The role of palmitoylation modifications in the regulation of bone cell function, bone homeostasis, and osteoporosis

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Osteoporosis a is a metabolic bone disease caused by an imbalance in bone homeostasis, which is regulated by osteoblasts and osteoclasts. Protein palmitoylation modification is a post-translational modification that affects protein function, localization, and targeting by attaching palmitoyl groups to specific amino acid residues of proteins. Recent studies have shown that protein palmitoylation is involved in the regulation of osteoclast overproduction, osteoblast migration, osteogenic differentiation, dysfunctional autophagy, and endocrine hormone membrane receptors in osteoporosis. Exactly to what extent palmitoylation modifications can regulate osteoporosis, and whether palmitoylation inhibition can delay osteoporosis, is a key question that needs to be investigated urgently. In this review, we observed that palmitoylation modifications act mainly through two target cells – osteoblasts and osteoclasts – and that the targets of palmitoylation modifications are focused on plasma membrane proteins or cytosolic proteins of the target cells, which tend to assume the role of receiving extracellular signals. We also noted that different palmitoyl transferases acting on different substrate proteins exert conflicting regulation of osteoblast function. We concluded that the regulation of osteocyte function, bone homeostasis, and osteoporosis by palmitoylation modifications is multidimensional, diverse, and interconnected. Perfecting the palmitoylation modification network can enhance our ability to utilize post-translational modifications to resist osteoporosis and lay the foundation for targeting palmitoyl transferases to treat osteoporosis in the future.

Article focus

- Palmitoylation modifications regulate protein binding to and transport to the cell membrane, and play a key role in protein function and cell signalling. However, to what extent do protein palmitoylation modifications actually regulate bone cell function and osteoporosis?
- The regulation of bone homeostasis by palmitoylation modifications is multidimensional and diverse. Are certain important signalling molecules driving the osteoporosis process, such as β-catenin, Notch, and STAT3, regulated by palmitoylation modifications?
- The lipotoxicity of palmitic acid on bone cells is widely recognized. Does palmitic acid cause this lipotoxicity by palmitoylation of certain key proteins?

Key messages

- A network of palmitoylated regulation of bone cell function and osteoporosis has been established through a comprehensive review of the relevant literature.
- It was found that STAT3 and NLRP3 may be palmitoylated and thus involved in the regulation of osteoblast function. Palmitic acid may manifest its lipotoxicity (dysfunctional autophagy and apoptosis) by regulating palmitoyltransferase expression, modulating palmitoylation of key molecules.

Strengths and limitations

- The mechanism of palmitoylation in osteoblast function is clearly described in this study, documenting the most comprehensive and up-to-date research in the field.
- Our results will help future researchers to assess the significance of in-depth studies of palmitoylation modifications

in the field of osteoporosis, and may even determine the potential for drug development against palmitoylated target proteins.
- This field of research is still in its infancy, and the small number of papers will limit our knowledge of palmitoylation modifications that regulate osteoblast function and osteoporosis.
- Our findings did not pay sufficient attention to palmitoylation modifications in osteocytes other than osteoblasts and osteoclasts, or to depalmitoylation.
- To date, there have been no reports of drug development for osteoporosis targeting palmitoylation sites. It is hoped that our research will promote more clinical practices targeting palmitoylation sites.

Introduction

Osteoporosis is a generalized disorder of the skeleton featuring decreased bone mineral density (BMD)[1] and microstructural deterioration, leading to enhanced bone fragility and susceptibility to fractures.[2] This condition can be classified into primary (age-related) and secondary (disease- or medication-induced) forms, both significantly elevating the susceptibility to hip and low back fractures.[3] Bone densitometry and CT techniques can identify the risk of fracture due to low bone mass.[4] It is well established that osteoblasts promote bone growth and morphology maintenance by remodelling units of bone-forming cells.[5] Abnormal ageing and postmenopausal hormonal changes lead to dysfunction of osteoblasts as well as an increase in the number and activity of osteoclasts,[6-9] which are considered to be the main causes of osteoporosis. Exploring the regulation of bone cell function can help us to recognize osteoporosis, which is beneficial in reducing the risk of fracture due to low bone mass in humans.

Post-translational modifications (PTMs) of proteins are thought to be extensively involved in the regulation of osteoblast and osteoclast function in the progression of osteoporosis. PTM is the destruction or generation of covalent bonds in the backbone or the side chains of amino acids of proteins after biosynthesis, yielding a variety of isomers with different biological functions.[10] PTMs are important in functional proteomics, where they can modulate the activity of enzymatic proteins,[11] alter the localization of cellular proteins,[12] facilitate or suppress interactions with other proteins, and tag proteins for degradation.[13] Protein ubiquitination modifications,[14,15] protein glycosylation modifications,[16,17] protein phosphorylation modifications,[18,19] protein citrullination modifications,[20] and protein small ubiquitin-related modifier (SUMO) modifications have been found to be extensively involved in the regulation of osteoporosis.[21]

Out of the multitude of post-translational modifications, protein palmitoylation modifications are very novel, unique, and important, and hence worth investigating. Recent studies have shown that protein palmitoylation modifications are involved in osteoclast differentiation and function,[22,23] as well as differentiation of bone marrow mesenchymal stem cells (BMSCs) to osteoblasts and secretion of mineral precursors during the ontogenetic and developmental stages of osteoporosis.[24-27]

Methods

Date source and searches

A comprehensive three-step search strategy was used to identify all relevant literature in English and Chinese, including published and grey literature. The initial search was conducted in PubMed, and the initial retrieval of literature helped to construct a broader search strategy. Multiple databases were comprehensively searched, including EMBASE, PubMed, Web of Science, CNKI (Chinese), and SinoMed (Chinese). The search period was from January 2000 to October 2024, as articles on palmitoylated modifications and bone homeostasis have only gradually appeared after 2000. In PubMed, we used a combination of free vocabulary and Mesh terms for the search, with search terms including [palmitate OR palmitoylation OR S-palmitoylation OR APT1 OR ZDHHC] AND [Osteoporosis OR "bone homeostasis"] AND [osteoclast OR osteoblast OR "bone cells"]. In addition, a manual search of the references of all papers that met the inclusion criteria was conducted. To ensure that the search was comprehensive and systematic, and to confirm that the literature was as up to date as possible, the search was conducted with reference to the PRISMA 2020 statement.[28] A total of 38 core articles were screened. A PRISMA literature screening flowchart is included as Supplementary Figure a, to clearly demonstrate the search process and results. In addition, this study was registered on the PROSPERO platform and obtained an official ID: CRD42024601362.

Inclusion and exclusion criteria

The review includes studies that met the following criteria: 1) research directed towards the effects of palmitate or palmitoylation modifications on bone cell function, bone homeostasis, and osteoporosis; 2) research on cell types focusing mainly on osteoblasts and osteoclasts, but also including other undifferentiated cells related to bone differentiation, e.g. human bone marrow mesenchymal stromal cells (hBMSCs) and mouse calvaria-derived osteoblast-like cell line (MC3T3) cells; 3) basic research related to biochemical mechanisms; and 4) publications in English. Exclusion criteria included failure to find the full text, lack of clear conclusions, and insufficient experimental evidence.

Bibliometric analysis of core literature

The retrieved core literature was analyzed econometrically. First, the trend of publications was analyzed (Figure 1a) and it was observed that although the overall number of publications was low, there was a general trend towards increasing publications in 2023, indicating that palmitoylation in osteoporosis is very much at the forefront of research and has great potential to be explored. Keyword clustering showed the frequency and association of palmitoylation with osteoporosis and bone homeostasis (Figure 1b). The top 15 journals in the overall rankings in which this literature was published demonstrated the value and recognition of the research (Figure 1c). National collaborative links demonstrate the interest and commitment of different countries to this type of research, with the USA, Australia, and China leading the way (Figure 1d). Author co-occurrence shows the most authoritative experts and scholars in the field and the links between them, with Duque et al[29–31] found to be authorities on the subject (Figure 1e).

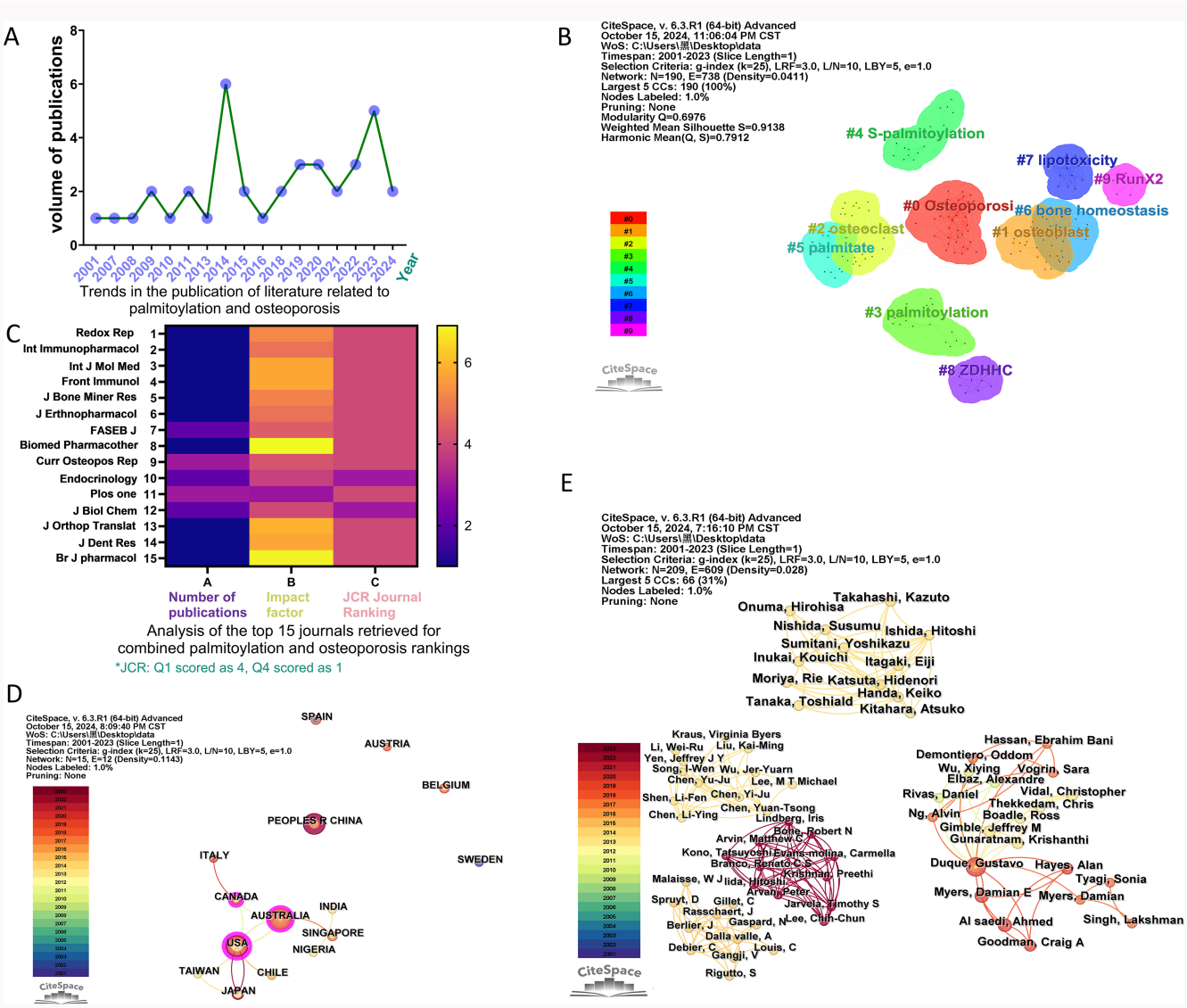

Fig. 1

Bibliometric analysis of final inclusion. a) Trends in publications since 2000 (no relevant literature retrieved between 2002 and 2006). b) Keyword clustering in the literature. c) Top 15 journals based on impact factor and number of articles published. d) Cooperation between countries. e) Authors' co-occurrence. a) and c) were created with GraphPad Prism version 10.1.2 (GraphPad Software, USA). b), d), and e) were created with CiteSpace.v.6.3.R1 (64-bit) Advanced (Drexel University, USA).

The phenomena of palmitoylation modification in bone homeostasis and osteoporotic processes have been observed in several physiological activities, including migration and differentiation of osteoclasts, differentiation and apoptosis of osteoblasts, palmitate (PA) toxicity, and endocrine membrane receptors in bone cells (Table I).

The dynamic remodelling of bone tissue is coordinated by two key cell types: osteoblasts and osteoclasts.[39] Orchestrating this continuous remodelling process, osteoblasts dedicate themselves to bone formation, meticulously synthesizing and laying down new bone matrix. Simultaneously, osteoclasts engage in targeted resorption, removing aged or damaged bone tissue. This coordinated process requires a delicate balance, achieved through mutual antagonism, where any disruption by internal or external factors can lead to skeletal dysfunction and potentially osteoporosis.[40] Thus, the effect of palmitoylation modifications on osteoblast and osteoclast function and differentiation inevitably affects bone remodelling, and even leads to osteoporosis.

Autophagy and apoptosis in osteoblasts are present during bone ageing and bone remodelling. At baseline, autophagy operates to meet cellular metabolic needs, renew specific organelles, and play a decisive role in maintaining cellular homeostasis. The regulation of autophagy has a dual impact on cells; excessive or swift autophagy can result in cell death through autophagy.[41] Ordinarily, autophagy suppresses the start of apoptosis. Nevertheless, in certain scenarios, autophagy or proteins associated with autophagy may contribute to the initiation of apoptosis or necrosis.[42] In the context of human osteoblasts, it has been documented that palmitate induces dysfunctional autophagy and apoptosis (Table I).[31] Moreover, modulation of autophagy in osteoblasts prevents apoptosis induced by palmitate.[43] Considering the strong link between palmitate and palmitoylation, we suggest that palmitate-induced dysfunctional autophagy in bone cells is mediated by palmitoylation modifications.

Table I. The role of palmitoylation modifications in the regulation of osteoporosis.

Study	Target cell	Inducer	Target points	Key findings	Treatment
Osteoclast palmitoylation					
Ma et al (2023)[22]	BMMs or osteoclast	Osteoclastic medium/RANKL	*c-Fos/NFATc1*, F-actin ring	2-bp inhibits c-Fos/NFATc1 and F-actin ring	2-BP
Brazier et al (2006);[32] Berzat et al (2005)[23]	Osteoclast; NIH 3T3 fibroblasts	RANKL; C255S mutation	RhoU/Wrch-1	Wrch-1 inhibition leads to abnormal proliferation of osteoclasts; inhibition of palmitoylation modification of Wrch-1 leads to incorrect subcellular localization	shRNA; 2-BP
Ory et al (2007)[33]	Osteoclast	RANKL	RhoU	The palmitoylation sequence of RhoU is not required for RhoU targeting focal adhesions	Replacement of cysteine residues 255 and 256 with serine
Osteoblast palmitoylation					
Ma et al (2023)[22]	MC3T3-E1 cells	Osteogenic medium	*Sp7/ Ibsp*	2-bp inhibits the transcription of *Sp7* and *Ibsp*	2-BP
Leong et al (2009)[24]	Osteoblast	PA	*Osterix*	2-BP negatively regulates p38 MAPK activation, resulting in decreased expression of osterix	2-BP
Tsukamoto et al (2013)[25]	MC3T3 cells	Osteogenic medium	IFITM5/BRIL	Inhibition of S-palmitoylation of IFITM5 leads to morphological distortion of bone nodules	2-BP
Gunaratnam et al (2014)[29]	Osteoblast	PA	*ALP, Runx2, OCN, BSP2, β-catenin,* and *Runx2*	PA negatively affected the expression of *ALP, Runx2, OCN,* and *BSP2* genes. The transcriptional activities of *β-catenin* and *Runx2* were decreased	None
Alsahli et al (2016)[34]	Osteoblast	PA and oleic acid	P1NP and osteocalcin	Inhibition of C16 ceramide accumulation can restore the mineralization activity of osteoblasts in PA-treated cells	Myriocin
Al Saedi et al (2020)[30]	Human primary osteoblasts	PA	smad1 -3,5, Runx2, and β-catenin	1,25 $(OH)_2D_3$ improves PA-induced decrease in Smad1-3,5, Runx2, and β-catenin transcription	1,25 $(OH)_2D_3$
Li et al (2023)[26]	hBMSCs	α-MEM medium	cAMP/PKA/CREB; *Runx2*	ZDHHC16 inhibits CREB phosphorylation	shRNA; 2-BP
Ji et al (2023)[27]	Osteoblast	Osteogenic medium	BMPR1A	Depalmitylation of BMPR1A activates the BMP/Smad pathway	oeRNA; LDN-1
PA and apoptotic autophagy					
Kim et al (2008)[35]	hFOB1.19 cell	PA	AMPK/ERK	Activation of AMPK rescues PA damage to ERK from apoptosis	Triacsin C; fumonisin B1; antioxidants
Gunaratnam et al (2013)[31]	Osteoblast	PA	Fas/JNK	PA activates autophagy and nuclear fragmentation in osteoblasts	SP600125; 3-MA
Al Saedi et al (2020)[30]	hOBs	PA	N/A	1,25 $(OH)_2D_3$ protects PA-activated osteoblast dysfunctional autophagy	1,25 $(OH)_2D_3$
The hormone membrane receptor palmitoylation					
Vinel et al (2016)[36]	Osteoblast; osteoclast	N/A	Membrane ERα	Membrane ERα loss of palmitoylation sites reduces ERαMISS signalling for bone benefit	ERα-C451A knock-in

(*Continued*)

(*Continued*)

Study	Target cell	Inducer	Target points	Key findings	Treatment
Gustafsson et al (2022)[37]	N/A	N/A	Membrane ERα	The SERM effect in the skeleton is membrane ERα-dependent	E_2; lasofoxifene
Kalyanaraman et al (2014)[38]	hOBs/ MC3T3 cells	T_3	30-kD TRα1	TH signalling of palmitoylated p30 TRα1 increases intracellular concentrations of calcium, NO, and cGMP	Rp-CPT-PET-cGMPS; U0126; LY294002

ALP, alkaline phosphatase; AMPK, AMP-activated protein kinase; BMMs, bone marrow macrophages; BMPR1A, bone morphogenetic protein receptor type 1A; 2-BP, 2-bromopalmitate; BSP2, bone sialoprotein 2; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ERα, oestrogen receptor alpha; ERαMISS, membrane-initiated steroid signalling mediated by ERα; hBMSC, human bone marrow mesenchymal stromal cell; hOBs, primary human osteoblasts; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; N/A, not applicable; NO, nitric oxide; OCN, osteocalcin; PA, palmitate; PKA, protein kinase A; P1NP, procollagen type 1 N-terminal propeptide; RANKL, receptor activator of NF-κB ligand; RhoU, Ras homolog family member U; Runx2, runt-related transcription factor 2; SERM, selective oestrogen receptor modulators; shRNA, short hairpin RNA; TH, thyroid hormone.

The regulation of bone metabolism involves the intricate interplay of various endocrine hormones. In women, premature ovarian failure or early menopause (occurring before the age of 40 years) results in a decline in oestrogen levels. In men, diminished androgen levels or dysfunctions such as sexual dysfunction or androgen resistance syndrome can similarly activate osteoclasts through the RANK-RANKL-OPG signalling pathway, contributing to bone loss.[44] Excessive thyroid hormone levels have the capacity to stimulate both osteoblast and osteoclast activity, leading to a concurrent increase in bone formation and resorption. However, the impact on bone formation is overshadowed by the magnitude of bone resorption, ultimately resulting in bone loss and the development of osteoporosis.[45] Furthermore, parathyroid hormone,[46] insulin, adrenal cortisol, growth hormone, and prolactin have been identified as factors involved in the intricate regulation of bone metabolism. Consequently, modification of endocrine hormone membrane receptor palmitoylation in bone cells directly intervenes in the regulation of bone metabolism.

The following section examines the role of palmitoylation in various physiological processes through a comparative analysis, with the aim of constructing a comprehensive framework for understanding its involvement in the regulation of osteoporosis.

Biochemical mechanism of S-palmitoylation

A systematic understanding of the biochemical mechanisms of palmitoylation is first needed to better understand the effects of palmitoylation modifications on different physiological activities in osteoporosis. Palmitoylation, also called S-palmitoylation, refers to the addition of 16-carbon long saturated palmitic acid to the cysteines of proteins via unstable thioester bonds.[47] This biochemical process controls the association and transport of proteins to the cell membrane, thus playing a crucial role in cellular signalling and protein function.[48] The instability of the high-energy thioester bond between palmitoyl and cysteine residues makes S-palmitoylation the sole reversible lipid modification of proteins.[49] Beyond aiding in targeting proteins to diverse organelle and cell membranes, palmitoylation is associated with aspects such as protein stability, conformation, isoforms, and heterogeneity.[49] Palmitoyl acyltransferases (PATs) catalyze the S-palmitoylation of most proteins (Figure 2). These PATs typically contain conserved aspartate-histidine-histidine-cysteine (DHHC) tetrapeptides and zinc-finger structural domains, and therefore genes encoding these enzymes are referred to as zinc-finger-containing DHHC (ZDHHC). A total of 23 different ZDHHCs have been identified in mammals (ZDHHC1-23).[47] The DHHC structural domain, characterized by its cysteine-rich structure, directly participates in palmitoyl transfer reactions.[50] Conversely, during depalmitoylation, acyl protein thioesterases (APTs), known as depalmitoylases, are responsible for removing palmitate from the cysteine site of the target protein.[51]

Protein palmitoylation regulates osteoclasts in osteoporosis

Classical signalling pathways of osteoclast differentiation

The RANK/RANKL/OPG axis is the most important signalling pathway in the process of inducing osteoclast differentiation, and most cytokines regulate the dynamic balance between osteoblasts and osteoclasts through this pathway.[52] The interaction between osteoblasts and osteoclast precursor cells involves the binding of receptor activating factor ligand (RANKL), a nuclear factor-κB (NF-κB) receptor activating factor produced by osteoblasts, to RANK (NF-κB receptor activating factor) on osteoclast precursor cells (Figure 2).[53] This binding activates NF-κB, leading to the promotion of osteoclast differentiation. Additionally, osteoblasts secrete osteoprotegerin (OPG), which functions as a receptor for RANKL. OPG competitively binds to RANKL, thereby inhibiting osteoclastogenesis.[54]

Inhibition of palmitoylation reduces bone resorption levels

Ma et al[22] first clarified the involvement of palmitoylation in osteoclast differentiation in osteoporosis. RANKL binds to RANK on osteoclasts and induces these bone resorbing cells to mature and become active.[55] Surprisingly, the palmitoylation inhibitor 2-bromopalmitate (2-BP) blocked RANKL-induced osteoclast generation in vitro.[22] As we know, in osteoclast differentiation and activation, RANKL induces the activation of NF-κB, PI3K-AKT, and mitogen-activated protein kinase (MAPK) pathways (including JNK, p38, and ERK),[56] which is accompanied by the increase of the transcription of some osteoclast-associated genes, such as *c-Fos* and *NFATc1* (Figure 2).[57]

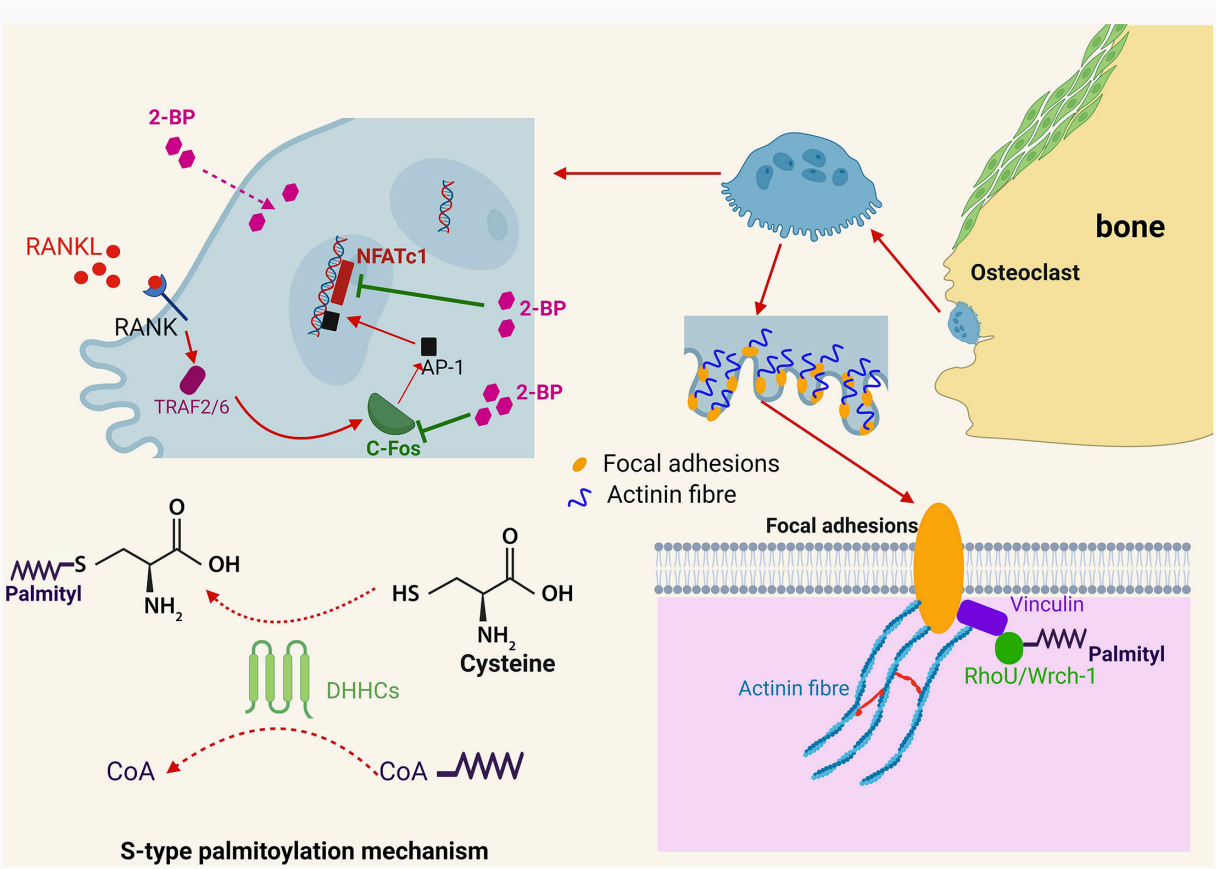

Fig. 2
S-type palmitoylation is the catalyzed attachment of palmitoyl groups to cysteine residues of proteins by palmitoyltransferases aspartate-histidine-histidine-cysteine (DHHCs). Protein palmitoylation modifications are involved in osteoblast differentiation and osteoclast migration. For example, the receptor activator of NF-κB ligand (RANKL) binds to the receptor activator of NF-κB (RNAK) on osteoclasts. By activating the signalling cascade via the linker proteins TRAF2/6, the transcription factor c-Fos/AP-1 is stimulated, thereby increasing the expression of the master transcription factor for osteoclast formation, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1). However, treatment with 2-BP inhibited *c-Fos* and *NFATc1* transcript levels. Furthermore, palmitoylation modification of Wrch-1 impaired osteoclast adhesion turnover and motility. Diagram created with BioRender (Canada). 2-BP, 2-bromopalmitate; RhoU, Ras homolog family member U.

The treatment of 2-BP did not obviously affect the phosphorylation level of signalling molecules related to the RANKL-induced NF-κB, AKT, and MAPK pathways, but palmitoylation of inhibitory proteins significantly inhibited the transcriptional level of *c-Fos* and *NFATc1*. This is the key to 2-BP blocking RANKL signalling. Furthermore, a new study found that 2-BP treatment inhibited RANKL-induced F-actin ring formation (Figure 2).[33] The study failed to go further in clarifying what the palmitoylated proteins are, which remains to be clarified. It is worth mentioning that messenger RNA (mRNA) levels of ZDHHC 1, 5, 8, 15, and 17 were significantly elevated in differentiated osteoclasts (Figure 3).[22]

Interestingly, we found that STAT3 may be a target of palmitoylation in osteoblastic differentiation.[22] Specific knockdown of the mouse STAT3 gene showed reduced NFATc1 expression and decreased osteoclast differentiation,[58,59] and STAT3 could drive its transcription by binding to the promoter of *NFATc1*. In addition, STAT3 inhibitor (Stattic) suppresses RANKL-induced activation of the STAT3 pathway,[60] as well as RANKL-induced expression of the transcription factors c-Fos and NFATc1. STAT3 undergoes reversible S-palmitoylation on cysteine 108 and promotes its membrane recruitment and phosphorylation.[61] These experimental findings reveal the association of palmitoylation, STAT3, and osteoblastic differentiation.

Inhibition of palmitoylation impairs osteoclast migration

Palmitoylation of proteins performs a crucial function in influencing membrane targeting and cell migration of osteoclasts (Figure 2). RhoU/Wrch-1 is a member of the Rho GTPase family (a family of regulators of cell adhesion structures), which functions to induce actin reorganization and filamentous pseudopod formation.[33] RhoU/Wrch-1 is localized to osteoclast adhesion structures, and by regulating its number and distribution can alter adhesion turnover and cell migration rates and increase cell motility.[33] During RANKL-induced osteoclastogenesis, RhoU/Wrch-1 gene expression is strongly upregulated.[32] Wrch-1 inhibition severely impairs cell fusion and leads to abnormal spreading of osteoclasts. Berzat et al[23] found that Wrch-1 was modified by palmitate, and that pharmacological inhibition of protein palmitoylation resulted in erroneous membrane binding and subcellular localization of Wrch-1. However, one study disputes that the palmitoylated sequence of RhoU is unnecessary for RhoU to target focal adhesions (an osteoclast adhesion structure).[33] The two aforementioned opposing perspectives remain controversial to this day.

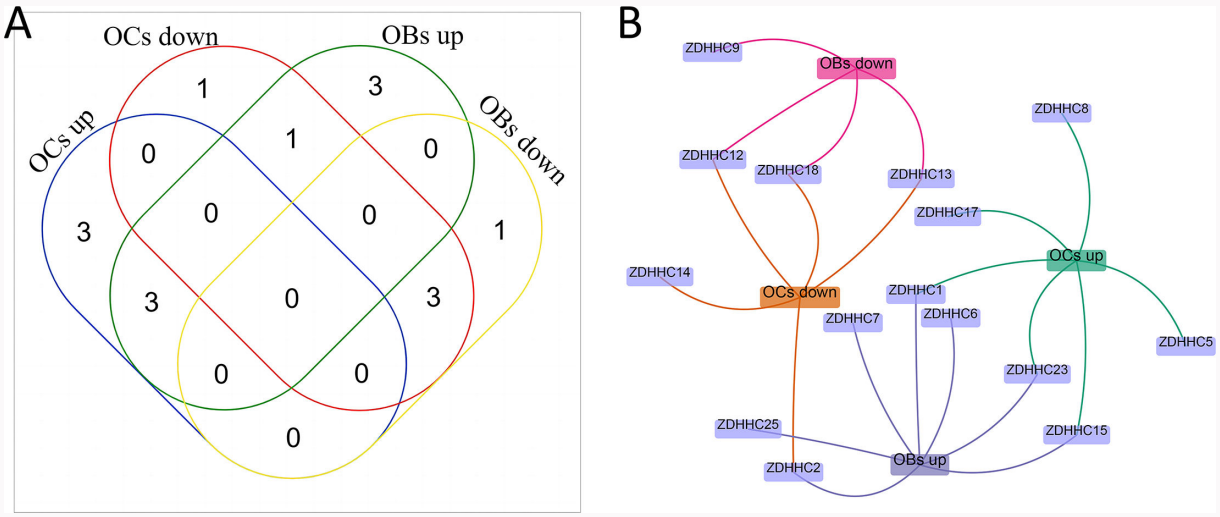

Fig. 3
a) Wayne diagram of reduced or upregulated palmitoyltransferase expression during osteoblast (OB) or osteoclast (OC) differentiation. b) Visualization of palmitoyltransferase expression differences and associations during osteoblast or osteoclast differentiation. Diagrams created with omicshare.com (Guangzhou Genedenovo Biotechnology, China).

Protein palmitoylation regulates osteoblasts in osteoporosis

Classical signalling pathways of osteoblastic differentiation

Osteoblast differentiation undergoes complex regulation by multiple signalling pathways, encompassing transcription factors such as Runx2 and osterix, along with BMP, Wnt, PAK2, and Notch signalling.[62] Runx2 has a critical function in this process by overseeing the expression of bone alkaline phosphatase (ALP) and fostering the differentiation of osteogenic progenitor cells into osteoblastic precursor cells.[63] Osterix, positioned downstream of Runx2, dictates the transformation of osteoblastic precursors into fully mature osteoblasts.[64] BMP signalling induces the activation of the Smad1/5/8 pathway, resulting in the phosphorylation of Smad1/5/8. Subsequently, the phosphorylated forms bind with Smad4 and translocate into the nucleus, orchestrating the regulation of BMP target genes such as *Runx2* and *Osterix* (Figure 4). This process serves to enhance osteoblast differentiation (Figure 4).[65] The Wnt/β-catenin pathway operates by preventing the phosphorylation of β-catenin, leading to increased stability and nuclear translocation of β-catenin. Once in the nucleus, β-catenin works synergistically with the transcription factor to stimulate the transcription of osteogenic target genes, thereby promoting differentiation of osteoblasts.[66]

Early studies suggest palmitoylation in the osteogenic pathway to avoid osteoporosis

Early researchers believed that protein palmitoylation was beneficial for bone formation, which favoured bone homeostasis and avoided osteoporosis.[24,25,67] Leong et al[24] were the first to find that inhibiting palmitoyltransferase (PAT) resulted in reduced osteoblast differentiation and mineralization. Bone morphogenetic protein (BMP), a member of the transforming growth factor-beta (TGF-β) family, stimulates DNA synthesis and cell replication, promoting the directed differentiation of mesenchymal stem cells (MSCs) into osteoblasts.[68] BMP2 was reported to transactivate osterix via either the BMP-Smad1/5/8 pathway or the non-classical BMP-MAPK pathway.[68] Osterix, a transcription factor specific to osteoblasts, plays a significant role in osteoblast differentiation.[69] Further investigation revealed that protein palmitoylation activated the p38 MAPK pathway but not the classical BMP-Smad pathway.[24] In summary, the PAT inhibitor 2-BP negatively regulates p38 MAPK activation, leading to reduced osterix expression and impaired osteogenic differentiation. Intriguingly, reverse transcription polymerase chain reaction (RT-PCR) analysis showed that 20 out of 23 PATs were detected in osteoblasts, with DHHC1, 2, 6, 7, 15, 23, and 25 upregulated in differentiated osteoblasts, and DHHC9, 12, 13, and 18 slightly downregulated, providing valuable insights for future research (Figure 3). Unfortunately, this study did not delve into the specific palmitoylated target proteins that regulate osteoporosis. It was only with advances in palmitoylation detection that subsequent studies identified the first palmitoylated target protein.

Tsukamoto et al[25] identified the first palmitoylated target protein in the osteogenesis mechanism, interferon-induced transmembrane protein 5 (IFITM5). IFITM5, also called bone restrictive IFITM-like protein (BRIL), belongs to the interferon-induced transmembrane (IFITM) family.[70] It forms a complex with FK506-binding protein 11 (FKBP11) in osteoblasts. BRIL is enriched during osteoblast mineralization, coinciding with the onset of matrix mineralization.[71,72] Researchers discovered that the transmembrane helix 1 (TM1) structural domain of IFITM5 and cysteine residues in the CP ring undergo S-palmitoylation, facilitating its interaction with FKBP11 (Figure 4). Inhibition of S-palmitoylation of IFITM5 resulted in morphological aberrations of the bone nodules. In summary, palmitoylated modified IFITM5 promotes bone mineralization.

Saleem et al[67] conducted a study utilizing a ZDHHC13 mutant mouse model to investigate the repercussions of protein palmitoylation deficiency. The model uncovered a direct association between palmitoyltransferase deficiency and the regulation of multiple physiological functions.

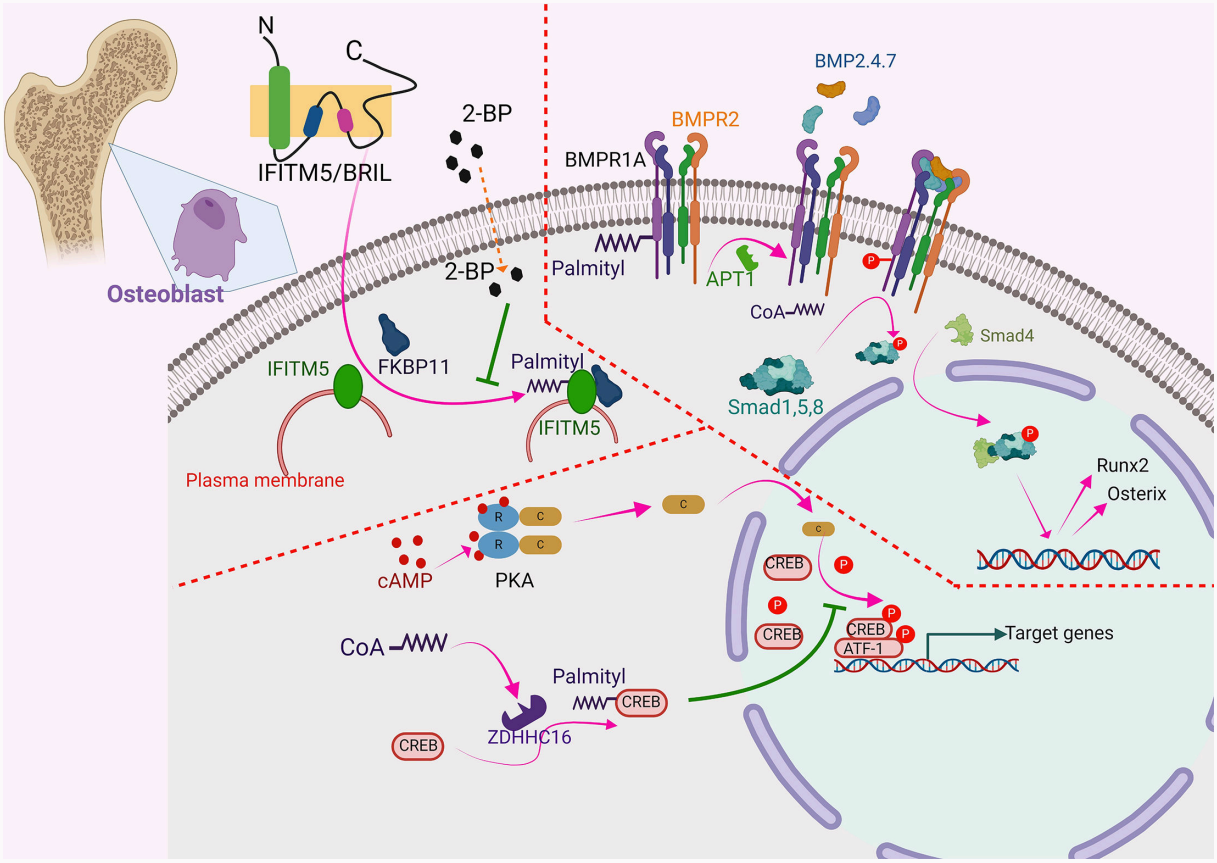

Fig. 4
Protein palmitoylation modifications are involved in regulating osteogenic matrix mineralization and differentiation signals. The transmembrane protein, bone-restricted interferon-induced transmembrane protein-like (BRIL), is palmitoylated to preferably bind to FKBP11 to form a complex that promotes osteogenic matrix maturation and mineralization, whereas 2-bromopalmitate (2-BP) inhibits palmitoylation of BRIL. The palmitoylase ZDHHC16 modulates CREB phosphorylation by altering the extent of CREB palmitoylation, thereby participating in the regulation of the cAMP/PKA/CREB/ATF-1 signalling pathway to suppress the expression of target genes. The acyl-protein thioesterases (APT) target and activate bone morphogenetic protein receptor 1A (BMPR1A), which then binds to BMPR2 and the ligand BMP2.4.7 to activate SMAD1/5/8, which binds to SMAD4 and enters the nucleus to activate genes such as runt-related transcription factor 2 (*Runx2*) and *osterix*. Diagram created with BioRender.com (Canada).

ZDHHC13 deficiency has been found to lead to severe systemic phenotypes, especially osteoporosis.[67] Confirmation of the osteoporotic condition in the affected mice was evident from severe kyphosis, markedly increased spinal angulation, a reduced number of femoral trabeculae, and lower BMD. The palmitoyltransferase ZDHHC13 is also a mediator of Mg^{2+} transport.[73] The fact that ZDHHC13 mutant mice possess normal magnesium-calcium levels, yet are greatly reduced in their ability to palmitoylate known substrates Huntington's proteins,[74] rigorously demonstrates that the phenotypes of the mutant mice of ZDHHC13 are due to downregulation of the level of protein palmitoylation. Interestingly, mutant mice with ZDHHC13 manifested abnormal phenotypes, while heterozygous mutants presented as phenotypically normal.

Lipotoxicity of palmitate on the osteogenic pathway

The perception of the relationship between palmitoylation and osteoporosis shifted as the mechanisms of lipotoxicity of palmitate on human osteoblasts were discovered. Gunaratnam et al[29] found that palmitate had inhibitory effects on osteoblast bone nodule formation and extracellular matrix mineralization (Table I). This inhibition was evident through the negative impact on the expression of key genes such as *ALP*, *Runx2*, *OCN*, and *BSP2*. Palmitate also significantly reduced the transcriptional activity of *β-catenin* and *Runx2*, crucial factors in osteoblast function. The increased expression of the Runx2-Smad complex, which activates osteogenic potential following BMP2 treatment of cells,[75] was interfered with by palmitate. While the lipotoxic mechanism of palmitate on osteoblasts was not directly shown to be mediated through protein palmitation, the study observed a dose-dependent downregulation of the palmitoyl transferases ZDHHC12 and ZDHHC1.

Alsahli et al[34] conducted further research on palmitate, confirming that the enrichment of palmitic acid led to pronounced reductions in levels of the bone formation markers procollagen type IN-terminal propeptide (P1NP) and osteocalcin (OC) in a high-fat diet (HFD) rat model. The unique aspect of this study was the focus on palmitate accumulation increasing C16 ceramides in osteoblasts. Ceramides, primarily derived from palmitate, are synthesized de novo through serine palmitoyltransferase and dihydroceramide synthase.[76] Inhibition of C16 ceramide accumulation was found to restore osteoblast mineralizing activity in PA-treated cells. Scholars have explored interventions to mitigate palmitate lipid toxicity, as demonstrated by the study conducted by Al

Saedi et al.[43] They compared the effects of vitamin D and PA-treated osteoblasts with those treated with PA alone (Table I). 1,25$(OH)_2D_3$ treatment reversed key osteogenic signalling proteins, including β-catenin, Runx2, and SMAD1-3,5. Moreover, 1,25$(OH)_2D_3$ rescued the decline of three palmitoyl acyltransferases (ZDHHC1, ZDHHC2, and ZDHHC12) as well as osteogenic genes (*alkaline phosphatase* and *osteocalcin*).

Palmitoylation promotes osteoporosis in the osteogenic pathway

Recent studies of palmitoylation of key proteins in the bone remodelling pathway have tended to highlight its negative impact on osteogenic differentiation.[26,27] This contradicts previous findings and underscores the complexity of the effects of protein palmitoylation on osteogenesis, suggesting that the outcomes may be influenced by different palmitoyl transferases and palmitoylated substrate proteins.

Li et al[26] found that palmitic acid inhibited *Runx2* mRNA expression in human bone marrow mesenchymal stem cells (hBMSCs), and identified ZDHHC16 as a potential palmitoyl transferase involved in this inhibitory signalling. The cAMP luciferase activity was significantly reduced in the ZDHHC16 overexpression group, suggesting that the cAMP pathway may be regulated by ZDHHC16 during osteogenesis. Previous literature found that the cAMP-PKA-CREB signalling pathway facilitates activation of *Runx2*, which plays an osteogenic role in hBMSCs.[77,78] Based on the link between the two, Li et al[26] measured protein expression and modification of the cAMP/PKA/CREB pathway in the ZDHHC16 overexpression group. ZDHHC16 was found to have no effect on the protein expression of PKA and CREB and did not affect the phosphorylation of PKA, but regulated the phosphorylation of CREB. Decreased palmitoylation of CREB was observed in ZDHHC16 knockdown hBMSCs; at the same time, phosphorylation of CREB was increased (Figure 4). CREB1 has also been reported to maintain cartilage homeostasis,[79] and whether palmitoylated modifications of CREB1 are involved in the progression of osteoarthritis is an unknown and interesting line of inquiry.

Compared with the regulation of osteogenic differentiation by palmitoylation, Ji et al[27] focused more on the study of osteogenic differentiation by depalmitoylation. Acyl protein thioesterases (APTs) play a role in mediating protein depalmitoylation modifications, with APT1 being the primary enzyme involved in this process.[80] The binding of bone morphogenetic protein receptor type 1A (BMPR1A) and BMPR2 to the ligand BMP2.4.7 activates SMAD 1, 5, and 8 (Figure 4). This activated protein complex is then translocated to the nucleus, where it regulates the activity of a variety of genes including *Runx2* and *osterix*, which in turn affects cell growth and division.[81] Wegleiter et al[82] found that the level of palmitoylation affected the membrane localization and transport of BMPR1a, leading to alterations in BMP signalling. Based on these observations, Ji et al[27] further showed that the depalmitoylase APT targets BMPR1a. This action served to activate the BMP/Smad pathway, promoting osteoblast differentiation and thereby mitigating osteoporosis in mice (Figure 4).

Palmitic acid lipotoxicity and dysfunctional autophagy

Potential link between palmitate lipotoxicity and palmitoylation

Our study suggests that damage to osteoblasts by palmitate is likely to be mediated through palmitoylation modifications. In the previous paragraph, we discussed that palmitate is lipotoxic to the osteogenic pathway and contributes to the development of osteoporosis. High levels of palmitate in the osteoblast microenvironment mean that key signalling molecules are more likely to be palmitoylated, and many studies have shown that palmitoylation tends to inhibit osteogenesis, which is consistent with the findings of two independent studies.[29,34] Palmitate is the most common fatty acid produced by bone marrow adipocytes, and bone marrow adipose infiltration during skeletal ageing secretes large amounts of fatty acids, leading to lipotoxic outcomes.[35,43,83] The lipotoxicity of palmitate is manifested by detrimental differentiation and mineralization of osteoblasts, and even induces osteoblast death.[30,31] The lipotoxic effects of palmitate can be prevented by adding the fatty acid (FA) synthase inhibitor cerulenin to the culture medium.[84] Additionally, astaxanthin is thought to inhibit oxidative stress and thus improve bone quality in PA-induced osteoporotic mice.[85] Gunaratnam et al[29] have documented changes in the expression levels of 23 major PAT genes after the action of palmitate on osteoblasts (Figure 3). It is highly likely that PA affects palmitoylation levels by adjusting (up- or down-regulating) palmitoyltransferase expression. Moreover, palmitate is sufficient to induce osteoclast differentiation and increase osteoclast activity even in the absence of RANKL (Table I).[86]

Mechanisms of dysfunctional autophagy stimulated by palmitate PA in osteoporosis

Palmitate can induce autophagy and apoptosis in osteoblasts in vitro.[30,31] Palmitic acid is an adipose-secreted factor that is thought to deleteriously affect osteoblasts and bone remodelling due to impaired ERK activation,[87] which induces osteoblast apoptosis.[35] However, activation of AMP-activated protein kinase (AMPK) enhances ERK activation and thus inhibits palmitic acid-induced apoptosis.[35] Apoptosis of osteoblasts after PA activation in osteoblasts showed a significant increase in Fas ligand (FasL), significant decrease in Bcl2, stable Bax expression, and significant increase in Bax/Bcl2 ratio under western blot analysis.[31] PA activation of autophagy and nuclear fragmentation in osteoblasts, a form of dysfunctional autophagy, was also found, and the application of the autophagy inhibitor 3-methyladenine (3-MA) inhibited autophagy in osteoblasts and prevented apoptosis. Al Saedi et al[30] investigated the protective effect of 1,25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) against dysfunctional autophagy in PA-activated osteoblasts. Interestingly, the use of 1,25 $(OH)_2D_3$ raised the Mitotracker (Thermo Fisher Scientific, USA) fluorescence intensity, suggesting an increase in mitochondrial mass. This seems to suggest that we can link the mechanism of PA lipotoxicity to osteoblasts to mitochondrial autophagy. These phenomena confirm the harmful effects of PA on osteoblasts and bone formation, which may lead to the development of osteoporosis.

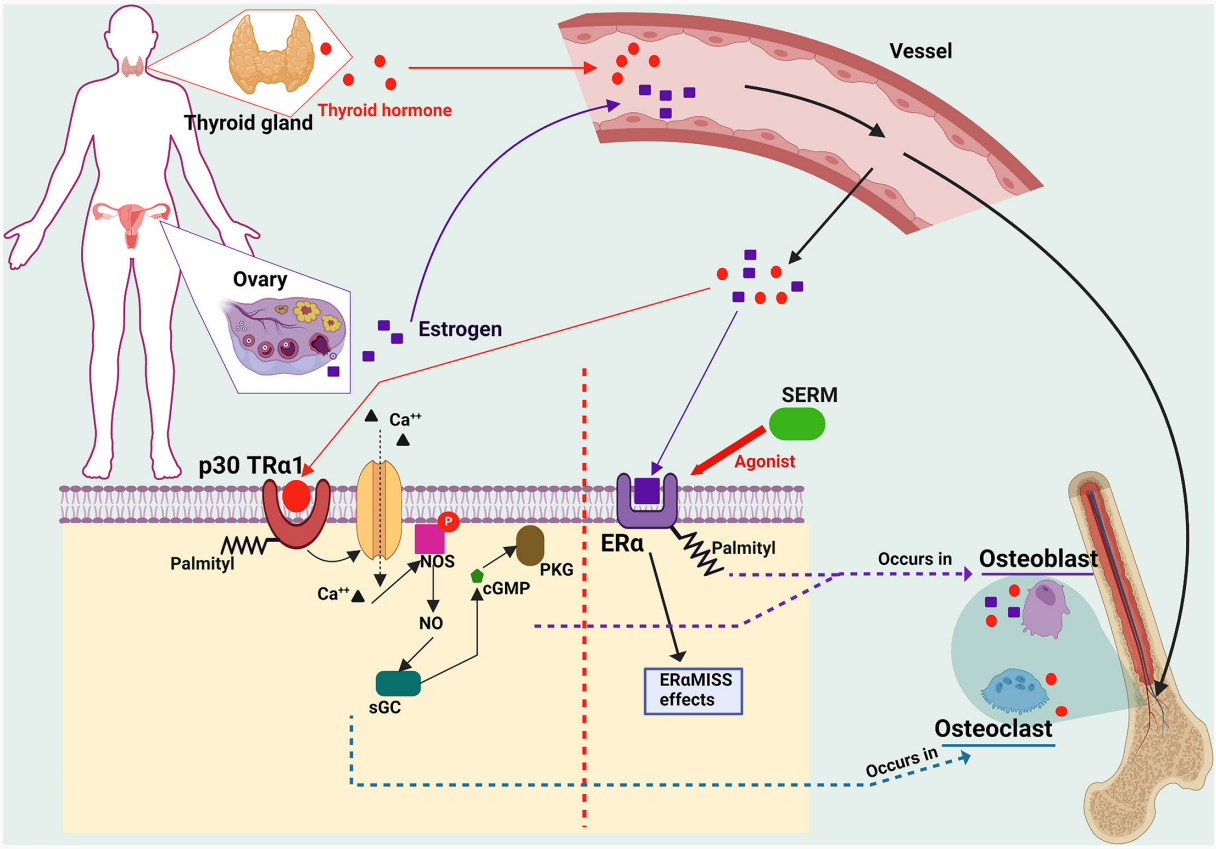

Fig. 5
The E_2 receptor oestrogen receptor alpha (ERα) and the 30-kD thyroid hormone receptor-α (p30 TRα1), which are localized in the cell membrane, can be modified by palmitoylation to enhance membrane targeting and promote downstream signalling. Selective estrogen receptor modulators (SERM) act as agonists on membrane ERα to promote ERα membrane-initiated steroid signalling (ERαMISS) effects. However, the lack of palmitoylation of ERα reduces the benefits of ERα membrane-initiated steroid signalling on bone density, bone cortex, and cancellous bone. Palmitoylation of p30 TRα1 promotes an increase in Ca^{2+} concentration, which leads to the activation of the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signalling cascade, promotes increased bone mass, and inhibits bone cell apoptosis. NOS, nitric oxide synthase; sGC, soluble guanylate cyclase. Diagram created with BioRender.com (Canada).

Understanding how protein palmitoylation stimulates autophagy in the context of osteoporosis represents a potential research avenue. The references mentioned earlier suggest that excessive palmitate leads to changes in palmitoyl transferases, as well as the palmitoylation of protein substrates.[24,29,30] The link between protein palmitoylation and autophagy is an exciting research topic.[88,89] For instance, Zhou et al[88] demonstrated that S-palmitoylation of nucleotide-binding oligomerization domain 2 (NOD2) by ZDHHC5 reduced the binding capacity of NOD2 to sequestosome-1 (p62). This inhibition of the autophagic degradation of NOD2 promoted NOD2-mediated inflammatory responses. Wang et al[89] showed that ZDHHC12-mediated palmitoylation of NLR family pyrin domain containing 3 (NLRP3) inflammatory vesicles facilitated degradation via the chaperone-mediated autophagy (CMA) pathway. This led to a downregulation of intracellular NLRP3 protein levels and served to inhibit NLRP3-induced persistent inflammation.

Palmitoylation modifications affect bone and endocrine hormone crosstalk

Palmitoylation of membrane oestrogen receptor-α (ERα) impairs osteoblast function, whereas palmitoylation of 30-kD thyroid hormone receptor-α (p30 TRα1) enhances osteoclast function, both of which have important implications for bone homeostasis and osteoporosis.[37,38]

Palmitoylation-dependent modification of membrane oestrogen receptors in osteoblasts

17β-oestradiol (E_2) acts on osteoclasts, osteoblasts, and BMSCs through multiple signalling pathways, affecting their value-addition, metabolism, differentiation, and apoptosis.[90,91] This is extremely important in regulating the balance between bone resorption and bone formation. The protection of bones by E_2 relies on the involvement of ERα.[92] Partial subpopulations of the transcription factor nuclear ERα are also present on or near the plasma membrane and induce membrane-initiated steroid signalling (MISS) effects.[93] Both nuclear ERα and membrane ERαMISS contribute to E_2 regulation of bone, with the noteworthy observation that selective activation of ERαMISS is more inclined toward protecting cortical bone mass.[94] Vinel et al[36] discovered that mutations in the C451A palmitoylation site of membrane ERα eliminated the membrane localization of ERα, significantly reducing the benefits of ERαMISS signalling on BMD and bone cortex and cancellous bone (Figure 5). Interestingly, ERαMISS signalling was experimentally found to specifically regulate osteoblasts, with no significant effect on osteoclast profiles. Treatment with

selective oestrogen receptor modulators (SERMs) that prevent osteoporosis increased trabecular and cortical bone mass in the control group.[37,95] However, the efficacy of SERM treatment was compromised when the C451A palmitoylation site of membrane ERα was mutated, suggesting a membrane ERα-dependent effect of SERMs in the skeleton. In conclusion, palmitoylated oestrogen receptors are detrimental to osteogenesis.

Palmitoylation-dependent modification of the 30-kD thyroid hormone receptor in osteoclasts

Thyroid hormone (TH) has a crucial effect in regulating bone growth and remodelling, stimulating bone resorption by osteoclasts and promoting new bone production by osteoblasts (Figure 5).[96] It has long been thought that TH stimulates target gene expression through both thyroid hormone receptor-α (TRα) and TRβ subtypes of the thyroid nuclear receptor. However, similar to the oestrogen receptor mentioned earlier, TH also exerts non-genomic effects.[97] Kalyanaraman et al[38] specifically focused on non-genomic TH signalling and identified the 30-kD and 33-kD TRα1 isoforms as crucial for TH to exert non-genomic effects. Notably, 30-kD TRα1 (p30 TRα1) undergoes palmitoylation at the Cys^{254} and Cys^{255} sites, a process essential for its membrane targeting and signalling. The mechanism can be summarized as palmitoylation of TRα1 subtype receptors promotes TH signalling, leading to increased intracellular calcium and NO/cGMP concentrations. These signalling events affect downstream processes associated with bone formation and osteoblast apoptosis (Figure 5). In conclusion, palmitoylated thyroid hormone receptor subtypes favour normal osteoclast function.

Discussion

In recent years, an increasing number of palmitoylated substrate proteins have been found to be involved in the regulation of osteoporosis. Key proteins in osteoblast development, differentiation, and bone homeostasis signalling pathways are palmitoylated to assume functions such as membrane targeting, membrane localization, and signal transduction. Our analysis indicates that the palmitoylation modification network remains to be improved, and a large number of palmitoylated membrane proteins and mechanisms involved in bone homeostasis await discovery. Palmitoylation and depalmitoylation are mutually reversible processes, and recent studies agree that palmitoylation is detrimental to osteogenic differentiation and depalmitoylation is beneficial to osteogenic differentiation; however, researchers have focused less on depalmitoylation than on palmitoylation in the mechanism of osteoporosis. Similarly, much less attention has been paid to osteoclasts than osteoblasts. Our summary also suggests that palmitoylation modifications tend to promote osteoclast development and function, and impair osteoblast differentiation and development, to promote osteoporosis progression. Unfortunately, however, the palmitoyl transferases acting in different experiments are not at all consistent, which adds to the difficulty of developing anti-osteoporosis drugs based on the transferases.

Bone metabolic balance has a complex and varied regulatory system. As reviewed in this article, palmitoylation plays a role in regulating the differentiation of osteoblasts and osteoclasts. However, few reports have concluded that osteocytes are modified by palmitoylation. One previous study has shown that various immune cells interact with osteoblasts and osteoclasts through intercellular contacts or paracrine mechanisms.[98] Palmitoylation modifications of immune cells have been studied,[61,99] but unfortunately there is a lack of research linking these findings with bone cells. In other words, whether palmitoylation-modified immune cells alter secretory regulation of osteoblasts is a research direction worth exploring. From our summary, endocrine hormones acting on osteoblasts or osteoclasts can be signal-transduced through membrane hormone receptors; whether palmitoylation modification of membrane receptors determines the efficiency of signal transduction, and whether insulin, thyroid hormone, and glucocorticoids also have palmitoylation involved in the effects of osteoporosis, are all to be further investigated in depth.

In conclusion, the refinement of the network of palmitoylated mechanisms involved in the regulation of osteoporosis provides possible targets for osteoporosis treatment, and helps to bridge the gap between the preclinical and clinical phases of anti-osteoporosis protein modification therapy. Jiang et al[100] have explored the intraperitoneal delivery of inhibitors specifically targeting palmitoyltransferase for hepatocellular carcinoma in preclinical studies. Similarly, we expect that future research will develop specific inhibitors against palmitoylated targets for the treatment of osteoporosis.

Supplementary material

PRISMA flowchart.

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