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Novel approaches for two and three dimensional multiplexed imaging of osteocytes

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ABSTRACT

Although osteocytes have historically been viewed as quiescent cells, it is now clear that they are highly active cells in bone and play key regulatory roles in diverse skeletal functions, including mechanotransduction, phosphate homeostasis and regulation of osteoblast and osteoclast activity. Three dimensional imaging of embedded osteocytes and their dendritic connections within intact bone specimens can be quite challenging and many of the currently available methods are actually imaging the lacunocanalicular network rather than the osteocytes themselves. With the explosion of interest in the field of osteocyte biology, there is an increased need for reliable ways to image these cells in live and fixed bone specimens. Here we report the development of reproducible methods for 2D and 3D imaging of osteocytes in situ using multiplexed imaging approaches in which the osteocyte cell membrane, nucleus, cytoskeleton and extracellular matrix can be imaged simultaneously in various combinations. We also present a new transgenic mouse line expressing a membrane targeted-GFP variant selectively in osteocytes as a novel tool for in situ imaging of osteocytes and their dendrites in fixed or living bone specimens. These methods have been multiplexed with a novel method for labeling of the lacunocanalicular network using fixable dextran, which enables aspects of the osteocyte cell structure and lacunocanalicular system to be simultaneously imaged. The application of these comprehensive approaches for imaging of osteocytes in situ should advance research into osteocyte biology and function in health and disease.

Introduction

Osteocytes have historically been viewed as quiescent cells that are inactive compared to other bone cell types, such as osteoblasts and osteoclasts. However, the last decade has seen these cells come to the forefront of mineralized tissue research to be recognized as a highly active bone cell type that plays multifunctional roles in skeletal homoeostasis (reviewed in [1–3]). Osteocytes make up about 90–95% of the cells in adult bone and can live as long as decades in the skeleton. They have a stellate morphology with long cytoplasmic processes (dendrites) extending out from the cell body. They also have a unique location, embedded in bone within a mineralized lacuna, with the dendrites extending through narrow channels in the bone matrix, called canaliculi.

Accumulating research is revealing that, far from being quiescent, osteocytes play key roles in a number of diverse skeletal functions, including mechanosensation, regulation of osteoblast and osteoclast function and regulation of mineral homeostasis (reviewed in [1–5]). Sclerostin, a protein that is highly expressed by osteocytes, has been shown to be a potent negative regulator of bone mass [6] and inhibition of sclerostin has become a major target for development of new bone anabolic therapies for treatment of osteoporosis [7–10]. Osteocytes have also recently been shown to be a major source of receptor activator of nuclear factor kappa-B ligand (RANKL) in bone and to be key players in the regulation of osteoclastic bone resorption [11,12]. Exciting advances in the field over the past decade have shown that osteocytes play an important role in phosphate and calcium homeostasis. They express dentin matrix protein-1 (DMP1) and phosphate regulating endopeptidase homolog, X-linked (PHEX), which control phosphate metabolism through regulation of fibroblast growth factor 23 (FGF23) ([13,14]; and reviewed in [3,5]). Moreover, the osteocyte network actually appears to function as an endocrine organ by secreting FGF23 into the circulation. This FGF23 then acts on the gut and kidney to regulate phosphate uptake and reabsorption. Another mechanism by which osteocytes can regulate ion homeostasis is through remodeling of their perilacunar matrix [15,16], which can release calcium and phosphate from the lacunae and canaliculi into the circulation. Not only do viable osteocytes play multiple roles in diverse skeletal functions, but the dying (apoptotic) osteocyte is also thought to play important regulatory roles in controlling osteoclast activity [17]. Overall, an exciting new

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paradigm is emerging of the osteocyte as a central orchestrator within the skeleton that may serve to integrate mechanical, hormonal and growth factor signals to regulate bone mass.

Osteocytes have been difficult to isolate in sufficient quantities for biochemical assays and it can be quite challenging to image embedded osteocytes and their dendritic connections in three dimensions within intact bone specimens. Many of the current methods for imaging osteocytes, such as traditional ground sections, procion red staining, FITC staining and acid etched electron microscopy are actually imaging the lacunocanalicular network rather than the osteocytes themselves [14,18,19]. With the explosion of interest in the field of osteocyte biology, there is an increased need for reliable ways to image these cells in live and fixed bone specimens.

Kamioka and colleagues developed methods for 3D confocal imaging of osteocytes using phalloidin staining of the actin cytoskeleton [20,21]. Building on this work, here we report the development of reproducible methods for 2D and 3D visualization of osteocytes in situ using multiplexed imaging approaches in which the osteocyte cell membrane, nucleus, cytoskeleton, and extracellular matrix can be imaged simultaneously in various combinations. We also present a new transgenic mouse line expressing a membrane targeted-GFP variant selectively in osteocytes as a novel tool for in situ imaging of osteocytes and their dendrites in fixed or living bone specimens. These methods have also been multiplexed with a novel method for labeling of the lacunocanalicular network using fixable dextran, which enables aspects of the osteocyte cell structure and lacunocanalicular system to be simultaneously imaged. The application of these comprehensive approaches for imaging of osteocytes in situ should advance research into osteocyte biology and function in health and disease.

Materials and methods

Preparation of fixed bone specimens

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at the University of Missouri, Kansas City and conformed to relevant federal guidelines. The UMKC animal facility is operated as a specific pathogen free facility and is AAALAC approved. Animal care and husbandry conform to the Guide for the Care and use of Laboratory Animals (8th Edition), National Research Council.

For experiments on whole mount calvaria, the calvaria were harvested from humanely euthanized 5–7 day-old mice and washed with phosphate buffered saline (PBS). The mice were either (i) wild-type C57BL/6 mice; (ii) transgenic mice with osteocyte-targeted expression of a membrane targeted GFP (Dmp1-memGFP mice); or (iii) transgenic mice expressing a GFP-collagen fusion protein (GFP-collagen mice) (see below for a description of these transgenic mice). For widefield epifluorescence imaging of osteocytes in whole calvaria, the periosteum was carefully removed under a dissection microscope and surface osteoblasts were gently removed using a rubber policeman. For confocal microscopy, it is not necessary to strip the periostea or remove osteoblasts, since the osteocytes can be imaged through the osteoblast layer. The specimens were fixed in cold 4% paraformaldehyde in PBS overnight at 4 °C with gentle rocking. Fixed half calvaria were then whole mount stained by immunofluorescent staining or using combinations of other fluorescent stains as outlined below, either with or without prior decalcification in 14% ethylenediaminetetraacetic acid (EDTA) pH 7.2 for 3 days.

For imaging of osteocytes in long bone specimens, femurs from C57BL/6 or GFP-collagen transgenic mice (ages specified in figure legends) were fixed for 48 h in 4% paraformaldehyde then decalcified in 14% EDTA. The decalcified bones were washed in PBS three times at 4 °C for 15 min with shaking and were then equilibrated at 4 °C in PBS containing 15% sucrose, followed by PBS/30% sucrose as a cryoprotectant. The bone specimens were embedded in OCT embedding medium (Tissue-Tek, PA, USA). Thick sections (50 or 100 μm) were cut using a Leica CM3050S cryomicrotome (Leica Microsystems, Wetzlar, Germany), and were collected into a 24-well plate containing PBS. The thick sections were washed twice with PBS and were whole mount stained by immunofluorescent staining or using other fluorescent stains as outlined below.

Antibodies and reagents

Antibodies to the osteocyte membrane protein E11/gp38 [22] (also known as podoplanin) included a hamster monoclonal (gift from Dr. Andrew Farr, University of Washington, Seattle) and a polyclonal goat antibody against podoplanin (R&D systems, Pittsburgh, PA). Phalloidin conjugated to Alexa Fluor 488, Texas red or Alexa Fluor 633 was obtained from Molecular Probes/Invitrogen Corporation (Carlsbad, California) and was used to stain F-actin. The membrane dye 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI), the nuclear stain 4′-6-Diamidino-2-phenylindole (DAPI) and a Texas red conjugated 10 kDa lysine-fixable dextran, used to image the lacunocanalicular system, were all obtained from Invitrogen Corporation. Detection antibodies and reagents used for immunostaining included Cy3 conjugated anti-hamster and anti-goat antibodies and a biotinylated anti-hamster antibody used in conjunction with FITC-streptavidin (Jackson Immunoresearch, Westgrove, PA).

Generation of transgenic mice expressing GFP selectively in osteocytes

A transgenic mouse line expressing the *topaz* variant of green fluorescent protein (GFP*tpz*) under control of an 8 kb fragment of the dentin matrix protein-1 (Dmp1) promoter has been previously described by Kalajzic and colleagues [23]. These mice express a GFP that is localized within the cytoplasm and this mouse line has been proven very useful for imaging/lineage tracing of osteocytes in vitro and in vivo [23,24] as well as for the generation of immortalized cell lines that recapitulate osteocyte differentiation [25]. However, in order to more clearly image living and fixed osteocytes and better resolve their dendrites in situ within bone, we have generated a new transgenic mouse line expressing a membrane targeted GFP variant (AcGFP1-mem) in osteocytes using the 9.6 kb fragment of the dentin matrix protein-1 (Dmp1) promoter to drive expression. The pAcGFP1-mem vector was obtained from Living Colors/Clontech Laboratories, Inc. (Mountainview, CA). The AcGFP-mem cDNA was retrieved from the pAcGFP1-Mem plasmid by XmaI and NotI restriction endonucleases, and was blunted at the NotI end. It was then subcloned into the XmaI and blunted XbaI sites of the pGL3-basic vector (Promega Corporation, Madison, WI) to replace the cDNA encoding firefly luciferase. The resultant construct was designated pGL-AcGFP1-Mem. The 14 kb *Dmp1* regulatory sequence, containing a 9.6 kb fragment of the *Dmp1* promoter region together with exon 1, intron 1 and the noncoding region of exon 2, was released from pSK vector by KpNI and XmaI (vector kindly provided by Dr. Jerry Feng, Texas A&M University Baylor College of Dentistry). This promoter has been previously shown to be highly expressed in osteocytes [26]. The promoter fragment was subcloned into the KpNI and XmaI sites of the pGL-AcGFP1-Mem plasmid to generate a 9.6 kb *Dmp1* promoter AcGFP1-Mem construct. This construct was designated pDmp1-AcGFP1-Mem. The transgene was released by SalI restriction endonuclease, separated from the vector backbone by agarose gel electrophoresis, and purified using Elutip-D columns (Whatman Schleicher & Schuell Bioscience, Inc. Keene, NH). Transgenic mice were generated on a C57BL/6N genetic background by pronuclear injection at the Transgenic Technology Center at the University of Texas Southwestern Medical Center, Dallas, TX. Founder mice were identified by PCR of tail DNA samples using the following primers: forward primer, 5′-CCAAGCCCTG AAAATCACAGA-3′, located on the *Dmp1* intron 1; and reverse primer, 5′-TCGCCGCTCACGCTGAACTT-3′, located on AcGFP1-Mem cDNA. AcGFP-Mem protein expression was confirmed by examining tail clip

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