

Chapter 34

TWEAK and TNF Regulation of Sclerostin: A Novel Pathway for the Regulation of Bone Remodelling

David M. Findlay and Gerald J. Atkins

Introduction

Bone remodelling is the critical process, by which skeletal mass and integrity are controlled. This process is disrupted in a number of bone pathologies, such as osteoporosis, and the focal bone loss that occurs in rheumatoid arthritis, around artificial joint prostheses or that induced by osteolytic tumours. A common feature of conditions that result in a net loss of bone is the presence of elevated levels of pro-inflammatory mediators and evidence has accumulated to show that a chronic inflammatory environment is both catabolic for bone and may also suppress bone formation. Here, we focus on the possible anti-anabolic roles in bone of TNF α and TNF-like weak inducer of apoptosis (TWEAK).

TWEAK

TWEAK, a member of the TNF ligand superfamily designated TNFSF12 [1], has pleiotropic effects, which include induction of pro-inflammatory mediators in a number of target cell types, including fibroblasts and synoviocytes obtained from RA and advanced OA patient tissues [2]. The receptor for TWEAK, fibroblast growth factor-inducible gene-14 (Fn14/TNFRSF12/TWEAK R), is widely expressed [3] and is up-regulated in the context of tissue injury and inflammatory responses [4–7]. Ligation of TWEAK to Fn14 has been shown to activate a limited set of signalling pathways involved in cell proliferation and differentiation [8], including NF κ B activation, which is associated with its pro-inflammatory effects [3, 9]. TWEAK has also been shown to activate the mitogen-activated protein kinases (MAPK), JNK [10], ERK [10, 11] and p38 MAPK [5].

G.J. Atkins (✉)

Bone Cell Biology Group, Discipline of Orthopaedics and Trauma, The University of Adelaide, Adelaide 5000, SA, Australia
e-mail: gerald.atkins@adelaide.edu.au

A Role for TNF Family Members in Bone Remodeling

The TNF family members and their cognate receptors, RANKL/RANK/OPG and TNF/TNFR1, have central and well-established actions in bone remodelling [12]. Physiologically, RANKL expression by osteoblastic stromal cells mediates osteoclast formation by binding to RANK expressed by osteoclast precursors. Evidence suggests that RANKL and TNF are together involved in inflammatory bone remodelling [13–15]. Although the mechanisms remain to be fully elucidated, TNF can both enhance RANKL expression and sensitize preosteoclasts to RANKL [12]. TNF expression is markedly up-regulated in peri-prosthetic osteolysis [16], in association with polyethylene (PE) particles produced by wear of the prosthesis [17]. We [18] and others [19] have reported a significant role for TWEAK in the inflammatory bone remodelling seen in the mouse CIA model of rheumatoid arthritis. Serum TWEAK was elevated in CIA mice [18] and a neutralising TWEAK antibody significantly reduced the disease severity. Together, the data suggest that TWEAK may play roles in both joint inflammation and tissue damage in the context of RA. The extent to which TWEAK and TNF, also a known mediator of joint pathology in inflammation-driven bone remodelling, each contribute to the pathogenesis of CIA remains an open question. We have reported that human osteoblasts express Fn14 and that TWEAK exposure inhibited their expression of the key osteoblast gene, osteocalcin [18, 20], implying a role for TWEAK in osteogenesis. Ando and colleagues [11] reported that an initial effect of TWEAK on the mouse osteoblast cell line, MC3T3-E1, is to induce RANTES production and RANKL expression. The work described below suggests that TWEAK may contribute to physiologic human osteoblast function and/or their differentiation and so may be a key regulator of bone remodelling.

Human Osteoblasts and Osteoblast-Like Cell Lines Express Fn14 and TWEAK

We have found that all human primary osteoblast [20] and osteoblast-like cell lines (Fig. 34.1a–c) tested express high basal levels of cell surface Fn14. Human osteoblasts also express intracellular TWEAK, suggesting that TWEAK could have an autocrine role in osteoblast activity [20]. In addition, TWEAK mRNA levels may be regulated in an inflammatory milieu, since exposure of human osteoblasts to TNF and IL-1 β decreased the expression of TWEAK mRNA in this cell type (Fig. 34.1d).

TWEAK Inhibits In Vitro Mineralisation and Antagonizes the Osteogenic Effect of TNF

The ability to form a mineralised matrix over time is a key functional indicator of osteoblast activity and osteogenesis. We have shown that TWEAK inhibits in vitro mineralisation by human osteoblasts and that this inhibitory effect could be reversed by concomitant incubation with a TWEAK neutralizing antibody. Conversely, TNF

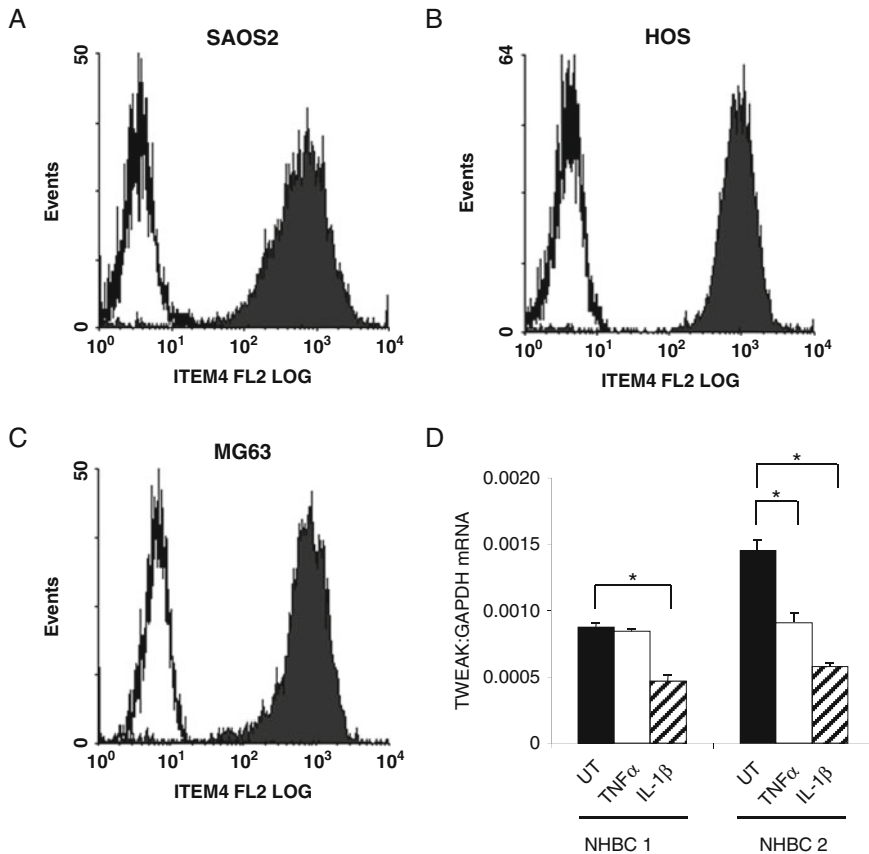


Fig. 34.1 Human osteoblast expression of Fn14 and TWEAK. All human osteoblastic-like cell lines examined expressed high levels of Fn14, including (a) SAOS2, (b) HOS and (c) MG63 cells. Immunofluorescence and flow cytometry were performed essentially as described previously [20]. (d) TWEAK expression in NHBC was regulated by a 48-h incubation with either recombinant human TNF α or IL-1 β (R&D Systems). Real-time RT-PCR was performed as described previously [20]. Oligonucleotide primers designed in-house to amplify human TWEAK were forward primer: 5'-ATCGCTGTCCGCCAGGAGC-3' and reverse primer 5'-CTGTCTGGGGATTTCAGTCCG-3', which amplify a 86-bp product. Data shown are means of triplicate reactions \pm SD, and are representative of two independent experiments. Asterisks denote significant difference compared to untreated (UT) ($p < 0.01$)

promoted in vitro mineralisation by human osteoblasts. Interestingly, TWEAK antagonized this effect in a dose-dependent fashion [20]. These results suggest that TWEAK is a negative regulator of osteoblast differentiation and osteogenesis.

Effect of TWEAK and TNF α on Osteoblast Proliferation

Osteoblast proliferation is another important determinant of the osteogenic effect and is inversely related to osteoblast maturation. We have described the use of

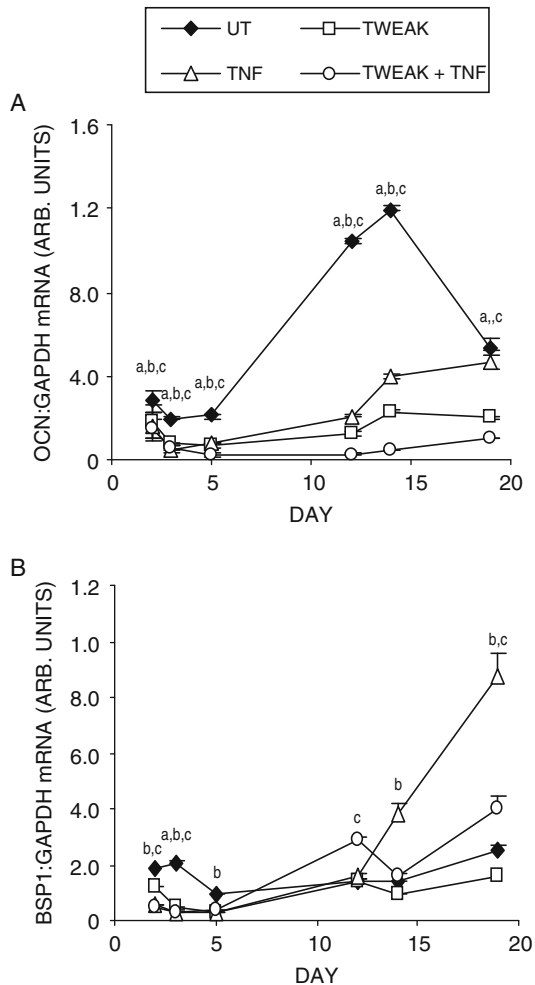
carboxyfluorescein diacetate succinimidyl ester (CFSE) to measure proliferation of human osteoblasts [21, 22]. CFSE-labelled human osteoblasts exhibited enhanced proliferation in response to TWEAK, showing reduced numbers of cells in the parental population and increased numbers of cells having three or more cell divisions. TNF α is also mitogenic for NHBC and this effect is enhanced in the presence of TWEAK, to the extent seen with TWEAK alone [20].

Effect of TWEAK and TNF on Osteoblast Osteogenic Gene Transcription

We have also investigated the effects of TNF and TWEAK on the expression of a number of osteoblast-associated genes under two sets of conditions, viz. basal conditions for 3 days and conditions permissive of mineralization (the latter containing supplemented phosphate levels) over 3 weeks. Under basal conditions, the osteoblast transcription factors, RUNX2 and osterix, together govern osteoblast lineage commitment and their subsequent differentiation [23]. We found that TWEAK significantly inhibits RUNX2 mRNA expression, in the co-presence or absence of TNF, although TNF alone had no discernible effect on RUNX2 levels [20]. TWEAK dose responsively increased osterix mRNA levels by up to ninefold, consistent with it holding osteoblasts at an immature stage of differentiation. In contrast, TNF alone had no apparent effect on osterix expression, the combined effect of TWEAK/TNF on osterix being similar to the effect of TWEAK alone [20]. These data implicate TWEAK as an important controller of osteoblast differentiation. The effect of TWEAK exposure on RUNX2 is consistent with the emerging negative role of RUNX2 in controlling osteoblast proliferation [24, 25]. Also consistent with the effect of TWEAK on osteoblast proliferation is the stimulatory effect on osterix expression, which has been shown itself to promote cell proliferation and decrease expression of osteoblast genes such as osteocalcin and alkaline phosphatase [26]. TWEAK dose-dependently down-regulated the expression of genes associated with osteoblast differentiation and mineralisation, including osteocalcin, BSP1 [20], alkaline phosphatase and osteopontin (Atkins, unpublished data). TNF had qualitatively similar effects on these genes, although quantitatively it varied from considerably more potent than TWEAK (e.g. on the osteocalcin gene) to approximately equipotent with respect to other genes (e.g. BSP1) [20].

Under conditions permissive of osteoblast differentiation and *in vitro* mineralization, we found that, while osteogenic genes such as OCN, BSP1 and OPN were more potently down-regulated by TNF in short-term dose-response studies compared with TWEAK, a different pattern of gene responsiveness emerged during a 3-week differentiation period. Early strong suppression of OCN transcription by TNF, for example, was followed by partial release from this suppression, consistent with our finding of an overall positive effect of TNF α on mineral apposition by

Fig. 34.2 Effect on osteogenic gene expression as a function of time of recombinant human (rh) TWEAK (50 ng/ml) [18], rhTNF α (1 ng/ml; R&D Systems, MN, USA) or a combination of both cytokines. Human osteoblasts were cultured for up to 3 weeks under mineralising conditions and analysed at the times indicated by real-time RT-PCR for (a) OCN and (b) BSP1 using a published protocol [20]. Significant differences to control (untreated) levels are indicated by *a* for TWEAK, *b* for TNF and *c* for the combination treatment ($p < 0.05$). Data shown are normalised to GAPDH mRNA levels and are means \pm SD of triplicate reactions. Similar results were obtained in two independent experiments



human osteoblasts (Fig. 34.2a). TWEAK, on the other hand, suppressed OCN transcription throughout the culture period (Fig. 34.2a), in fact antagonizing the TNF effect on OCN transcription. Similarly, both TWEAK and TNF suppressed BSP1 mRNA expression, a gene associated with mineral deposition, in the early phase of the mineralising cultures. However, expression was later induced by TNF to a level above that seen in control cultures, again consistent with an osteogenic effect of TNF, but this was not seen in cultures exposed to either TWEAK or TWEAK/TNF (Fig. 34.2b). Thus, while the short-term effects of TWEAK resemble those of TNF, these cytokines have divergent effects on osteoblast differentiation, with TWEAK exerting the more dominant inhibitory effect.

Interaction Between TWEAK and TNF

TNF ligand family members are capable of modulating each others' activities. For example, TNF synergizes with RANKL in the induction of osteoclast formation [16, 27]. Given that the microenvironment encountered by cells during physiologic or pathologic bone remodelling, for example in RA, consists of a complex cytokine milieu, such interactions are likely highly relevant and important. For example, we recently reported that TWEAK is a mediator of joint erosion in the mouse CIA model, a model in which TNF is also expressed and is implicated in the mechanism [18]. Our data indicate that TWEAK and TNF modulate each other's activities with respect to human osteoblast behaviour, and the functional outcome likely depends on the relative expression of each cytokine and their respective receptors. It is also likely that there are species-specific effects of TWEAK, as neither TWEAK nor TNF caused detectable phosphorylation of p38 MAPK, or activation of the Akt survival pathway, in human osteoblasts in contrast to effects of TWEAK in mouse MC3T3-E1 osteoblast-like cells [11].

The Wnt Signalling Pathway and Inhibitors: Sclerostin

In investigating the mechanisms for the inhibitory effects of TWEAK on osteoblast differentiation and function, it is important to consider the possibility of interaction with a central regulator of this process, the Wnt signalling pathways. The canonical Wnt-signalling pathway is the best described of these [28, 29]. In this pathway, Wnt ligands bind to frizzled (Fzd) and LRP5/6 co-receptors on target cells, preventing the proteosomal degradation of β -catenin and promoting the formation of transcription complexes with TCF/LEF transcription factors, resulting in the downstream transcription of osteogenesis-related genes. Several inhibitors of the Wnt pathway have been identified, including Dickkopf 1 (DKK1), secreted Frizzled-related protein (sFRP) and sclerostin [28]. It is noteworthy that impaired osteoblast function on bone surfaces adjacent to sites of inflammation, in a murine model of rheumatoid arthritis, was associated with up-regulated expression of a number of Wnt inhibitors, including DKK1 and sFRP1 [30]. Sclerostin is the product of the *SOST* gene, mutations in which cause conditions with high bone mass, and is a key negative regulator of bone formation [29, 31, 28]. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength [32]. A major activity of sclerostin appears to be the inhibition of Wnt/ β -catenin signalling [33].

TWEAK Alone and in Combination with TNF Induce Sclerostin Expression

We have reported that TWEAK can induce the expression, at both the mRNA and protein levels, of sclerostin in a dose-dependent fashion [20]. Sclerostin mRNA levels remained elevated relative to control conditions throughout the mineralisation

period, increasing further towards the end of the 3-week differentiation period. TNF by itself had a mild effect but combined with TWEAK to further increase sclerostin expression. The induction of sclerostin transcription by TWEAK and TWEAK/TNF was highly dependent on JNK and ERK1/2 phosphorylation [20]. Sclerostin expression in normal bone is virtually restricted to mature osteocytes embedded in mineralised bone, with some expression by osteoblasts [31]. Our unpublished observations concur with this, that sclerostin expression normally coincides with *in vitro* mineralisation by NHBC (Atkins et al., unpublished), and in some cases where mineralisation is increased, for example by the anabolic drug, strontium ranelate [34], sclerostin levels also increase both as a marker for osteocytes arising in the cultures and in an apparent bid to regulate the anabolic effect. However, the expression pattern of sclerostin *in vivo* has not been tested under inflammatory conditions. It is possible that less mature osteoblasts may be induced to express sclerostin under inflammatory conditions. Notably, while TWEAK induced proliferating cells to express sclerostin, as judged by their expression of the nuclear proliferation antigen Ki67 [20], there was an inverse relationship between intracellular sclerostin expression and the proliferative potential of primary osteoblasts following TWEAK treatment (Fig. 34.3). It is possible that by inducing sclerostin expression TWEAK may cause premature differentiation into a mature osteocyte-like phenotype, thereby bypassing the mineral apposition phase of osteoblast differentiation [35] and explaining the observed inhibitory effect on mineralisation [20]. This possibility is the subject of ongoing research.

Our preliminary data indicated that the exposure of human osteoblasts to recombinant human sclerostin has remarkably similar effects to treatment with TWEAK and TWEAK/TNF, with respect to the suppression of osteocalcin and RUNX2 expression [20]. We are currently exploring in greater detail the involvement of sclerostin in TWEAK-mediated effects on osteoblasts, using human cell line models. Notably, blockage of sclerostin activity is currently being explored as a novel treatment option for osteoporosis [36]. A recent report [37] identified TNF-mediated induction of DKK1 expression in a mouse model of inflammatory arthritis and in human RA.

Concluding Remarks

Taken together, these data provide a number of potential mechanisms for the anti-anabolic effects of an inflammatory environment in bone. The induction of expression of inhibitors of the Wnt and/or BMP signalling pathways is a novel potential mechanism, by which TWEAK alone, or in concert with TNF, might regulate physiologic osteoblast differentiation and mineralisation, and suppress these processes in chronic inflammatory disease states. In an inflammatory setting, TWEAK or TWEAK/TNF may act to regulate osteoblast proliferation, inhibit the osteogenic activity of osteoblasts and perhaps also promote the formation and activity of osteoclasts, as depicted in (Fig. 34.4). TNF has been shown to both directly and indirectly activate osteoclast activity [12, 27]. A single report has implicated

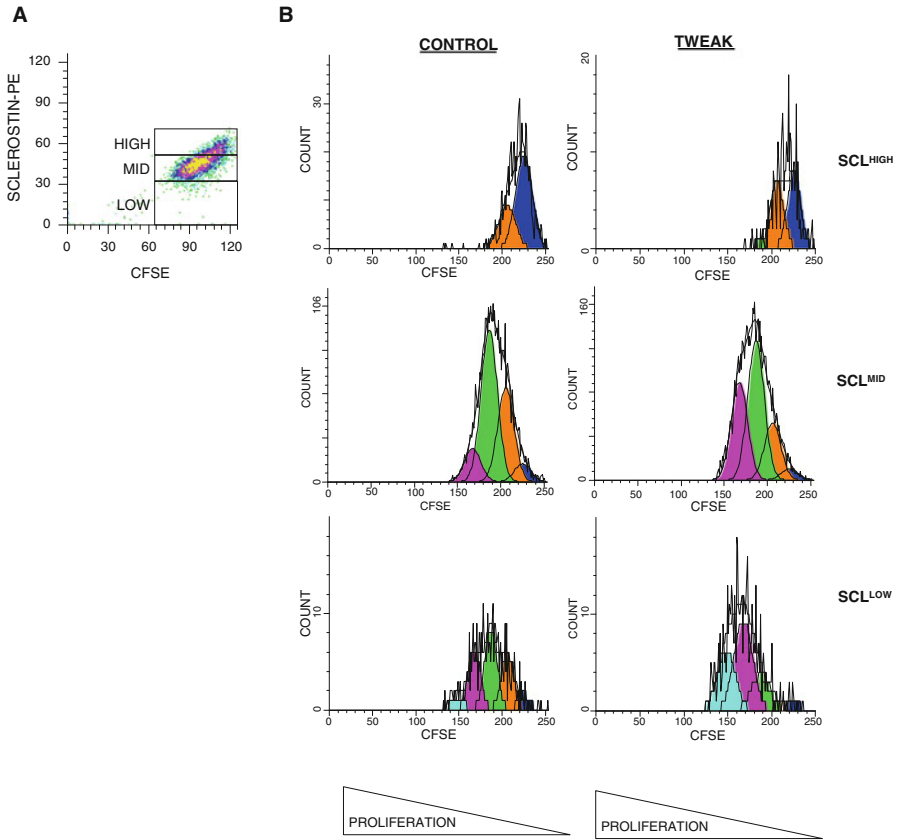


Fig. 34.3 Sclerostin (SCL) expression is inversely related to cell proliferation. NHBC were labelled with CFSE and cultured for 3 days in the presence or absence of recombinant human TWEAK (50 ng/ml), as described [20]. Cells were analysed by flow cytometry for intracellular expression of sclerostin by a published method [20], using a commercially available monoclonal antibody (MAb) against human sclerostin (R&D Systems) or an isotype-matched negative control MAb. Data were analysed using ModfitTM software (Verity, NJ, USA). (a) Cells were gated on low, mid and high levels of expression of sclerostin, the 'low' gate completely overlapping the level of staining generated by the negative control MAb. (b) Cell divisions were calculated as described [20], the proportion of cells having undergone successive divisions represented by coloured peaks, with the *dark blue* peak representing undivided cells and the *light blue* peak the cells that had undergone four divisions. Even though TWEAK induces sclerostin expression [20], cells expressing high levels (SCL^{HIGH}) proliferated the least while the cells expressing low levels of intracellular sclerostin (SCL^{LOW}) displayed the highest proliferative potential

TWEAK as a direct regulator of osteoclast activity in the absence of evidence for Fn14 expression [38]. This has yet to be confirmed by others, and evidence from our laboratories instead implies that the effect is indirect, via effects on osteoblastic stromal cells (Atkins, unpublished data). Together, these observations give new insight

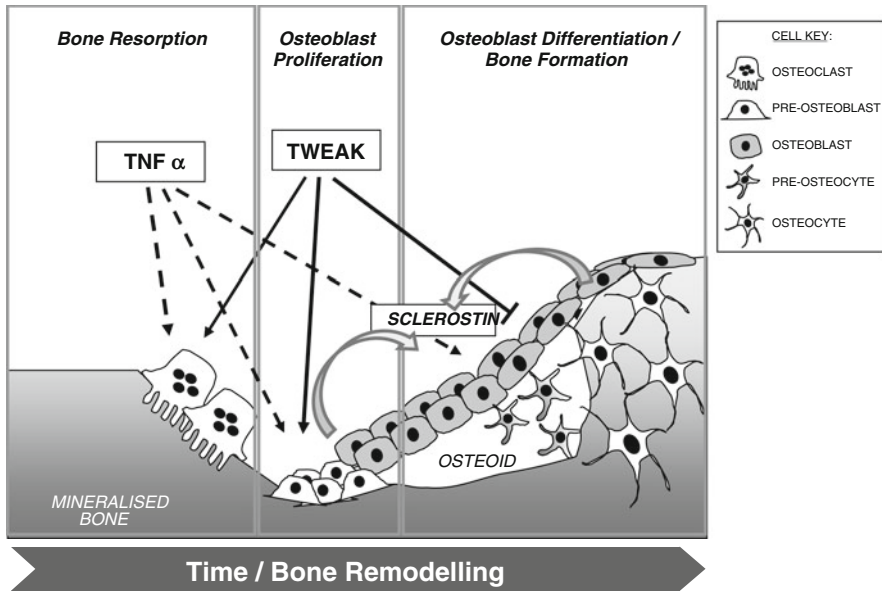


Fig. 34.4 Cartoon depicting the anti-anabolic actions of TWEAK in bone remodelling, here divided into the phases of bone resorption, osteoblast proliferation and osteoblast differentiation accompanying bone formation, as inferred from the work described in this manuscript and that reported by others and discussed in the text. In an inflammatory environment, TWEAK may act (*solid arrows*) on cells of the osteoblast lineage to stimulate osteoblast proliferation and also produce inhibitors of bone formation, including sclerostin (*curved arrows*), which acts back on bone forming osteoblasts to limit both osteoblast differentiation and the production of new bone. $TNF\alpha$ also has mitogenic effects on osteoblasts and induces the production of sclerostin (*dashed arrows*) but the overall effect is positive on bone formation perhaps because of the transient nature of these effects. $TNF\alpha$ together with TWEAK modulate each other's activity and the net effect would depend on their relative abundance. TWEAK may also activate bone resorption by promoting the formation and activity of osteoclasts, as has been reported for $TNF\alpha$

into the mechanism of physiologic bone remodelling and the bone loss and lack of repair that are observed in a number of clinically important bone loss pathologies, including osteoporosis and RA.

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