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Biological Regulation of Bone Quality

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Abstract

The ability of bone to resist fracture is determined by the combination of bone mass and bone quality. Like bone mass, bone quality is carefully regulated. Of the many aspects of bone quality, this review focuses on biological mechanisms that control the material quality of the bone extracellular matrix (ECM). Bone ECM quality depends upon ECM composition and organization. Proteins and signaling pathways that affect the mineral or organic constituents of bone ECM impact bone ECM material properties, such as elastic modulus and hardness. These properties are also sensitive to pathways that regulate bone remodeling by osteoblasts, osteoclasts, and osteocytes. Several extracellular proteins, signaling pathways, intracellular effectors, and transcription regulatory networks have been implicated in the control of bone ECM quality. A molecular understanding of these mechanisms will elucidate the biological control of bone quality and suggest new targets for the development of therapies to prevent bone fragility.

Keywords

Bone quality; extracellular matrix; elastic modulus; fragility; nanoindentation; TGFβ; bone remodeling; osteocyte; perilacunar remodeling; osteocyte osteolysis; mineralization; collagen; crosslinking; transcription factor; signaling pathway

Introduction

Bone quality comprises features of bone across multiple length scales and includes bone geometry, microarchitecture, and the material quality of bone extracellular matrix, among others[1]. Aspects of bone quality are site-specific – such that bone ECM material properties differ throughout the body[2, 3], are sensitive to developmental and environmental factors - such as bone geometry[4, 5], and are affected by disease processes – such as bone microarchitecture[6]. Relative to bone mass however, the biological mechanisms that regulate

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Conflict of Interest
T Alliston declares no conflicts of interest.

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bone quality are less well elucidated. This article focuses on the biological mechanisms that specify aspects of bone quality, with a focus on those regulating the material quality of the extracellular matrix (ECM).

Biological control of bone ECM composition and organization

The material quality of bone ECM is critically dependent on its mineral and organic constituents. Both the composition and the organization of these constituents can affect bone ECM material properties. In many cases, mutations or disease processes that disrupt the normal composition and organization of bone ECM compromise the ability of bone to resist fracture, independently of changes in bone mass. Therefore, the biological control of bone quality includes mechanisms that control the composition and organization of bone ECM.

Bone ECM mineralization

Mineral concentration is a major determinant of the elastic modulus of bone matrix. As the mineral fraction of the bone ECM increases, so too does the elastic modulus[7], generally at the expense of the work to fracture or post-yield behavior of the bone[2]. The control of biomineralization is dynamic and complex with diverse theories describing the responsible mechanisms. Many factors have been implicated as agonists and antagonists of mineralization – the deregulation of which can lead to pathological extra-skeletal mineralization. Among these, enzymes that regulate levels of inorganic pyrosphosphate (PPi), a potent inhibitor of mineralization, have been implicated in the control of bone ECM quality. Osteoblast and osteocyte-derived matrix vesicles control extracellular PPi levels with a host of factors, including tissue nonspecific alkaline phosphatase (TNAP) and the progressive ankylosis protein (ANK)[8]. TNAP is an enzyme that hydrolyzes and inactivates PPi. Normally expressed at sites of mineralization during development, loss of TNAP function results in hypomineralized bone[9–11].

Conversely, ANK is expressed in non-mineralizing tissues where it transports PPi to the extracellular space to antagonize mineralization. Loss of function mutations in ANK cause hypermineralization[12]. Importantly, ANK levels are sensitive to vitamin D[13], a factor that impacts bone quality at multiple levels[14, 15]. To maintain systemic mineral homeostasis, the vitamin D receptor can induce ANK gene expression. These elevated ANK levels limit the deposition of calcium into the bone ECM[13]. The extent to which ANK can directly impact bone quality remains to be established. Nonetheless, these studies highlight factors that regulate PPi levels as a possible target of signaling pathways known to control bone quality.

Non-collagenous proteins

Although non-collagenous proteins comprise only 10% of the total bone protein, they play a critical role in bone quality[16]. Osteocalcin and osteopontin are two of the most abundant (and most well-studied) non-collagenous proteins. In addition to their regulation of cellular function[17–20], both osteopontin and osteocalcin influence the deposition of mineral within the collagen fibril-rich bone ECM. In vitro studies implicate osteocalcin and osteopontin in the control of hydroxyapatite nucleation, size, shape, and orientation[21, 22]. These highly phosphorylated proteins also have the capacity to dissipate energy through numerous weak

sacrificial intra- and intermolecular bonds[23]. Although the overall skeletal phenotypes of mice deficient in these proteins are quite mild, the relevance of these in vitro findings is supported by recent in vivo studies by Poundarik, et al., who found that osteopontin and osteocalcin colocalize at the interface between hydroxyapatite crystals in dilatational bands along mineralized collagen fibrils[24]. At the nanometer scale, these bands dissipate energy to toughen bone and limit crack propagation. Accordingly, bone deficient in either or both osteopontin or osteocalcin has severely impaired fracture toughness[24, 25].

Several other non-collagenous proteins have also been implicated in the control of bone quality, including biglycan[26], DMP1[27], Phex[28], and fibrillins[29]. Key bone regulatory pathways control the expression or activity of each of these proteins. For example, osteopontin expression is regulated by TGFβ, vitamin D, PTH, and glucocorticoids[30–33], all of which have been shown to influence some aspect of bone quality[14–15, 34–37]. Consequently, by targeting non-collagenous proteins, these signaling pathways can impact bone quality directly by toughening bone and indirectly by regulating the organization of mineralized collagen fibrils or bone cellular activity.

Collagen

The organic composition of bone matrix is dominated by collagen I. Defects in this fundamental constituent of the bone ECM exert their effects on bone quality across many length scales. This is most apparent in the diverse irregularities in bone from individuals with osteogenesis imperfecta, or from mouse models of this disease[38–4142, 43]. Collectively these studies show that collagen mutations can deregulate the coupled remodeling of bone by osteoblasts and osteoclasts, the maintenance of bone mass, and the organization of mineralized collagen fibrils. The disorganized collagen fibrils lower the threshold for crack initiation in OI bone, whereas defects in the lamellar structure limit the ability to prevent crack growth[39]. In some forms of OI, adaptive remodeling of the bone microarchitecture and geometry compensates for the inferior OI bone quality to accommodate mechanical load[42, 43]. The multitude of pathological and adaptive responses of bone to collagen mutations shows the critical importance of this protein, as well as the presence of feedback loops that couple the mechanical and biological homeostasis of bone.

Collagen undergoes extensive post-translational modification, including crosslinking by enzymatic and non-enzymatic mechanisms[44]. As collagen matures, the lysl oxidase-generated interfibrillar crosslinks (pyridinoline and pyrrole) predominate over the lysly hydroxylase-generated intrafibrillar crosslinks (dehydrodihydroxynorleucine and dehydrohydroxy-lysinonorleucine). Non-enzymatically generated crosslinks, advanced glycation end products (AGEs) and pentosadine, accumulate with age. Conditions that limit bone remodeling effectively increase bone tissue age[45], which often increases the level of AGEs in bone ECM[46]. In aging or irradiated bone, AGE levels are increased as the toughness of the bone declines[47]. The inverse correlation of AGE levels with fracture toughness has been attributed to the inability of glycated collagen, and possibly other proteins, to support microdamage formation or crack deflection. With the diminished function of these toughening mechanisms[48], cracks that initiate in aged or irradiated bone are much more likely to grow unchecked and cause bone fracture[49, 50]. Accumulation of

AGEs in diabetes is also consistent with the loss of bone toughness in diabetic rats. Thus, AGEs may contribute to bone fragility in human diabetes, which is not otherwise explained by changes in mineral content of material properties[51].

Bone remodeling and the control of bone quality

Each of the three predominant cell types in bone, the osteoclast, the osteoblast, and the osteocyte, participate in the remodeling of bone extracellular matrix. Of the many signaling pathways shown to regulate the remodeling activity of these cell types, some have been directly implicated in the control of bone ECM material properties. Here we discuss these mechanisms, with the idea that other bone remodeling regulatory pathways may prove to participate in the maintenance of bone quality, or its deregulation in disease.

Remodeling by osteoclasts and osteoblasts

Osteoclast-mediated resorption of bone ECM not only leads to the loss of bone mass, but it also changes the dynamics of bone remodeling, which in turn, affects bone ECM age and material properties[52, 53]. Because bone resorption and deposition are normally coupled, biological factors that inhibit osteoclast activity also suppress osteoblast function. Inhibition of bone remodeling increases the existing bone matrix age, which in turn increases the maturity of mineral crystals and collagen crosslinks[45, 46]. As described above, these changes in the mineral and organic constituents of the bone ECM cause an increase in the bone matrix elastic modulus and hardness[54].

Consequently, the many signaling pathways and therapeutic agents that affect the dynamics of bone remodeling also have the potential to regulate bone ECM material properties and bone quality. For example, hyperactive bone remodeling, as in hyperparathyroidism or vitamin D deficiency[35, 55], leads to the deposition of a hypomineralized and disorganized bone ECM with a low elastic modulus and hardness. Conversely, bisphosphonates inhibit bone remodeling and increase mineral and collagen crosslink maturity and ECM elastic modulus[56]. The overall benefit of bisphosphonate therapy in preventing the loss of bone mass overrides the potential side effects of a somewhat more brittle bone matrix. However, the rare but catastrophic failure of femora following bisphosphonate exposure suggests that, in some cases, the bone ECM material properties are compromised to the extent that increased bone mass no longer protects against fracture[57].

Osteocyte-mediated perilacunar remodeling

Several lines of evidence point to the critical role of osteocytes in maintaining bone ECM material properties. Recent molecular studies reinvigorate decades-old observations that osteocytes remodel the local bone ECM[58]. This process is called osteocyte osteolysis or perilacunar remodeling (PLR)[59–62]. In PLR, osteocytes secrete several proteases, including MMP2, MMP13, MMP14, and cathepsin K, to dynamically resorb and then replace the perilacunar ECM[61–66]. PLR was originally implicated in the maintenance of mineral homeostasis in lactation, hibernation, and other metabolically demanding states in vertebrates[67–68]. By studying MMP13-deficient bone, we found that PLR is also essential for the maintenance of bone quality[61]. MMP13-deficiency limits osteocyte-mediated bone

resorption in lactating animals. Even in non-lactating or male mice, MMP13-deficiency abrogates PLR and significantly compromises fracture toughness. The fragility of MMP13-deficient bone may derive from its heterogeneous mineralization, collagen and canalicular disorganization, variable ECM material properties, and increased collagen crosslinking. Recent high-resolution imaging studies strikingly show the relationship of the lacunar-canalicular network to ECM mineralization and collagen fibril alignment[69, 70], supporting an active role for osteocytes in the control of ECM organization. Together, these and other studies strongly suggest that PLR is a constitutive homeostatic mechanism that operates to maintain bone quality.

Maintenance of healthy bone ECM requires other osteocyte-derived enzymes in addition to MMP13. The canalicular network is also disrupted in MMP2 and MMP14-deficienct bone[64, 65], suggesting their role in PLR. MMP2-deficient bone ECM is hypomineralized with a lower ECM elastic modulus (assessed by nanoindentation) and impaired bone strength in macromechanical tests[71]. Recently, cathepsin K was elegantly implicated in lactation-induced PLR[62]. An earlier study demonstrated the fragility of cathepsin K-deficient bone, in spite of elevated bone mass[72]. Although the cellular mechanisms responsible for these defects were unclear at the time, the severely disorganized collagen organization and reduced bone quality suggests that cathepsin K is required for the PLR-dependent maintenance of bone ECM organization and material properties. Bone fragility also associates with hallmarks of defective PLR in bone from mice deficient in NF1, a GTP-ase activating protein that regulates important signaling intermediates such as Ras. Among the many skeletal phenotypes of NF1-deficient mice, the defects in bone quality relate to the heterogeneous ECM mineralization and the disorganized collagen and canalicular networks[73]. However, a precise role of NF1 in PLR remains to be elucidated.

The clinical significance of PLR in bone quality is perhaps most evident in glucocorticoid treated bone. Patients treated with glucocorticoids exhibit bone fragility that is unexplained by the loss of bone mass, pointing to a defect in bone quality[74]. Using scanning probe microscopy, Lane, et al., showed a localized glucocorticoid-dependent reduction in perilacunar bone ECM elastic modulus and mineralization[37]. Glucocorticoids exert a multitude of effects on osteocytes, osteoblasts, and osteoclasts[75]. Several of these contribute to the reduced ability of glucocorticoid-exposed bone to resist fracture. Nonetheless, the dramatic effect of glucocorticoids on perilacunar remodeling deserves additional attention to better understand the mechanisms by which osteocytes contribute to bone fragility.

Molecular mechanisms controlling bone quality

Each of the cellular processes that regulate bone quality responds to signaling by numerous endocrine, paracrine, and autocrine pathways. The activity of signaling pathways is regulated at multiple levels. This hierarchical control provides cells the opportunity to generate a specific and integrated response to the diverse cues in the cellular microenvironment. Ligands, receptors, and intracellular effectors are subject to transcriptional, translational, and post-translational control. This regulation can alter the magnitude, duration, or nature of the cellular response to the same ligand. The molecular infrastructure of signaling pathways has been elucidated using model organisms (i.e. *S.*

cerevisiae, *D. melanogaster*, *C. elegans*) and in vitro cell culture systems. Genetically modified mice provide the opportunity to apply this insight to understand the molecular control of bone ECM material properties and other aspects of bone quality. When applied to study the molecular control of bone mass, this strategy yielded new anabolic therapies for osteoporosis. Likewise, understanding the mechanisms controlling bone quality will yield novel therapeutic strategies to reduce bone fragility.

Many signaling pathways act on multiple cell populations such that they coordinate osteoblast, osteoclast, and/or osteocyte activity. In this way, the same protein can independently regulate different aspects of bone quality or bone mass. For example, cathepsin K-deficient mice exhibit increased bone mass because of impaired osteoclast activity; but inferior ECM material properties, likely due to defects in osteocyte-mediated perilacunar remodeling[72]. Furthermore, the activity of bone cell populations is coupled. Mutations that interfere with signaling pathway function in one cell type most often impact other cell types as well. Therefore, interpreting the biologic and mechanical phenotypes of genetically modified mice requires careful consideration of this cellular complexity and the potential for independent control of bone mass and bone quality. This can be particularly challenging when only using macromechanical tests to evaluate bone quality, some of which may not have the sensitivity in mouse bone to distinguish the effect of a mutation on bone mass from its effects on bone quality. While macromechanical tests are essential to establish physiological relevance, the select use of methods that evaluate smaller length scales can be helpful in deciphering the biological mechanisms controlling of bone quality.

Control of bone quality through hierarchical regulation of signaling pathways

The most detailed understanding of biological mechanisms regulating bone quality derives from study of the TGFβ pathway. TGFβ directs the proliferation, differentiation, and apoptosis of cells in the osteoblast and osteoclast lineages. TGFβ plays a vital role in coupling the activity of these cell types to maintain bone homeostasis[76]. Consequently, human mutations in several components of the TGFβ pathway have been implicated in skeletal dysplasia and disease. Likewise, bone from genetically modified mice with altered TGFβ ligand, receptor, or effector function showed dramatic and complex bone phenotypes (Reviewed in[77]).

In vitro studies have elucidated mechanisms by which TGFβ exerts its effects on each bone cell type[78]. In osteoblasts, activated TGFβ ligands bind to a heterotetrameric complex of type I and type II TGFβ receptors (TβRI and TβRII). Upon formation of this ligand/receptor complex, the receptors phosphorylate one another, as well as downstream effectors Smad2 and Smad3. Phosphorylated Smads translocate to the nucleus to regulate the expression of genes required for osteoblast differentiation[79, 80]. To determine if TGFβ employed the same pathway to control bone quality as it did to regulate osteogenic differentiation, we evaluated ECM material properties in mouse bone with altered TGFβ ligand, receptor, and effector activity. Bone ECM elastic modulus and hardness showed an inverse dose-dependent correlation with the level of TGFβ signaling[34]. This result was particularly striking given the otherwise complex bone phenotypes of these mouse strains. Pharmacologic regulators of TGFβ signaling replicate these findings[81, 82], in which TGFβ signaling was modified

genetically. Interestingly, several other proteins implicated in the control of bone ECM material properties, including fibrillins, biglycan, and MMPs, functionally interact with the TGFβ pathway[78]. The extent to which the bone quality phenotypes of these mouse strains is TGFβ-dependent remains unclear. Overall, these studies show that, at least in the case of TGFβ signaling, the same molecular effectors control both osteoblast differentiation and bone ECM material properties.

Ligands and receptors

Of the biological molecules implicated in the control of bone quality, we know most about the ligands. As mentioned above, vitamin D, glucocorticoids, and parathyroid hormone modulate the material properties of bone ECM, at least in part through their effects on bone remodeling. Osteoblast-derived IGF-1, but not systemic growth hormone[83], is sufficient to protect several aspects of bone quality, including the bone ECM material properties measured by nanoindentation, from the deleterious effects of a low protein diet[84]. Although growth hormone increased the cross-sectional area of cortical bone, this change in bone geometry did not produce the expected increase in macromechanical behavior. This result suggests that growth hormone compromises the material quality of bone ECM through mechanisms that remain unclear[84].

Cells respond to ligands through specific receptors. Receptors have also been implicated in the control of bone ECM material quality. Leptin receptor-deficient mice have a reduced breaking force in macromechanical tests, which is attributed in part to reduced cortical bone thickness and a slight but significant reduction in the bone ECM elastic modulus as measured by nanoindentation[85]. Though many other ligands and receptors also impact the macro-mechanical behavior of bone, it can be difficult to distinguish whether these changes are due to bone ECM material properties or other aspects of bone quality such as bone geometry or microarchitecture.

Transcriptional regulators of bone quality

Taking these observations a step further in the cell, we found that Runx2 is a requisite target of TGFβ/Smad3 in the control of bone ECM quality as it is in the control of osteoblast gene expression[86]. The Runx2/Smad3 complex recruits histone deacetylases (HDACs) to DNA, which modify chromatin to repress transactivation of osteogenic genes such as osteocalcin[87]. Interestingly, mice with HDAC3-deficient osteoblasts have hypomineralized bone ECM with reduced hardness and elastic modulus, as measured by nanoindentation[88]. The extent to which the many other Runx2-regulatory pathways also impact the material quality of bone matrix remains unclear. Also unclear are the specific transcriptional targets of Smad3 and Runx2 that confer differences in ECM composition, organization, and material quality.

Other transcription factors implicated in the control of bone ECM material properties include ATF4, GATA1, and NF-E2. ATF4 is a critical regulator of osteoblast gene expression[89]. Deficiency in ATF4 is associated with a higher mineral to collagen ratio and reduced fracture toughness, suggesting that the transcriptional targets of ATF4 directly or indirectly act to maintain bone ECM organization and bone quality[90]. Bones deficient in

GATA1 or NF-E2, transcription factors required for megakaryocyte development, have increased macromechanical stiffness and peak load. Differences in bone mass and ECM mineralization are insufficient to explain the macromechanical behavior, suggesting the role of these transcription factors in regulating expression of proteins that impact the material properties of the bone matrix[100].

Beyond elucidating the specific proteins employed to control bone quality, these studies collectively show that cells employ the same ligand, receptor, intracellular effectors, and transcriptional regulatory networks to regulate the material quality of the bone ECM as they do to control other critical cellular decisions. We propose a model in which, not only in bone but in other tissues as well, signaling pathways target the function of linage specific transcription factors to control the material quality of the ECM[86]. In this case, the same mechanisms that define the tissue-specific expression of ECM proteins would also define the material properties and physical microenvironment of the tissue, a model that would illuminate our understanding of development as well as of disease.

Future Directions

In addition to those outlined above, many critical questions about the regulation of bone ECM quality remain.

How are bone ECM material properties regulated by biochemical, physical, and chemical cues?

Undoubtedly, many additional pathways and proteins will be implicated in the biological regulation of bone quality. In addition to understanding the role of pathways activated by biochemical ligands and receptors, it will be intriguing to explore the extent to which bone ECM material properties respond to physical cues such as mechanical loading or chemical cues such as hypoxia.

Which downstream targets of signaling pathways and transcription factors are responsible for differences in bone ECM quality?

So far, the causal link has yet to be made between a signaling pathway or transcription factor and the downstream target genes that confer changes in bone ECM material properties. Many attractive candidates arise from molecular interactions identified in vitro. For example, osteopontin, osteocalcin, and MMP13 gene expression are regulated by TGFβ, Smad3, and Runx2[30, 80, 91]. As described above, each of these has the capacity to impact bone ECM material properties. However, the necessity of specific proteins in the regulation of bone ECM quality by TGFβ (or other pathways) has yet to be established.

What are the mechanisms by which bone mass, bone ECM material properties, and other aspects of bone quality are coordinately regulated?

Systems biology approaches have already yielded fascinating insights about the integrated regulation of many aspects of bone quantity and quality. Whether using mice[92], baboons[93], or zebrafish, these genetic models are powerful approaches to identify genetic networks that allow co-adaptation of bone mass, matrix composition and bone quality to prevent fragility.

What are the functional implications of biologically controlled bone ECM material properties?

Vertebrates have evolved sophisticated mechanisms to calibrate the material quality of the bone ECM. The precise regulation of bone ECM elastic modulus is essential for hearing[86], such that a 1 GPa reduction in cochlear bone ECM elastic modulus corresponds to a 1.84 Db hearing loss. However, the selective advantage that derives from the specification of anatomically distinct bone ECM material properties at other sites is less clear. In addition, the physical mechanisms by which bone ECM material properties prevent or exacerbate bone fragility across length scales must be more firmly established.

How does the regulation of bone ECM material properties affect the cellular microenvironment of the niche?

Cells generate cytoskeletal tension to adapt to physical features of their microenvironment such as ECM material properties and topography[94]. Cytoskeletal tension influences fundamental cellular processes from proliferation and differentiation to migration and lineage selection. Therefore, ECM material properties in the cellular microenvironment represent a powerful cue that directs cell behavior. Additional research is needed to determine if the hematopoietic stem cell, mesenchymal stem cell, or tumor niche in bone has specific ECM material properties, as well as the biological mechanisms through which they are regulated. Recent studies suggest that osteocytes sense the physical microenvironment and respond by generating cytoskeletal tension to modify their function[95]. Given the importance of cellular tension in defining the maintenance of HSC and MSC pluripotency, lineage selection, and differentiation[96, 97], it is critical to understand the mechanisms that regulate bone ECM material properties.

Conclusions

Research in these emerging areas will elucidate the control of bone ECM quality, its role in the mechanical function of bone, as well as the ability of bone ECM material properties to act as an instructive cue in the cellular microenvironment. These studies will lead to the discovery of new mechanisms that couple the biological and physical homeostasis of bone and mechanisms by which it is disrupted in disease.

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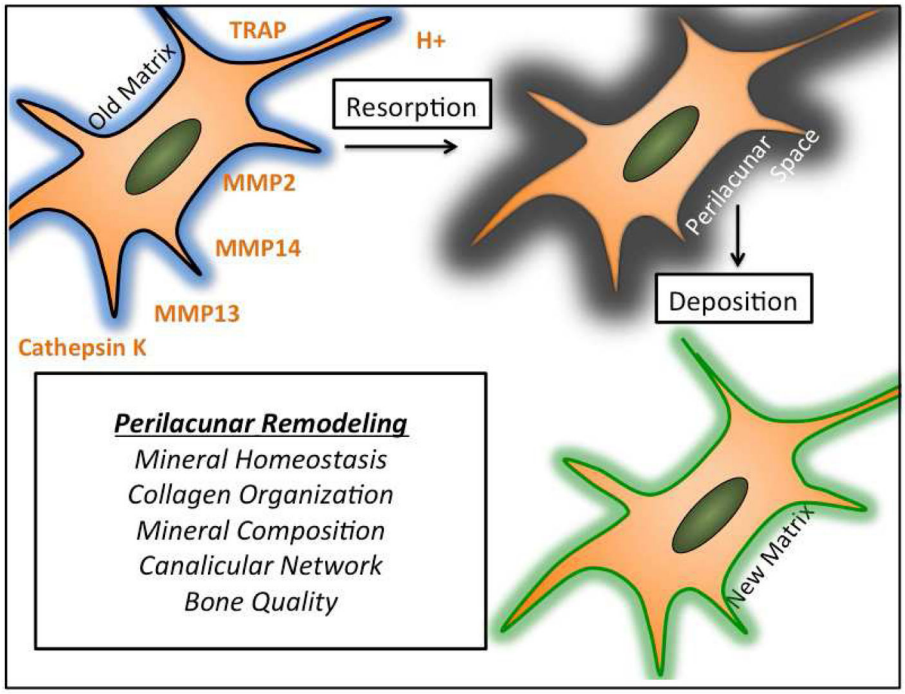

Figure 1. Perilacunar Remodeling by Osteocytes

Bone health is maintained through dynamic remodeling executed by osteoclasts, osteoblasts, and osteocytes. While osteocytes direct osteoclast and osteoblast activity, osteocytes also play a direct role in remodeling bone matrix. Through a dynamic process called perilacunar remodeling (PLR), osteocytes secrete protons and proteases to resorb the perilacunar bone matrix, often to accommodate metabolic demands. After mineral homeostasis is restored, osteocytes refill the lacunar spaces with new bone matrix. Several osteocyte-derived proteins have been implicated in PLR including cathepsin K, MMP13, MT-1MMP, MMP2, TRAP, the Na/H+ exchanger, but not the osteoclast marker, RANK. Recent studies using genetically modified mice demonstrate that MMP13-dependent PLR is an essential constitutive process that actively maintains bone quality.

Table 1

Extracellular proteins implicated in the control of bone ECM material properties

Protein	Reference
Ligands	
vitamin D	13 14
TGFb	34
PTH	35 36
glucocorticoids	37
growth hormone	83, 84
IGF-1	83
Secreted Proteases	
MMP2	71
MMP13	61
MMP9	71
Cathepsin K	72
Extracellular Matrix Proteins	
osteocalcin	24
osteopontin	24, 25, 98, 99
collagen I	38–43
biglycan	26
Fibrillin	29
DMP1	27

Table 2

Cellular proteins implicated in the control of bone ECM material properties

Protein	Reference
Receptors	
TβRI	81, 82
TβRII	34, 86
Leptin Receptor	85
Transcriptional Regulators	
Smad3	34
Runx2	86
ATF4	90
GATA1	100
HDAC3	87
Effector	
NF1	73
Transporter	
ANK	8
Enzymes	
TNAP	9–11
Phex	28