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REVIEW

The role of midkine in skeletal remodelling

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Bone tissue is subjected to continuous remodelling, replacing old or damaged bone throughout life. In bone remodelling, the coordinated activities of bone-forming osteoblasts and bone-resorbing osteoclasts ensure the maintenance of bone mass and strength. In early life, the balance of these cellular activities is tightly regulated by various factors, including systemic hormones, the mechanical environment and locally released growth factors. Age-related changes in the activity of these factors in bone remodelling can result in diseases with low bone mass, such as osteoporosis. Osteoporosis is a systemic and age-related skeletal disease characterized by low bone mass and structural degeneration of bone tissue, predisposing the patient to an increased fracture risk. The growth factor midkine (Mdk) plays a key role in bone remodelling and it is expressed during bone formation and fracture repair. Using a mouse deficient in Mdk, our group have identified this protein as a negative regulator of bone formation and mechanically induced bone remodelling. Thus, specific Mdk antagonists might represent a therapeutic option for diseases characterized by low bone mass, such as osteoporosis.

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Abbreviations
Ank, progressive ankylosis; BMD, bone mineral density; DKK, dickkopf; ECM, extracellular matrix; Enpp1, ectonucleotide pyrophosphatase/phosphodiesterase 1; LRP, low-density lipoprotein receptor-related protein; M-CSF, macrophage colony-stimulating factor; Mdk, midkine; OPG, osteoprotegerin; PPi, inorganic pyrophosphate; Ptn, pleiotrophin; PTH, parathyroid hormone; RANKL, receptor activator of NF-κB ligand; Rptpz, receptor-type protein tyrosine phosphatase zeta; SOST, sclerostin; TNAP, tissue non-specific alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase

Introduction

Bone is a dynamic, metabolically active organ that is subjected to a continuous remodelling process in vertebrates throughout life (Harada and Rodan, 2003; Karsenty, 2003; Pogoda *et al.*, 2005a; Hadjidakis and Androulakis, 2006; Zaidi, 2007; Zuo *et al.*, 2012). Remodelling is necessary because bone needs to adapt to its changing mechanical environment and because old and damaged bone has to be replaced to ensure structural stability. Remodelling is essential for maintaining skeletal functions, including provision of mechanical support and muscle attachment sites as a prerequisite to mobility, protection of vital organs such as the brain and bone marrow, and providing storage for calcium and phosphate required for metabolism. Two important compartments are involved in preservation of these functions: cortical bone for protection and mechanical strength, and trabecular bone for strength and as the major site for metabolic functions. Because turnover rates are much higher in trabecular bone than in cortical bone, remodelling-related metabolic bone diseases, such as osteoporosis, represent themselves predominantly in trabecular bone.

Osteoporosis is associated with an increased fracture risk, particularly for fragility fractures of the vertebrae and the hip that can result in chronic pain, disability and post-fracture mortality (Delmas, 2002; Pietschmann *et al.*, 2009). Because

most of the available drugs for osteoporosis only have a moderate ability to increase bone density and to lower fracture risk, and as their application is associated with toxic side effects, there is great need for new therapeutic strategies in the prevention and treatment of osteoporosis (Marie and Kassem, 2011; Lim and Clarke, 2012).

Bone remodelling

In bone remodelling, bone-forming osteoblasts and bone-resorbing osteoclasts are the key cells whose well-coordinated activities are necessary for maintaining bone mass and strength. Active osteoblasts form new unmineralized bone matrix (osteoid) that is mainly composed of collagen type I and further includes many matricellular proteins, supporting the bone matrix and serving as biological modulators (Alford and Hankenson, 2006; Zaidi, 2007; Edwards and Mundy, 2011; Karsenty and Oury, 2012; Zuo *et al.*, 2012). During early life, the balance between bone formation and bone resorption is strictly regulated, locally by growth factors, such as members of the TGF-β superfamily, and cytokines such as IL-1, IL-6 and TNF-α, deposited in the extracellular matrix (ECM) and released during resorption, as well as systemically by hormones and vitamins, including parathyroid hormone (PTH), oestrogen and vitamin D respectively. In addition, the CNS has been demonstrated to participate in the regulation of bone remodelling, which was revealed for the first time with the adipocyte-derived hormone leptin (Amling *et al.*, 2000; Ducy *et al.*, 2000; Pogoda *et al.*, 2006; Karsenty and Oury, 2010, 2012). All of these factors have marked effects on both osteoblast and osteoclast lineage activities, including replication of undifferentiated cells, recruitment of cells and function of differentiated cells. During adult life, a disturbed cellular function can lead to an excessive resorption and bone loss, as seen in Paget's bone disease, tumour osteolysis and osteoporosis.

The local regulation of bone remodelling occurs through cell-to-cell communication in temporary anatomical structures known as basic multicellular units, which particularly comprise osteocytes, osteoblasts and osteoclasts (Eriksen, 2010; Nakahama, 2010; Raggatt and Partridge, 2010; Trouvin and Goeb, 2010). Terminally differentiated osteoblasts, the osteocytes, are embedded in the mineralized bone matrix. They form a network through canaliculi, ensuring communication with the osteoblasts (bone-lining cells) located on the bone surface. Bone damage is sensed by osteocytes, initiating differentiation of osteoclast precursors through the monocyte/macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL) whose expression is increased in osteoblast/stromal cells. Osteoprotegerin (OPG), similiar to RANK, the receptor of RANKL, is a receptor of the TNF family and acts as a decoy receptor for RANKL, thereby inhibiting osteoclast differentiation and activity. The balance of RANKL/OPG is regulated by numerous osteotropic factors, including oestrogen, PTH, IL-1, IL-17 and TNF-α.

Because OPG is a target molecule of wingless-int (WNT) signalling in osteoblasts, this pathway appears to have a crucial role in maintaining bone homeostasis (Glass and Karsenty, 2006; Kramer *et al.*, 2010; Regard *et al.*, 2012; Baron and Kneissel, 2013). The discovery that loss-of-function and gain-of-function mutations in the gene encoding the WNT co-receptor low-density lipoprotein receptor-related protein-5 (LRP5) caused osteoporosis–pseudoglioma syndrome and a high bone mass phenotype, respectively, implicated the WNT/β-catenin signalling pathway as a key regulating pathway in bone remodelling (Gong *et al.*, 2001; Zhang *et al.*, 2004). Mouse models with the corresponding mutations reproduced the human bone phenotypes (Kato *et al.*, 2002; Babij *et al.*, 2003) and demonstrated the important role of gene-targeted mouse models as an experimental tool in understanding skeletal physiology. Small animal models, such as the mouse (Gunther and Schinke, 2000; Schilling *et al.*, 2001; Priemel *et al.*, 2002; Pogoda *et al.*, 2005b; Johnson *et al.*, 2009) as well as large animal models, particularly sheep (Claes *et al.*, 2012; Oheim *et al.*, 2012; 2013), are indispensable techniques for understanding the pathophysiology of skeletal disorders. Using various transgenic models, the powerful influence of WNT signalling on bone homeostasis has been demonstrated (Rawadi, 2008; Schulze *et al.*, 2010; Wagner *et al.*, 2011). WNT/β-catenin-pathway-associated proteins, such as secreted WNT antagonists of the dickkopf family (DKK1 and DKK2), secreted frizzled-related proteins, WNT inhibitory factors and sclerostin (SOST), impair osteoblast function and reduce bone formation (Westendorf *et al.*, 2004; Hoeppner *et al.*, 2009). Thus, therapeutic approaches to increase bone mass by targeting antagonists of WNT signalling in patients suffering from diseases with low bone mass, such as osteoporosis, have already been tested (Ke *et al.*, 2012; Monroe *et al.*, 2012; Baron and Kneissel, 2013).

Bone remodelling is also regulated by mechanical load, stimulating bone formation and suppressing bone resorption by paracrine and endocrine signalling (Liedert *et al.*, 2006; Robling *et al.*, 2006; Rubin *et al.*, 2006; Ozcivici *et al.*, 2010; Price *et al.*, 2011; Robling, 2012). Mechanical stimuli are sensed mainly by osteocytes, which express mechanically sensitive key membrane receptors, such as the integrins, connecting cells with the ECM, adherence junctions, interlinking the cytoskeleton between neighbouring cells and stretch-activated cation (Ca^{2+}) channels. The mechanically induced response in bone cells is dependent on the activity and interaction of numerous molecular local and systemic factors. Growth factor-induced signalling, including WNT and insulin-like growth factor signalling that is influenced by systemic hormones, for example, oestrogen, PTH and leptin, regulates cell proliferation and differentiation as well as cell fate, thereby maintaining bone mass.

Osteoporosis

Osteoporosis is the most common metabolic bone disorder worldwide (Pietschmann *et al.*, 2009; Li *et al.*, 2010; Feng and McDonald, 2011). It is a systemic and age-related disease characterized by low bone mineral density (BMD) and structural degeneration of the bone tissue, predisposing to high fracture risk. Osteoporosis results from an imbalance of bone remodelling, consequently uncoupling bone formation from bone resorption. An increase in the rate of bone resorption accompanied by an insufficient increase of bone formation

leads to a high-turnover osteoporosis, whereas a diminished bone formation lagging behind resorption causes a low-turnover osteoporosis. Osteoporosis-associated high-fragility fractures occur mainly at the hip, wrist and the spine.

According to the World Health Organization (WHO), a femoral neck BMD that is 2.5 standard deviations below the young female adult mean (T-score) is the diagnostic marker of osteoporosis (NIH Consensus Development Panel, 2001; Feng and McDonald, 2011). Growth of bone mass during childhood and early adolescence determines the extent of later bone loss and development of osteoporosis. Thus, a suboptimal bone mass (no peak bone mass) may result in osteoporosis, which must not be associated with accelerated bone loss. There are two types of osteoporosis that are traditionally distinguished: (i) primary osteoporosis, which can occur in both sexes and at all ages, but often arises after menopause by oestrogen deficiency in women (type I osteoporosis) or is associated with age in women and men (type II osteoporosis, also termed as age-related or senile osteoporosis), and (ii) secondary osteoporosis, which results from prolonged therapeutic interventions (e.g. glucocorticoids), certain medical disorders (e.g. hypogonadism, coeliac disease) or changes in physical activity.

BMD is a complex quantity that is influenced by physical activity, environmental factors, adequate nutrition, as well as genetic factors. A large number of genes have been demonstrated to be associated with BMD and osteoporosis (Richards *et al.*, 2009; Rivadeneira *et al.*, 2009; Li *et al.*, 2010). Clusters of these genes have been shown to belong mainly to the vitamin D endocrine pathway, the oestrogen endocrine pathway, the WNT pathway and the RANKL/RANK/OPG pathway. Thus, it is not surprising that these pathways have been found to be interesting targets for the prevention and treatment of osteoporosis (Canalis, 2010; Baron and Hesse, 2012). Because 25-hydroxyvitamin D increases BMD through its effects to promote calcium and phosphate absorption, it is used in the first stage of osteoporosis prevention and treatment (von Domarus *et al.*, 2011; Yoshida and Stern, 2012). Pharmacological agents with anti-resorptive effects, including aminobisphosphonates, oestrogen and selective oestrogen receptor modulators, are the most commonly used drugs for the treatment of osteoporosis (Boyce *et al.*, 2012). Newer anti-resorptive agents act by neutralizing RANKL or inhibiting the digestive enzyme cathepsin K in osteoclasts (Canalis, 2010).

In contrast, only a limited number of anabolic agents, which target osteoblastic cells to increase bone formation and strength, are currently available (Marie and Kassem, 2011; Lim and Clarke, 2012). In fact, PTH analogues (PTH 1–34, also known as teriparatide and PTH 1–84) are currently the only approved anabolic drugs for the treatment of osteoporosis. If administered intermittently, they increase BMD effectively in humans by enhancing osteoblast proliferation and function. PTH directly activates survival signalling in osteoblasts and delays osteoblast apoptosis, at least in mice (Jilka, 2007). Because WNT signalling is one of the main mechanisms regulating bone formation and bone mass, activation of this pathway is a promising strategy in the treatment of osteoporosis (Marie and Kassem, 2011; Lim and Clarke, 2012; Kim *et al.*, 2013). Animal studies with monoclonal antibodies to neutralize the antagonists SOST and DKK1 have demonstrated enhanced bone formation, bone strength and BMD after application (Ke *et al.*, 2012). Clinical trial studies are currently underway, examining the safety and efficacy of anti-SOST and anti-DKK1 antibodies, for example, in postmenopausal women (Kim *et al.*, 2013). However, unfocused overactivation of WNT signalling, specifically promoting the renewal and proliferation of stem cells, is associated with elevated cancer risk in non-skeletal tissue. Indeed, activating mutations of WNT signalling mediator genes are associated with several types of cancer, including colorectal cancer and leukaemia. Possible solutions to reduce tumourigenic risk in the future might include activation of WNT signalling, using a calibrated dose of antagonist of this pathway for a limited duration and specifically in skeletal tissue.

Midkine (Mdk) and pleiotrophin (Ptn)

The growth factors Mdk and Ptn are the two members of a unique family of heparin-binding growth and differentiation factors, and their physiological functions have been elucidated by various *in vitro* studies, but also *in vivo* through the generation and phenotypic analysis of mouse models with genetic deletion of the factors (Nakamura *et al.*, 1998; Amet *et al.*, 2001). Neither Mdk- nor Ptn-deficient mice displayed severe anatomical abnormalities, but did show alterations in the post-natal development of the hippocampus, as well as in their working memory and behaviour. Mice deficient in both Mdk and Ptn exhibited very severe auditory deficits, and female mice were characterized by infertility (Muramatsu *et al.*, 2006; Zou *et al.*, 2006). In previous studies, Ptn- and Mdk-deficient mice have been used to clarify the role of the two proteins in the skeleton. Thus, Ptn-deficient mice displayed low bone formation and osteopenia as well as resistance to disuse-induced bone loss (Imai *et al.*, 2009). These findings were compatible with the phenotype of transgenic mice overexpressing Ptn displaying enhanced bone formation (Masuda *et al.*, 1997; Tare *et al.*, 2002; Imai *et al.*, 2009). In contrast, our own skeletal analysis of Ptn-deficient mice did not reveal any changes in skeletal development and bone remodelling, presumably explained by the use of a different genetic background (Lehmann *et al.*, 2004; Neunaber *et al.*, 2010). We did however observe a remarkable high bone mass phenotype in Mdk-deficient mice, suggesting a physiological role in regulating bone formation (Neunaber *et al.*, 2010). These data were not only consistent with earlier findings from various investigators, but also provided the basis for additional experiments regarding the role of Mdk in the skeleton, all of which are discussed below.

Midkine in skeletal development and regeneration

In the 1990s, studies from Kadomatsu and Muramatsu provided the first evidence for a remarkable function of Mdk in skeletal development (Kadomatsu *et al.*, 1990; Mitsiadis *et al.*, 1995). Expression analysis in the tooth germ of mouse embryos demonstrated Mdk transcripts in the mesenchyme of the developing bone, and tooth morphogenesis and differentiation was inhibited in the presence of a neutralizing

anti-Mdk antibody. Likewise, studies with *Xenopus laevis* demonstrated restricted expression of the homologue to Mdk (XMK, X-PTF-α) in adult organs, including spinal cord and bone respectively (Sekiguchi *et al.*, 1995; Tsujimura *et al.*, 1995). Using a standardized closed murine diaphyseal tibia fracture model, Ohta *et al.* (1999) showed the expression of Mdk in spindle-shaped mesenchymal stem cells at the fracture site on day 4 and in chondrocytes in the area of enchondral ossification on day 7 after fracture. In addtion, Mdk overexpression in chondrogenic cells promoted their differentiation, as shown by enhanced synthesis of sulfated glycosaminoglycans, aggrecan and collagen type II. Mdk has also been detected in the inflammatory synovitis of rheumatoid arthritis (RA) and osteoarthritis (OA), two destructive and progressive inflammatory diseases of the joints (Takada *et al.*, 1997). Interestingly, Mdk-deficient mice with arthritogenic antibody-induced arthritis showed a decreased migration of inflammatory leukocytes (Maruyama *et al.*, 2004). Using an *in vitro* assay, it was shown that recombinant human MDK promoted the differentiation of osteoclasts, which play a key role in the aetiology of RA. The osteoclast-inducing activity of Mdk together with RANKL was as strong as that of RANKL in combination with M-CSF. Moreover, inhibition of Mdk expression by Mdk-specific siRNA or inhibition of Mdk activity by chondroitin sulfate E, which impeded the differentiation of osteoclasts, suppressed the development of antibody-induced arthritis (Yamamoto *et al.*, 2006).

Midkine in bone remodelling

Because there was experimental evidence from transgenic mouse models that Ptn, the second member of this heparin-binding growth factor family, is a physiologically relevant stimulator of bone formation by osteoblasts (Imai *et al.*, 2009), it came as a surprise that we observed a high bone mass phenotype in mice lacking Mdk (Figure 1). More specifically, while the trabecular bone mass was not signficantly different between wild-type and Mdk-deficient littermates at 4 months of age, there was a nearly twofold increase of trabecular bone mass in 12- or 18-month-old Mdk-deficient mice, thereby demonstrating that Mdk deficiency protects against ageing-induced bone loss. Histomorphometric analysis further revealed that the high bone mass phenotype of Mdk-deficient mice was fully explained by an increased bone formation rate, that is, by a higher osteoblast activity. A second pathology, albeit by far less pronounced, was related to cortical bone, where we observed a significantly increased porosity, only at 18 months of age. This phenotype was explained by an increased number of osteoclasts in the cortical compartment, yet interestingly, the number of osteoclasts per trabecular bone surface was significantly decreased compared to wild type littermates.

While the latter result was consistent with the observations that (i) Mdk administration to primary osteoblasts caused an induction of RANKL expression and (ii) that RANKL serum levels were reduced in 18 months old Mdk-deficient mice, it is still unclear why Mdk deficiency causes increased osteoclastogenesis in the cortical compartment. Because serum levels of the osteoclast marker, tartrate-resistant acid phosphatase (TRAP), were significantly increased compared with wild-type littermates at 12 and 18 months of age, it appears that the deduced negative influence of Mdk on osteoclastogenesis was outweighing the effect on RANKL expression, and that overall Mdk deficiency caused high bone turnover. That this situation is associated with high bone mass is fully explainable by the strong increase of bone formation in Mdk-deficient mice. Taken together, these results revealed that the major physiological function of Mdk is not related to skeletal development or growth, but to a regulation of bone remodelling, especially in older mice.

Based on these findings, we analysed whether the anabolic response of bone to mechanical loading is influenced by Mdk deficiency (Liedert *et al.*, 2011). Therefore, we used the *in vivo* ulna loading model that has been demonstrated to promote endosteal and periosteal cortical bone formation in mice (Lee *et al.*, 2002). In this model, an axial compression is applied to the ulna, which results in medial-to-lateral bending due to the natural curvature of the ulna. For histomorphometric analysis, mice were injected consecutively with the fluorochrome derivatives calcein green and alizarin red, which incorporate into bone matrix surfaces that

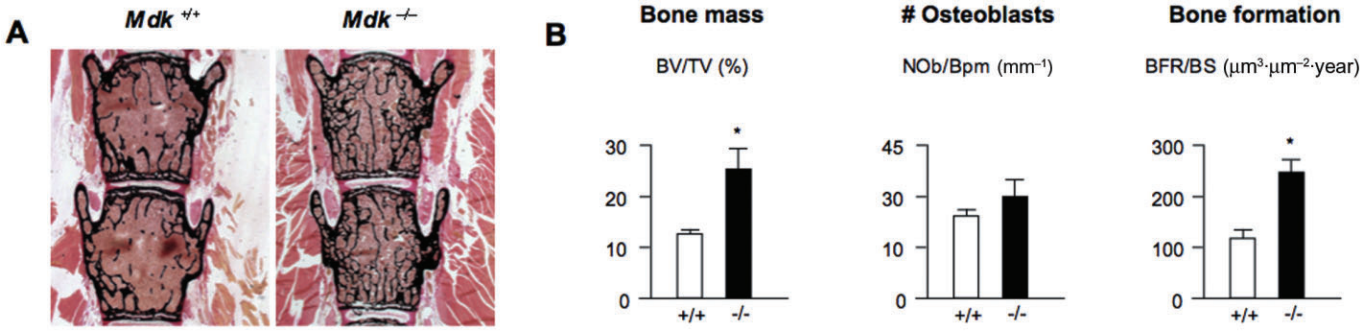

Figure 1

Increased bone formation in Mdk-deficient mice. (A) Undecalcified histology of spine sections from 1-year-old wild-type and Mdk-deficient mice (Mdk-/-) demonstrates an increased amount of trabecular bone (stained black) in the latter ones. (B) Histomorphometric quantification reveals that Mdk-deficient mice display significantly increased bone mass and bone formation rate with a normal number of osteoblasts.

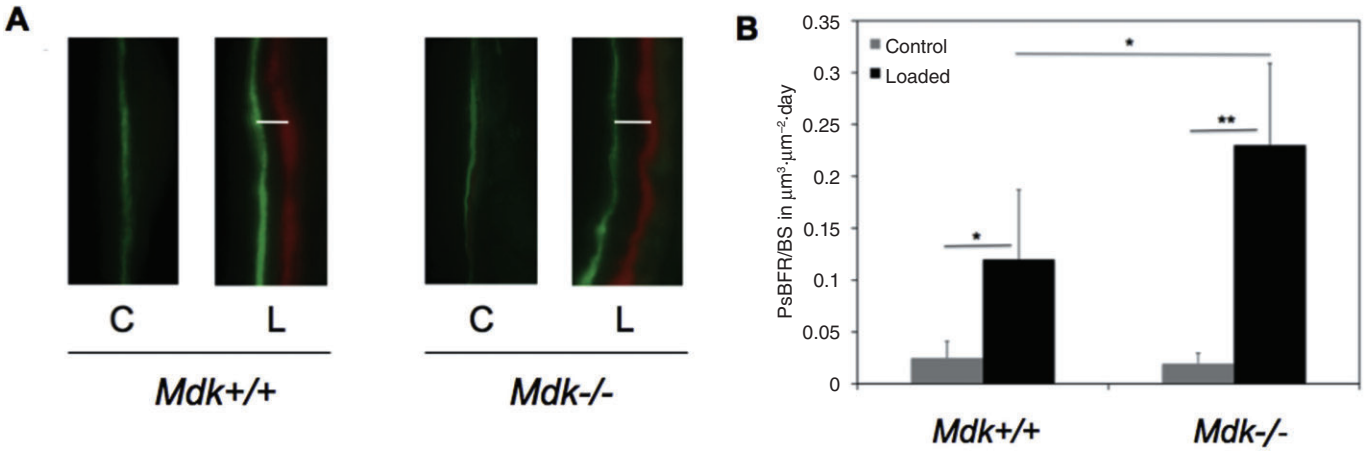

Figure 2

Increased bone formation in Mdk-deficient mice after mechanical loading. (A) Histomorphometric analysis using calcein green and alizarin red labelling in wild-type and Mdk-deficient mice (Mdk-/-) reveals increased bone formation in mid-diapphyseal cross sections at the periosteal surface of the ulnae of Mdk-deficient mice compared with control: ulnae without mechanical loading [C], and ulnae of wild-type mice (Mdk+/+) after mechanical loading [L]. (B) Increased periosteal bone formation rate per bone surface (PsBFR/BS) in ulnae of Mdk-deficient mice compared with wild-type mice after mechanical loading.

undergo mineralization and allow the determination of the onset time and location of ossification (van Gaalen *et al.*, 2010). The ulnae of wild-type mice and of Mdk-deficient mice without mechanical loading showed an almost single-labelled fluorescence line at the periosteal bone surface, whereas the ulnae of wild-type and Mdk-deficient mice after mechanical loading displayed a double fluorescence labelling at the periosteal surface (Figure 2A) (Liedert *et al.*, 2011). Interestingly, in Mdk-deficient mice, the distance between the two labellings was greater, which signified an increased bone formation rate in these mice compared with wild-type mice (Figure 2B). These results provide further evidence of a relevant role of Mdk as an inhibitor of osteoblast function, that is, bone formation (Figure 3).

Molecular effects of midkine on bone-forming cells

The Mdk receptor has been proposed to be a complex consisting of Rptpz (chondroitin sulfate proteoglycan receptor protein tyrosine phosphatase, type zeta), Lrp6, as well as α4β1-integrin and α6β1-integrin (Muramatsu *et al.*, 2004). A strong binding of Mdk requires over-sulfated structures in chondroitin sulfate and heparan sulfate in glycosaminoglycan chains (Zou *et al.*, 2003). Several *in vitro* studies have demonstrated that the migration of various cell types, including macrophages and osteoblastic cells, is stimulated by Mdk (Maeda *et al.*, 1999; Hayashi *et al.*, 2001; Qi *et al.*, 2001; Muramatsu, 2002), and this effect, at least in osteoblasts, has been shown to be mediated by Rptpz (Maeda *et al.*, 1999; Qi *et al.*, 2001; Muramatsu, 2010). In addition, increased tyrosine phosphorylation of paxillin seems to be involved in the migration response of osteoblastic cells to Mdk (Muramatsu *et al.*, 2004). Mdk has also been shown to interact with

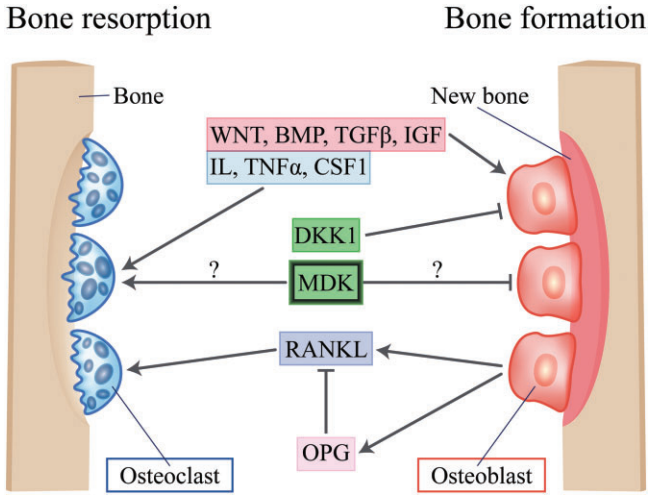

Figure 3

Supposed role for Mdk in bone remodelling.

Abbreviations: BMP, bone morphogenic protein; CSF1, macrophage colony-stimulating factor-1; DKK1, Dickkopf-1; IGF, insulin-like growth factor; MDK, midkine; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; TGFβ, transforming growth factor-β. Modified according to Logothetis and Lin (2005).

members of the syndecan receptor family, such as syndecan-1 and syndecan-3, and the latter one has been considered to have a role as an Mdk receptor in neurite outgrowth and neuronal cell migration (Nakanishi *et al.*, 1997).

In our own studies regarding the role of Mdk as an inhibitor of bone formation, we have not yet identified the relevant receptor molecule but, unexpectedly, we observed that aged Rptpz-deficient mice displayed decreased bone formation (Schinke *et al.*, 2008). We did however perform genome-wide

expression analysis of primary osteoblasts following short-term treatment with recombinant Mdk to identify immediate target genes, potentially explaining the effects of Mdk on bone formation. Here, we found that only a small number of genes were regulated by Mdk, and interestingly, these included two genes (*Enpp1* and *Ank*) responsible for raising the level of extracellular inorganic pyrophosphate (PPi). PPi is a physiologically relevant inhibitor of mineralization, and while the enzyme encoded by *Enpp1*, ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) is required for PPi generation, the transmembrane protein Ank (encoded by the gene *Ank* and mutated in progressive ankylosis) mediates PPi secretion into the extracellular space. Together with the PPi-degrading enzyme, tissue non-specific alkaline phosphatase (TNAP), these genes play a key role in the regulation of matrix mineralization (Harmey *et al.*, 2004; Orimo, 2010), and their inactivation in mice and humans causes ectopic calcification (Ho *et al.*, 2000; Terkeltaub, 2001; Rutsch *et al.*, 2003). In addition, heterozygous inactivating mutations in the human *ANKH* gene [homo sapiens ankylosis, progressive homologue (mouse)], the human orthologue of the murine Ank gene, cause craniometaphyseal dysplasia that is associated with hyperostosis and sclerosis of the craniofacial bone and abnormal modelling of the metaphysis of long bones (Nurnberg *et al.*, 2001). Likewise, the tiptoe-walking mouse (ttw/ttw), carrying a mutation in the *Enpp1* gene, is characterized by ossification of the spinal ligaments, as well as articular and arterial calcification (Okawa *et al.*, 1998). This suggests that a lower expression of Ank and/or Enpp1 in Mdk-deficient osteoblasts can explain, at least in part, the increased trabecular bone mass of Mdk-deficient mice, albeit there is still a need to confirm this hypothesis *in vivo*.

An alternative explanation for the mode of Mdk action in osteoblasts comes from our findings related to the increased anabolic response to loading in Mdk-deficient mice. Because it is known from previous studies that Rptpz and Lrp6, together with α4β1- and α6β1-integrins, are able to form a signalling receptor complex for Mdk (Muramatsu *et al.*, 2004), and because Rptpz is involved in the regulation of tyrosine dephosphorylation of β-catenin (Meng *et al.*, 2000), we investigated whether canonical WNT/β-catenin signalling could be regulated in a Mdk- and Rptpz-dependent manner. Therefore, we transfected osteoblastic cells with a WNT3a-inducible reporter plasmid and co-transfected the cells with expression plasmids for Rptpz, WNT3a and/or Mdk. We chose WNT3a, a member of the highly conserved WNT glycoprotein family, as it was known to stimulate osteoblastic differentiation in murine embryonic mesenchymal cells (An *et al.*, 2010). By these experiments, we were able to demonstrate that Mdk has a negative effect on WNT3a-induced transcription (Liedert *et al.*, 2011). Moreover, this negative effect was enhanced by the additional expression of Rptpz. We further demonstrated that Mdk is involved in increasing serine phosphorylation of β-catenin, representing an important step to inactivate WNT/β-catenin signalling. In conlusion, Mdk promoted a negative effect on WNT signalling and the extent of this negative effect was dependent on Rptpz expression. Using an *in vitro* model of mechanical loading with osteoblastic cells, we additionally demonstrated that Mdk repressed the expression of at least two WNT target proteins, c-Fos and c-Myc, involved in osteoblast proliferation (Onyia *et al.*, 1995). In fact, both proteins were down-regulated in the presence of Mdk and this effect was even more pronounced by mechanical load (Liedert *et al.*, 2011).

In any case, if Mdk affects gene expression in osteoblasts, it remains to be established which cell type is the relevant source of Mdk in this context. We have previously found that Mdk is differentially expressed in primary osteoblasts and that Mdk-deficient bone marrow cells display increased osteogenic potential *ex vivo*. Taken together, these data suggested that the increased bone formation phenotype of Mdk-deficient mice is related to a cell-autonomous impairment of inhibitory mechanisms. In order to test this hypothesis *in vivo*, we would need to inactivate Mdk in specific cell types (osteoblasts and others), using Cre-lox technology.

Therapeutic implications

Inhibitors of Mdk activity, such as antibodies, aptamers, glycosaminoglycans, peptides and low molecular weight compounds, are currently under development (Muramatsu, 2011). It has been shown that Mdk deficiency attenuated experimental autoimmune encephalomyelits, a mouse model of multiple sclerosis, and that an RNA aptamer targeted to Mdk effectively suppressed symptoms of MS in wild-type mice (Wang *et al.*, 2008). Moreover, because Mdk has been demonstrated to play a fundamental role in the pathogenesis of other inflammatory diseases, such as atherosclerosis and diabetic nephropathy (Weckbach *et al.*, 2011), an inhibition of Mdk might be a therapeutic option in these patients as well.

With respect to bone remodelling, it is reasonable to suggest that Mdk antagonists might be useful as stimulators of osteoblast activity in individuals with bone loss disorders. To provide a proof-of-principle here, we have already addressed the question of whether Mdk deficiency would protect against ovariectomy-induced bone loss (Neunaber *et al.*, 2010). More specifically, bilateral ovariectomy was performed in 3-month-old wild-type and Mdk-deficient littermates and skeletal analysis was perfomed at 6 months of age. Here, we found, as expected, that the trabecular bone volume of wild-type mice was significantly reduced by the surgical procedure, whereas there was no significant difference between sham-operated and ovariectomized Mdk-deficient mice. This demonstrated that Mdk deficiency, at least in mice, protects not only against ageing-associated bone loss but also against ovariectomy-induced bone loss. It is therefore conceivable to speculate that Mdk-specific antagonists, such as anti-Mdk antibodies, might be new anabolic drugs for the treatment of osteoporosis and other low bone mass diseases in the future.

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Conflict of interest

None of the authors have any conflicts of interest.

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