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Molecular genetics of too much bone

Katrien Janssens and Wim Van Hul*

Department of Medical Genetics, University of Antwerp, 2610 Antwerp, Belgium

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Bone remodelling is an important process both throughout growth and in adult life. The homeostasis of bone tissue is maintained by the balanced processes of bone resorption and formation. Imbalance can give rise to a broad spectrum of skeletal pathologies, of which osteoporosis, characterized by a decrease in bone density leading to increased fracture risk, is the best known because of its high prevalence and consequently high socio-economic impact. At the opposite end of the spectrum, several genetic conditions displaying too much bone are situated. Mainly because of their monogenic nature—in contrast to the multifactorial character of osteoporosis—the underlying molecular genetic causes for several of these conditions have been revealed recently. In this review, the most important gene identifications of the last years and their impact on the understanding of bone biology are discussed.

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Bone tissue, the major constituent of the human skeleton, has many metabolic and mechanical functions. Abnormalities affecting this tissue can therefore lead to an extended number of pathological conditions. Many osteochondrodysplasias involve a disturbed skeletal morphogenesis due to abnormalities in bone patterning and growth during development. In an important group of conditions, however, only or mainly the homeostasis of bone tissue throughout life is affected. This homeostasis is maintained by two balanced processes: bone resorption by osteoclasts and bone formation by osteoblasts. In early life, the latter predominates over the former until the so-called peak bone density is reached between the ages of 25 and 35 years. Later in life, the rate of bone formation cannot fully compensate for the bone lost by resorption, resulting in a net loss of bone tissue.

Osteoporosis, by far the most common disorder affecting the skeleton, is defined by an abnormal decrease in bone mass leading to an increased fracture risk. The ethiology of this condition is multifactorial, with both genetic and environmental factors being involved. Therefore, the mainly monogenic conditions located at the other end of the spectrum are of great interest, since they nicely prove that an abnormality in one gene can dramatically influence bone balance, as illustrated in Figure 1. Three different pathogenic mechanisms can give rise to conditions with an increased bone density: decreased bone resorption, increased bone formation, or a disturbed balance between bone resorption and formation. The recent progress of the Human Genome Project, combined with the further development of molecular genetic technologies, has allowed the identification of the genes and associated pathogenic mechanisms underlying several of these conditions.

DECREASED BONE RESORPTION

The cell responsible for the resorption of bone tissue is the osteoclast. This cell type is of hematopoietic origin and differentiates in a late phase from the monocyte/macrophage cell lineage to form a giant, multinucleated cell that can attach to mineralized bone tissue. Specific receptors are implicated in this attachment at the clear or sealing zone. The process of resorption itself takes place at the ruffled border with its highly infolded plasma membranes. The resorption of mineralized bone tissue is a two-step procedure involving the dissolution of bone mineral and the enzymatic degradation of the organic bone matrix. For both processes, an acidic environment is needed, which is created in a sealed compartment between the osteoclast and the bone surface. Recent discoveries have illustrated that impairing any of these two processes results in different forms of osteopetrosis or in pycnodysostosis, respectively.

Osteopetrosis: defects in the acidification process of the extracellular compartment

The osteopetroses are a heterogeneous group of conditions characterized by an increased bone density due to impaired bone resorption. In analogy with the existence of a large number of spontaneous osteopetrotic animal models, different human forms of osteopetrosis have been described. Currently, the underlying molecular genetic defects have been identified for three of these. Interestingly, all three genes play a role in the acidification process of the extracellular compartment located between the osteoclast and the bone tissue (Fig. 2).

An autosomal recessive form of osteopetrosis associated with renal tubular acidosis and cerebral calcifications (MIM 259730)

*To whom correspondence should be addressed at: Department of Medical Genetics, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium. Tel: +32 38202585; Fax: +32 38202566; Email: vhul@uia.ua.ac.be

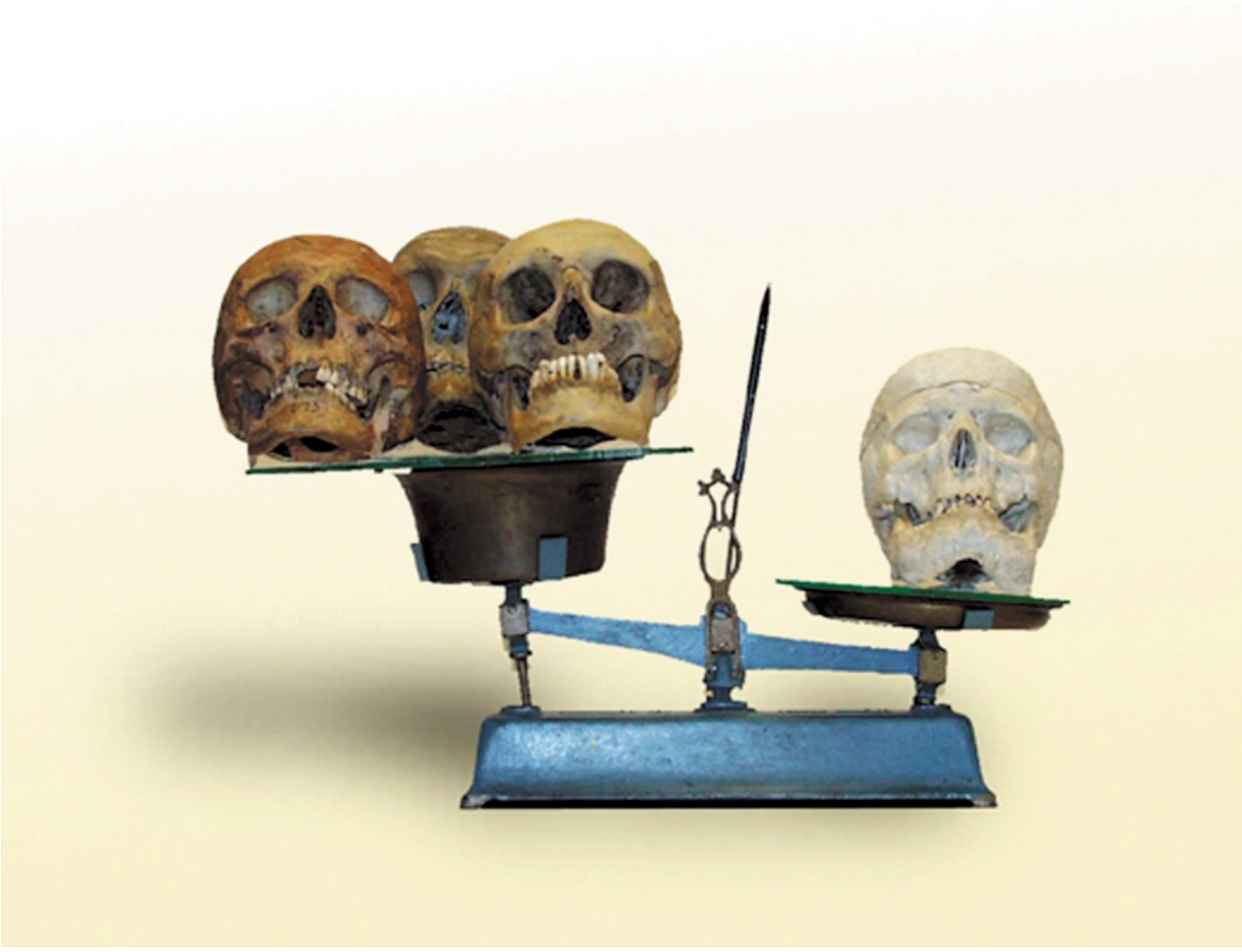

Figure 1. Picture illustrating the impressive increase in weight of a skull of a Van Buchem's disease patient. The weight is close to four times that of a normal skull.

is caused by mutations in the carbonic anhydrase II (*CA II*) gene (1). This enzyme catalyzes the intracellular conversion of CO_2 and H_2O to HCO_3^- and protons (H^+). This cytoplasmic mechanism provides a source of protons, which are transferred over the plasma membrane by a proton pump to acidify the extracellular compartment. Recently, positional cloning efforts for the severe, autosomal recessive form of osteopetrosis (MIM 259700) revealed that loss-of-function mutations in the *ATP6i* gene encoding a transmembrane 116 kDa subunit of the vacuolar (V) H^+-ATPase proton pump are present in a subset of patients with this condition (2,3). In a few patients with the same condition, loss-of-function mutations have been found in the gene encoding the chloride channel ClCN7 (4,5). The function of this osteoclastic Cl^- channel is to provide an electrical shunt for the proton pump antagonizing the generation of a membrane potential built up by the transport of protons. While complete absence of Cl^- transport results in the severe form of osteopetrosis, the milder, autosomal dominant form of osteopetrosis (Albers–Schönberg disease; MIM 166600) has recently been associated with mutations in the *ClCN7* gene as well (4). As chloride channels are known to function as dimers, the missense mutations identified in this type of osteopetrosis exert a dominant-negative effect (Table 1).

In conclusion, all three genes involved in a human form of osteopetrosis take part in the mechanism of acidification. Absence of CA II results in a relatively mild form of osteopetrosis, while a more severe form is found in the case where either a functional proton pump or chloride channel is absent from the osteoclast. In the case of the *ClCN7* gene, a dominant-negative effect caused by missense mutations leads to the mild, autosomal dominant form of osteopetrosis.

While some animal models of osteopetrosis are caused by a differentiation defect of osteoclasts (6–8), this is, at present, not the case for any human form. However, it cannot be excluded that a currently unidentified human osteopetrosis gene plays a role in this mechanism.

Pycnodysostosis: defect in the degradation of organic bone matrix

After the demineralization process, the second step in bone resorption implies the enzymatic degradation of organic bone

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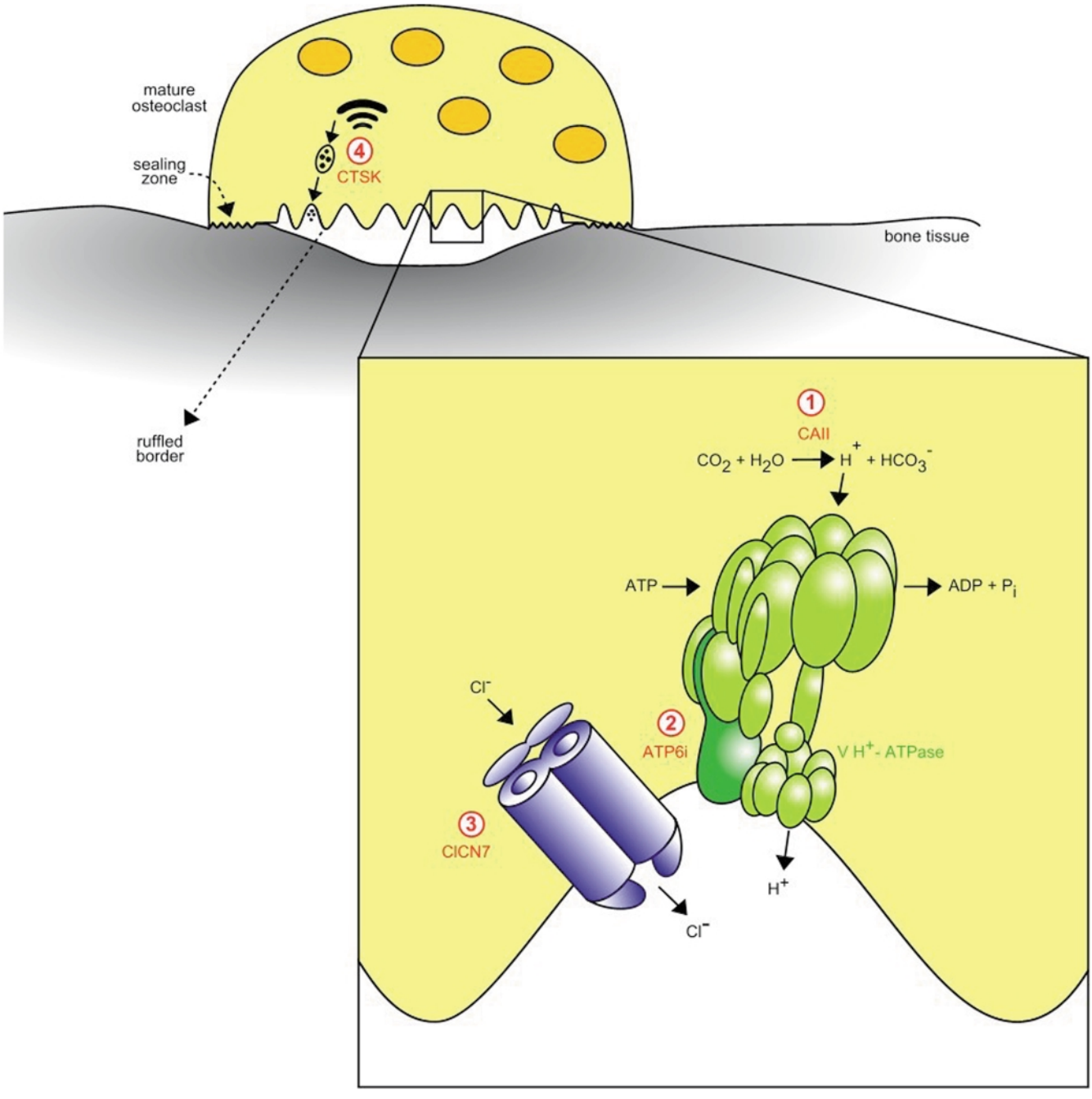

Figure 2. Schematic representation of the osteoclast attached to bone tissue, with enlargement of part of the ruffled border. Proteins involved in disorders with decreased bone resorption are marked in red: [1]–[4]. Carbonic anhydrase II [1] catalyzes the formation of protons in the cytoplasm of the osteoclast. The vacuolar (V) H^+-ATPase, of which the ATP6i subunit [2] is mutated in osteopetrosis, shuttles these protons to the extracellular compartment. Together with the Cl^- ions, transported by ClCN7 [3], the acidity of the extracellular environment, needed for the demineralization process, is established. CTSK [4] is the major collagenase of the osteoclast, responsible for enzymatic degradation of the organic bone matrix.

matrix. Cathepsin K (CTSK) is one of the most prominent acid lysosomal enzymes in osteoclasts, and is secreted into the subosteoclastic space (Fig. 2) after autocatalytic activation intracellularly as the osteoclast approaches bone (9). It cleaves collagen type I, osteopontin and osteonectin at low pH (10–13). By positional cloning, the *CTSK* gene was identified as the gene causing pycnodysostosis (14) (MIM 265800), an autosomal recessive bone dysplasia characterized mainly by a short stature and increased bone density but with a predisposition to fractures (15,16). The mutations found affect normal transcription and/or activation of this protein, leading to ablation of collagenase activity.

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Table 1. List of currently known genes underlying sclerosing bone dysplasias

No.[a]	Gene	Chromosomal locus	Disorder	Mode of inheritance[b]	Effect of mutation	Pathogenic mechanism[c]
Disorders with decreased bone resorption						
1	*CA* II	8q22	Malignant osteopetrosis	AR	Loss-of-function	Impaired acidification of EC compartment OC
2	*ATP6i*	11q12–q13	Malignant osteopetrosis	AR	Loss-of-function	Impaired acidification of EC compartment OC
3	*ClCN7*	16p13	Malignant osteopetrosis	AR	Loss-of-function	Impaired acidification of EC compartment OC
			Albers–Schönberg disease	AD	Dominant-negative effect	Reduced acidification of EC compartment OC
4	*CTSK*	1q21	Pycnodysostosis	AR	Loss-of-function	Loss of collagenase activity
Disorders with increased bone formation						
5	*SOST*	17q12–q21	Sclerosteosis	AR	Loss-of-function	Impaired inhibition of BMP signaling pathway
			Van Buchem's disease	AR	Impaired or loss-of-function	Impaired inhibition of BMP signaling pathway
6	*LRP5*	11q12–q13	High bone mass	AD	Gain-of-function	Overinduction of Wnt signaling pathway
7	*ANK*	5p14–p15	Craniometaphyseal dysplasia	AD	Unclear	Changed shuttling of PP_i in OB
Disorders with disturbed balance between bone formation and resorption						
8	*RANK*	18q21	Familial expansile osteolysis	AD	Gain-of-function	Increased NFkB signaling
			Expansile skeletal hyperphosphatasia	AD	Gain-of-function	Increased NFkB signaling
9	*p62*	5q35–qter	Paget's disease of bone	AD	Gain-of-function	Increased NFkB signaling
10	*OPG*	8q24	Juvenile Paget's disease	AR	Loss-of-function	Increased NFkB signaling
11	*TGFB1*	19q13	Camurati–Engelmann disease	AD	Gain-of-function, dominant-negative affect	Overinduction of TGF-β signaling pathway

[a]These are the numbers used in Figures 2 and 3.
[b]AR, autosomal recessive; AD, autosomal dominant.
[c]EC, extracellular compartment; OC, osteoclast; OB, osteoblast.

INCREASED BONE FORMATION

Osteoblasts, cells of mesenchymal origin, are the bone-forming cells. They produce an organic matrix, mainly composed of type I collagen, which is subsequently mineralized. Both the differentiation of osteoblasts and the bone formation rate by differentiated osteoblasts are under the control of local and endocrine factors. Recent efforts to clone the genes underlying conditions with phenotypic evidence of increased bone formation have led to the identification of three genes.

Sclerosteosis and Van Buchem's disease: increased BMP signaling

Sclerosteosis (MIM 269500) and Van Buchem's disease (MIM 239100) are clinically related conditions characterized by an impressive increase in the amount of bone tissue (Fig. 1). The normal structure of the bone results in increased bone strength. Genetic linkage studies suggested that both conditions are allelic, caused by mutations in the same gene (17,18). Recently, loss-of-function mutations in a previously unknown gene (*SOST*) were found in sclerosteosis patients (19,20). No mutations were found in patients with Van Buchem's disease, the milder of the two conditions, but a deletion of about 52 kb is located 30 kb downstream of the *SOST* gene (21). Most likely, the expression of *SOST* is suppressed by the presence of this deletion. Functional evidence for the *SOST* gene product, Sclerostin, was initially based on homology (19,20) with a recently identified gene family including *DAN*, *Gremlin* and *Cerberus* (22). The proteins that they encode have antagonistic effects on the activity of bone morphogenetic proteins (BMPs) by binding extracellularly to these BMPs, thus inhibiting the binding of the BMPs to their receptor complex and the induction of the BMP signaling pathway (23). Preliminary experimental data indicate that Sclerostin indeed binds with high affinity to a subset of BMPs and by doing so can block BMP-induced bone formation *in vitro* (24) (Fig. 3).

High-bone-mass phenotype: increased Wnt signaling

In 1997, Johnson *et al.* (25) were able to localize a gene with a major influence on bone density on chromosome 11q12–13 by linkage analysis in a family with a high-bone-mass (HBM) phenotype (MIM 601884), but without any clinical implications. Recently, Gong *et al.* (26) identified the gene underlying the osteoporosis–pseudoglioma syndrome (OPS)—with a bone phenotype opposite to the HBM phenotype—from the same chromosomal region. Loss-of-function mutations in this gene, encoding the low-density lipoprotein receptor-related protein 5 (LRP5), result in the decreased bone formation rate of OPS. Not unexpectedly, the same gene turned out to be involved in the HBM phenotype, since a missense mutation (G171V) was found in these patients (27). LRP5 is a co-receptor for

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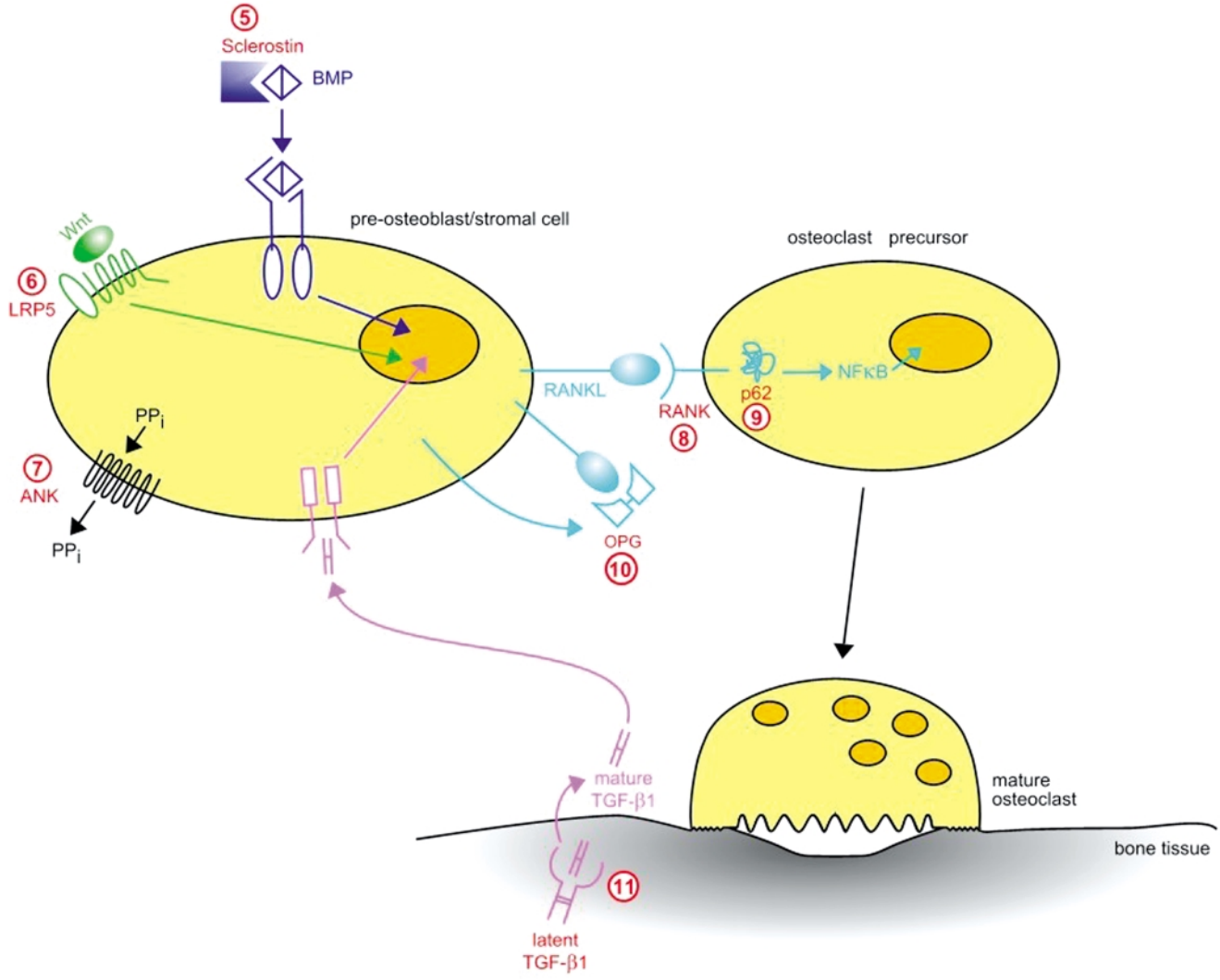

Figure 3. Schematic representation of the coupling process between pre-osteoblast/stromal cell and osteoclast precursor. Proteins involved in disorders with increased bone formation, [5]–[7], or imbalance between bone resorption and formation, [8]–[11] are marked in red. Sclerostin [5] is a BMP antagonist, inhibiting the BMP signaling pathway that promotes osteoblast differentiation, matrix production and mineralization. LRP5 [6] induces signaling of the Wnt pathway, which plays an important role in bone formation. ANK [7], a transmembrane protein expressed on the osteoblast, shuttles PP_i, an inhibitor of calcification, bone mineralization and bone resorption, across the cell membrane. RANK [8], a transmembrane receptor on the osteoclast membrane, induces, after interaction with RANKL, the NFκB signaling pathway, thus stimulating differentiation and activation of osteoclasts. p62 [9] is one of the downstream effectors of this RANK pathway. OPG [10], secreted by the osteoblast, is a decoy receptor of RANKL, thus inhibiting RANK/RANKL interaction. Finally, TGF-β1 [11] decreases RANKL and increases OPG expression, thereby modulating the OPG/RANK/RANKL system detrimentally to osteoclast differentiation.

Wnt proteins, which are secreted factors with many important functions in early developmental processes (Fig. 3). Genetic studies in OPS and HBM as well as biological evidence from the *Lrp5* knockout mouse indicate that Lrp5-induced Wnt signaling plays an important role in osteoblastic bone formation postnatally (28), and consequently significantly influences the peak bone density obtained in adult life (26). Experimental data indicate that the increased bone formation rate in HBM individuals is due to the inability of the mutated LRP5 to bind its antagonist Dickkopf (29). Dickkopf plays a regulatory role in this pathway, since binding to LRP5 inhibits the interaction of LRP5 with the Wnt–receptor complex and the induction of the Wnt signaling pathway (30).

Craniometaphyseal dysplasia: abnormal osteoblastic shuttling of inorganic pyrophosphate (PP_i)

The *ANK* gene, the human ortholog of the mouse progressive ankylosis gene, codes for a multipass transmembrane protein that shuttles inorganic pyrophosphate (PP_i) across the cell membrane (31) (Fig. 3). PP_i is a major inhibitor of calcification, bone mineralization and bone resorption (32). In 2001, two groups identified missense mutations in *ANK* (33,34) in patients with the autosomal dominant form of craniometaphyseal dysplasia (CMD; MIM 123000). This condition is characterized by hyperostosis and sclerosis of the skull associated with metaphyseal flaring of the long bones (35). How the missense

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mutations cause an increased bone formation rate in these patients is still controversial. A dominant-negative effect could impair the functioning of ANK, thus generating a decreased extracellular level of PP_i and an increased bone mineralization (34). Nürnberg *et al.* (33), on the other hand, postulated a gain-of-function effect of the mutations, making the channel 'leaky' and resulting in an increase in extracellular PP_i levels. Under this assumption, it cannot be excluded that the CMD phenotype is partially explained by the antiresorptive capacity of PP_i. Further *in vivo* and *in vitro* studies of the mutated ANK are needed to explain the increased bone density in this condition.

DISTURBED BALANCE BETWEEN BONE FORMATION AND RESORPTION

Bone remodelling and the balance between bone formation and resorption are regulated by both systemic hormones and local factors. An extended list of factors has been suggested to have an influence on bone formation, bone resorption or both. Furthermore, the existence of a coupling mechanism between osteoblasts and osteoclasts that plays a major role in keeping the balance between both processes has long been postulated. The unravelling of the OPG/RANK/RANKL system (see below) provided the long-sought answer to the question of how crosstalk between preosteoblastic/stromal cells and osteoclast precursors is established. Several cytokines and hormones, including IL-1, TNF-α, TGF-β, PTH and vitaminD_3, can modulate this system.

Familial expansile osteopetrosis, expansile skeletal hyperphosphatasia, Paget's disease of bone and juvenile Paget's disease: increased NFκB signaling

The OPG/RANK/RANKL molecules play a central role in the regulation of bone turnover (Fig. 3). RANKL, the receptor activator of nuclear factor κB (RANK) ligand, is a member of the tumor necrosis factor (TNF) ligand family expressed on the surface of the preosteoblastic/stromal cell and binds to RANK on the osteoclast precursor (36,37). This activates the NFκB signaling pathway, inducing the differentiation, maturation and activation of osteoclast precursors (38,39). Osteoprotegerin (OPG), a soluble member of the TNF receptor family secreted by the preosteoblastic/stromal cell, can block the RANK/RANKL interaction by acting as a decoy receptor of RANKL (40,41). During osteoblast differentiation, RANKL is downregulated and OPG is upregulated, so that osteoclast development is no longer stimulated (42).

Recently, several related clinical conditions have been associated with mutations in the OPG/RANK/RANKL pathway. The clinical similarities are explained by the shared pathogenic mechanism of increased NFκB signaling causing increased bone resorption, consequently compensated by increased bone formation.

Familial expansile osteolysis (FEO; MIM 174810) is a rare autosomal dominant condition characterized by focal skeletal lesions (43). After the early stage of increased resorption, both osteoclast and osteoblast activity are increased leading to expansion of the bone, deformities and an increased fracture rate. Hughes *et al.* (44) characterized a disease-causing tandem duplication in the signal peptide of the *TNFRSF11A* gene encoding RANK. *In vitro* assays showed that as a result of defective signal peptide cleavage, RANK accumulates intracellularly, leading to receptor self-association and thus increased constitutive RANK signaling. Recently, Whyte and Hughes (45) were able to prove that a similar duplication underlies expansile skeletal hyperphosphatasia, which is distinguishable from FEO despite strong similarities. A duplication in the RANK signal peptide was also found in a Japanese family diagnosed with Paget's disease of bone (PDB; MIM 602080) (44). This condition is a rather common disease characterized by focal areas of increased bone resorption combined with increased but disorganized bone formation. With the exception of this somewhat atypical family, no cases where shown to be caused by mutations in the *RANK* gene (46). Recently however, Laurin *et al.* (47) described the occurrence of a missense mutation (P392L) in the gene coding for p62 (sequestosome 1), causing 16% of sporadic and 46% of familial cases of PDB. Several divergent functions have been assigned to the p62 protein; one of these is its role as a downstream effector of the RANK/RANKL regulatory system, leading to NFκB activation (48). The gain-of-function mutation thus leads to increased bone resorption compensated by increased formation.

The inhibiting effect of OPG on the RANK/RANKL system suggests that loss-of-function mutations in the *TNFRSF11B* gene encoding OPG could also resort in an activating effect on NFκB signaling. In line with this assumption, Cundy *et al.* (49) found a deletion of one amino acid in OPG in a family with Juvenile Paget's disease (MIM 239000). This condition was initially considered to be an allelic variant of PDB (50), but currently it is well established that it is a separate disorder with an earlier onset and worse outcome. By *in vitro* studies, they showed that the mutant OPG could no longer suppress bone resorption.

In conclusion, activating mutations in *RANK* and *p62* and inactivating mutations in *OPG* were shown to increase NFκB signaling, giving rise to different but pathologically related conditions.

Camurati–Engelmann disease: increased TGF-β1 signaling

Transforming growth factor (TGF)-β1 is abundant in skeletal tissue, where it is stored in the bone matrix (51). Although data are sometimes conflicting, depending on the culture system and conditions used, it is generally assumed that TGF-β1 is a coupling factor between bone resorption and formation, acting through the following sequence of events. Resorbing osteoclasts can activate TGF-β1 in the low-pH environment of their ruffled border (52) (Fig. 3). Active TGF-β1 then decreases RANKL and increases OPG expression in osteoblasts (53,54). Consequently, fewer RANKL/RANK interactions occur, leading to an inhibition of osteoclast differentiation and activation. On the other hand, TGF-β1 stimulates osteoblast chemotaxis, proliferation and differentiation, thus promoting new bone formation (55,56).

In 2000, mutations in *TGFB1* were found to cause Camurati–Engelmann disease (MIM 131300) (57,58). This is a rare, autosomal dominant craniotubular hyperostosis with bilateral symmetrical affection of the diaphyses of the long bones on

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both the periosteal and endosteal sides. The majority of the mutations are located near the disulfide bridges between the latency-associated peptides (LAPs). It is postulated that the mutations weaken these bonds, thereby promoting premature activation of TGF-β1, leading to an imbalance between bone resorption and formation (59).

CONCLUSIONS AND FUTURE PROSPECTS

Genetic factors play an important role in determining bone mineral density (BMD) by influencing both peak bone mass and age-related bone loss. Low BMD is a major risk factor for the development of osteoporosis. Considering its high prevalence, it is not surprising that so much effort has been expended in the development of treatments. Because of the multifactorial character of osteoporosis, the identification of the genes responsible has been shown to be a tedious task. Several approaches are currently being used, including linkage studies in humans and animals and candidate gene studies. Genome-wide searches have identified loci on different chromosomal regions, but so far, the underlying genetic defects remain to be elucidated. Only in some rare cases where osteoporosis is inherited as a Mendelian trait, such as OPS and osteogenesis imperfecta, could the responsible gene be identified. Candidate gene studies have mainly focused on regulators of bone metabolism, such as cytokines and growth factors, bone matrix proteins and calciotropic hormones. Associations have been found with polymorphisms in *COL1A1* (60), *CTR* (61,62), *VDR* (63), *TGFB1* (64,65), *ESR* (66,67), *PTH* (68), *IL6* (69,70) and other genes, but in most cases confirmation of results in other populations has been problematic.

In this review, we illustrate that the approach of identifying genes relevant to bone metabolism and homeostasis based on the study of monogenic skeletal dysplasias has been successful in recent years, leading to the identification of previously unknown genes and the illustration of the involvement of already-known genes and pathways in bone metabolism (Table 1). Association studies using natural variants of these genes are on their way, and will give an indication of the influence of these proteins on bone density in the general population.

These new discoveries can pave the way to the development of novel therapeutic tools for osteoporosis. Currently, the most frequently used drugs are the antiresorptive agents, comprising estrogens, estrogen-receptor modulators, bisphosphonates, calcitonin, calcitriol, calcium and vitamin D. With the recent characterization of several proteins that play a role in the resorption process, more directed research can be performed to influence the activity of these proteins. For example, major efforts are ongoing to develop potent and selective inhibitors of CTSK (71–73). Despite the accomplished decrease in fracture risk, antiresorptives are unable to reverse the microarchitectural damage in advanced disease. In the future, it is therefore not inconceivable that a combination of an antiresorptive and a bone-forming agent will be used. During the last few years, much attention is drawn to anabolic agents, such as parathyroid hormone (PTH), fluoride, growth hormone (GH), insulin-like growth factor (IGF)-I and statins, which have the great advantage of stimulating bone formation dramatically. With the identification of *SOST* and *ANK* and the assignation of a major role to LRP5 and the Wnt signaling pathway in bone accrual, new potential drug targets are available. Finally, the members of the NFκB signaling pathway, including OPG, RANK, RANKL and p62, are interesting targets as well, since they interact in the crosstalk between osteoblasts and osteoclasts. Preliminary experiments with recombinant OPG have shown that this protein might indeed be used as a therapeutic agent, decreasing the bone turnover rate (74).

In conclusion, the newly identified players in bone metabolism are without doubt of major relevance to further understanding of and potential influences on bone homeostasis.

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