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Chapter 4. Osteocytes

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INTRODUCTION

In the adult skeleton, osteocytes make up >90–95% of all bone cells compared with 4–6% osteoblasts and ~1–2% osteoclasts. These cells are regularly dispersed throughout the mineralized matrix, connected to each other and cells on the bone surface through dendritic processes generally radiating toward the bone surface and the blood supply. The dendritic processes travel through the bone in tiny canals called canaliculi (250–300 nm), whereas the cell body is encased in a lacuna (15–20 μm; Figs. 1 and 2). Osteocytes are thought to function as a network of sensory cells mediating the effects of mechanical loading through this extensive lacuno-canalicular network. Not only do these cells communicate with each other and with cells on the bone surface, but their dendritic processes extend past the bone surface into the bone marrow. Osteocytes have long been thought to respond to mechanical strain to send signals of resorption or formation, and evidence is accumulating to show that this is a major function of these cells. Recently, it has been shown that osteocytes have another important function, to regulate phosphate homeostasis; therefore, the osteocyte network may also function as an endocrine gland. Defective osteocyte function may play a role in a number of bone diseases, especially glucocorticoid-induced bone fragility and osteoporosis in the adult, aging skeleton.

OSTEOCYTE ONTOGENY

Osteoprogenitor cells reside in the bone marrow before differentiating into plump, polygonal osteoblasts on the bone surface.[1,2] By an unknown mechanism, some of these cells are destined to become osteocytes, whereas some become lining cells and some undergo programmed cell death known as apoptosis.[3] Osteoblasts, osteoid–osteocytes, and osteocytes may play distinct roles in the initiation and regulation of mineralization of bone matrix, but Bordier et al.[4] first proposed that osteoid-osteocytes are major regulators of this process. Oste-

Dr. Bonewald has received graduate student support from and has consulted for Procter & Gamble. She also holds a patent on MLO cell lines.

Key words: osteocytes, mechanical load, phosphate metabolism, apoptosis, bone disease

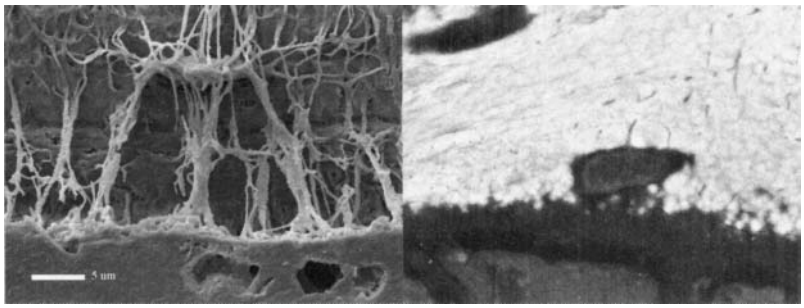

FIG. 1. The embedding osteocyte retains its connectivity with cells on the bone surface. The image on the right is of acid-etched plastic embedded murine cortical bone. With this technique, resin fills the lacuno-canalicular system, osteoid, and marrow, but cannot penetrate mineral. Mild acid is used to remove the mineral leaving behind a resin cast relief. Note the canaliculi connecting the lacunae with the bone surface at the bottom of the image. The image on the right is a from transmission electron microscopy showing a fully embedded osteocyte and an osteoid-osteocyte becoming surrounded by mineral (white). The osteoid is black and the osteoblasts are at the bottom of the image.

oid–osteocytes actively make matrix while simultaneously calcifying this matrix. The osteoblast cell body reduces in size ~30% at the osteoid–osteocyte stage, whereas cytoplasmic processes are forming and are ~70% with complete maturation of the osteocyte (Fig. 1).

Whereas numerous markers for osteoblasts have been identified such as cbfa1, osterix, alkaline phosphatase, and collagen type 1, few markers have been available for osteocytes until recently.[2] In 1996, the markers described for osteocytes were limited to low or no alkaline phosphatase, high casein kinase II, high osteocalcin protein expression, and high CD44. Osteocyte markers such as E11/gp38, phosphate-regulating neutral endopeptidase on chromosome X (Phex), dentin matrix protein 1 (DMP1), and sclerostin have recently been identified (Table 1). Some of these markers are overlapping in expression with osteoblasts, but some have been identified for specific stages of differentiation. Promoters for specific markers have been used to drive green fluorescent protein (GFP) to follow osteoblast to osteocyte differentiation in vivo. Collagen type 1–GFP is strongly expressed in both osteoblasts and osteocytes, osteocalcin–GFP is expressed in a few osteoblastic cells lining the endosteal bone surface and in scattered osteocytes, and the osteocyte-selective promoter, the 8-kb DMP1 driving GFP showed exclusive expression in osteocytes.[5] The actin-bundling proteins, villin, α-actinin, and fimbrin, were shown to be markers for osteocytes with strong signals of fimbrin at branching points in dendrites.[6] It is likely that these actin reorganizing proteins play a role in osteocyte cell body movement within its lacuna and the retraction and extension of dendritic processes.[7]

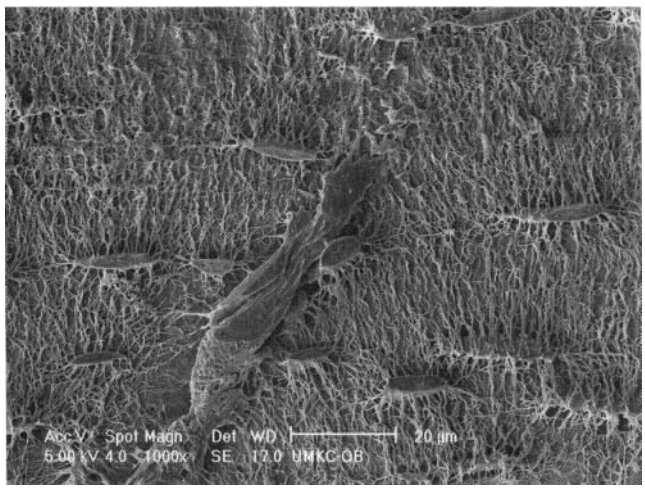

FIG. 2. The osteocyte lacuno-canalicular network is intimately associated with the blood vessel network in the bone matrix. The white marker points to an osteocyte lacunae intimately associated with the blood vessel.

OSTEOCYTES AS ORCHESTRATORS OF BONE (RE)MODELING

Considerable evidence is mounting that osteocytes can conduct and control both bone resorption and bone formation. Some of the earliest supporting data for the theory that osteocytes can send signals of bone resorption were observations that isolated avian osteocytes can support osteoclast formation and activation in the absence of any osteotropic factors[8] as can the osteocyte-like cell line, MLO-Y4.[9] It was suggested that expression of RANKL along exposed osteocyte dendritic processes provides a potential means for osteocytes within bone to stimulate osteoclast precursors at the bone surface.

One of the major means by which osteocytes may support osteoclast activation and formation is through their death. Osteocyte apoptosis can occur at sites of microdamage, and it is proposed that dying osteocytes are targeted for removal by osteoclasts. The expression of anti-apoptotic and pro-apoptotic molecules in osteocytes surrounding microcracks was mapped, and it was 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.[10] Therefore, those osteocytes that do not undergo apoptosis are prevented from doing so by protective mechanisms, whereas 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.[11] Injection of a single dose of diphtheria toxin eliminated ~70% of osteocytes in cortical bone in these mice, leading to dramatic osteoclast activation. Therefore, viable osteocytes are necessary to prevent osteoclast activation and maintain bone mass (Fig. 3).

The osteocyte-like cell line MLO-Y4 not only supports osteoclast formation but also osteoblast differentiation,[12] and surprisingly, mesenchymal stem cell differentiation.[13] It is most likely (but remains to be proven) that primary osteocytes can perform all three functions, therefore possessing the unique capacity to regulate all phases of bone remodeling.

OSTEOCYTE CELL DEATH AND APOPTOSIS

It has been proposed that the purpose and function of osteocytes is to die, thereby releasing signals of resorption. Osteocyte cell death can occur in association with pathological conditions, such as osteoporosis and osteoarthritis, leading to increased skeletal fragility.[14] Such fragility is considered to be caused by loss of the ability to sense microdamage and/or signal repair. Several conditions have been shown to result in osteocyte cell death such as oxygen deprivation as occurs during immobilization, withdrawal of estrogen, and glucocorticoid treatment.[14] TNFα and interleukin-1 (IL-1) have been re-

TABLE 1. OSTEOCYTE MARKERS

Marker	*Expression*	*Function*
E11/gp38	Early, embedding cell[37]	Dendrite formation?[37]
CD44	More highly expressed in osteocytes compared with osteoblasts[38]	Hyaluronic acid receptor associated with E11 and linked to cytoskeleton[39]
Fimbrin	All osteocytes[5]	Dendrite branching?
Phex	Early and late osteocytes[40,41]	Phosphate metabolism[42]
OF45/MEPE	Late osteoblast through osteocytes[43]	Inhibitor of bone formation[43]/regulator of phosphate metabolism[44]
DMP1	Early and mature osteocytes[45]	Phosphate metabolism and mineralization[34]
Sclerostin	Late embedded osteocyte[46]	Inhibitor of bone formation[47]
FGF23	Early and mature osteocytes[35]	Induces hypophosphatemia[42]

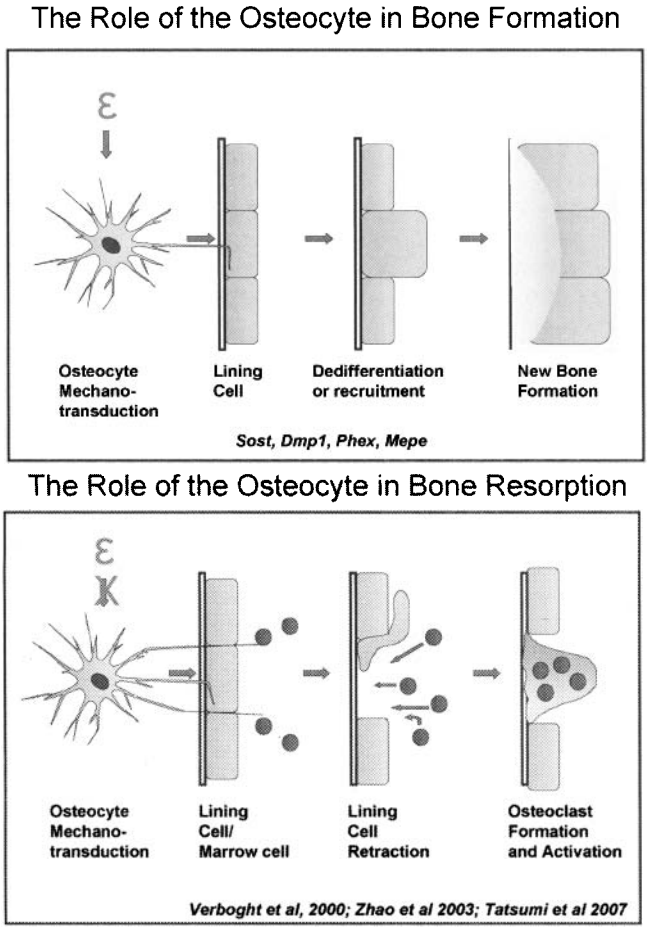

FIG. 3. Osteocytes as orchestrators of bone (re)modeling. Osteocytes play a role in bone formation and mineralization as promoters of mineralization (such as Dmp1 and Phex) and inhibitors of mineralization and bone formation (such as Sost/sclerostin and MEPE/OF45) are highly expressed in osteocytes (top). These supporters and inhibitors of bone formation and mineralization are most likely exquisitely balanced to maintain equilibrium to maintain bone mass. Osteocytes also appear to play a major role in the regulation of osteoclasts, by both inhibiting and activating osteoclastic resorption. It has recently been shown that with loading, the osteocytes send signals inhibiting osteoclast activation (bottom).[11] In contrast, compromised, hypoxic, apoptotic or dying osteocytes especially with unloading appear to send unknown signals to osteoclasts/preosteoclasts on the bone surface to initiate resorption. Therefore, osteocytes within the bone regulate bone formation and mineralization and inhibit osteoclastic resorption, whereas having the capacity to also send signals of osteoclast activation under specific conditions.

ported to increase with estrogen deficiency and also induce osteocyte apoptosis.[2]

Several agents have been found to reduce or inhibit osteoblast and osteocyte apoptosis. These include estrogen, selective estrogen receptor modulators, bisphosphonates, calcitonin, CD40 ligand, calbindin-D28k, monocyte chemotactic proteins MCP1 and 3, and recently mechanical loading through the release of prostaglandin.[2] Osteocyte viability clearly plays a significant role in the maintenance of bone homeostasis and integrity. However, whereas blocking osteocyte apoptosis may improve diseases such as bone loss caused by aging or glucocorticoid therapy, osteocyte apoptosis may be essential for normal damage repair and skeletal replacement. Any agents that block this process may exacerbate conditions in which repair is required. The death processes and consequently the resorption signals sent by dying osteocytes in an aging or glucocorticoid-treated skeleton may be distinct from those in a normal, healthy skeleton in response to microdamage. It will be important to identify and characterize these differences.

OSTEOCYTE MODIFICATION OF THEIR MICROENVIRONMENT

Almost 100 yr ago, it was proposed that osteocytes may resorb their lacunar wall under particular conditions.[15] The term "osteolytic osteolysis" was initially used to describe the enlarged lacunae in patients with hyperparathyroidism[16] and later in immobilized rats[17]. Osteolytic osteolysis has a negative connotation because it was confused with osteoclastic bone resorption. When resorption pits similar to those observed with osteoclasts were not observed with primary avian osteocytes seeded onto dentin slices, it was concluded that osteocytes cannot remove mineralized matrix. Removal of mineral by osteocytes would not be detectable using this approach because these cells are within a lacuna and do not form the characteristic sealed osteoclast resorption lacuna that rapidly decalcifies bone. In contrast to lacunar enlargement by the embedded, mature osteocyte, enlarged lacunae with renal osteodystrophy may be caused by defective mineralization during embedding of the osteoid–osteocyte during bone formation.[2]

In addition to enlargement of the lacunae, changes can take place in the perilacunar matrix. The term "osteocyte halos" was used to describe perilacunar demineralization in rickets[18] and later to describe periosteocytic lesions in X-linked hypophosphatemic rickets.[19] Glucocorticoids in addition to having effects on osteocyte apoptosis seem to also cause osteocytes to not only enlarge their lacunae but to also remove mineral from the perilacunar space thereby generating "halos" of hypomineralized bone.[20] Glucocorticoids may therefore alter or com-

promise the metabolism and function of the osteocyte and not just induce cell death.

The capacity to deposit or remove mineral from lacunae and canaliculi has important implications with regard to (1) mineral homeostasis, (2) magnitude of fluid shear stress applied to the cell, and (3) mechanical properties of bone. The surface area of the osteocyte lacuno-canalicular system is several order of magnitudes greater than the bone surface area; therefore, removal of only a few angstroms of mineral would have significant effects on circulating, systemic ion levels. Enlargement of the lacunae and canaliculi would reduce bone fluid flow shear stress, thereby reducing mechanical loading on the osteocyte. As holes in a material act as stress concentrators, enlargement of lacunae would enhance this effect in bone. Therefore, changes in lacunar size and matrix properties could have dramatic effects on bone properties and quality in addition to osteocyte function.

Over three decades ago, it was suggested that the osteocyte not only has matrix destroying capability but also can form new matrix.[21] Osteocyte lacunae were shown to uptake tetracycline, called "periosteocytic perilacunar tetracycline labeling," indicating the ability to calcify or form bone. Therefore, the osteocyte may be capable of both adding and removing mineral from its surroundings.

MECHANOSENSATION AND TRANSDUCTION

Mechanical strain is required for postnatal but not for prenatal skeletal development and maintenance. The postnatal and adult skeleton is able to continually adapt to mechanical loading by the process of adaptive remodeling where new bone is added to withstand increased amounts of loading and bone is removed in response to unloading or disuse. The parameters for inducing bone formation or bone resorption in vivo are fairly well known and well characterized. Frequency, intensity, and timing of loading are all important parameters. Bone mass is influenced by peak applied strain[22] and bone formation rate is related to loading rate.[23] When rest periods are inserted, the loaded bone shows increased bone formation rates compared with bone subjected to a single bout of mechanical loading and improved bone structure and strength is greatest if loading is applied in shorter versus longer increments.[24]

The major challenge in the field of mechanotransduction has been to translate these well-characterized in vivo parameters of mechanical loading to in vitro cell culture models. Theoretical models and experimental studies suggest that flow of bone fluid is driven by extravascular pressure as well as applied cyclic mechanical loading of osteocytes.[25] Mechanical forces applied to bone cause fluid flow through the canaliculi surrounding the osteocyte inducing shear stress and deformation of the cell membrane. It has also been proposed that mechanical information is relayed by cilia, a flagellar-like structure found on every cell.[26,27] Osteocytes may use a combination of means to sense mechanical strain.[28] Theoretical modeling predicts osteocyte wall shear stresses resulting from peak physiologic loads in vivo to be in the range of 8–30 dynes/cm^2,[25] but this has not been confirmed in vivo. It will be a significant advance in the field to be able to actually measure bone fluid flow within the lacuno-canalicular system.

ROLE OF GAP JUNCTIONS AND HEMICHANNELS IN OSTEOCYTE COMMUNICATION

A means by which osteocytes communicate intracellularly is through gap junctions, transmembrane channels that connect the cytoplasm of two adjacent cells, through which molecules with molecular weights <1 kDa can pass. Gap junction channels are formed by members of a family of proteins known as connexins, and Cx43 is the major connexin in bone cells. Much of mechanotransduction in bone is thought to be mediated through gap junctions.

Primary osteocytes and MLO-Y4 osteocyte-like cells[29,30] express large amounts of Cx43, suggesting that Cx43 has another function in addition to being a component of gap junctions. Recently, it has been shown that connexins can form and function as unapposed halves of gap junction channels called hemichannels. Hemichannels directly serve as the pathway for the exit of intracellular PGE_2 in osteocytes induced by fluid flow shear stress[31] and function as essential transducers of the anti-apoptotic effects of bisphosphonates.[32] Hemichannels are now one of several types of openings or channels to the extracellular bone fluid that also includes channels such as calcium, ion, voltage, stretch-activated channels, and others.[33] Therefore, gap junctions at the tip of dendrites seem to mediate a form of intracellular communication, and hemichannels along the dendrite (and perhaps the cell body) seem to mediate a form of extracellular communication between osteocytes.

POTENTIAL ROLE OF OSTEOCYTES IN BONE DISEASE

Osteoid osteocytes play a role in phosphate homeostasis. Once the osteoblast begins to embed in osteoid, molecules such as Dmp1, Phex, and Mepe are elevated (Table 1). Autosomal recessive hypophosphatemic rickets in patients is caused by mutations in Dmp1.[34] Dmp1-null mice have a similar phenotype to hyp mice carrying a Pex mutation, that of osteomalacia and rickets caused by elevated fibroblast growth factor 23 (FGF23) levels in osteocytes.[34,35] The osteocyte lacuno-canalicular system should be viewed as an endocrine organ regulating phosphate metabolism. The unraveling of the interactions of these molecules should lead to insight into diseases of hyper- and hypophosphatemia (see chapter on Phex/FGF23).

The connectivity and structure of the osteocyte lacuno-canalicular system may play a role in bone disease. Osteocyte dendricity may change depending on orientation and with static and dynamic bone formation and has been shown to be disrupted in bone disease.[36] In osteoporotic bone, there is disorientation of the canaliculi and a marked decrease in connectivity, which increases in severity. In contrast, in osteoarthritic bone, a decrease in connectivity is observed, but orientation is intact. In osteomalacic bone, the osteocytes seem viable with high connectivity, but the processes are distorted and the network is chaotic.[36] Variability in complexity and number of dendrites and canaliculi could have a dramatic effect on osteocyte function and viability and on the mechanical properties of bone.

Osteocyte cell death may be responsible for some forms of osteonecrosis. Osteonecrosis is "dead" bone containing empty osteocyte lacunae that does not remodel but can remain in the bone for years. As reviewed above, viable osteocytes are necessary to send signals of (re)modeling. Early proposed mechanisms responsible for osteonecrosis include the mechanical theory, where osteoporosis and the accumulation of unhealed trabecular microcracks result in fatigue fractures; the vascular theory, where ischemia is caused by microscopic fat emboli; and a newer theory of osteocyte apoptosis, where agents induce osteocyte cell death resulting in dead bone that does not remodel.[14] Osteocyte health, compromised status, viability,

and capacity to regulate its own death most likely play a highly significant role in the maintenance and integrity of bone. Bone loss in osteoporosis may be caused in part by pathological and not physiological osteocyte cell death.[3] It will be important to develop therapeutics that maintain both osteocyte viability and physiological osteocyte cell death that leads to normal bone repair.

In conclusion, it is most likely that osteocytes use undiscovered specific molecules to regulate bone (re)modeling. With the dramatic increases or maintenance of bone mass being observed with neutralizing antibody to sclerostin, an osteocyte-specific marker, greater effort is being made to identify additional markers and to unravel the mysteries surrounding osteocyte function. It is also likely that new functions will be discovered for these cells, making them a target of investigation, not only to understand basic bone physiology, but also to understand and treat bone disease.

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Chapter 5. Connective Tissue Pathways That Regulate Growth Factors

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INTRODUCTION

Different connective tissues perform different physiological functions. To perform these functions, connective tissue cells secrete distinct sets of extracellular matrix (ECM) proteins that are arranged within the individual connective tissue in discrete patterns. The relative abundance of ECM proteins within a tissue and the histological patterns in which these proteins are organized endow connective tissues with their specific developmental, physiological, and homeostatic properties. For example, in bone, type I collagen is the most abundant ECM protein constituent, and type I collagen fibers are organized in long, thick bundles that are consistent with the mechanical properties required of bone.

The contributions of ECM proteins such as collagens and proteoglycans to the mechanical properties of cartilage and bone are better understood today than the contributions of many of the minor constituents of the ECM. However, recent knowledge of the function of a fibril-forming ECM protein named fibrillin[1] indicates important roles for fibrillin microfibrils in the development, growth, and maintenance of skeletal elements. Recent studies of fibrillin microfibrils have created a new, and still emerging, paradigm for understanding the extracellular regulation of growth factor signaling. This chapter summarizes current knowledge about fibrillin microfibrils, their roles in the regulation of growth factor signaling, their molecular partners in the connective tissue, and their relevance to skeletal biology.

THE FIBRILLINOPATHIES

The importance of fibrillin to the skeleton was first appreciated when a mutation in the gene for fibrillin-1 (FBN1) was identified as the cause of the Marfan syndrome.[2] Individuals with Marfan syndrome (OMIM 154700) display major disease features in the skeleton: tall stature and arachnodactyly, scoliosis and chest deformities, joint hypermobility and muscle wasting, pes planus, and craniofacial abnormalities, including a highly arched palate. Skeletal features of Marfan syndrome are thought to be largely caused by the overgrowth of long bones. Multiple features in other organs (cardiovascular, ocular, skin, lung, and central nervous system) also characterize Marfan syndrome, reflecting the ubiquitous tissue distribution of fibrillin-1 and the importance of fibrillin-1 to the affected tissues.

Mutations in fibrillin-2 cause Beals syndrome or congenital contractural arachnodactyly (CCA).[3] Features of CCA (OMIM 121050) include contractures of the small and large joints, crumpled ears, and arachnodactyly. The more limited nature of disease features caused by mutations in fibrillin-2 is thought to reflect the low to null expression levels of FBN2 mRNA in postnatal tissues and the compensatory high levels of FBN1 mRNA in postnatal tissues.

Mutations in FBN1 also cause autosomal dominant Weill-Marchesani syndrome.[4] Skeletal features of Weill-Marchesani syndrome are the opposite of those found in the Marfan syndrome. Individuals with Weill-Marchesani syndrome (OMIM 608328) display short stature, brachydactyly, hypermuscularity, and joint stiffness. Whereas these skeletal features are the opposite of Marfan, ectopia lentis (resulting from a weakness in the suspensory ligament of the lens) is typical of both syndromes.

FIBRILLIN MICROFIBRILS

Fibrillin was first identified as the major protein component of small (10 nm) diameter microfibrils that are ubiquitous in the extracellular space.[1] At the ultrastructural level, fibrillin microfibrils can be distinguished from collagen fibers by their uniform small diameter and a characteristic beaded or hollow

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