Published in final edited form as:

Bone. 2013 June ; 54(2): 296–306. doi:10.1016/j.bone.2012.09.040.

In vitro and in vivo approaches to study osteocyte biology

Ivo Kalajzic^{1,*}, Brya G. Matthews¹, Elena Torreggiani¹, Marie A. Harris², Paola Divieti Pajevic³, and Stephen E. Harris²

¹Department of Reconstructive Sciences, University of Connecticut Health Center, Farmington, Connecticut, USA

²Department of Periodontics, University of Texas Health Science Center School of Dentistry, San Antonio, Texas 78299, USA

³Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Thier 1101, 50 Blossom Street, Boston, Massachusetts 02114, USA

Abstract

Osteocytes, the most abundant cell population of the bone lineage, have been a major focus in the bone research field in recent years. This population of cells that resides within mineralized matrix is now thought to be the mechanosensory cell in bone and plays major roles in regulation of bone formation and resorption. Studies of osteocytes had been impaired by their location, resulting in numerous attempts to isolate primary osteocytes and to generate cell lines representative of the osteocytic phenotype. Progress has been achieved in recent years by utilizing *in vivo* genetic technology and generation of osteocyte directed transgenic and gene deficiency mouse models.

We will provide an overview of the current *in vitro* and *in vivo* models utilized to study osteocyte biology. We discuss generation of osteocyte-like cell lines and isolation of primary osteocytes and summarize studies that have utilized these cellular models to understand the functional role of osteocytes. Approaches that attempt to selectively identify and isolate osteocytes using fluorescent protein reporters driven by regulatory elements of genes that are highly expressed in osteocytes will be discussed.

In addition, recent *in vivo* studies utilizing overexpression or conditional deletion of various genes using dentin matrix protein (Dmp1) directed Cre recombinase are outlined. In conclusion, evaluation of the benefits and deficiencies of currently used cell lines/genetic models in understanding osteocyte biology underlines the current progress in this field. The future efforts will be directed towards developing novel *in vitro* and *in vivo* models that would additionally facilitate understanding the multiple roles of osteocytes.

Keywords

osteocyte; cell lines; transgenic mice; Dmp1; Sost; Cre-recombinase; GFP

© 2012 Elsevier Inc. All rights reserved.

*Corresponding author: Ivo Kalajzic, Department of Reconstructive Sciences, MC 3705, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06032. Tel.: 860-679-6051; Fax: 860-679-2910; ikalaj@neuron.uchc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Osteocytes represent more than 95% of the cellular component of mature adult bone [1–3]. They are stellate shaped cells enclosed within the skeletal lacuno-canalicular network. Morphologically they are characterized by numerous elongated cell processes and dendrites extending into channels in the matrix called canaliculi (~250–300 nm in diameter) [4]. The shape of embedded osteocytes is dependent on their location with cells present in trabecular bone exhibiting rounded morphology whereas osteocytes from cortical bone are elongated [5]. These cells display polarity in terms of the distribution of their cell processes with the majority coming from the cell membrane facing the bone surface [6]. Osteocytes are connected, via gap junctions, to neighboring osteocytes, to cells on the bone surface such as lining cells, osteoclasts and osteoblasts [7] and to pericytes of capillaries, which supply nutrients and oxygen to osteocytes and other bone cells [8]. Furthermore, Kamioka et al., suggest the existence of a direct signaling system between the osteocytes and the bone marrow compartment without involvement of the osteoblast/lining cells [9]. Dynamic imaging studies showed that the dendritic connections between cells in the bone matrix, and into marrow spaces, appear to be able to extend and retract [10, 11]. Osteocytes also appear to show undulating motion of their cell bodies within their lacunae, suggesting that they are not inactive cells, as previously thought.

During the transition from osteoblast to osteocyte, the cell undergoes a dramatic transformation that requires extensive restructuring of the cytoskeletal and intracellular machinery. Cells at the early stages of osteocyte differentiation are larger than mature osteocytes and have numerous ribosomes, a well-developed endoplasmic reticulum and a wide Golgi complex [3]. Once the osteoid mineralizes, they decrease protein synthesis and secretion [12]. Moreover, osteocytes lose the apical and basolateral plasma membrane polarization normally seen in osteoblasts [13]. The study of different markers shows that osteocyte differentiation is accompanied by the progressive reduction of numerous bone markers (bone sialoprotein, collagen type I, alkaline phosphatase (ALP), osteocalcin, Runx2), the maintenance of some others (osteopontin and E11/gp38 antigen), and the appearance of new markers (dentin matrix protein 1 (DMP1), CD44, matrix extracellular phosphoglycoprotein (MEPE), phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), sclerostin (SOST) and fibroblast growth factor 23 (FGF23)) [3, 12, 14]. Osteocytes have proved difficult to isolate and study due to their localization within the mineralized matrix, and the fact that they are terminally differentiated post-mitotic cells. This review will discuss *in vitro* and *in vivo* techniques and tools that have enabled great advances in our understanding of osteocyte biology in recent years.

Primary culture of osteocytic cells

Over the last 20 years a number of methods have been developed to study osteocytes *in vitro* and this has greatly expanded our knowledge on the autocrine and paracrine functions of these inaccessible cells. Van der Plas and Nijweide [15] were the first to report a method to isolate and culture osteocytes from embryonic chicken calvaria. After releasing cells by serial collagenase/EDTA digestion, they purified osteocytes by immunomagnetic separation with a monoclonal antibody MAb OB 7.3 later shown to target Phex [16]. Cells isolated with this technique are post-mitotic and show stellate morphology with extensive processes. This method generates a relatively pure culture of osteoid osteocytes (as the calvaria is hypomineralized at the age of extraction) that produce low levels of ALP and high osteocalcin [17, 18]. However, any contaminating fibroblastic cells will overgrow the mitotically inactive osteocytes making these culture suitable only for relatively short term experiments, and cell yield using this method is low [17].

Other investigators have used different fractions of cells digested from newborn rat calvaria, or outgrowth cells from predigested tissue to isolate osteocytes based on their morphology

when cultured on matrigel or type I collagen-coated surfaces [19, 20]. A similar methodology has also been employed using human alveolar bone and neonatal rodent long bones [13, 21]. The osteocyte-enriched fractions show lower ALP activity and higher osteocalcin expression than osteoblast-enriched fractions, however the osteocyte-like cultures show proliferation after a week in culture suggesting the presence of contaminating proliferative cells [19]. More recently a method has been reported for culturing long bone osteocytes from adult mice which should enable *in vitro* investigation of age-related changes in osteocyte function as well as characterization of isolated osteocytes from various genetically modified mice [22].

Osteocyte-like cell lines

Development of osteocyte-like cell lines has greatly aided investigations of osteocyte biology. A summary of the sources and phenotypic characteristics of cell lines that have been reported is shown in Table 1. The most widely used cell lines, MLO-Y4 and MLO-A5, were developed by Lynda Bonewald's group [23, 24]. These are immortalized cells and undergo proliferation which is not a characteristic of osteocytes, however they show stellate morphology and expression of selected osteocyte marker genes. MLO-Y4 cells appear to have an osteocytic phenotype, with low ALP, high osteocalcin, E11 and connexin 43 expression, however, they do not mineralize or produce Sost [25]. These cells have proved useful for studies of modulation of osteocyte apoptosis and cell death [26–33], effects of fluid flow and other mechanical stimuli [30, 34–36], and the function and regulation of gap junctions and hemichannels [37–39]. MLO-Y4 cells also support osteoclastogenesis in the absence of osteotropic factors [40–42] and modulate the activity of osteoblasts [43], mesenchymal progenitor cells [44], and endothelial cells [45]. MLO-A5 cells are considered to be late osteoblastic/preosteocytic cells, and form mineralized sheets very rapidly in culture, even in the absence of added ascorbate and β -glycerophosphate which are usually added to induce mineralization [23]. They have therefore been useful in studies of osteoblast to osteocyte transition, mineralization, and the effects of mechanical stimuli on these processes [11, 46–48].

Bodine et al reported the development of a human osteocyte-like cell line, HOB-01-C1 that is conditionally transformed depending upon the temperature of culture [49]. These cells mineralize rapidly, and are responsive to parathyroid hormone (PTH), TNF α and IL1 β , however they have not been used in further studies. Divieti et al. also produced three lines of conditionally transformed osteocyte-like cells from PTH/PTH related protein (PTHrP) receptor (PTH1R) knockout mice that showed high binding affinity to the C-terminal end of PTH [50]. These cells have not been further utilized in the study of osteocyte biology, but the absence of PTH1R makes them suitable to study PTH signaling through other receptors. The newest osteocyte-like cell line reported, IDG-SW3, was isolated from mice carrying the Dmp1-GFP reporter transgene and a conditionally immortalizing, temperature sensitive SV40 Tag (Immortomouse) [51]. Cells derived from these animals can be expanded at 33°C in the presence of IFN γ (Tag permissive temperature), but they stop proliferating and start to differentiate in non-permissive conditions (37–39°C). IDG-SW3 appear to progress from a late osteoblastic phenotype, to a mature osteocyte-like phenotype with production of mineralized matrix. Induction of various osteocyte marker genes including Dmp1, MEPE, Sost and Fgf23 is observed over 3–4 weeks in culture at 37°C [51]. These cells should prove useful for further study of osteocytes at various differentiation stages. However, it should be noted that these cultures still contain a mixture of osteoblasts and osteocytes at different stages of differentiation and for studies requiring enriched osteocytes, the Dmp1-GFP cells will need to be isolated by flow cytometry (FACS).

Use of osteocyte-like cell cultures to understand mechanobiology in bone

In healthy individuals bone accrues in response to weight bearing activity, and is lost in the absence of mechanical stimuli. The osteocyte network is thought to be the main mechanosensor orchestrating this process, most likely by sensing changes in fluid flow that occur in the canalicular network in response to loading [52, 53]. It is not clear how this occurs, but it may involve the dendritic processes [54, 55], the cell body and/or the primary cilium [56, 57]. Modeling *in vivo* mechanical conditions with *in vitro* models is not trivial, but cell culture experiments have demonstrated molecules and pathways that are likely to be important in the response to mechanical signals. Primary chicken osteocytes and MLO-Y4 cells respond rapidly to fluid flow shear stress (FFSS), releasing nitric oxide (NO), prostaglandin E₂ (PGE₂), Ca²⁺ and ATP [36, 38, 58–61]. These responses tend to occur to a greater degree, or at lower magnitudes of stress in osteocytes than in comparable osteoblast cultures, suggesting that osteocytes are particularly sensitive to this type of stimulus. For example, primary chicken osteocytes produced greater amounts NO and PGE₂ than osteoblasts under the same conditions [58–60, 62], and nuclear translocation of β -catenin occurred at lower levels of stress in MLO-Y4 than in 2T3 cells [63]. In addition, Bacabac et al. found that partially adherent rounded MLO-Y4 cells showed much greater NO response to mechanical force than flat adherent cells suggesting that studies of adherent cells may underestimate the differences in responsivity to mechanical signals between osteoblasts and osteocytes [64]. Rapid release of signalling molecules such as PGE₂ can occur via connexin 43 hemichannels [35] whereas stretch-activated cation channels and integrins have been shown to be important for Ca²⁺ fluxes [65, 66]. Conditioned medium from osteocytes exposed to FFSS inhibits osteoclastogenesis and resorption compared to medium from static cultures [40, 67]. Conditioned medium from primary osteocytes exposed to FFSS also inhibited osteoblast proliferation and stimulated ALP production, while medium from static cultures had no effect [68]. FFSS has also been shown to protect osteocytes but not osteoblasts from undergoing apoptosis [30, 32, 65, 69]. *In vitro* study of osteocytic cells is now an active field in numerous labs, and has greatly contributed to our knowledge of the extensive activities these cells are likely to be performing *in vivo*.

Transgenic approaches to identify and characterize osteocytes

Osteoprogenitor cell differentiation is a process that is much easier to document *in vitro* than in an *in vivo* context [70]. Becoming an osteocyte represents terminal differentiation of the osteoprogenitor lineage. Until recently, osteocyte isolation was performed using only few osteocyte-specific antibodies such as monoclonal antibodies MAb OB7.3 [71], MAb SB5 [72] and MAb OB37.11 [73], specific for avian osteocytes. Breakthroughs in osteocyte isolation were made thanks to the identification of new osteocyte selective markers such as Dmp1, Mepe, Phex, E11/gp38 and Sost. Dmp1 belongs to the SIBLING (small integrin-binding ligand N-linked glycoprotein) family and is thought to be involved in the regulation of matrix mineralization and phosphate homeostasis [74]. Sclerostin is a secreted glycoprotein that appears to be highly expressed in mature but not early osteocytes [14]. It functions as a Wnt antagonist by binding Lrp4/5/6, acting as a regulator of bone mass [75–77].

In the last decade, a number of visual transgenes have been developed that have been very useful for dissecting different populations of osteoblast lineage cells including osteocytes. Early studies utilized collagen type I promoters (Col3.6kb and Col2.3kb) to drive expression of green fluorescent proteins (GFP) in preosteoblasts and osteoblasts/osteocytes respectively [78]. This approach was followed by generation of other bone directed promoter-GFP reporters like osteocalcin (Oc-GFP) that labeled a population of mature osteoblasts [79, 80]. Despite the usefulness of these transgenic mice in bone biology, they

did not show enough specificity to target partially embedded (preosteocytes) or matrix embedded cells (osteocytes).

Dmp1 gene expression is characteristic of odontoblasts, but in bone, expression is restricted to preosteocytes/osteocytes as assessed by in situ hybridization in chicken, rat and murine models [81, 82]. To study the activation of Dmp1 in the osteoblast lineage, different lengths of Dmp1 promoter have been utilized to drive transgene expression (GFP or lacZ) [83]. Their temporal activation was evaluated in an *in vitro* model using BMP-induced differentiation in 2T3 osteoblast-like cells. Constructs containing larger Dmp1 promoter fragments –9624bp (10kb) or –7892bp (8kb) showed a dramatic increase in expression following the onset of mineralization. The majority of 2T3 cells harboring the –2433 (2.5kb) promoter showed little or no GFP expression. In addition, FFSS activated the endogenous Dmp1 gene and the 8kb Dmp1-GFP in mineralized 2T3 cells, suggesting that Dmp1 is responsive to mechanical loading. Further *in vivo* studies utilizing the 8kb Dmp1 promoter show activation of the reporter gene following mechanical loading of ulnae [83].

Subsequently, to utilize the advantage of the strong expression with the onset of mineralization of 8kbDmp1 promoter in 2T3 cells, a transgenic model was generated that expressed a topaz variant of eGFP protein (excitation 514nm, emission 527nm) under the control of the 8kbDmp1 promoter [82]. The expression of GFP was restricted to populations of preosteocytes and osteocytes within the bone (Figure 1A–B). Interestingly, expression was also detected in cells with dendritic processes within the brain (Figure 1C), and in peritubular areas of the kidney (Figure 1D). Dmp1-GFP expression is also activated in primary bone marrow stromal cell cultures under osteogenic conditions in cells residing within mineralized nodules. They exhibit an osteocyte-like phenotype characterized by an extensive network of dendritic processes (Figure 2A) [82].

New transgenic reporter mice for studying osteocyte biology

New recombineering approaches have been used to make transgenic mice for studying the complete cis-regulatory regions of genes expressed in osteocytes. Patients with Van Buchem disease lack mutations in SOST coding regions, but they have a homozygous 52kb noncoding deletion downstream of SOST gene and upstream of the MEOX1 gene. The first study to utilize the human SOST BAC sequences confirmed that this deletion affected a SOST-specific regulatory element resulting in a phenotype with characteristics of Van Buchem disease [84].

Recently, we have generated transgenic mice for studying the complete cis-regulatory regions of the Dmp1 and Sost genes using different variants of fluorescent proteins (Fig 3A). Cortical long bone histology from a mouse containing 160Kb of the Sost gene with an EGFP reporter [85], and a Dmp1 BAC clone containing 165Kb of the Dmp1 gene with a DsRed reporter, is shown in Fig 3B–C. Notably, only a fraction of the osteocytes are positive for the Sost-GFP but many more osteocytes are positive for the Dmp1-DsRed. These results suggest that for any bone or condition, there is heterogeneity in expression of osteocyte-selective or specific genes within the osteocyte population.

These double reporter mice have been used to develop a calvarial osteocyte culture and a long bone cortical osteocyte culture system for *ex vivo* studies of osteocytes [85]. There is evidence that mineralized matrix around the osteocyte is required to maintain osteocyte characteristics. Many of the osteocyte cell models, such as MLO-Y4 make no mineralized matrix and express few osteocyte signature genes at any appreciable level. When bone chips are cultured, Dmp1 labeled cells outgrow and lose Dmp1 expression, showing evidence for dedifferentiation and loss of osteocytic phenotype *in vitro* [86]. Therefore the hypothesis was that to maintain the most “natural” osteocyte state, leaving them in the bone matrix and

removing other cellular elements was a reasonable approach to study osteocytes in mature cortical bone. To achieve this a “bone tube” system was employed. Briefly, following dissection and cleaning of the attached muscles, cortical bones from femur, tibia, and humerus were isolated, the growth plate region was removed, and the bone marrow and periosteal and endosteal osteoblasts were eliminated by five serial collagenase-trypsin digests. The SostBAC-GFP and Dmp1BAC-DsRed reporters could be monitored and demonstrated that the osteocytes remained alive and functional for up to 10 days in these *ex vivo* cultures [85].

The “bone tube” osteocyte system can also be used to image osteocytes under different conditions. An example of this approach is shown in Figure 4. Tibial bone tubes from Sost-GFP mice were loaded to 3N and underwent imaging in culture. The load was removed and samples reimaged. We have used a DISMAP approach and evaluated GFP labeled osteocytes, before and after loading [87, 88]. The strain fields could be calculated using the position of the osteocytes as a coordinate map. It was observed that the local strains throughout the bone are very discontinuous, with strains as high as 10,000 μ strain around the osteocyte lacunar region. We anticipate that in future bones from these transgenic mice will be used with high resolution confocal microscopy to map deformation changes at the osteocyte-lacunar-canalicular level in physiological or genetic mouse models [85].

The osteoblast versus the osteocyte

The mechanism of transition of osteoblasts into osteocytes remains poorly defined. To better understand this process and define the gene expression changes between osteoblasts and osteocytes, dual transgenic mice expressing Dmp1-GFP (topaz) and Col2.3CFP (cyan, excitation 433nm, emission 475nm) were utilized. Osteocytes were identified as Dmp1-GFP⁺ cells, while osteoblasts are represented by the Col2.3CFP⁺/Dmp1-GFP⁻ population [89]. This combinatorial approach allows for more detailed histological analysis (Figure 2B) and allows for additional characterization and cell isolation by FACS. FACS-separated osteocytic and osteoblastic populations from calvarial digests were used for microarray analysis. The genes most characteristic of osteoblasts included keratocan (Kera) and tenomodulin (Tnmd), while reelin, Dmp1 and Phex were strongly expressed in osteocytes [89]. In addition, this study points to the high expression levels of selected muscle-related genes, neuronal regulators such as Npy, and ion channels in the osteocytic population. The expression of these genes might be important in the maintenance of the osteocyte phenotype (dendritic extensions) and intercellular communication.

Models to evaluate effects of osteocyte-targeted gene deletion or overexpression

In the past decade the use of the Cre/loxP technology became a popular method to evaluate gene deletion or to target genes to tissues or particular stages within specific cell lineages. The efficacy and specificity of the recombination depends on the promoter utilized as well as the particular gene locus that is targeted. To direct recombination to the osteoprogenitor lineage, multiple promoter-Cre transgenic mice have been generated. Cre has been utilized to target earlier stages of differentiation within the bone lineage (Osterix-Cre, Col3.6kb-Cre, Col2.3kb-Cre, Oc-Cre) [90–92]. Most of these transgenes are also expressed in the osteocytes, and as osteoblast lineage cells eventually mature into osteocytes the gene of interest will also be deleted in osteocytes. Due to these complexities, the interpretation of these experiments requires extensive work, and it can be very difficult to clarify which stage(s) of the lineage are primarily responsible for the effects of the gene deficiency. A number of Cre recombinase transgenes targeting osteocytes have been reported and their specificity will be discussed here.

Evaluation of the 10kbDmp1-Cre using Ai9 reporter mice

Based on the expression of Dmp1 in osteocytes, Lu et al. utilized a 10kb fragment of the Dmp1 promoter (−9624 ~ +4439) to direct expression of Cre recombinase [93]. Using a Rosa26R reporter model in which a lacZ expression is activated following excision of a stop cassette, recombination was detected in osteocytes and odontoblasts [94]. The authors did not observe any Cre activity during embryonal development, and little or no expression in other tissues like kidney and brain. This result could be due to low Cre efficiency or be specific to the floxed gene locus (Rosa26R). In addition, detection of recombination is highly dependent on the variability of the β -galactosidase detection methodology. The incomplete penetration of fixative, shorter staining to avoid background and variable length of the development of the lacZ reaction, can significantly affect the extent of the staining and intensity.

To evaluate the Cre activity we have utilized a different reporter model in which a recombination event causes activation of tdTomato fluorescent protein (Jackson labs, #007905, termed Ai9). The Rosa-CAG-LSL-tdTomato-WPRE targeting vector contains a CMV-IE enhancer/chicken beta-actin/rabbit beta-globin promoter (CAG), a loxP-flanked STOP cassette, tdTomato sequence, and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE; to enhance the mRNA transcript stability), followed by a polyA signal. This entire construct was inserted between exons 1 and 2 of the Gt(ROSA)26Sor locus [95]. This design provides additional sensitivity for recombination detection. In addition, it allows straightforward detection of the tdTomato using frozen sections and epifluorescent imaging, without the requirement for additional staining.

Utilizing 10kbDmp1-Cre, recombination was detected within brain and kidney (data not shown), muscle tissue, including mature myofibers, and in the majority of the osteoblasts on both trabecular and endocortical surfaces (Figure 5C–D, arrows indicate osteoblasts). tdTomato expression was also observed within a population of bone marrow cells (Figure 5C–D, indicated by arrowheads). Despite improved detection of the Dmp1-Cre directed recombination using tdTomato reporter mice, a number of potential difficulties can be experienced using fluorescent reporter genes. Detection of GFP in formalin-fixed, paraffin embedded tissue sections can be obtained using only certain GFP variants (topaz, emerald), and in high-expressing transgenic lines. Tissue autofluorescence can be an obstacle, which can be eliminated by careful selection of fluorescent filters [96, 97]. Use of cryosectioning as a gold standard for GFP detection significantly improves transgene detection.

Evaluation of the 8kbDmp1-Cre using Ai9 reporter mice

Due to the potential issue of non-specific expression of 10kbDmp1-Cre, and based on the more osteocyte-restricted expression of 8kbDmp1-GFP, another construct had been recently developed that harbors the 8kbDmp1 promoter directing Cre [98]. Similar to the 10kbDmp1-Cre model, Cre activity in the 8kbDmp1-Cre was observed within osteocytes. However, a proportion of osteoblasts showed Cre activity on the endocortical and cancellous surfaces in the 8kbDmp1-Cre (Figure 5E–F).

Use of the inducible 10kbDmp1-CreERT2 to target osteocytes

Another approach used to avoid non-specific expression of 10kbDmp1-Cre potentially present during developmental stages, is to allow for control of the Cre activity using a tamoxifen-inducible Cre system (CreERT2) in which a mutated estrogen ligand-binding domain has been fused to the Cre recombinase [99]. In this model, Cre activates following induction with tamoxifen treatment. Histological evaluation showed the presence of spontaneous (non-tamoxifen induced) Cre activity in a small proportion of osteocytes (Figure 6C–D, see arrowheads). No spontaneous activation was detected in the osteoblasts

on bone surface (Figure 6C–D, see arrows). To test whether the various dosing of tamoxifen can modulate Cre activity specifically to osteocytes and not osteoblasts, tamoxifen was used as a single dose of two concentrations, high, 75 μ g/g ip and low 7.5 μ g/g ip. Two days after induction with high dose tamoxifen, the majority of osteocytes and osteoblasts were tdTomato positive (data not shown), while the lower concentration showed more restricted Cre expression to osteocytes (Figure 6E–F). Similar findings were previously reported by Powell et al. [99] using Rosa26R reporter mice (Figure 6A–B). Interestingly, under both treatment regimens we did not observe any Cre activity within the muscle. The presence of transient expression of the Dmp1 promoter during developmental stages could be a potential explanation, as this activation is circumvented using the inducible system. As mentioned above, the differences in Cre-recombinase activity using different reporter mice highlight the importance of carefully assessing not only the expression but also the functional activity of any gene of interest that is targeted for ablation.

Methods to evaluate the osteocyte specificity of a gene deletion

Use of osteocyte enrichment by serial digestion of bone

To evaluate the specificity of gene deletion in osteocytes, Kramer et al. utilized serial digestions of long bones [100]. The third digestion presented an osteoblastic phenotype, while the sixth digestion contains cells that expressed Dmp1 and Sost, defining it as an osteocyte population [100, 101]. This approach can be utilized to evaluate the effects of Cre recombination on the expression of a targeted gene within osteocyte and osteoblast populations.

Breeding Dmp1-GFP into the Dmp1-Cre targeted deletion model

To confirm that Dmp1-Cre deletion was targeted specifically to osteocytes, breeding with the Dmp1-GFP mouse was completed [98, 99]. This allows for a combination of histological examination of the Dmp1-GFP expression, and cell isolation by FACS to confirm that Dmp1-GFP negative cells do not express Cre transgene.

Immunohistochemical detection of recombination

Some of the studies utilized immunohistochemistry to detect osteocyte-specific deletion of the gene of interest [98]. As recombination effects of Cre are also locus dependent [102–104], confirmation of the deletion of the gene at protein level serves as ultimate proof of recombination efficiency. This approach represents an important step to test whether Dmp1-Cre targeted gene deletion is specific to osteocytes.

Strategies to target overexpression or gene deletion in osteocytes

Deleting genes in a lineage-specific manner using the Cre/loxP system has provided insights into numerous aspects in bone biology. Following reports using Oc-Cre and Col2.3kb-Cre models that act on osteoblasts and osteocytes, the Dmp1 promoter has become the main tool to target the expression or deletion of genes to the most mature stages of the osteoblast lineage (Table 2). Transgenic mice expressing a constitutively active PTH receptor (caPTH1R) under the control of the 8kbDmp1 (8kbDmp1-caPTH1R) promoter exhibit increased bone mass and bone remodeling. They show reduced expression of sclerostin resulting in increased Wnt signaling, increased osteoclast and osteoblast number, and decreased osteoblast apoptosis [105]. This phenotype bears similarities to mice with Col2.3kb-directed caPTH1R expression without showing accumulation of the fibrotic cells within bone marrow. The striking difference within these two models is a marked stimulation of periosteal bone formation in the long bones and calvaria of 8kbDmp1-caPTH1R mice [106]. In the Col2.3-caPTH1R, periosteal bone formation is reduced and

calvarial thickness is decreased [107]. In addition, despite increased numbers of osteoblastic cells within bone marrow, activation of PTH signaling in osteocytes does not expand hematopoietic stem cells (HSCs) [108]. This is in contrast to the mice with constitutive activation of the PTH1R in immature and maturing osteoblasts (Col2.3-caPTH1R) which exhibited increased osteoblastic cell numbers with a corresponding expansion of HSCs. In line with this, osteoblast ablation directed by the Col2.3kb promoter revealed the importance of the earlier stages of the osteoblast lineage on hematopoietic lineage regulation [109].

A number of studies have utilized DMP1-Cre transgenic models to evaluate the effects of activation or deletion of Wnt-signaling related molecules in mature osteoblast/osteocytes. A useful model to study the role of SOST was developed by overexpressing human SOST in osteocytes and evaluating the response to mechanical loading [110]. Load-induced bone formation was reduced in Dmp1-SOST transgenic mice, due to lower Wnt signaling in osteoblast lineage cells. This suggests that downregulation of Wnt signaling modulator Sost in osteocytes is required for the response to mechanical loading. An additional insight into regulation of the Wnt pathway has been provided by studies in which Lrp5, a co-receptor in canonical Wnt signaling has been deleted using Dmp1-Cre. While activation of a high bone mass mutation in Lrp5 by Dmp1-Cre caused an increase in bone mass, Dmp1-Cre targeted deletion of Lrp5 resulted in bone loss. This work provided compelling evidence that Lrp5 signaling functions locally through mature osteoblast/osteocyte populations [111].

To evaluate the role of β -catenin-dependent canonical Wnt signaling, a study was completed using Dmp1-Cre directed deletion of β -catenin [101]. Similar to the conditional deletion of β -catenin in osteoblasts using Col1a1-Cre or Oc-Cre, Dmp1-directed deletion of β -catenin resulted in low bone mass due to increased osteoclast activity [112, 113]. The phenotype progressively developed in early adulthood, suggesting a lack of Dmp1-Cre expression prior to formation of mature bone and osteocytes. Increased osteoclastogenesis was due to decreased OPG expression by osteocytes, while changes in RANKL expression were minimal [101]. More recent work by the O'Brien and Takayanagi groups evaluated the role of the osteocyte in RANKL production and presented evidence that osteocyte-derived RANKL is a critical regulator of osteoclastogenesis [114, 115]. The deficiency of these studies is their heavy reliance on Dmp1-Cre to target RANKL specifically in osteocytes and not in osteoblasts. In addition, the ability to detect gene deletion is limited by a lack of good Rankl antibodies. In a recent webinar <http://www.nature.com/bonekey/webinars/index.html?key=webinar15>, Dr. O'Brien summarized his findings and outlined the importance of generating new Cre transgenes that are more osteocyte specific.

Acknowledgments

We would like to thank Dr. Jerry Feng for providing us with 10kbDmp1-Cre mice. The loading of bone tubes is a result of collaboration with of Dan Nicollella at Southwest Research Institute in San Antonio, TX. We thank Dr. Teresita Bellido and Dr. Lilian Plotkin for important comments and suggestions.

References

1. Marotti G. The structure of bone tissues and the cellular control of their deposition. *Ital J Anat Embryol.* 1996; 101:25–79. [PubMed: 9203871]
2. Parfitt, AM. Bone forming cells in clinical conditions. In: Hall, BK., editor. *Bone: A Treatise, Vol1, The Osteoblast and Osteocyte.* New Jersey: Telford Press; 1990. p. 351-429.
3. Franz-Odenaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. *Dev Dyn.* 2006; 235:176–90. [PubMed: 16258960]
4. Donahue HJ. Gap junctions and biophysical regulation of bone cell differentiation. *Bone.* 2000; 26:417–22. [PubMed: 10773579]
5. Currey JD. The many adaptations of bone. *J Biomech.* 2003; 36:1487–95. [PubMed: 14499297]

6. Palumbo C, Palazzini S, Zaffe D, Marotti G. Osteocyte differentiation in the tibia of newborn rabbit: an ultrastructural study of the formation of cytoplasmic processes. *Acta Anat (Basel)*. 1990; 137:350–8. [PubMed: 2368590]
7. Bonewald LF. Generation and function of osteocyte dendritic processes. *J Musculoskelet Neuronal Interact*. 2005; 5:321–4. [PubMed: 16340122]
8. Noble BS, Reeve J. Osteocyte function, osteocyte death and bone fracture resistance. *Mol Cell Endocrinol*. 2000; 159:7–13. [PubMed: 10687847]
9. Kamioka H, Honjo T, Takano-Yamamoto T. A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy. *Bone*. 2001; 28:145–9. [PubMed: 11182371]
10. Veno P, Nicolella DP, Sivakumar P, Kalajzic I, Rowe D, Harris SE, Bonewald L, Dallas SL. Live imaging of osteocytes within their lacunae reveals cell body and dendrite motions. *J Bone Min Res*. 2006; 21(Suppl 1)
11. Dallas SL, Veno PA, Rosser JL, Barragan-Adjemian C, Rowe DW, Kalajzic I, Bonewald LF. Time lapse imaging techniques for comparison of mineralization dynamics in primary murine osteoblasts and the late osteoblast/early osteocyte-like cell line MLO-A5. *Cells Tissues Organs*. 2009; 189:6–11. [PubMed: 18728354]
12. Cameron DA, Paschall HA, Robinson RA. Changes in the fine structure of bone cells after the administration of parathyroid extract. *J Cell Biol*. 1967; 33:1–14. [PubMed: 6033936]
13. Gu G, Nars M, Hentunen TA, Metsikko K, Vaananen HK. Isolated primary osteocytes express functional gap junctions in vitro. *Cell Tissue Res*. 2006; 323:263–71. [PubMed: 16175387]
14. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW, Reeve J. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J*. 2005; 19:1842–4. [PubMed: 16123173]
15. van der Plas A, Nijweide PJ. Isolation and purification of osteocytes. *Journal of Bone and Mineral Research*. 1992; 7:389–396. [PubMed: 1609628]
16. Westbroek I, De Rooij KE, Nijweide PJ. Osteocyte-specific monoclonal antibody MAb OB7.3 is directed against phex protein. *Journal of Bone and Mineral Research*. 2002; 17:845–853. [PubMed: 12009015]
17. van der Plas A, Aarden EM, Feijen JHM, Deboer AH, Wiltink A, Alblas MJ, Deleij L, Nijweide PJ. Characteristics and properties of osteocytes in culture. *Journal of Bone and Mineral Research*. 1994; 9:1697–1704. [PubMed: 7863820]
18. Aarden EM, Wassenaar AMM, Alblas MJ, Nijweide PJ. Immunocytochemical demonstration of extracellular matrix proteins in isolated osteocytes. *Histochemistry and Cell Biology*. 1996; 106:495–501. [PubMed: 8950608]
19. Mikuni-Takagaki Y, Kakai Y, Satoyoshi M, Kawano E, Suzuki Y, Kawase T, Saito S. Matrix mineralization and the differentiation of osteocyte-like cells in culture. *Journal of Bone and Mineral Research*. 1995; 10:231–242. [PubMed: 7754802]
20. Kawata A, Mikuni-Takagaki Y. Mechanotransduction in stretched osteocytes -temporal expression of immediate early and other genes. *Biochemical and Biophysical Research Communications*. 1998; 246:404–408. [PubMed: 9610372]
21. Sekiya H, Mikuni-Takagaki Y, Kondoh T, Seto K. Synergistic effect of PTH on the mechanical responses of human alveolar osteocytes. *Biochemical and Biophysical Research Communications*. 1999; 264:719–723. [PubMed: 10543998]
22. Stern AR, Stern MM, Van Dyke ME, Jahn K, Prideaux M, Bonewald LF. Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. *BioTechniques*. 2012; 52:361–73. [PubMed: 22668415]
23. Kato Y, Boskey K, Spevak L, Dallas M, Hori M, Bonewald LF. Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. *Journal of Bone and Mineral Research*. 2001; 16:1622–1633. [PubMed: 11547831]
24. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. *Journal of Bone and Mineral Research*. 1997; 12:2014–2023. [PubMed: 9421234]
25. Rosser J, Bonewald LF. Studying osteocyte function using the cell lines MLO-Y4 and MLO-A5. *Bone Research Protocols*. 2012:67–81.

26. Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *The Journal of clinical investigation*. 1999; 104:439–46. [PubMed: 10449436]
27. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*. 2001; 104:719–30. [PubMed: 11257226]
28. Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. *Endocrinology*. 2003; 144:1761–9. [PubMed: 12697681]
29. Plotkin LI, Manolagas SC, Bellido T. Glucocorticoids induce osteocyte apoptosis by blocking focal adhesion kinase-mediated survival. Evidence for inside-out signaling leading to anoikis. *The Journal of biological chemistry*. 2007; 282:24120–30. [PubMed: 17581824]
30. Plotkin LI, Mathov I, Aguirre JI, Parfitt AM, Manolagas SC, Bellido T. Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs. *American journal of physiology Cell physiology*. 2005; 289:C633–43. [PubMed: 15872009]
31. Jia J, Yao W, Guan M, Dai W, Shahnazari M, Kar R, Bonewald L, Jiang JX, Lane NE. Glucocorticoid dose determines osteocyte cell fate. *FASEB Journal*. 2011; 25:3366–3376. [PubMed: 21705669]
32. Kitase Y, Barragan L, Qing H, Kondoh S, Jiang JX, Johnson ML, Bonewald LF. Mechanical induction of PGE(2) in osteocytes blocks glucocorticoid-induced apoptosis through both the beta-Catenin and PKA pathways. *Journal of Bone and Mineral Research*. 2010; 25:2381–2392.
33. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *Journal of Clinical Investigation*. 1999; 104:1363–74. [PubMed: 10562298]
34. Aguirre JI, Plotkin LI, Gortazar AR, Millan MM, O'Brien CA, Manolagas SC, Bellido T. A novel ligand-independent function of the estrogen receptor is essential for osteocyte and osteoblast mechanotransduction. *The Journal of biological chemistry*. 2007; 282:25501–8. [PubMed: 17609204]
35. Cherian PP, Siller-Jackson AJ, Gu SM, Wang X, Bonewald LF, Sprague E, Jiang JX. Mechanical strain opens connexin 43 hemichannels in osteocytes: A novel mechanism for the release of prostaglandin. *Molecular Biology of the Cell*. 2005; 16:3100–3106. [PubMed: 15843434]
36. Lu XL, Huo B, Chiang V, Guo XE. Osteocytic network is more responsive in calcium signaling than osteoblastic network under fluid flow. *Journal of Bone and Mineral Research*. 2012; 27:563–574. [PubMed: 22113822]
37. Plotkin LI, Manolagas SC, Bellido T. Transduction of cell survival signals by connexin-43 hemichannels. *The Journal of biological chemistry*. 2002; 277:8648–57. [PubMed: 11741942]
38. Jiang JX, Cherian PP. Hemichannels formed by connexin 43 play an important role in the release of prostaglandin E-2 by osteocytes in response to mechanical strain. *Cell Communication and Adhesion*. 2003; 10:259–264. [PubMed: 14681026]
39. Yellowley CE, Li ZY, Zhou ZY, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. *Journal of Bone and Mineral Research*. 2000; 15:209–217. [PubMed: 10703922]
40. You L, Temiyasathit S, Lee P, Kim CH, Tummala P, Yao W, Kingery W, Malone AM, Kwon RY, Jacobs CR. Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. *Bone*. 2008; 42:172–179. [PubMed: 17997378]
41. Kurata K, Heino TJ, Higaki H, Vaananen HK. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. *Journal of Bone and Mineral Research*. 2006; 21:616–625. [PubMed: 16598382]
42. Zhao S, Kato Y, Zhang Y, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *Journal of Bone and Mineral Research*. 2002; 17:2068–2079. [PubMed: 12412815]

43. Taylor AF, Saunders MM, Shingle DL, Cimbala JM, Zhou Z, Donahue HJ. Mechanically stimulated osteocytes regulate osteoblastic activity via gap junctions. *American Journal of Physiology-Cell Physiology*. 2007; 292:C545–C552. [PubMed: 16885390]
44. Heino TJ, Hentunen TA, Vaananen HK. Conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts. *Experimental Cell Research*. 2004; 294:458–468. [PubMed: 15023534]
45. Cheung W-Y, Liu C, Tonelli-Zasarsky RML, Simmons CA, You L. Osteocyte apoptosis is mechanically regulated and induces angiogenesis in vitro. *Journal of Orthopaedic Research*. 2011; 29:523–530. [PubMed: 21337392]
46. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, Manolagas SC, Jilka RL. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology*. 2005; 146:4577–83. [PubMed: 16081646]
47. Barragan-Adjemian C, Nicolella D, Dusevich V, Dallas MR, Eick JD, Bonewald LF. Mechanism by which MLO-A5 late osteoblasts/early osteocytes mineralize in culture: Similarities with mineralization of lamellar bone. *Calcified Tissue International*. 2006; 79:340–353. [PubMed: 17115241]
48. Prideaux M, Loveridge N, Pitsillides AA, Farquharson C. Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation. *PLoS ONE*. 2012; 7:e36786. [PubMed: 22586496]
49. Bodine PV, Vernon SK, Komm BS. Establishment and hormonal regulation of a conditionally transformed preosteocytic cell line from adult human bone. *Endocrinology*. 1996; 137:4592–604. [PubMed: 8895322]
50. Divieti P, Inomata N, Chapin K, Singh R, Juppner H, Bringham FR. Receptors for the carboxyl-terminal region of PTH(1-84) are highly expressed in osteocytic cells. *Endocrinology*. 2001; 142:916–25. [PubMed: 11159865]
51. Woo SM, Rosser J, Dusevich V, Kalajzic I, Bonewald LF. Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates bone formation in vivo. *Journal of Bone and Mineral Research*. 2011; 26:2634–2646. [PubMed: 21735478]
52. Price C, Zhou X, Li W, Wang L. Real-Time Measurement of Solute Transport Within the Lacunar-Canalicular System of Mechanically Loaded Bone: Direct Evidence for Load-Induced Fluid Flow. *Journal of Bone and Mineral Research*. 2011; 26:277–285. [PubMed: 20715178]
53. You J, Yellowley CE, Donahue HJ, Zhang Y, Chen Q, Jacobs CR. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. *Journal of Biomechanical Engineering-Transactions of the Asme*. 2000; 122:387–393.
54. Han YF, Cowin SC, Schaffler MB, Weinbaum S. Mechanotransduction and strain amplification in osteocyte cell processes. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:16689–16694. [PubMed: 15539460]
55. Burra S, Nicolella DP, Francis WL, Freitas CJ, Mueschke NJ, Poole K, Jiang JX. Dendritic processes of osteocytes are mechanotransducers that induce the opening of hemichannels. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:13648–13653. [PubMed: 20643964]
56. Temiyasathit, S.; Jacobs, CR. Osteocyte primary cilium and its role in bone mechanotransduction. In: Zaidi, M., editor. *Skeletal Biology and Medicine*. 2010. p. 422-428.
57. Bonewald LF. The amazing osteocyte. *J Bone Miner Res*. 2011; 26:229–38. [PubMed: 21254230]
58. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation. *Biochemical and Biophysical Research Communications*. 1995; 217:640–8. [PubMed: 7503746]
59. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH. Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB Journal*. 1995; 9:441–445. [PubMed: 7896017]

60. Ajubi NE, Klein-Nulend J, Nijweide PJ, Vrijheid-Lammers T, Alblas MJ, Burger EH. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes - A cytoskeleton-dependent process. *Biochemical and Biophysical Research Communications*. 1996; 225:62–68. [PubMed: 8769095]
61. Genetos DC, Kephart CJ, Zhang Y, Yellowley CE, Donahue HJ. Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. *Journal of Cellular Physiology*. 2007; 212:207–214. [PubMed: 17301958]
62. Ajubi NE, Klein-Nulend J, Alblas MJ, Burger EH, Nijweide PJ. Signal transduction pathways involved in fluid flow-induced PGE(2) production by cultured osteocytes. *American Journal of Physiology-Endocrinology and Metabolism*. 1999; 276:E171–E178.
63. Kamel MA, Picconi JL, Lara-Castillo N, Johnson ML. Activation of beta-catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE(2): Implications for the study of mechanosensation in bone. *Bone*. 2010; 47:872–881. [PubMed: 20713195]
64. Bacabac RG, Mizuno D, Schmidt CF, MacKintosh FC, Van Loon JJWA, Klein-Nulend J, Smit TH. Round versus flat: Bone cell morphology, elasticity, and mechanosensing. *Journal of Biomechanics*. 2008; 41:1590–1598. [PubMed: 18402963]
65. Tan SD, Kuijpers-Jagtman AM, Semeins CM, Bronckers ALJJ, Maltha JC, Von den Hoff JW, Everts V, Klein-Nulend J. Fluid shear stress inhibits TNF alpha-induced osteocyte apoptosis. *Journal of Dental Research*. 2006; 85:905–909. [PubMed: 16998129]
66. Miyauchi A, Notoya K, Mikuni-Takagaki Y, Takagi Y, Goto M, Miki Y, Takano-Yamamoto T, Jinnai K, Takahashi K, Kumegawa M, Chihara K, Fujita T. Parathyroid hormone-activated volume-sensitive calcium influx pathways in mechanically loaded osteocytes. *Journal of Biological Chemistry*. 2000; 275:3335–3342. [PubMed: 10652322]
67. Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Elverts V, Klein-Nulend J. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone*. 2007; 41:745–751. [PubMed: 17855178]
68. Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J. Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. *Biochemical and Biophysical Research Communications*. 2006; 348:1082–1088. [PubMed: 16904067]
69. Bakker A, Klein-Nulend J, Burger E. Shear stress inhibits while disuse promotes osteocyte apoptosis. *Biochemical and Biophysical Research Communications*. 2004; 320:1163–1168. [PubMed: 15249211]
70. Dacic S, Kalajzic I, Visnjic D, Lichtler AC, Rowe DW. Coll1a1-driven transgenic markers of osteoblast lineage progression. *J Bone Miner Res*. 2001; 16:1228–36. [PubMed: 11450698]
71. Nijweide PJ, Mulder RJ. Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes. *Histochemistry*. 1986; 84:342–7. [PubMed: 3522495]
72. Bruder SP, Caplan AI. Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. *Bone*. 1990; 11:189–98. [PubMed: 2202356]
73. Nijweide PJ, van der Plas A, Olthof AA. Osteoblastic differentiation. *Ciba Found Symp*. 1988; 136:61–77. [PubMed: 3068017]
74. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connect Tissue Res*. 2003; 44 (Suppl 1):33–40. [PubMed: 12952171]
75. van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, Hamersma H, Papapoulos SE, ten Dijke P, Lowik CW. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med*. 2004; 199:805–14. [PubMed: 15024046]
76. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, Wu D. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem*. 2005; 280:19883–7. [PubMed: 15778503]
77. Leupin O, Piters E, Halleux C, Hu S, Kramer I, Morvan F, Bouwmeester T, Schirle M, Bueno-Lozano M, Fuentes FJ, Itin PH, Boudin E, de Freitas F, Jennes K, Brannetti B, Charara N,

- Ebersbach H, Geisse S, Lu CX, Bauer A, Van Hul W, Kneissel M. Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. *J Biol Chem*. 286:19489–500. [PubMed: 21471202]
78. Kalajzic I, Kalajzic Z, Kaliterna M, Gronowicz G, Clark SH, Lichtler AC, Rowe D. Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. *J Bone Miner Res*. 2002; 17:15–25. [PubMed: 11771662]
 79. Kalajzic Z, Liu P, Kalajzic I, Du Z, Braut A, Mina M, Canalis E, Rowe DW. Directing the expression of a green fluorescent protein transgene in differentiated osteoblasts: comparison between rat type I collagen and rat osteocalcin promoters. *Bone*. 2002; 31:654–60. [PubMed: 12531558]
 80. Bilic-Curcic I, Kronenberg M, Jiang X, Bellizzi J, Mina M, Marijanovic I, Gardiner EM, Rowe DW. Visualizing levels of osteoblast differentiation by a two-color promoter-GFP strategy: Type I collagen-GFPcyan and osteocalcin-GFPtpz. *Genesis*. 2005; 43:87–98. [PubMed: 16149065]
 81. Toyosawa S, Shintani S, Fujiwara T, Ooshima T, Sato A, Ijuhin N, Komori T. Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. *J Bone Miner Res*. 2001; 16:2017–26. [PubMed: 11697797]
 82. Kalajzic I, Braut A, Guo D, Jiang X, Kronenberg MS, Mina M, Harris MA, Harris SE, Rowe DW. Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. *Bone*. 2004; 35:74–82. [PubMed: 15207743]
 83. Yang W, Lu Y, Kalajzic I, Guo D, Harris MA, Gluhak-Heinrich J, Kotha S, Bonewald LF, Feng JQ, Rowe DW, Turner CH, Robling AG, Harris SE. Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. *J Biol Chem*. 2005; 280:20680–90. [PubMed: 15728181]
 84. Loots GG, Kneissel M, Keller H, Baptist M, Chang J, Collette NM, Ovcharenko D, Plajzer-Frick I, Rubin EM. Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. *Genome research*. 2005; 15:928–35. [PubMed: 15965026]
 85. Harris SE, Harris MA, Gluhak-Heinrich J, Yang W, Potter RS, Bonewald L, Nicolella D. Use of a Sost-GFP Mouse Model to Determine the Role of the AKT-GSK3 β in Osteocyte Function and to Map Local Strain Fields around Osteocytes with Loading. *J Bone Miner Res*. 2010; 25:Fr287.
 86. Pejda S, Kizivat T, Fatahi M, Igwe JC, Kalajzic I. Tracking Osteocyte Lineage Plasticity In Vitro. *J Bone Miner Res*. 2010; 25:SA286.
 87. Nicolella DP, Moravits DE, Gale AM, Bonewald LF, Lankford J. Osteocyte lacunae tissue strain in cortical bone. *Journal of biomechanics*. 2006; 39:1735–43. [PubMed: 15993413]
 88. Nicolella DP, Nicholls AE, Lankford J, Davy DT. Machine vision photogrammetry: a technique for measurement of microstructural strain in cortical bone. *Journal of biomechanics*. 2001; 34:135–9. [PubMed: 11425075]
 89. Paic F, Igwe JC, Nori R, Kronenberg MS, Franceschetti T, Harrington P, Kuo L, Shin DG, Rowe DW, Harris SE, Kalajzic I. Identification of differentially expressed genes between osteoblasts and osteocytes. *Bone*. 2009; 45:682–92. [PubMed: 19539797]
 90. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, Carmeliet G, Kronenberg HM. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Developmental cell*. 2010; 19:329–44. [PubMed: 20708594]
 91. Liu F, Woitge HW, Braut A, Kronenberg MS, Lichtler AC, Mina M, Kream BE. Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. *Int J Dev Biol*. 2004; 48:645–53. [PubMed: 15470637]
 92. Zhang M, Xuan S, Boussein ML, von Stechow D, Akeno N, Faugere MC, Malluche H, Zhao G, Rosen CJ, Efstratiadis A, Clemens TL. Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. *J Biol Chem*. 2002; 277:44005–12. [PubMed: 12215457]
 93. Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res*. 2007; 86:320–5. [PubMed: 17384025]
 94. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–1. [PubMed: 9916792]

95. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* 2010; 13:133–40. [PubMed: 20023653]
96. Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, Rowe DW. Histological analysis of GFP expression in murine bone. *J Histochem Cytochem.* 2005; 53:593–602. [PubMed: 15872052]
97. Swenson ES, Price JG, Brazelton T, Krause DS. Limitations of green fluorescent protein as a cell lineage marker. *Stem Cells.* 2007; 25:2593–600. [PubMed: 17615263]
98. Bivi N, Condon KW, Allen MR, Farlow N, Passeri G, Brun LR, Rhee Y, Bellido T, Plotkin LI. Cell autonomous requirement of connexin 43 for osteocyte survival: consequences for endocortical resorption and periosteal bone formation. *J Bone Miner Res.* 2012; 27:374–89. [PubMed: 22028311]
99. Powell WF Jr, Barry KJ, Tulum I, Kobayashi T, Harris SE, Bringhurst FR, Pajevic PD. Targeted ablation of the PTH/PTHrP receptor in osteocytes impairs bone structure and homeostatic calcemic responses. *J Endocrinol.* 2011; 209:21–32. [PubMed: 21220409]
100. Halleux C, Kramer I, Allard C, Kneissel M. Isolation of mouse osteocytes using cell fractionation for gene expression analysis. *Methods in molecular biology.* 2012; 816:55–66. [PubMed: 22130922]
101. Kramer I, Halleux C, Keller H, Pegurri M, Gooi JH, Weber PB, Feng JQ, Bonewald LF, Kneissel M. Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis. *Mol Cell Biol.* 2010; 30:3071–85. [PubMed: 20404086]
102. Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, Hennighausen L, Wynshaw-Boris A, Deng CX. Conditional mutation of *Brcal* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet.* 1999; 22:37–43. [PubMed: 10319859]
103. Li W, Qiao W, Chen L, Xu X, Yang X, Li D, Li C, Brodie SG, Meguid MM, Hennighausen L, Deng CX. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in *Smad4/Dpc4* conditional knockout mice. *Development.* 2003; 130:6143–53. [PubMed: 14597578]
104. Coumoul X, Shukla V, Li C, Wang RH, Deng CX. Conditional knockdown of *Fgfr2* in mice using Cre-LoxP induced RNA interference. *Nucleic Acids Res.* 2005; 33:e102. [PubMed: 15987787]
105. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH, Jilka RL, Weinstein RS, Manolagas SC, Bellido T. Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS One.* 2008; 3:e2942. [PubMed: 18698360]
106. Rhee Y, Allen MR, Condon K, Lezcano V, Ronda AC, Galli C, Olivos N, Passeri G, O'Brien CA, Bivi N, Plotkin LI, Bellido T. PTH receptor signaling in osteocytes governs periosteal bone formation and intracortical remodeling. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2011; 26:1035–46. [PubMed: 21140374]
107. Calvi LM, Sims NA, Hunzelman JL, Knight MC, Giovannetti A, Saxton JM, Kronenberg HM, Baron R, Schipani E. Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *The Journal of clinical investigation.* 2001; 107:277–86. [PubMed: 11160151]
108. Calvi LM, Bromberg O, Rhee Y, Weber JM, Smith JN, Basil MJ, Frisch BJ, Bellido T. Osteoblastic expansion induced by parathyroid hormone receptor signaling in murine osteocytes is not sufficient to increase hematopoietic stem cells. *Blood.* 2012; 119:2489–99. [PubMed: 22262765]
109. Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J, Aguila HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood.* 2004; 103:3258–64. [PubMed: 14726388]
110. Tu X, Rhee Y, Condon KW, Bivi N, Allen MR, Dwyer D, Stolina M, Turner CH, Robling AG, Plotkin LI, Bellido T. Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading. *Bone.* 2012; 50:209–17. [PubMed: 22075208]

111. Cui Y, Niziolek PJ, MacDonald BT, Zylstra CR, Alenina N, Robinson DR, Zhong Z, Matthes S, Jacobsen CM, Conlon RA, Brommage R, Liu Q, Msee F, Powell DR, Yang QM, Zambrowicz B, Gerrits H, Gossen JA, He X, Bader M, Williams BO, Warman ML, Robling AG. Lrp5 functions in bone to regulate bone mass. *Nature medicine*. 2011; 17:684–91.
112. Glass DA 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell*. 2005; 8:751–64. [PubMed: 15866165]
113. Holmen SL, Zylstra CR, Mukherjee A, Sigler RE, Faugere MC, Bouxsein ML, Deng L, Clemens TL, Williams BO. Essential role of beta-catenin in postnatal bone acquisition. *J Biol Chem*. 2005; 280:21162–8. [PubMed: 15802266]
114. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med*. 2011; 17:1235–41. [PubMed: 21909103]
115. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med*. 2011; 17:1231–4. [PubMed: 21909105]
116. Xiao Z, Dallas M, Qiu N, Nicolella D, Cao L, Johnson M, Bonewald L, Quarles LD. Conditional deletion of Pkd1 in osteocytes disrupts skeletal mechanosensing in mice. *FASEB J*. 2011; 25:2418–32. [PubMed: 21454365]
117. Lieben L, Masuyama R, Torrekens S, Van Looveren R, Schrooten J, Baatsen P, Lafage-Proust MH, Dresselaers T, Feng JQ, Bonewald LF, Meyer MB, Pike JW, Bouillon R, Carmeliet G. Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization. *The Journal of clinical investigation*. 2012; 122:1803–15. [PubMed: 22523068]
118. Kramer I, Baertschi S, Halleux C, Keller H, Kneissel M. Mef2c deletion in osteocytes results in increased bone mass. *J Bone Miner Res*. 2012; 27:360–73. [PubMed: 22161640]

Summary

We have described the *In vitro* and *In vivo* models that are currently available to study osteocyte biology. The identification of several osteocyte specific genes has allowed the generation of a plethora of new data on osteocyte functions. Researchers have shown that the Dmp1 promoter can be successfully utilized to overexpress or delete genes in the most mature cells of the osteoblast lineage. Currently, a DMP1-GFP model is used to isolate osteocyte cell populations, while few models are available for targeting gene deletion including 10kbDmp1-Cre, 8kbDmp1-Cre and a tamoxifen inducible 10kbDmp1-CreERT2. It is important to note that the use of restricted promoters rarely achieves absolute specificity and efficiency. Moreover, detection of Cre recombinase activity using reporter mice should be complemented with the demonstration of the deletion of the gene of interest in order to assure successful tissue/cell specific gene ablation. At present, Dmp1 directed expression represents a useful approach in combination with other Cre models where promoters are targeted to earlier stages of the osteoblast lineage. In conclusion, we have presented the pros and cons or benefits and deficiencies of currently available cell lines and genetic models used to investigate and understand osteocyte biology. We have outlined current progress in this field. Future efforts will be directed towards the generation and development of novel osteocyte-specific approaches that will enhance our understanding of the multiple roles and functions of osteocytes.

Figure 1. Histological evaluation of 8kbDmp1-GFP expression

6–8 week old 8kbDmp1-GFP transgenic mice were sacrificed and cryosections of different tissues prepared. GFP expression was observed in osteocytes within cortical and trabecular bone (A–B, arrowheads). Osteoblasts on bone surface were GFP negative (A–B, arrows). We have detected GFP expression in brain in cells that have dendritic processes (C), and in the cells located in the peritubular areas of the kidney (D). (BM, bone marrow; CB, cortical bone) Tissue samples were dissected, fixed in 10% formalin, bones decalcified for 1 week in 15% EDTA, placed in 30% sucrose overnight and embedded in cryomedium. Sectioning has been completed using a cryostat and a tape transfer system.

Figure 2. Osteocytic expression of Dmp1-GFP in vitro and in vivo

Mesenchymal progenitor cells in primary bone marrow stromal cultures differentiate into mineralized nodules. Osteocyte-like cells expressing Dmp1-GFP can be detected within the mineralized colonies. Cells exhibit dendritic features reassembling osteocytes (A). Histological analysis of cortical bone derived from Col2.3cyan/Dmp1-GFP^{topaz} dual transgenic mice (B). The extensions from osteocytes (green-arrowhead) connect to osteoblasts on bone surface (blue-arrows). Images 2A and 2B were a part of published studies [82, 89].

Figure 3. SostBAC-EGFP and Dmp1BAC-DsRed transgenic reporter mice

BAC constructs used to generate reporter mice (A). Confocal image of cryosectioned cortical bone of the femoral diaphysis from one-month old dual transgenic animals (B). Dmp1 red cells are overlayed with the Sost green cells. Nuclei are stained with DAPI. The same image showing Sost but not Dmp1 reporter (C).

Figure 4. Mechanical loading of tibia from SostBAC-EGFP reporter mice

Reporter mice at 2 months of age were used to generate *ex vivo* osteocyte-enriched tibial “bone tubes” with Sost-EGFP expressing osteocytes. The tibiae were loaded to 3N while cultured in 10% FCS/ α MEM. Bone tubes were imaged and the load removed and reimaged. Using DISMAPP, a computational tool to map strain fields, the osteocytes were used as landmarks and the local deformation of the bone was calculated. Note the high strains around the osteocytes exceeding 10,000 μ strain, and the discontinuous strains throughout the bone at this local level.

Figure 5. Evaluation of Dmp1-Cre directed recombination

Histological sections from Dmp1-Cre negative mice showing background fluorescence signal using dual filters (TRITC/FITC) (A–B). 10kbDmp1-Cre activity was evaluated using cryosections of adult femurs of dual transgenic mice. Dmp1-Cre-activated tdTomato expression was detected in the majority of osteocytes within cortical (CB) and trabecular bone (C–D). Osteoblasts on the bone surface are tdTomato+ (C–D, arrows). Cre activity was observed within the muscle and in cells within bone marrow (C–D, see arrowheads). The expression of 8kbDmp1-Cre is similar to the 10kbDmp1-Cre activity, and is present in muscle, osteocytes, and in a proportion of cells on the bone surface and within bone marrow (BM) (E–F).

Figure 6. Evaluation of Dmp1 directed inducible Cre system

X-gal staining of tibia sections from Rosa26:10KbDmp1-CreERT2-positive mice (A-B). Animals were injected with a low dose of tamoxifen (7.5 μ g/g) at days 3, 5, 7, 14 and 21 days postnatal and sacrificed at 23 days (2 days after last tamoxifen injection). Images of osteocyte staining in cortical (A) and trabecular (B) bone are shown.

Cre activation of tdTomato reporter without tamoxifen treatment in cortical and trabecular areas (C-D). 10kbDmp1-Cre activity was induced in four-week old mice by a single dose of tamoxifen (7.5 μ g/g) and mice were sacrificed two days later (E-F). Cre activation was observed in osteocytes, and some osteoblasts. Arrows indicate osteoblasts, and arrowheads osteocytes.

Osteocyte cell lines

Table 1

Cell line	Source	Method of immortalization	Proliferation	Mineralization	ALP	OC	E11	Cx43	Dmp1	Sost	FGF2 3	Ref.
MLO-Y4	14 d.o mouse long bone	OC-SV40 TAg ^b	Yes	No	+/-	++	+++	+++	++	-	+	[24]
MLO-A5	14 d.o mouse long bone	OC-SV40 TAg ^b	Yes	Yes	+++	+++	++	++	++	+	+	[23]
HOB-01-C1	Human trabecular bone	Adenoviral SV40 TAg 209 ^c	No ^d	Yes	+/-	++	ND	ND	ND	ND	ND	[49]
OC1/14/59	E18.5 mouse calvaria, PTH1R-/-	Immortomouse ^e	No ^d	Yes	+/-	+++	ND	+++	ND	ND	ND	[50]
IDG-SW3	3 m.o mouse long bone, Dmp-GFP+	Immortomouse ^e	No ^d	Yes	++	ND	+++	ND	+++	+++ ^f	+	[51]

^a ND: not determined; - not expressed; +/- very low expression; + low expression; ++ intermediate expression; +++ high expression. Note that only some of these genes were directly compared to each other.

^b Cells derived from a mouse expressing the SV40 T antigen under the control of an osteocalcin promoter [24]

^c Thermolabile SV40 T antigen construct, active at 34°C, inactive at 39-40°C allowing conditional immortalization

^d At non-permissive temperature (37-40°C)

^e Mice have a IFNγ-inducible promoter driving thermolabile SV40 T antigen expression allowing conditional immortalization

^f Expression low/absent initially but present after 1-2 weeks in differentiation conditions

Table 2

Transgenic mice targeting osteocyte.

Gene	Symbol	Type	Effect	Bone phenotype	Reference
Parathyroid hormone receptor	ca PTHR1	8kbDmp1	Expression	↑ bone mass/decrease in SOST expression	[105]
β-catenin	Ctnnb1	10kbDmp1-Cre	Deletion	↓ bone mass/increase	[101]
low-density lipoprotein receptor-related protein 5	Lrp5	10kbDmp1-Cre	Deletion/Activation	↑ bone mass with deletion/↓ bone mass with activation of high bone mass mutation	[111]
Polycystin 1	Pkd1	10kbDmp1-Cre	Deletion	↓ bone mass/lack of anabolic response to loading	[116]
Vitamin D receptor	Vdr	10kbDmp1-Cre	Deletion	No change in bone mass on normal calcium diet	[117]
Receptor activator of nuclear factor κB ligand	Tnfrsf11	10kbDmp1-Cre	Deletion	↑ bone mass/regulation of osteoclast function	[114, 115]
Parathyroid hormone and parathyroid hormone related protein receptor	Pth/Phrp receptor (PPR)	10kbDmp1-CreERT2	Deletion	↓ bone mass/increase in SOST expression	[99]
Myocyte enhancer factor 2	Mef2c	10kbDmp1-Cre	Deletion	↑ bone mass/decrease in SOST expression	[118]
Connexin 43	Cx43	8kbDmp1-Cre	Deletion	↑ osteocyte apoptosis/widening of long bones	[98]
Sclerostin	SOST	8kbDmp1	Expression	↓ bone mass/modulation of response to loading	[110]