution (HBSS) containing 5 U/ml of Alexa 488 conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) as described by Paltauf-Doburzynska et al. [23]. The bone explants were washed once with HBSS. To visualise nuclei in live osteocytes *in situ*, the bone explants were then incubated with 1 μM of SYTO 85 orange fluorescent nucleic acid stains (Invitrogen) in HBSS for 10 min at room temperature. They were then washed once in HBSS before visualisation by using confocal laser scanning microscopy as described below.

Paxillin staining. Bone explants were fixed with 4% paraformaldehyde (Merck, Whitehouse Station, NJ, USA) in PHEM buffer made up of 60 mM Pipes (Sigma, St. Louis, MO, USA), 25 mM Hepes (Sigma), 5 mM EGTA (Sigma), 1 mM $MgCl_2$ (Merck), 3% sucrose, and 0.1% Triton X-100 (Serva, Heidelberg, Germany) for 20 min in the dark at 37 °C as described earlier [24]. After washing thrice for 5 min each with PBS, bone explants were blocked in blocking buffer (PBS containing 5% horse serum, 5% glycine, and 0.1% Triton X-100) for 2 h at room temperature in the dark. Subsequently bone explants were stained overnight for paxillin using monoclonal phospho-paxillin (tyr 118) primary antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:40 in blocking buffer in the dark at room temperature. The explants were then washed thrice for 5 min each with PBS and incubated with 1:250 dilution of donkey anti-rabbit IgG Alexa 488 (Invitrogen) for 1 h in blocking buffer at RT. The explants were washed in PBS thrice for 5 min each and mounted in vectashield for imaging.

Confocal laser scaning microscopy

Image acquisition. Confocal images of osteocytes were obtained by using BioRad MRC-1000 UV Leica confocal system attached to a Leica inverted microscope (Leica Microsystems, Wetzlar, Germany). A Leica 63× water immersion objective lens was used with a numerical aperture of 1.2 and a working distance of 170 μm. The fibular and calvarial bone explants were excited by using a krypton/argon laser at 488 nm and the emission was collected at 520 nm. Sequences of x–y optical slices were collected as z-stacks, separated by 0.25–0.50 μm on the z-axis. A single image of maximum projection was obtained from the z-stacks by using Leica confocal software (Leica Microsystems).

Results

In order to compare the osteocyte cytoskeleton in fibula and calvaria we studied the organisation of actin fibres in live osteocytes and distribution of paxillin protein in fixated osteocytes *in situ* in the two bone types. The actin cytoskeleton of cell bodies of fibular osteocytes was elongated and aligned parallel to the principle mechanical loading on fibula, which is longitudinal (Fig. 1A). The actin fibres were evenly distributed across the cell bodies. The fibular bone matrix appeared heavily interspersed with the osteocyte cell processes. Actin rich processes originating from the cell bodies branched and rebranched in different planes to form intercellular connections with the surrounding osteocytes (Fig. 1B). Interestingly, the nucleus