Bone ECM Signaling

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Bone mineral density, a bone matrix parameter frequently used to predict fracture risk, is not the only one to affect bone fragility. Other factors, including the extracellular matrix (ECM) composition and microarchitecture, are of paramount relevance in this process. The bone ECM is a noncellular three-dimensional structure secreted by cells into the extracellular space, which comprises inorganic and organic compounds. The main inorganic components of the ECM are calcium-deficient apatite and trace elements, while the organic ECM consists of collagen type I and noncollagenous proteins. Bone ECM dynamically interacts with osteoblasts and osteoclasts to regulate the formation of new bone during regeneration. Thus, the composition and structure of inorganic and organic bone matrix may directly affect bone quality. Moreover, proteins that compose ECM, beyond their structural role have other crucial biological functions, thanks to their ability to bind multiple interacting partners like other ECM proteins, growth factors, signal receptors and adhesion molecules. Thus, ECM proteins provide a complex network of biochemical and physiological signals.

ECM,bone fragility,fracture risk,bone disease,ECM signaling

1. Introduction

The bone mineralized extracellular matrix (ECM) is predominantly responsible for bone's resistance to fracture, defined as bone strength. Bone formation or internal reconstruction will determine not only the spatial structure of the tissue but its mechanical properties. Bone mass has been used as a predictor of bone fragility; however, it is only a partial correspondent. Indeed, the skeleton derives its resistance to fracture from multiple components regulated across several levels of hierarchical organization. That way, the relative composition, organization, and maturity of the mineral and organic matrix have a paramount relevance on how bones respond to mechanical demand.

2. Bone Extracellular Matrix Composition

Bones involve living cells embedded in a mineralized matrix, consisting of organic and inorganic phase[1]. While the inorganic matrix is responsible for the ability to resist deformation (bone strength and stiffness), organic matrix allows energy absorption (toughness)[2]. The cellular component of bone is in constant interaction with the surrounding ECM, which affects cellular function by regulating different signaling pathways. All in all, different cells and molecules that compose bone matrix are involved in bone strength and, therefore, alterations in either fraction may affect bone composition and mechanical properties, determining fracture risk[3].

2.1. Inorganic Matrix

The inorganic (or mineral) fraction of bone tissue, composed of a combination of calcium and phosphorus salts, (predominantly in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)), is of ultimate importance to bone strength and stiffness. Crystals of calcium phosphate, produced by osteoblasts, are laid down in precise amounts within the fibrous matrix, leading to bone mineralization (also known as calcification). Mineral is initially deposited between the ends of collagen fibrils of the matrix, whilst during bone maturation hydroxyapatite crystals grow and aggregate[4].

When the maturation process occurs, expressed proteins regulate ordered deposition of mineral by regulating the amount and size of hydroxyapatite crystals formed. Two proteins appear essential in bone mineralization: type I collagen, which constitutes the scaffold upon which mineral is deposited, and alkaline phosphatase, that hydrolizes pyrophosphate (a strong inhibitor of mineralization) plus modifies the phosphorylation status of osteopontin (OPN), a factor implicated in bone remodeling[5]. Other bone matrix proteins are also known to regulate the mineralization process such as proteoglycans[6], matrix Gla-protein[7] and various phosphate-regulating proteins. Bone mineralization is also controlled by systemic hormones such as parathyroid hormone (PTH) and vitamin D[8]. PTH, the principal regulator of calcium homeostasis, enhances the release of calcium from the large reservoir contained in the bones[9] whilst, vitamin D stimulates the intestinal absorption of calcium and phosphorus to achieve enough calcium concentration[10]. Even more, the later also promotes differentiation of osteoblasts, stimulating the expression of bone crucial players; such as bone-specific alkaline phosphatase, osteocalcin (OC) and osteonectin (ON), among others.

Finally, the degree of mineralization, closely linked with bone strength[11], is mostly determined by the rate of bone turnover[12]. High bone turnover decreases the overall bone mineralization leading to lower bone stiffness. On the contrary, a reduced bone turnover leads to the accumulation of older and more extensively mineralized bone[12], with the consequent biomechanical drawbacks: it makes bone more brittle[13] and leads to the accumulation of damaged (aged) bone with reduced elastic properties, facilitating microcrack and fracture occurrence. Therefore, adequate balance between bone formation and resorption is crucial for bone quality[14].

2.2. Organic Matrix

Proteins that compose bone ECM can be divided into collagen and, to a minor extent, other noncollagenous proteins (NCPs). Bone-forming cells (osteoblasts) secrete the main compound of the organic matrix: type I collagen, which constitutes about 85–90% of the total bone protein content. Type I collagen, encoded by *COL1A1* and *COL1A2* genes, not only plays a major structural role in bone but also contributes to tissue organization and therefore to its mechanical properties[15]. Type I collagen is first synthesized as the precursor procollagen, being subsequently stabilized by post-translational modifications and disulfide bonds. Then, it is secreted into the ECM, cleaved of the N- and C-terminals, and processed until native triple helix collagen is obtained.

NCPs, such as proteoglycans, SIBLING proteins (small integrin-binding ligand, N-linked glycoproteins), glycosylated proteins, γ-carboxylated proteins, and other serum-derived proteins, are present in the bone matrix taking part in collagen assembly and crosslink formation[16] affecting the mechanical properties of collagen. This way, abnormalities in collagen crosslinks have been associated with increased fracture risk[17][18].

All in all, the correct synthesis and fiber orientation of collagen are mandatory to obtain a healthy bone matrix able to withstand bone tensile strength. As such, it is not surprising that defects in type I collagen have dramatic effects on the skeleton.

2.3. Cellular Components

Bone is additionally composed of four different cell types that are in constant interaction with the surrounding ECM [19]. First, osteoprogenitor cells have the capacity to divide and differentiate into different bone cells. These cells, also known as mesenchymal stem cells (MSCs), differentiate to osteoblasts under osteogenic conditions. Osteoblasts are bone forming cells that synthesize and secrete the collagen matrix plus accomplish the mineralization of bone matrix. Then, when the secreted matrix surrounding the osteoblast calcifies, the osteoblast becomes trapped within it. As a result, it changes in morphology, becoming an osteocyte, the primary cell of mature bone that maintains the bone tissue. Finally, osteoclasts, multinucleated cells derived from hematopoietic progenitors, are the responsible for bone tissue degradation. Since bone is a dynamic tissue, bone remodeling is tightly regulated by both osteoblasts and osteoclasts: while osteoblasts form new bone, osteoclasts resorb it.

3. Bone Structure: Microarchitecture

Overall, the human skeleton is composed of bones grouped in four categories: long bones (femur, tibia, clavicles), short bones (for instance carpal and tarsal bones), flat bones (such as the ribs, mandible and skull) and irregular bones (such as vertebrae). All of them are composed of two types of bone tissue which can be distinguished macroscopically, differing in their architecture but similar in molecular composition: cortical (or compact) bone and trabecular (or cancellous) bone (80% and 20% of human skeleton, respectively)[20]. Although composed by the same components, mainly hydroxyapatite, collagen and water, trabecular bone is less mineralized (it has lower calcium content and higher water content), presenting lower tissue density and mineral content compared to cortical bone [21]. Consequently, cortical bone is densely packed, providing the strength and rigidity to bones. On the contrary, trabecular bone, responsible for the most bone turnover[22], is a porous material composed of a network of trabeculae organized to optimize load transfer, dispersing the energy of loading[23]. The cortical to trabecular ratio in each bone varies depending on the bone type and the specific skeletal site of that bone. Thus, cortical bone is mainly present in shafts of long bones and outer surfaces of flat bones, whereas trabecular bone is found at the end of long bones, vertebral bodies and the inner part of flat bones.

Alterations in bone ECM components can disrupt ECM-bone cell signaling leading to deterioration of bone mineral density (BMD) (the content of calcium in a certain volume of bone) and/or bone microarchitecture, (the organization of bone components in space), the two main parameters determining bone strength. In vivo quantification of

cortical and trabecular BMD, geometry and microarchitecture can be analyzed at the same time by quantitative computed tomography methods, rendering the amount of cortical and trabecular bone tissue and features of trabecular (trabecular number, trabecular thickness, trabecular separation) and cortical (cortical thickness and porosity) bone microarchitecture.

4. Biophysical Properties of Bone Extracellular Matrix

A growing body of evidence in ECM biology points at biophysical properties of the bone ECM (mineral crystal size, their crystallinity (the degree of structural order) and the degree and type of collagen crosslinking,) as important determinants of cell behavior. Indeed, every cell in its anatomical localization has to balance the external forces dictated by the mechanical properties of its environment, which results from the compression exerted by neighboring cells as well as the stiffness of the surrounding ECM.

Regarding the biophysical properties of the mineralized matrix that surrounds bone cells, not only does the degree of mineralization matter so does the individual characteristics of the hydroxyapatite crystals (their size and shape) and crystallinity. Indeed, excessive crystal growth damages collagen fibers, affecting the tissue mechanical properties. Moreover, bone strength seems to be favored by greater mineral crystal size heterogeneity[24].

The biophysical properties of collagen type I fibers affect cellular behaviors[25], since cells respond differently to denatured collagen than to mature, crosslinked collagen fibrils[26]. Collagen crosslinking is a major post-translational modification which determines biophysical properties such as tensile strength and viscoelasticity[17]. Crosslinks can be divided into enzymatic and nonenzymatic. Enzymatic crosslinking is a process in which the ends of the collagen molecules are linked (so their number is greatly limited), acquiring a more stable, trivalent, nonreducible conformation[27]. When mature crosslinks accumulate, collagen fibril remodeling is inhibited and stiffness of the fibril increased, providing strength to the tissue[28]. That way, enzymatic crosslinking bears beneficial effect on the mechanical properties of collagen[17]. Conversely, nonenzymatic crosslinking does not involve any enzymes, and are found at any position along the collagen molecule to connect either collagen molecules or fibrils. Nonenzymatic glycation results in the formation of intermediate products (advanced glycation end-products (AGEs)) that undergo additional reactions to create crosslinks that form within and across collagen fibers. Thus, nonenzymatic crosslinking results in a brittle collagen network that, when accumulated or when its spatial distribution is altered leads to deteriorated bone mechanical properties[29][30]. In summary, while enzymatic crosslinking of collagen is generally considered to have a positive effect on bone´s mechanical properties, nonenzymatic crosslinking can lead to deteriorated bone mechanical properties.

5. Bone Extracellular Matrix Signaling

As previously mentioned, the majority of bone ECM is composed by collagen type I, reaching up to 90% of the protein content. However, proteomic analysis of decalcified bone has identified the minority presence of more than 100 ECM proteins in bone, different from collagen, reflecting the complexity of bone ECM[31][32].

In addition to structural role and thanks to their ability to bind multiple interacting partners like other ECM proteins, growth factors, signal receptors and adhesion molecules[33], the diverse set of ECM proteins also reveal other crucial biological functions. ECM components thus, provide a complex network of biochemical and physiological signals that contribute to bone metabolism, affecting fundamental cellular processes (such as proliferation, differentiation, migration and survival) via the integration of a number of signals that constitute the matrix-to-cell signaling[33]. This way, ECM regulates both, the osteoblast-lineage (for instance progenitors, mature osteoblasts, and osteocytes) and osteoclast-lineage (including precursors and mature osteoclasts), including also the crosstalk between them[34]. Besides, external influences can exert changes in these complex signaling systems as for instance vitamins[35] hormones[36][37] and/or minerals[38] intake.

In this section, we will highlight the main pathways that are involved in bone ECM signaling to offer a better understanding of how cell-matrix signaling occurs and the relevance of thereof in pivotal biological processes.

5.1. Integrin-Dependent Cell Adhesion Structures in Cell-ECM Signaling

Cell migration, essential for embryonic development, tissue renewal and immune response among other key processes, becomes crucial for correct bone remodeling. The formation of new bone needs the migration and differentiation of MSCs, an event tightly controlled by sequential activation of diverse transcription factors which regulates the expression of specific genes responsible for this transition[39]. The activation of these signaling cascades, and thus cell fate, is governed by the integration of all the signals that the cell receives from its environment through the ECM and intercellular adhesions.

Integrin-dependent cell adhesion structures allow cells to be attached to the ECM, binding intracellular actin fibers to extracellular proteins like fibronectin. This connection also transmits the mechanical force and regulatory signals between the ECM and the cytoskeleton of the cells.

Integrins are heterodimeric transmembrane receptors formed by one α and one β subunit. There are several subunit isoforms (eighteen α and eight β) that can be noncovalently assembled into 24 combinations[33] and the exact subunit combination dictates their binding specificity to different ECM components. Within the cell, the intracellular domain do not bind directly to the cytoskeleton, they do so via adapter proteins such as talin, α-actinin, filamin, vinculin and tensin[40][41], which transmit the applied forces on ECM to the actin cytoskeleton. Conversely, forces applied to actin, the so-called ‘traction forces’, are also transmitted to the ECM through the same mechanism[42].

As mentioned, integrins can be assembled into several combinations that are different in their mechanosensitivity and elicited cellular responses. Mechanosensation depends on ECM material properties, being broadly demonstrated that ECM stiffness determines cellular response during MSC differentiation into osteoblasts[43][44][45]. Furthermore, the communication also works the other way around; cellular response alter ECM´s mechanical stiffness as well[46].

5.2. MMPs as Signal Regulators

The main function of matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, is to degrade the proteins of the ECM, cleaving structural components such as collagen and gelatin.

MMPs expression and activity are regulated at multiple levels; inactive proenzyme transcription, translation and secretion, as well as proenzyme activation or inactivation via signaling of different factors like cytokines, growth factors or even ECM proteins. Normally, secreted MMPs are synthesized as proenzymes which are activated by proteolytic cleavage of the N-terminal prodomain by serine proteases or by active MMPs. Classic activators of MMPs include the activator protein-1, nuclear factor kappa B, tumor necrosis factor- α, and the transforming growth factor beta (TGF-β) together with some interleukins. There is growing evidence showing the importance of balance amongst MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) and the membrane anchored gly coprotein RECK, for MSC fate and stage-specific expression during bone cells differentiation [47].

MSCs from different organs have shown differential expression and secretion of MMPs/TIMPs[48][49]. In fact, the treatment of these cells with a broad spectrum of MMP inhibitors leads to alterations in migration, proliferation, and osteoblastic differentiation, supporting that these processes are MMP dependent[48][50]. Mauney J. and Volloch V. showed that bone marrow MSCs undergoing adipogenic differentiation, express more MMPs than TIMPs[51]. However, under osteogenic differentiation conditions, cells express more TIMPs than MMPs, reinforcing the key role of MMP/TIMP balance for matrix modulation and MSC differentiation[52].

MMPs, apart from ECM degrading enzymes, have a central role regulating several signaling pathways by cleaving many circulating, cell surface and pericellular proteins irreversibly. Among the molecules that are released by MMPs, TGF-β stands out, responsible of MSCs migration to resorbed sites promoting bone formation[53]. MMP-mediated activation and release of TGF-β has been described as a negative feedback mechanism to limit MMP expression and further TGF-β release[54]. Osteoclast secretion of MMP-9 activates trapped TGF-β in the ECM, and this TGF-β may downregulate cathepsin K and MMP-9 expression; thereby controlling the amount of bone resorption that occurs by mature osteoclasts[52]. However, TGF-β can also lead to an increase in MMP-13 expression, which is related with increased osteoclast differentiation and activation[55][56]. Altogether, this evidence underlines the required tight regulation and interconnection between TGF-β and MMPs pathways to achieve a correct bone homeostasis.

5.3. TGF-β Signaling Pathway

As stated previously, TGF-β pathway plays a crucial role in bone metabolism regulating bone mass and quality[57]. There are more than 40 members in the TGF superfamily, including bone morphogenetic proteins (BMPs), growth and differentiation factors, activins, nodal, and Müllerian inhibitory substance[58], in addition to TGF-β1, TGF-β2 and TGF-β3 isoforms, being TGF-β1 one of the most abundant cytokines in the bone matrix[59].

In bone, TGF-β is produced as large precursor molecule by bone-forming osteoblasts, being composed of mature TGF-β and latency-associated protein (LAP). TGF-β remains sequestered in the ECM as an inactive, latent form since LAP remains noncovalently bound to mature TGF-β as it is secreted. However, upon osteoclastic resorption,

LAP is cleaved, releasing the active TGF-β. A gradient of active TGF-β promotes the recruitment of MSCs to the recently resorbed bone surface by inducing chemotaxis and proliferation[60]. Once MSCs reach these sites, they differentiate into osteoblasts in response to environmental factors (such as bone-matrix-derived insulin-like growth factor 1)[61].

In addition to regulating the proliferation and differentiation of MSCs, active TGF-β has shown to be also an important regulator for osteoclastogenesis in a dose-dependent manner. High concentrations of active TGF-β generated at resorption areas inhibit the recruitment of osteoclast precursors, protecting it from further resorption during bone formation process[62]. Instead, low concentrations of active TGF-β induce the migration of osteoclast precursors[63]. This dual effect of TGF-β is also important in osteoclast differentiation. Low TGF-β levels stimulate osteoclast differentiation, whereas high levels inhibit such differentiation by regulating receptor activator of nuclear factor κβ ligand (RANKL)/osteoprotegerin (OPG) ratio[64]. In normal conditions, osteoblasts and osteocytes secrete RANKL, which binds to its receptor in osteoclasts (RANK) and promotes their differentiation. However, TGF-β can induce the expression of OPG in osteoblasts, a cytokine that acts as a decoy receptor for RANKL[65], thus inhibiting osteoclasts differentiation.

More recently, it has been shown that TGF-β presents both inhibitory and stimulatory effects in human osteoclast differentiation via Smad1 and Smad3 signaling, respectively[66]. These facts points out the complexity of TGF- β signaling governing the regulation of a wide range of bone metabolisms cellular functions.

Other pivotal members of TGF superfamily are BMPs. BMPs induce MSCs differentiation into bone[67][68] via the interaction with their cell surface receptors (BMPRs) in a canonical pathway similarly to TGF-β, leading to the activation of Smads. Like TGF-β, BMPs also activate several non-Smad signaling transducers, namely, mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinases (ERKs), c-Jun amino terminal kinase (JNK), p38 MAPK, the IκB kinase, phosphatidylinositol-3 kinase and Akt, as well as Ras homolog family GTPases.

Several studies have demonstrated that following TGF-β/BMP induction, both the Smad and p38 MAPK pathways converge at the runt-related transcription factor 2 (*Runx2*) gene to control mesenchymal precursor cell differentiation [69][70]. *Runx2* promotes the differentiation of progenitor cells into osteoblast, preventing adipogenesis[71] and exhibiting its essential role in MSC fate determination.

5.4. Wnt Signaling Pathway

Wingless-type mouse mammary tumor virus integration site family (Wnt) is essential for skeletal formation and development, being involved in a variety of processes like differentiation, proliferation and synthesis of bone matrix by osteoblasts as well as osteoclasts differentiation and function[72][73]. In fact, alterations not only in the intensity, but amplitude, and duration of Wnt signaling pathways affects skeletal formation during development, in addition to bone remodeling, regeneration, and repair during the lifespan [74].

Wnts can trigger several signaling cascades, among them, the most studied is the canonical Wnt/B-catenin pathway. Briefly, Wnt elicits the stabilization and nuclear translocation of β-catenin, which is a transcription coregulator. In the absence of Wnt, β-catenin is phosphorylated by a large protein complex (adenomatous polyposis coli/Axin/glycogen synthase kinase -3β-complex), leading to its ubiquitination and proteasomal degradation through the β-TrCP/Skp pathway. However, when Wnt is secreted, it binds to membrane Frizzled receptors and triggers a cascade of several intracellular events, allowing β-catenin translocation to the nucleus, activating Wnt target genes expression[75].

Canonical Wnt signaling pathway promotes MSCs differentiation into osteoblasts by preventing apoptosis in both; osteoblast progenitor cells and differentiated osteoblast[76]. As expected, Wnt signaling is also involved in cellular lineage dichotomy; more precisely Wnt10a, Wnt10b and Wnt6 favor osteogenesis at the expense of adipogenesis; suppressing the differentiation of MSCs to adipocytes while facilitating their differentiation to osteoblasts through the canonical Wnt pathway[77][78].

As stated throughout the present review, osteoclast progenitor differentiation is tightly regulated by osteoblasts and osteocytes. In normal conditions, osteoblasts and osteocytes express RANKL, which binds to osteoclasts receptor RANK, promoting their differentiation. However, the canonical activation of Wnt signaling pathway in osteoblast-lineage cells enhances the expression of OPG, a decoy receptor of RANKL, suppressing osteoclast differentiation and thus bone resorption[79].

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