Monday Morning (8am-Noon)

Mechanotransduction and the Osteocyte Network  
(L. Bonewald, Chair) Continental Room of the Sun Valley Inn

(1) Dendrite formation in osteocytes – L. Bonewald (UMKC)  
(2) DMP1 and MEPE expression: what are they and why are they important – J. Feng (UMKC)  
(3) Primary cilia as osteocyte strain sensors – D. Quarles (UMKC)  
(4) PTH and osteocytes – P. Divieti (Mass General)  
(5) Glucocorticoids and osteocyte function – N. Lane (UCSF)
Generation and Function of Osteocyte Dendritic Processes

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Osteocytes in vivo possess a distinctive morphology—that of dendricity—connecting osteocyte to osteocyte creating the osteocyte syncytium and also connecting osteocytes with cells on the bone surface (See figure on right). It is thought that bone fluid surrounding the dendrite within the canaliculi is responsible for the transmission of mechanical strain through fluid flow shear stress. Dendrites may be essential for osteocyte function, viability, and response to load.

The cell processes of osteocytes are connected with each other via gap junctions [1], thereby allowing direct cell-to-cell coupling. Osteocytes and MLO-Y4 osteocyte-like cells [2] express large amounts of Connexin 43, the component of gap junctions. But as these cells are only in contact through the tips of their dendritic processes, this raises the question, what is the function of Cx 43 located on the rest of the cell membrane? Recently it has been shown that connexins can complex and function in the form of un-apposed halves of gap junction channels called hemichannels. These channels are localized at the cell surface, independent of physical contact with adjacent cells [3]. Recently evidence of functional hemichannels formed by Cx43 has been reported in neural progenitors and neurons, astrocytes, heart, and especially, osteoblasts and osteocytes. The opening of hemichannels appears to provide a mechanism for ATP and NAD+ release, which raises intracellular Ca2+ levels and promotes Ca2+ wave propagation in astrocytes, bone cells, epithelial cells, and outer retina. Hemichannels expressed in bone cells such as MLO-Y4 cells appear to function as essential transducers of the anti-apoptotic effects of bisphosphonates[4] and act as a portal for the extracellular release of PGE2 in osteocytes upon exposure to fluid flow shear stress[5]. Therefore, in osteocytes, gap junctions at the tip of dendrites appear to mediate a form of intracellular communication and hemichannels along the dendrite appear to mediate a form of extracellular communication.
Osteocytogenesis has been thought to be a passive process whereby some osteoblasts become passively encased in osteoid that passively mineralizes. However, Holmbeck and colleagues[6] have shown osteocytogenesis to be an active invasive process requiring cleavage of collagen and potentially other matrix molecules. Osteocytes in mice null for the metalloproteinase MT1-MMP have significantly reduced number and length of dendritic processes. MT1-MMP is a membrane-anchored proteinase that can cleave collagens type I, II, and III, fibrin, fibronectin, and other matrix molecules. In this mouse model, the almost complete lack of dendritic processes did not appear to affect viability or density of osteocytes in contrast to studies by Zhao and coworkers[7] where osteocytes in a mouse model of collagenase resistant type I collagen did show increased apoptosis. However, it was impossible to determine the effect of a lack of dendritic processes on either osteocyte function or effects on the skeleton as the MT1-MMP null mouse exhibits multiple defects such as dwarfism due to a lack of MT1-MMP in other skeletal tissues[8]. These investigators also showed an increase in dendricity of osteocytes in mice between 3 and 4 months of age suggesting that the embedded osteocyte can generate new canaliculi containing new dendrites. Our preliminary studies also suggest increased dendricity with age.

The early formation of dendrites by embedding osteoid-osteocytes is polarized towards the mineralization front to which cellular processes are oriented. Cellular processes towards blood vessels only began to appear when the mineralization begins to spread around the cell[9]. Osteocyte dendricity changes depending on orientation and with static and dynamic bone formation [10]. In undiseased bone, osteocyte connectivity is high and the processes are oriented in the direction of the blood supply[11]. In osteoporotic bone there is a marked decrease in connectivity as well as disorientation of the dendrites which increases in severity. In contrast, in osteoarthritic bone, a decrease in connectivity is observed, but the orientation is intact. In osteomalacic bone, the osteocytes appear viable with high connectivity, but the processes are distorted and the network chaotic[11]. Changes in osteocyte dendricity could not only have a dramatic effect on osteocyte function and viability, but also on the mechanical properties of bone. An equilibrium must be met between number and branching of dendrites to preserve function and viability versus the number that would decrease bone strength.

We propose that a molecule known as E11/gp38 plays a role in the formation of dendritic processes. The earliest description of the gene for E11 was in 1990 as an unknown phorbol ester inducible gene in MC3T3 osteoblast-like cells, called OTS-8[12]. Since that time this gene/protein has become known by several names depending on tissue expression. It is expressed in the choroid plexus, ciliary epithelium of the eye, intestine, kidney podocytes, thyroid, esophagus, type I alveolar lung cells, lymphatic endothelium and osteocytes in bone. The gene cloned from murine peripheral lymphoid tissue was called Gp38[13], from rat type I epithelial alveolar lung cells, ‘Talpha’[14], the protein as RTI40[15], and in mouse kidney, as ‘podoplanin’ as it localizes to the foot processes of podocytes[16]. Expression occurs on the apical surface of lung epithelial cells which are the thin, flat, polarized cells that form the air-blood barrier[14, 17]. Deletion of this gene results in mice that die at birth due to respiratory failure as their lungs cannot be inflated to normal volumes[18], due to a failure of type II alveolar lung cells to differentiate into type I alveolar lung cells.
There has been considerable speculation regarding the function of E11. As the molecule is highly negatively charged and resistant to proteases, it may provide a physical barrier protecting cells from environmental agents[19]. The fact that E11 is found in cells that are exposed to an external or internal fluid compartment further supports this hypothesis. Accumulating evidence suggests that the major function of E11/gp38 may be in the formation of dendritic processes. Antibody to podoplanin (E11) causes rapid flattening of podocytes and proteinuria suggesting that the molecule maintains the shape of the podocyte foot processes[20]. Ectopic over-expression of the gene in keratinocytes induces plasma membrane extensions[21] and overexpression in endothelial cells promotes formation of long and thin tube-like structures on Matrigel[22], major reorganization of the actin cytoskeleton and relocalization of ezrin to cell projections[21]. The molecule colocalizes with ezrin, radixin, and moesin, ERMs[21], molecules that are concentrated in cell-surface projections where they link the actin cytoskeleton to plasma membrane proteins and are involved in cell motility[23]. E11/gp38 was also found to be physically associated with CD44 in tumor vascular endothelial cells[24, 25]. Together this data suggest that E11/gp38 associates with CD44 and the ERMs to induce and regulate the formation of dendritic processes. In osteocytes, the ‘gp38/podoplanin/T1alpha’ molecule is known as E11, the name given by Wetterwald and co-workers[26]. The E11 antigen was only found on the dendritic processes of osteocytes, not on osteoblasts in vivo[27]. Over-expression in an osteoblast-like cell line led to the generation of extended cytoplasmic processes[28]. Other than this information, very little is known about the function of E11 in mineralized tissues.

We have performed studies reproducing previous observations that E11 is expressed in osteocytes and not osteoblasts and have generated E11 null mice that die at birth. We have also shown that E11 is regulated by mechanical strain both in vivo and in vitro [29]. MLO-Y4 cells express large amounts of E11 protein and gene expression is increased in response to fluid flow shear stress. We had shown previously that fluid flow shear stress increased dendrite lengthening [30] and now have data showing that this increase in dendrite formation can be blocked using siRNA to E11. In the mouse ulna loading model, E11 expression is elevated not only in osteocytes near the cell surface, but also in cells embedded deep in the bone matrix [29]. The fact that dendrite formation is an active, not passive process, suggests that E11 may be critical for the generation of dendritic processes not only upon embedding in osteoid but also while the cell is embedded in a mineralized matrix. Therefore, dendrite formation may be essential for normal bone function.


DMP1 is Essential for Osteocyte Formation and Function

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DMP1 is an acidic phosphorylated extracellular matrix protein that was originally identified from a rat incisor cDNA library and thought to have a primary function in regulation of dentinogenesis [1]. Dmp1 shares similar sequence, biochemical, and genomic DNA organizational properties with a family of proteins, called SIBLINGs for Small; Integrin-Binding Ligand, N-linked Glycoprotein. The members of this family include bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP) and Matrix extracellular phosphoglycoprotein (MEPE). All of these family members contain an RGD sequence for integrin binding and can bind to hydroxyapatite [2, 3]. BSP, OPN and DMP1 can also bridge complement Factor H to cell surface receptors, an alternative complement pathway in order to prevent cell lysis [2, 3]. OPN and DMP1 [4] have been demonstrated to bind to CD44, a membrane bound protein thought to interact with the ERM (ezrin, radixin, moesin) family of adapter proteins that link to actin in the cytoskeleton.

Our recent observations on the expression pattern of DMP1 and of the phenotype in mice lacking the DMP1 gene underscore the critical importance of DMP1 in osteocyte function. These findings include; 1) although DMP1 is expressed in all tissues that undergo mineralization, its expression in osteocytes is much higher than in any other cell type as determined by in situ hybridization, lac Z knock-in expression, and immunostaining; 2) by immunostaining, DMP1 appears to be highly abundant in the dendritic processes of osteocytes and by immuno-EM appears to be localized on the canalicular walls along the lamina limitans; 3) a dramatic increase in Dmp1 expression is observed in osteocytes in response to mechanical loading both in vitro and in vivo; 4) Dmp1 null mice show major abnormalities in osteocyte morphology, with a 2-fold increase in lacunar size, abnormal “buckling” of the membrane surface of the dendrites, loss of integrity of the lamina limitans and obliteration of the canalicular space; 5) Dmp1 null mice show a severe impairment in mineralization, with patchy and poorly organized mineral and an apparent delay in the transition from osteoblasts to osteocytes; 6) mechanical loading of the ulna from Dmp1 null mice at 60 Hz produces strains 1.7 times higher than the strains in wild type and heterozygous littermates, indicating a significant change in the material (elastic moduli) and/or structural (stiffness) properties of the bones; and 7) a further striking observation in these mice is the progressive change in the skeletal properties with age with bony protrusions forming over time, appearing primarily at sites of muscle insertion, suggesting an abnormal response to mechanical loading in postnatal animals. We propose that osteocytes in the Dmp1 null mice exist in a hyperstimulated state, due to the mechanically compromised skeleton in these mice and that the formation of abnormal bony protrusions over time reflects an abnormal adaptive response that is attempting to restore skeletal homeostasis.

Our laboratory has generated transgenic mice that lack the gene for Dmp1 [5]. Although these animals appear normal at birth, a profound defects is observed in dentinogenesis [6], chondrogenesis [7], and craniofacial development (Ye et al, in press, appendix). However, the
most striking phenotype is mineralization and osteocyte dysfunction. *Dmp1* is almost exclusively expressed in osteocytes and shows a restricted localization along the dendrites and/or canicular walls. We have developed several techniques to analyze morphological changes in osteocytes from *Dmp1* KO mice. First, a combination of injection of calcein/Alizarin Red in conjunction with DAPI nuclear counterstaining allows visualization of the mineralization front and its relationship with osteocytes. In the WT animals, there are three discrete lines of labeling, which are clearly separated from the osteocytes. In contrast, the labeling in the KO is absent in some areas and is highly diffuse in other areas, making it difficult to distinguish discrete lines of labeling. Osteocytes are surrounded by patchy fluorochrome label. These data suggest defective and disorganized mineralization in the *Dmp1* KO mice.

Second technique we have used to examine the lacuno-canicular system in *Dmp1*-KO mice is injection of procian red. This small molecular dye, when injected into the tail vein (0.8 %, 0.01ml/g), fills in lacunae and canaliculi but does not enter the cell. Thus the dye can be used to give a visual representation of the organization of the lacuno-canicular system within the skeleton. WT osteocyte lacunae are highly organized and spaced apart regularly, generally in linear arrays. The canaliculi are generally straight and run perpendicular to the long axis of the osteocyte. In contrast, the osteocyte lacunae in *Dmp1* KO mice appear much larger, the distribution of the osteocytes appears less organized and the canaliculi are less straight and more randomly oriented.

To quantify the lacunar area we have used atomic force microscopy (AFM) together with measurement by image analysis. This confirmed an approximately two fold increase in osteocyte lacunar size in *Dmp1* KO mice. By TEM, striking abnormalities were observed in the canaliculi of *Dmp1* KO mice compared to WT controls. In WT mice the membrane surface of the dendritic processes was smooth, there was a clear space between the membrane and the canicular wall, and the wall was defined by a clearly visible lamina limitans (arrowhead). In contrast in KO mice, the membrane surface of the dendritic processes appeared irregular and buckled, the canicular spaces surrounding the dendritic processes were poorly delineated, the lamina limitans was absent and the canicular space appeared to be obliterated by collagen fibrils. These observations suggest that DMP1 may play an essential role in formation and/or maintenance of the canaliculi and canicular space.

Another technique we have established for analyzing the morphology of the lacuno-canicular system is acid-etched resin casting of the bones. In this technique, a polished surface from a resin embedded bone is etched with acid to remove mineral, leaving a relief cast of the non-mineralized areas that have been penetrated by resin. Using this technique, striking differences were observed in the appearance of the lacunae and canaliculi of *Dmp1* KO mice compared to WT controls, consistent with the abnormalities seen by TEM (See Figure 1). Thus, the lacunae were larger and the surface of the lacunae and canaliculi appeared highly irregular and rough compared to the smooth surfaces of the lacunae and canaliculi in WT mice. There also appeared to be reduced numbers of canaliculi in the KO mice.
Figure 1. Scanning electron microscopy image of acid etched resin embedded cortical bone from wild-type (left) and Dmp-1 null mice (right). Note the smooth inner surfaces of canaliculi and extensive branching with consistent, continuous canalaricular diameter in the wild-type osteocyte lacuno-canicular system. Note the rough and buckled appearance of the lacuna and canaliculi of osteocytes from Dmp1 null mice. Fewer canaliculi are also present.

Taken together the above observations suggest that DMP1 is a major regulator of mineralization and that it may play a role in the transition of osteoblasts/preosteocytes to osteocytes perhaps through maintenance of structure of the lacunae and canaliculi. DMP1 may also be an important regulator of osteocyte-mediated responses to mechanical loading perhaps through its role as a regulator of mineralization. We hypothesize that these abnormalities in the osteocyte canaliculi/dendritic processes result in impaired fluid flow through the canaliculi, thus affecting the ability of the osteocytes to respond to mechanical signals.

Reference:


Inactivating mutations of PKD1, the gene coding PC1, is cause autosomal dominant polycystic kidney disease (ADPKD), a disorder believed to result from impaired cilia function in renal epithelia. Recent investigations into the function of polycystin-1 (PC1), a transmembrane G-protein coupled receptor, indicates that this protein functions as a putative flow-responsive mechanosensor complexed with polycystin-2 (PC2), a receptor-activated calcium channel, in renal epithelial cilia.

PC1 is also expressed in embryonic mesenchyme and neural crest tissues, which give rise to endochondral and intramembranous bone. Initial insights into a possible function of PC1 in bone were derived from the observation that mice lacking Pkd1 have abnormal skeletogenesis. Osteocytes and osteoblasts are the key mechano-transducing cells in bone, but the molecular mechanisms mediating responses to mechanical strain are uncertain. We hypothesize that that PC1 might be an important mechanosensor in the skeleton. In particular, we propose that PKD1-PKD2 complexes on osteoblast and osteocyte cell surfaces might be activated by cilia through the back and forth pulsatile movement of extracellular fluid during walking or running cycles. We also suggest that PKD1-PDK2 activation will result in increments in intracellular calcium, calcineurin activation, and increments in Runx2-dependent gene transcription which mediate the osteoblastic response to cilia activation. If our hypothesis is correct, we would expect to observe reduced bone density in Pkd1 deficient mice (due to resistance to mechanical loading), expression of PKD1, PKD2 and ciliary genes in osteoblasts, activation of certain common pathways by Pkd1 and mechanical stress, resistance of osteoblasts with mutations in Pkd1 to flow-induced Ca\(^{2+}\) signaling, and failure of Pkd1 deficient mice to respond to mechanical strain in vivo.

To test our hypothesis we have initiated studies in Pkd1\(^{m1Bei}\) null mouse, which has a point mutation (T to G at 9248bp) causing a M to R substitution affecting the first transmembrane domain of PC1. We observed normal cartilage formation at E13.5 in both wild-type (WT) and Pkd1\(^{m1Bei}\) mice. By E14.5 WT mice began to form bone (stained red skeleton), but no calcification was found in Pkd1\(^{m1Bei}\) embryos. At E15.5, Pkd1\(^{m1Bei}\) embryos displayed evidence of calcification, but the amount of mineralized bone was less than wild-type control, indicating that Pkd1 deficient mice have defective osteoblast-mediated bone formation. Pkd1\(^{m1Bei}\) mice are embryonic lethal, but the heterozygous mice have normal survival. To determine the impact of loss of one functional Pkd1 allele in the adult, we assessed bone mineral density (BMD) in 6-week-old WT and Pkd1\(^{m1Bei}\) heterozygous mice. Pkd1\(^{m1Bei}\) deficient mice had a 7% reduction BMD, indicating Pkd1 mutation leads to osteopenia.

To determine if PC1 and PC2 transcripts are expressed in osteoblasts and osteocytes, we performed RT-PCR with specific primers for PC1 and PC2 using RNA from MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. We found that both PC1 and PC2 transcripts are highly expressed in both cell types. In addition, MC3T3-E1 and MLO-Y4 cells expressed transcripts...
for polaris, a protein present in cilia, consistent with the presence of cilia in osteoblasts/osteocytes.

Activation of PC1 signaling can be achieved by overexpression of the C-terminus of PC1. To assess the function of PC1 on Runx2 promoter activity in osteoblasts, we transiently co-transfected MC3T3-E1 osteoblasts with two mouse C-terminal PC1 constructs, PC1-LT containing the C-terminal 222 aa of PC1 or PC1-HT containing the C-terminal 193 aa of PC1 and the 1.4 kb Runx2 P1 luciferase promoter/reporter construct. Overexpression of either PC1-LT or PC1-HT in this osteoblastic cell line resulted in a 3-4 fold increase of the P1 promoter activity of Runx2, an essential transcription factor controlling osteoblastogenesis. Similar results are achieved with MLO-Y4 cells. These findings are consistent with a possible role of PC1 to function as a mechanosensing complex in bone that allows osteoblasts/osteocytes to sense and transduce mechanical forces into anabolic cell signals leading to new bone formation.
PTH and Osteocytes

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Osteocytes are the most abundant cells in bone, yet their functions still are poorly understood. Osteocytes express several membrane receptors, including the parathyroid hormone (PTH) type 1 receptor (PTH1R) (1), estrogen receptors (both α and β) (2-5), and, as we recently have reported, a novel receptor that specifically recognizes the carboxy-terminal region of PTH, the carboxy-terminal PTH receptor (CPTHR) (6, 7).

PTH is a single-chain polypeptide comprised of 84 amino acids and its main function is to maintain constant the serum calcium concentration. PTH accomplishes this via activation of a G-protein coupled receptor, the type 1 PTH/PTH-related peptide receptor (PTH1R), mainly expressed in kidney and bone (8).

The amino-acid sequence of PTH is highly conserved among species and this high degree of evolutionary conservation strongly suggests the possibility of additional, independent biological function(s) for the C-terminal region of the PTH molecule. Indeed, evidence of cellular receptors with specificity for the C-terminal portion of PTH(1-84) (“CPTHRs”) has accumulated steadily over the past 25 years, as recently reviewed (9). We reported abundant expression of CPTHRs (2-3 x 10^6/cell), detected using 125I-[Tyr^34]hPTH(19-84) as radioligand, on the surface of clonal osteocytic cells (“OC cells”) isolated from calvarial bone of fetal PTH1R-null mice, thus providing the first conclusive evidence that CPTHRs exist independently of PTH1Rs (6, 10, 11). Recently we have identified specific structural determinants of CPTHR binding (7) and we demonstrated that CPTHR activation in osteocytes leads to increased cell death, an effect opposite that reported for the PTH1R, and to an increase in cell-to-cell communication.

PTH1R and osteocytes

Direct actions of PTH upon osteocytes “in vivo” were first suggested by early experiments in which various adverse morphological changes (cellular retraction, mitochondrial swelling and cell death) were observed in osteocytes and osteoblasts by light electron microscopy within hours of administration of PTH extract (PTE) (12, 13). Other investigators demonstrated evidence of increased proteolytic activity associated with enlarged osteocytic lacunae in bone of animals given daily doses of PTE for several days. These findings initially suggested that the role of the osteocytes was osteolysis. The theory was quickly abandoned when it appeared evident that osteocytes in culture were unable to resorb bone (or mineralized matrix). Recently the concept of osteocytic osteolysis has found new support after the report of Tazawa et al., in which osteolysis was observed in lacunae of rats continuously infused with 80 g/kg/day of PTH(1-34) (14).

Direct evidence of PTH1R expression on osteocytes derived from work of Davidovitch et.al. (15), who demonstrated an increase in cAMP (detected immunohistochemically) in osteocytes of cats treated with PTE. Specific binding of iodinated PTH(1-34) was then shown both in vivo (1) and in vitro (16).
The localization of PTH1Rs on osteocytes, as well as the synergistic effects of mechanical stress and PTH, indicated an important role of the hormone in regulating the signal transduction induced by loading in osteocytes. Early studies of Duncan et al., demonstrated stretch-activated cation channels and their activation by PTH in UMR106 osteoblast-like cells (17, 18). Subsequent work of Miyauchi et al. revealed the presence of stretch-activated Ca channels synergistically activated by PTH and mechanical stimulation (hypo-osmotic stress) (19). Interestingly, another class of calcium channel, the L-type voltage-operated calcium channels (VOCCs), also was detected on MLOY4 osteocytic cells after stimulation with a very low dose (0.1 nM) of PTH(1-34). Similar effects were elicited by 17-estradiol and dexamethasone, suggesting that hormonal control might prime osteocytes to sense or respond to calcium. In line with this observation, in vivo studies in rats, showed that a single injection of PTH (80 g/Kg) increased the effect of loading, measured as histomorphometrical parameters. This effect of PTH was specifically inhibited by verapamil, a VOCC blocker. Lastly, Jilka et al. reported that PTH exerts its anabolic effect on bone by suppressing osteoblast and osteocyte apoptosis (20). They reported that the intermittent versus sustained effect of PTH is related to proteosomal degradation of Runx-2. Interestingly we have demonstrated that CPTHR activation on osteocytes exerts a pro-apoptotic effect, an action opposite that of PTH1R activation.

CPTHR and osteocytes

The high levels of CPTHR expression by OC cells enabled a reliable analysis, using OC59 cells and the CPTH radioligand $^{125}$I-[Tyr$^{34}$]-hPTH(19-84) (which does not bind to the PTH1R), of the structural determinants for ligand binding. As recently reported (7), N-terminally truncated human PTH peptides hPTH(7-84), [Tyr$^{34}$]hPTH(11-84), [Tyr$^{34}$]hPTH(13-84), [Tyr$^{34}$]hPTH(19-84) and [Tyr$^{34}$]hPTH(24-84) displaced the radioligand as effectively as hPTH(1-84) (IC$_{50}$s: 10-40 nM), whereas a group of shorter peptides, including hPTH(28-84), hPTH(34-84), hPTH(37-84), [Asn$^{76}$]hPTH(39-84) and hPTH(53-84), bound with significantly lower apparent affinity (IC$_{50}$s: 200-600 nM). Further minimal N-terminal truncation beyond position 53, as in hPTH(55-84), hPTH(57-84) and hPTH(60-84), effectively abolished measurable binding affinity for CPTHRs (IC$_{50}$s >> 10,000 nM) highlighting the presence of at least two domains required for maximal binding affinity – one within the sequence hPTH(24-27), (“binding domain 1; BD1”) and another represented by the dibasic sequence (Lys$^{53}$-Lys$^{54}$), termed “binding domain 2 (“BD2”). Further analysis of the intact hormone pointed to the presence of additional major determinants of binding affinity within the region hPTH(55-84), thereby defining a third “binding domain” (“BD3”). To identify key residues involved in the contribution of BD3 to overall CPTHR ligand binding affinity, clustered triple-alanine substitutions were introduced across the sequence of hPTH(53-84), to produce nine mutant hPTH(53-84) peptides. Three of these peptides, with substitutions at positions 71-74 (“M4”), 64-66 (“M6”) and 55-57 (“M9”) respectively, showed dramatic (roughly 100-fold) reductions in apparent affinity. Further analysis of additional peptides harboring single-alanine substitutions within these regions identified three key residues - Asn$^{57}$, Lys$^{65}$ and Lys$^{72}$ - that appear to be critical for high affinity binding to CPTHRs.

As osteocytes are terminally differentiated osteoblasts, it was of interest to determine if CPTHR activation might play a role in regulating apoptosis in the OC cells. We found that incubation of OC cells, which lack functional PTH1R genes, for 6 hr with 100 nM hPTH(1-84) led to
increased nuclear pyknosis and chromatin condensation, as revealed by DNA staining with Hoechst dye 33258. Increased apoptosis also was observed in response to the intact hormone or the short fragment hPTH(53-84) using a TUNEL immunocytochemical assay (7).

Our initial signaling studies demonstrated that CPTH activation induces a rapid influx of calcium from the extracellular compartment, likely via opening of calcium channels. Since calcium is a major regulator of the cytoskeleton, we examined the effect of CPTH-dependent calcium influx on cytoskeletal rearrangement. OC59 cells were treated with 100nM hPTH(53-84) for 2 and 10 min and then were examined by immuno-fluorescent staining of cytoskeletal components (vinculin and actin). OC59 cells treated with the CPTH fragment demonstrated a marked actin and vinculin condensation, suggestive of a rapid modification of the cytoskeleton. The specificity of this effect was examined by treating the cells with the mutant peptide [Ala\(^{55-57}\)]hPTH(53-84), which does not bind or activate calcium influx and, as expected, the mutant analog failed to induce any cytoskeletal changes in OC cells. The role of calcium influx was examined by blocking calcium influx with gadolinium chloride (GdCl\(_2\)) (1 and 10 M). When OC59 cells were treated with GdCl, hPTH(53-84) failed to induce cytoskeletal changes, indicating that calcium influx might play an important role in regulation of osteocyte cytoskeletal assembly and structure.

Thus, in summary, it seems possible that PTH might regulate osteocytic function via at least two receptor systems, the PTH1R and the CPTH, although many questions still remain. Frost had proposed that systemic hormones might alter bone remodeling by changing the thresholds at which mechanosensory cells in bone respond to differing intensities of mechanical stress or loading (21). It therefore will be important, in the future, to further define the effects of PTH, acting both via the PTH1R and the CPTH, on osteocytes undergoing mechanical stimulation.

Reference:
14. Tazawa K, Hoshi K, Kawamoto S, Tanaka M, Ejiri S, Ozawa H 2004 Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. J Bone Miner Metab 22:524-9
             & Mineral Research 9:1697-704
New observations on Bone Fragility with Glucocorticoid Treatment. Results from an in vivo animal model.

Nancy E. Lane, Co-investigators and Collaborators and Advisors: Wei Yao, Mehdi Balooch, Ravi K. Nalla, Guive Balooch, Stefan Habelitz, John H. Kinney Lynda F. Bonewald

Glucocorticoid treatment frequently is associated with an increase in the risk of bone fracture, especially in the spinal vertebrae and the femoral head (1,2,3). Individuals treated with glucocorticoids have alterations in bone remodeling (5,6). These alternations in bone remodeling include an increase in bone resorption and reductions in bone formation such that reductions in trabecular bone mass and architecture occur including reductions in trabecular thickness and trabecular number (5,6,7,8). The mechanism behind these changes have been proposed to be alterations in bone cell viability, ie, glucocorticoids reduce the lifespan of osteoblasts and osteocytes through apoptosis while increasing osteoclast viability (7,8). While these proposed alterations in bone cell lifespan could explain the reduction in bone formation markers and trabecular bone architecture, neither of these observations completely explains the increased bone fragility observed in glucocorticoid-induced osteoporosis when compared to postmenopausal osteoporosis (4).

Methods: We assessed the changes in the fifth lumbar vertebral body and or distal femur for trabecular bone structure (microCT and histomorphometry), elastic modulus of lumbar vertebrae trabeculae (Scanning Probe Microscopy), whole bone strength (compression testing), mineral to matrix ratio of the trabeculae (MicroRamen Spectroscopy), and bone turnover (serum and urine biochemical markers and histomorphometry) for prednisolone-treated mice and controls; and estrogen deficient mice and sham-operated controls after 21 days of treatment.

Results: We observed significant reductions in trabecular bone volume and whole bone strength in both prednisolone-treated and estrogen deficient mice compared to their controls after 21 days (p<0.05). In addition, significant changes within the trabecular bone surrounding the osteocyte lacunae was observed in the prednisolone-treated mice. The size of the osteocyte lacunae was increased, and elastic modulus was reduced around the lacunae. In addition, a “halo” of hypomineralized bone surrounding the lacunae was observed only in the prednisolone-treated mice and this was associated with reduced (nearly 40%) mineral to matrix ratio determined by Raman microspectroscopy.

Conclusions: Based on these results, we propose that glucocorticoids have direct effects on osteocytes, not only to induce cell death, (7,8) but more importantly, to induce viable cells to modify their microenvironment. By ‘leaching’ mineral from their surroundings, there is both enlargement of the lacunae and a sphere of hypomineralized bone that is generated. Together this may result in highly localized changes in bone strength. Based on these results, bone active agents that influence bone cell activity such as bisphosphonates and hPTH (1-34) might be useful agents to prevent or treat glucocorticoid-induced bone loss (9,10). These results also suggest that glucocorticoids produces localized changes in bone strength that are in some aspects similar to
estrogen deficiency (increased bone remodeling on the trabecular surface) but also different (hypomineralization around the osteocyte lacunae) that may help to explain why glucocorticoid-treated patients fracture at higher bone mineral densities than postmenopausal women with osteoporosis.

References (suggested readings, not meant to be extensive)