Advancing our understanding of osteocyte cell biology

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Abstract: Osteocytes were the forgotten bone cell until the bone community could become convinced that these cells do serve an important role in bone function and maintenance. In this review we trace the history of osteocyte characterization and present some of the major observations that are leading to the conclusion that these cells are not passive placeholders residing in the bone matrix, but are indeed, major orchestrators of bone remodeling.

Keywords: osteocytes, bone remodelling, parathyroid hormone

Osteocyte morphology and function

Osteocytes are stellate-shaped cells within the mineralized bone matrix that comprise 90–95% of all bone cells, therefore, they are the most abundant bone cell in the adult skeleton. The morphology of the osteoblast, a plump polygonal cell, changes dramatically as it becomes an osteocyte with reduced cytoplasm and numerous dendritic processes. Osteocytes are encased within lacunae, within the mineralized bone matrix, and are connected and networked to each other via dendrites that travel through canaliculi (Figure 1). The extracellular fluid in the lacunar-canalicular space is believed to be able to stimulate osteocytes by mechanical loading-induced fluid flow shear-stress [Weinbaum *et al.* 1994].

Due to the inaccessible nature of osteocytes in bone matrix, knowledge of their functions is still incomplete. Their special morphology implies multiple potential roles as mechanosensors and communicators. Years ago, sensitivity to mechanical stress was discovered in osteocytes by the rapid emission of prostaglandin (PGE₂) within 1 hour of fluid flow, compared to a much slower response in osteoblasts or fibroblasts [Klein-Nulend *et al.* 1995b]. Localized on both osteocytes and osteoblasts, functional gap-junctions are now known to be the channels through which osteocytes release their PGE₂ after fluid flow, and further upregulate connexin (Cx43) through PGE₂ EP-4 receptor and the PKA pathway in an autocrine manner [Cherian *et al.* 2003]. Recent studies of hemichannels also provide an alternative mechanism of release of PGE₂ to the extracellular space [Cherian *et al.* 2005; Jiang and

Cherian, 2003]. Osteoblasts, osteoclasts, and bone-marrow cells could potentially be regulated through gap junctions and hemichannels by signal molecules sent from osteocytes [Heino *et al.* 2004; Ilvesaro and Tuukkanen, 2003; Zhao *et al.* 2002; Yellowley *et al.* 2000].

Other channels and signaling molecules have been reported in osteocytes. Nitric oxide (NO) was suggested as another mechanical mediator correlating with PGE₂ release from osteocytes [Klein-Nulend *et al.* 1995a], which was supported by the detection of endothelial nitric oxide synthase (eNOS) in osteocytes [Zaman *et al.* 1999]. ATP and intracellular calcium can also be released from osteocytes in response to extracellular calcium or mechanical stimulation [Genetos *et al.* 2007; Kamioka *et al.* 1995]. Voltage-operated calcium channels (VOCC) that can be regulated by hormones, were shown to be expressed in osteoblasts and osteocytes [Shao *et al.* 2005; Gu *et al.* 2001b]. Expression of potassium (K⁺) channels during differentiation from osteoblasts to osteocytes leads to different K⁺ currents between osteocytes and osteoblasts [Gu *et al.* 2001a]. Because of their contribution to the maintenance of the cell-membrane potential, and their fast response in 20 msec, K⁺ channels and other ion channels may be involved in the earliest initiation of mechanical response in osteocytes. Expression of sodium-dependent glutamate transporters also supports the concept that a mechanism similar to neural synapse may exist in the osteocytes [Mason and Huggett, 2002]. Polycystins (PKD1, 2) have been shown to form calcium-permeable channels in response to tubular flow in primary cilia of

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Figure 1. Osteocyte lacuno-canalicular network. (a) Scanning electron micrograph of an acid-etched resin-embedded mouse ulna revealing a complex lacuno-canalicular system in bone. (b) In this diagram of osteoblast to osteocyte differentiation, the early osteoid osteocyte has reduced in cell body by around 30% and by the time it becomes a mature osteocyte, by 70%. The dendrites remain connected to cells on the bone surface mostly likely through gap junctions. Hemichannels are also present on the body and dendritic processes of osteocytes to release small signaling molecules in response to shear stress by the surrounding bone fluid.

kidney cells [Nauli *et al.* 2003], which could be another mechanical sensor pathway in osteocytes since cilia have been observed on the surface of osteocytes [Xiao *et al.* 2006; Tonna and Lampen, 1972].

Mechanical strains could be converted to biological signals through cytoskeletal deformation in the cytoplasm. Based on the abundance of actin filaments in osteocytes, it is reasonable to propose their direct involvement in the mechanical response. An above-threshold fiber-strain can

induce actin fiber-disassembly in osteoblasts [Sato *et al.* 2005]. Certain protein kinases, such as Rho, may mediate this process, [Sarasa-Renedo *et al.* 2006; Gerthoffer, 2005] however, their regulation of actin dynamics have not been reported in osteocytes. Since vimentin has been observed to be adjacent to gap junctions in osteocytes and osteoblasts [Shapiro *et al.* 1995], it is reasonable to hypothesize that the higher mechanical sensitivity of osteocytes compared to osteoblasts could be caused by their distinctive cytoskeleton configuration. Recent evidence further revealed that

cytoskeletal disruption in osteocytes inhibited PGE₂ response to fluid flow, while in osteoblasts it was enhanced [Mcgarry *et al.* 2005].

Residing within bone matrix means reduced nutrition and oxygen, and no space for proliferation of the cell. Therefore, osteocytes maintain the adaptive features of no cell-division, a long life-span, and resistance to apoptosis. Several apoptosis suppressors have been reported to support osteocyte survival under adverse conditions or apoptotic inducement. During the differentiation of osteoblasts to osteocytes, matrix metalloproteinases (MMPs) safeguard the cells from apoptosis [Karsdal *et al.* 2004]. Estrogen protects osteocytes against glucocorticoid-induced apoptosis [Gu *et al.* 2005]. Parathyroid hormone (PTH) prevents dexamethasone-induced apoptosis through PTH receptors expressed in osteocytes [Bringham, 2002]. Through expression of its receptor in osteoblasts and osteocytes, CD40 ligand blocks apoptosis induced by multiple apoptotic factors [Bonewald, 2004; Ahuja *et al.* 2003]. Deletion of a hypoxia protective gene, *klotho*, influenced spatial distribution of osteocytes, and accelerated aging and apoptosis of the cells [Suzuki *et al.* 2005]. Interestingly, mechanical stimulation inhibits, and disuse promotes osteocyte apoptosis [Plotkin *et al.* 2005; Bakker *et al.* 2004].

Flow of bone fluid through the lacuno-canalicular system

Deformation of calcified tissues is difficult under physiological loads. Strain in healthy bones of adult animals and humans was quantitatively measured to be less than 0.2% [Burr *et al.* 1996; Rubin, 1984], adequate to induce bone formation *in vivo* [Forwood, 1996]. However, *in vitro* studies showed that much higher deformations were needed to stimulate cultured osteoblast cells for any response [Murray and Rushton, 1990]. A well-known model proposed that osteocytes can sense the extracellular lacuno-canalicular fluid flow derived from the strain of physiological loads on bone [Cowin *et al.* 1991]. According to related experiments and calculations, a strain of 0.1% on bone would be magnified into a canalicular-fluid flow shear-stress of approximately 8–30 dyn/cm² because of the narrow canalicular space [Weinbaum *et al.* 1994]. This magnitude of flow is adequate to induce PGE₂, ATP and NO release from osteocytes [Genetos *et al.* 2007; Westbroek *et al.* 2000; Klein-Nulend *et al.* 1995a, b]. Fluid flow can also promote material exchange through the lacuno-canalicular network.

The signal molecules can be carried to cells on the bone surface, near the loaded area of bone. More sufficient nutrition and oxygen can also be provided to osteocytes in the loaded area [Kufahl and Saha, 1990]. This may be one of the reasons that mechanical loading has been shown to inhibit osteocyte apoptosis [Plotkin *et al.* 2005], and disuse promoted apoptosis [Bakker *et al.* 2004]. The osteocyte's response to canalicular fluid flow may play an important role in the regulation of bone remodeling. To understand the function of osteocytes, it is necessary to study the transcriptional and translational changes in osteocytes subjected to mechanical loading such as fluid-flow shear-stress.

Osteocyte cell models

Compared to osteoblasts and osteoclasts with clearly identified roles, little is known concerning osteocyte function. This is in part due to the difficulties in isolating sufficient numbers of osteocytes from the mineralized bone-matrix and maintaining their differentiated function *in vitro*. Primary cultures of osteocyte-like cells have been prepared by sequential alternating digestions of fetal rat and chick calvaria with collagenase and EDTA [Van Der Plas and Nijweide, 2005; Mikuni-Takagaki *et al.* 1996]. A population enriched for osteocytes can be released in late digests after the removal of fibroblasts and osteoblasts. However, the yields of osteocytes are low. There has been a lack of cell lines to represent osteocytes *in vitro*. Previously, our group had developed an osteocyte-like cell line, MLO-Y4 [Kato *et al.* 1997]. This cell line was derived from a transgenic mouse in which the immortalizing T-antigen was expressed under control of the osteocalcin promoter. MLO-Y4 cells exhibit properties of osteocytes including high expression of osteocalcin, connexin 43, and the antigen E11/gp38, while alkaline phosphatase is low. Also, the dendritic morphology of MLO-Y4 cells is similar to that of primary osteocytes.

Osteocyte markers

Osteocyte specific proteins start to be expressed when osteoblasts differentiate into osteocytes (Figure 2). E11/gp38 and CD44 have been reported to be expressed in osteocytes during the formation of dendritic processes [Zhang *et al.* 2006; Kato *et al.* 1997], and physical association of the two proteins may be essential for the function of E11/gp38 [Ohizumi *et al.* 2000]. It is believed that organized expression of tubulin, vimentin, and actin in cell bodies and dendrites

Figure 2. Markers of osteoblasts and osteocytes. Distinctive markers have been identified during the differentiation of osteoblasts to osteocytes. Osteoblasts are differentiated from mesenchymal stem cells expressing Stro 1, CD29, CD105 and CD166. Markers for osteoblasts include Cbfa1 and osterix for early osteoblasts, alkaline phosphatase and collagen for differentiating osteoblasts and osteocalcin for the mature osteoblast. Markers recently identified for osteocytes include E11/gp38, MEPE, PHEX, DMP1 and sclerostin. E11/gp38, MEPE and PHEX start to be expressed in late osteoblasts differentiating into osteocytes. DMP1 is expressed in early embedding osteocytes. Sclerostin is only expressed in deeply-embedded late osteocytes.

of osteocytes are crucial to maintaining their cytoskeletal shape which is distinct from the polygonal osteoblast [Tanaka-Kamioka *et al.* 1998]. Furthermore, dramatic differences in distribution of actin-binding proteins, such as fibrin, villin, filamin and spectrin, have been described accompanying the differentiation of osteoblasts to osteocytes [Kamioka *et al.* 2004]. A recent study of green fluorescent protein (GFP)-labeled osteocytes in calvaria [Kalajzic *et al.* 2004] also dynamically recorded frequent extension and retraction of dendrites, indicating constant changes of the embedded osteocyte cytoskeleton [Dallas *et al.* 2009].

E11/gp38/pdpn highly expressed in early embedding osteoid osteocytes

E11, also called podoplanin, OTS-8, gp38 or PA2.25, was first detected on the cell surface of osteocytes in rat bone [Schulze *et al.* 1999; Wetterwald *et al.* 1996], and odontoblasts in rat tooth [Schwab *et al.*, 1999]. It is also expressed in type I cells of rat lung and other tissues of brain, kidney, lymphatic tissue, and skin. E11/gp38 shares 87% cDNA homology between mouse and rat, and with similar distribution in tissues of the two species. Immunohistochemical staining of mouse bones indicated that E11/gp38 was strongly expressed in the newly embedded osteoid-osteocytes, but not in chondrocytes, osteoblasts or bone-marrow cells.

Expression was decreased in deeply embedded osteocytes [Zhang *et al.* 2006].

It was reported that E11/gp38 colocalizes with ERM (ezrin, radixin and moesin), and coimmunoprecipitated with ezrin and moesin in keratinocytes [Scholl *et al.* 1999]. In fibroblasts, ERM proteins crosslink actin and plasma membranes via the N-terminus of CD44 [Yonemura *et al.* 1998; Tsukita *et al.* 1997] which is a plasma-membrane protein associating with E11/gp38 [Ohizumi *et al.* 2000]. The binding of ERM proteins with the cytoplasmic tail of E11/gp38 triggered further downstream activation of RhoA GTPase to regulate actin cytoskeleton in kidney-epithelial cells [Martin-Villar *et al.* 2006].

It was recently revealed that the morphology and function of osteocytes could be related to E11/gp38, especially dendrite formation and response to mechanical stimulation. Application of fluid flow shear-stress on osteocyte-like MLO-Y4 cells increased the number and length of dendrites as well as the mRNA level of E11/gp38 [Zhang *et al.* 2006]. The response was blocked by small interfering RNA against E11/gp38 mRNA. Similarly, ulnae from heterozygote E11+/- with one allele replaced by lacZ showed increased X-gal staining after mechanical loading. However, the E11/gp38 knockout failed to generate viable homozygotes for a postnatal functional study because the newborns die at birth. This is possibly due to

respiratory failure when E11/gp38 is depleted from the type I cells in the lung [Schacht *et al.* 2003]. Therefore, a targeted deletion of E11/gp38 *in vivo* in osteocytes is needed to reveal its function in the skeleton.

Role of osteocyte-specific/highly-selective proteins in mineral metabolism

The osteocyte lacuno-canalicular system should be viewed as an endocrine organ regulating phosphate metabolism, as several osteocyte-specific or highly-selective proteins have been shown to function in phosphate metabolism (Table 1). Once the osteoblast begins to embed in osteoid, molecules such as Dmp1, Phex, and Mepe are expressed. Dentin Matrix Protein 1, (Dmp1), and Pex/Phex, (phosphate regulating neutral endopeptidase on chromosome X) are both highly expressed in osteocytes [Toyosawa *et al.* 2001]. Deletion or mutation of either Pex or Dmp1 results in hypophosphatemic rickets due to a dramatic elevation of FGF23 in osteocytes [Feng *et al.* 2006; Liu *et al.* 2006]. Elevated circulating levels of FGF23 are responsible for the human conditions of Autosomal Dominant Hypophosphatemic Rickets caused by inactivating mutations of *Pex* and Autosomal Recessive Hypophosphatemia caused by mutations in *Dmp1* [Feng *et al.* 2006; Lorenz-Depiereux *et al.* 2006; The Hyp Consortium, 1995]. FGF23 is a phosphaturic factor that prevents reabsorption of Pi by the kidney leading to hypophosphatemia. Dmp1 may have multiple functions. Unmineralized osteoid continues to surround osteocytes in Dmp1-null mice fed a high-phosphate diet in spite of a rescue of the length of the long bones [Feng *et al.* 2006]. These observations suggest that Dmp1 has both a systemic- (regulation of FGF23) and a local-function (regulation of osteocyte perilacunar mineralization). Based on the fact that osteocytes can express Phex, Dmp1,

and FGF23, we have proposed that the osteocyte network can function as an endocrine gland to regulate phosphate homeostasis.

The role of MEPE (matrix extracellular phosphoglycoprotein) in the regulation of phosphate metabolism is less clear. MEPE, also known as osteoblast/osteocyte factor 45, is highly-expressed in osteocytes as compared to osteoblasts. The proteases, Cathepsin D or B, have been shown to cleave MEPE, releasing the C-terminal phosphoprotein region, called the ASARM peptide, which has been shown to be a potent inhibitor of mineralization *in vitro* [Bresler *et al.* 2004] and high-ASARM peptide production by osteocytes correlates to an osteomalacia-type phenotype in the hyp mouse model. Deletion of this gene in mice, results in increased bone formation and bone mass and resistance to age-associated trabecular bone loss [Gowen *et al.* 2003]. As terminally differentiated osteoblasts become embedded in the bone matrix, MEPE expression increases, therefore osteocytes act directly on osteoblasts through MEPE to inhibit their bone forming activity. The unraveling of the interactions between these molecules and their fragments should lead to insight into the function of osteocytes.

Sost/sclerostin

Research on sclerosteosis and van Buchem disease revealed mutations of a new gene named *Sost* responsible for the defects in these diseases [Balemans *et al.* 2001; Brunkow *et al.* 2001]. Patients with mutant *Sost* showed high bone mass [Balemans *et al.* 2005]. Its protein, termed sclerostin, is highly expressed in mature osteocytes and acts as a negative regulator of bone formation [Poole *et al.* 2005]. It was originally considered to be a bone morphogenetic protein (BMP) antagonist but later discovered to be involved in the Wnt pathway as an antagonist against LRP5, a positive

Table 1. Proteins highly expressed in osteocytes.

Protein	Expression	Potential functions
E11/gp38	Early osteocytes (newly embedded)	Dendrite formation
DMP1	Early and mature osteocytes	Phosphate homeostasis
PHEX	Early and mature osteocytes	Phosphate homeostasis
MEPE	Early and mature osteocytes	Phosphate homeostasis
Sclerostin	Mature osteocytes (deeply embedded)	Osteoblast inhibitor

Functional studies have been done on a few osteocyte-specific proteins recently. E11/gp38 shows its highest expression in the newly embedded osteocytes [Zhang *et al.* 2006; Kato *et al.* 1997]. It is inferred that E11/gp38 may be important for osteocyte dendrite formation since it is also expressed on other dendritic cells. The functions of DMP1, PHEX, and MEPE are related to phosphate homeostasis [Feng *et al.* 2006; Liu *et al.* 2006; Rowe *et al.* 2000]. Sclerostin is expressed in mature osteocytes and functions as an osteoblast inhibitor [Poole *et al.* 2005].

regulator of bone mass [Li *et al.* 2005; Van Bezooijen *et al.* 2004]. Sclerostin is upregulated by osterix and runx-2 [Ohyama *et al.* 2004] which are downstream of BMP signaling pathways, indicating a negative feedback preventing excessive bone formation [Sutherland *et al.* 2004]. PTH has been reported to function as an inhibitor of sclerostin which partially explains the anabolic effect of PTH on bone formation [Silvestrini *et al.* 2007; Keller and Kneissel, 2005]. Similarly, another bone formation stimulator, mechanical loading, has been reported to reduce sclerostin expression [Robling *et al.* 2006]. An antibody to sclerostin is being considered as a new drug against postmenopausal osteoporosis [Lewiecki, 2009; Li *et al.* 2008] because of its specificity and its anabolic effect on bone formation.

Osteocytes as orchestrators of bone resorption and formation

After a review of the osteocyte markers as outlined above, it is clear that osteocytes can regulate bone formation, and there is also evidence that osteocytes regulate osteoclastic bone resorption. The osteocyte-like cell line MLO-Y4 supports osteoblast differentiation [Heino *et al.* 2002], and surprisingly, mesenchymal stem-cell differentiation [Heino *et al.* 2004]. In contrast, isolated avian-osteocytes can support osteoclast formation and activation [Tanaka *et al.* 1995] as can the osteocyte-like cell line, MLO-Y4 [Zhao *et al.* 2002] both in the absence of any osteotropic factors, unlike any other stromal or osteoblast cell. Kogianni and colleagues found that these cells produce 'apoptotic bodies' with the capacity to recruit osteoclasts due to the expression of RANKL on their surface [Kogianni *et al.* 2008]. Together these data suggest that osteocytes can act as orchestrators of bone remodeling.

Not only do viable osteocytes send signals of resorption, osteocytes appear to also support osteoclast activation and formation through their death, whether apoptotic or necrotic. Osteocyte apoptosis occurs at sites of microdamage where they send targeted signals for osteoclastic removal of damaged bone. Verborgt and co-workers mapped the expression of anti-apoptotic and pro-apoptotic molecules in osteocytes surrounding microcracks and found that pro-apoptotic molecules are elevated in osteocytes immediately at the microcrack locus, whereas anti-apoptotic molecules are expressed 1–2 mm from the microcrack [Verborgt *et al.* 2000]. Therefore those

osteocytes that do not undergo apoptosis are prevented from doing so by protective mechanisms while those destined for removal by osteoclasts undergo apoptosis. Targeted ablation of osteocytes was performed using the 10 kb *Dmp1* promoter to drive the diphtheria toxin receptor in mice [Tatsumi *et al.* 2007]. Injection of a single dose of diphtheria toxin eliminated approximately 70% of osteocytes in cortical bone in these mice leading to dramatic osteoclast activation. This suggests that viable osteocytes are necessary to prevent osteoclast activation and maintain bone mass. In summary, it appears that osteocytes can support mesenchymal cell-differentiation, osteoblast formation, and osteoclast resorption, thereby possessing the unique capacity to regulate all phases of bone remodeling.

Osteocyte genomics and proteomics for the future

Comprehensive genomic and proteomic profiling is needed to explore the details of osteocyte morphology, function as mechanosensors, their viability, and their regulation of other cells. Recently, transcriptional level screening with mRNA microarrays has been practiced to compare global patterns of osteocytes and osteoblasts [Yang *et al.* 2004]. At the transcription level, microarray results showed major differences in actin cytoskeleton and cell-communication systems. Genomic changes of osteocytes after mechanical loading were also studied [Yang *et al.* 2004]. However, mRNA transcripts do not always correlate with corresponding protein abundance, or with post-translationally modified (PTM) forms. Therefore, it is not enough to understand the function of osteocytes only at the transcription level. Proteins are vital molecules because they directly determine structure and function of the cell, and do the 'work' of the cell. Proteomics is the large-scale study of proteins which is usually considered the next step after genomic studies. Proteomics is more complicated than genomics because of the variations resulting from alternative splicing, translation regulations, post-translational modifications, and different fragmentations and degradations. These processes potentially change the function and activity of a protein. Therefore, proteomics provides directly relevant information regarding protein identification and potential structure and function. Therefore, a proteomic comparison and alternative validations are necessary not only to support the prior discoveries, but also to reveal new targets responsible for novel functional mechanisms in osteocytes.

Identification of osteocyte-selective proteins should allow us to identify and understand more about the function of osteocytes. Proteomic changes in fluid flow treated osteocytes should help us understand their role as mechanical sensors. This knowledge would form a foundation for understanding and treatment of defective osteocyte-related diseases.

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Conflict of interest statement

None declared.

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