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REVIEW

The role of autophagy in bone metabolism and clinical significance

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ABSTRACT

The skeletal system is the basis of the vertebral body composition, which affords stabilization sites for muscle attachment, protects vital organs, stores mineral ions, supplies places to the hematopoietic system, and participates in complex endocrine and immune system. Not surprisingly, bones are constantly reabsorbed, formed, and remodeled under physiological conditions. Once bone metabolic homeostasis is interrupted (including inflammation, tumors, fractures, and bone metabolic diseases), the body rapidly initiates bone regeneration to maintain bone tissue structure and quality. Macroautophagy/autophagy is an essential metabolic process in eukaryotic cells, which maintains metabolic energy homeostasis and plays a vital role in bone regeneration by controlling molecular degradation and organelle renewal. One relatively new observation is that mesenchymal cells, osteoblasts, osteoclasts, osteocytes, chondrocytes, and vascularization process exhibit autophagy, and the molecular mechanisms and targets involved are being explored and updated. The role of autophagy is also emerging in degenerative diseases (intervertebral disc degeneration [IVDD], osteoarthritis [OA], etc.) and bone metabolic diseases (osteoporosis [OP], osteitis deformans, osteosclerosis). The use of autophagy regulators to modulate autophagy has benefited bone regeneration, including MTOR (mechanistic target of rapamycin kinase) inhibitors, AMPK activators, and emerging phytochemicals. The application of biomaterials (especially nanomaterials) to trigger autophagy is also an attractive research direction, which can exert superior therapeutic properties from the material-loaded molecules/drugs or the material's properties such as shape, roughness, surface chemistry, etc. All of these have essential clinical significance with the discovery of autophagy associated signals, pathways, mechanisms, and treatments in bone diseases in the future.

Abbreviations: Δψm: mitochondrial transmembrane potential AMPK: AMP-activated protein kinase ARO: autosomal recessive osteosclerosis ATF4: activating transcription factor 4 ATG: autophagy-related β-ECD: β-ecdysone BMSC: bone marrow mesenchymal stem cell ER: endoplasmic reticulum FOXO: forkhead box O GC: glucocorticoid HIF1A/HIF-1α: hypoxia inducible factor 1 subunit alpha HSC: hematopoietic stem cell HSP: heat shock protein IGF1: insulin like growth factor 1 IL1B/IL-1β: interleukin 1 beta IVDD: intervertebral disc degradation LPS: lipopolysaccharide MAPK: mitogen-activated protein kinase MSC: mesenchymal stem cell MTOR: mechanistic target of rapamycin kinase NP: nucleus pulposus NPWT: negative pressure wound therapy OA: osteoarthritis OP: osteoporosis PTH: parathyroid hormone ROS: reactive oxygen species SIRT1: sirtuin 1 SIRT3: sirtuin 3 SQSTM1/p62: sequestosome 1 TNFRSF11B/OPG: TNF receptor superfamily member 11b TNFRSF11A/RANK: tumor necrosis factor receptor superfamily, member 11a TNFSF11/RANKL: tumor necrosis factor (ligand) superfamily, member 11 TSC1: tuberous sclerosis complex 1 ULK1: unc-51 like autophagy activating kinase 1

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Introduction

The skeletal bone acts as the scaffold of the body to support the morphological and movement of organisms. Disrupting bone homeostasis leads to osteoporosis and fractures, often associated with aging, trauma, and disease. Fracture nonunion occurs in up to 15% of cases and causes tremendous social and economic burdens. According to the institute for Health Metrics and Evaluation, Global Burden of Disease (2019), the worldwide burden of musculoskeletal disorders is 150.08 million Disability-adjusted life years lost [1]]. Age-related fractures in the United States are projected to reach about 3 million by 2025 [2]. In 2021 alone, the total cost of orthopedic diseases in China reached about 19.59 billion yuan. Therefore, there is an urgent need for effective therapeutic approaches for bone disorders.

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To develop an optimal treatment, it is necessary to understand the cellular and molecular mechanisms underlying bone physiology and pathology. The cellular components in bone consist of osteocytes, osteoblasts, and osteoclasts. Osteocytes are the most abundant cell type in bone tissue, which originate from osteoblasts and assemble into bone. Osteoblasts derived from multipotent mesenchymal stem cells are responsible for new bone production and contribute to bone matrix mineralization. Osteoclasts, in contrast, are large multinucleated cells responsible for resorbing bone. Physiologically, bone undergoes a life-long remodeling consisting of bone formation and resorption. The dynamic balance between osteoblasts and osteoclasts maintains the homeostasis of the skeletal system [3]. This osteoblasts-osteoclasts coupling is regulated by many cellular functions and molecular signaling pathways, among which autophagy is a recently emerging mechanism in bone remodeling and regeneration.

Autophagy is a complex dynamic process involved in the degradation and correction of intracellular proteins and organelles under different physiological or pathological conditions, a vital function to maintain cell homeostasis in response to starvation, hypoxia, infection, and other stressful stimuli. Three types of autophagy have been identified in mammals: macroautophagy, microautophagy, and chaperone-mediated autophagy [4]. As the most common form, macroautophagy is closely related to cell physiology, biological behaviors, and diseases in bone and is referred to as autophagy in this review.

Autophagy is highly involved in the metabolism of bone tissue. Autophagy has been reported to play necessary roles in the homeostasis of bone marrow mesenchymal stem cells (BMSCs) derived cells (including adipocytes, chondrocytes, osteoblasts, osteocytes, etc.) [5]. Interestingly, Nuschke et al. [6]. observed an accumulation of numerous undegraded autophagic vacuoles with halted autophagic flux in undifferentiated mesenchymal stem cells (MSCs). During early-stage osteogenic differentiation, the expression of autophagosome marker LC3-II in MSC is reduced within 12 h, suggesting that these accumulated autophagic vacuoles may serve as a source of rapidly produced energy substrates to support MSC differentiation [7]. Osteoblasts are specialized mesenchymal-derived cells involved in bone formation, and impaired autophagy in osteoblasts leads to decreased bone mass [8]. It is also worth noting that inhibiting autophagy in osteocytes results in bone tissue senescence [9]. Autophagic proteins are also necessary for osteoclast-directed bone resorption [10]. Moreover, autophagy contributes to pre-osteoblast differentiation, osteoblast-osteocyte transition, and the genesis and functioning of osteoclasts [11]. All these findings suggest that autophagy plays a decisive role in bone remodeling and regeneration, which could be further explored.

In the current review, authors summarized the recent findings on the role of autophagy regulation on osteogenesis under different physiological and pathological conditions, to facilitate a better understanding of the mechanisms of autophagy in bone regeneration, therefore shedding new light on new diagnostic tools and therapeutic approaches for bone associated diseases in the future.

Overview of autophagy and mitophagy

Autophagy mainly involves the formation of autophagosomes by endoplasmic reticulum (ER) and mitochondria-derived monolayer or bilayer membranes. Then it fuses with lysosomes to form autolysosomes, which contain hydrolases to promote the degradation of related substrates [12]. Autophagy is a highly evolutionarily-conserved cellular process mediated by *ATG* (autophagy related) genes. *ATG* genes and ATG proteins are considered the core mechanism for autophagosome biogenesis. For example, the yeast Atg13-Atg1-Atg17 complex initiates autophagy [13]; in mammals, phosphatidylinositol 3-kinase (PtdIns3K) and BECN1 are involved in the localization of ATG proteins on phagophore membrane [13]. The ATG12–ATG5-ATG16L1 complex and LC3-II participate in phagophore cell membrane extension [14] (Figure 1). Additionally, ATG proteins are involved in coordinating cell homeostasis, regulating the cell cycle, modulating the immune response, and inflammation, etc [15].

Mitophagy is a selective form of autophagy that effectively removes the damaged and dysfunctional mitochondria in response to various metabolic stresses, such as inflammation, hypoxia, genotoxicity, and nutritional deficiencies [16,17]. Mitochondria are primary subcellular organelles that generate ATP, which is essential for the survival and function of cells. In addition, they are signaling centers that regulate Ca^{2+}, energy metabolism, and oxidative stress and determine the fate of bone tissue. Mitochondria suffer structural and functional damage when they are exposed to both endogenous (oxidative toxicants and DNA damage) and exogenous (environmental) stress [18]. Hence, mitophagy serves as a critical mitochondrial quality-control mechanism to maintain mitochondrial function and homeostasis, and a protective approach to reduce the intracellular reactive oxidative species (ROS) level to prevent the cell from oxidative damage [16,17].

The damaged/dysfunctional mitochondria can result in host cell death/dysfunction in at least three ways: 1) ROS are produced during mitochondrial oxidative phosphorylation, and excessive accumulation of ROS can damage mitochondrial DNA, leading to mitochondrial cycle collapse and premature apoptosis of cells. 2) Damaged/dysfunctional mitochondria produce and promote inflammatory signaling. ROS produced by mitochondria activates the inflammasome composed of NLRP3, receptor protein PYCARD/ASC, and CASP1 (caspase 1), which occurs on the mitochondria-associated endoplasmic reticulum. 3) Damaged/dysfunctional mitochondrial outer membrane permeation releases mitochondrial intermembrane proteins leading to apoptosis [19]. Autophagy is activated to isolate and digest parts of the cytoplasm, mitochondria, and other organelles in response to excessive ROS or DNA damage. Notably, mitochondria play a crucial role in autophagy because they provide biofilms to develop autophagosomes. ULK1 is one of the core proteins related to human autophagy, which receives signals from MTOR and AMP-activated protein kinase (AMPK) to initiate autophagy and recruit downstream ATG proteins to autophagosome formation sites. In mitochondria, especially under hypoxia conditions, ULK1 can translocate into mitochondria, and ULK1 interacts with FUNDC1 to enhance the binding of

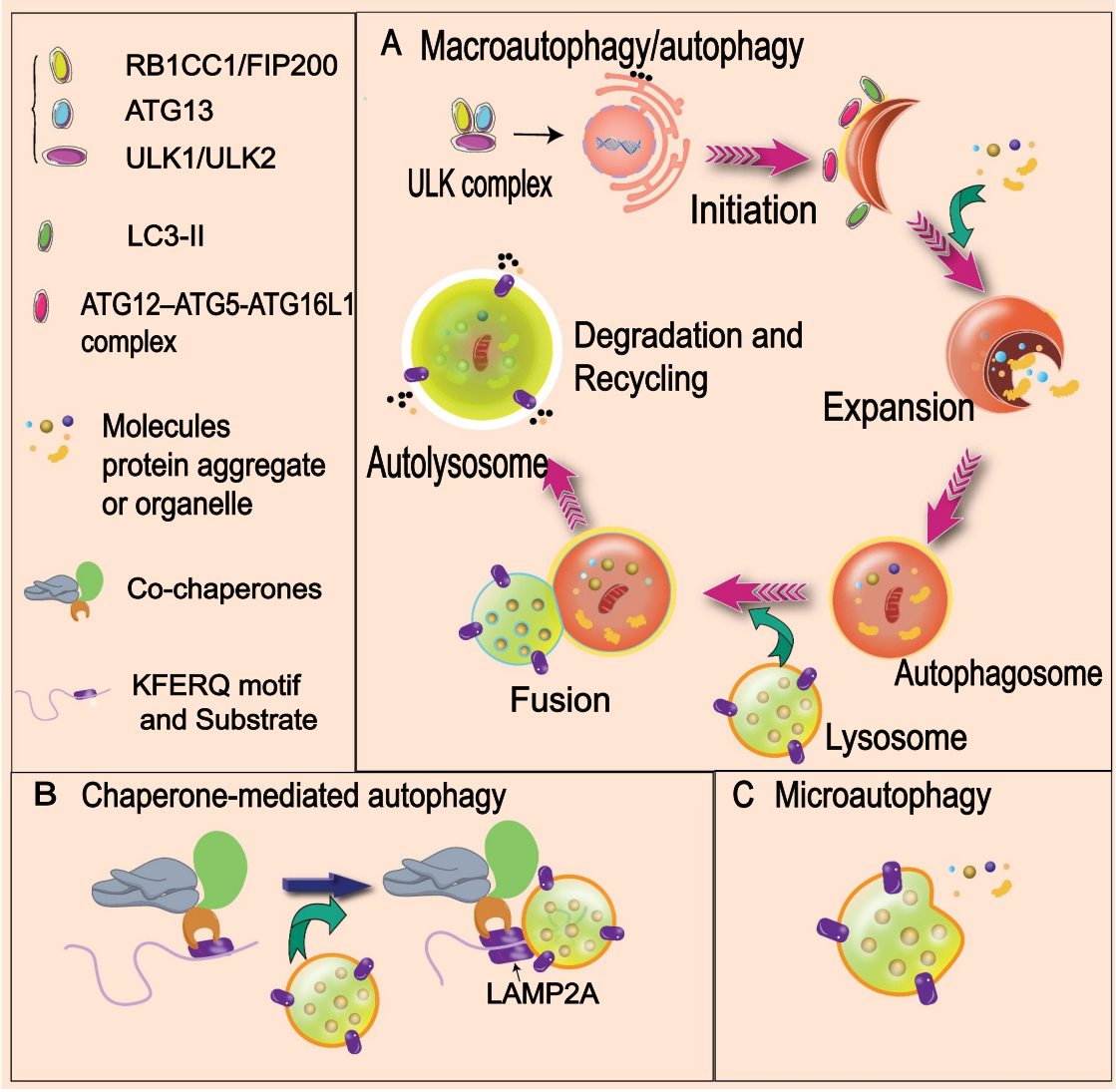

Figure 1. Schematic diagram of the three primary types of autophagy. (A) Macroautophagy/autophagy. (B) Chaperone-mediated autophagy. (C) Microautophagy. The main stages of macroautophagy are presented, including initiation, nucleation, expansion, closure and maturation, fusion, and degradation. All these processes help to complete the recycling of "waste" in the body.

FUNDC1 to LC3 and induce mitophagy [20]. Accordingly, ULK1 deficiency results in defective mitophagy, abnormal mitochondrial morphology, and mitochondrial transmembrane potential ($\Delta\psi m$) in primary hepatocytes [21]. Another protein, BNIP3, can trigger mitophagy by competitively disrupting the inhibition between BCL2 and BECN1 [22]. The entry of PINK1 (a key mitophagy protein) into healthy mitochondria depends on $\Delta\psi m$. Mitochondrial damage leads to PINK1 accumulation and recruits the ubiquitin ligase PRKN/parkin, inhibiting mitochondrial fusion and promoting mitophagy [23]. The interaction between different mechanisms of mitophagy is still unclear. Still, almost all of them control the mitochondria quality through mitophagy and avoid the adverse effects of damaged mitochondrial accumulation to effectively prevent diseases.

Lack of mitophagy is indeed linked to various human disorders, including aging, cardiac diseases, senile dementia, and degenerative diseases such as IVDD and OA, all of which are related to dysfunction of mitophagy maintenance of cellular energy homeostasis and functions [24]. Accumulating evidence reveals that mitophagy deficiency is detrimental to BMSCs survival and osteogenic differentiation. Accordingly, mitophagy can be induced to protect BMSCs from apoptosis or necrosis and allow them to survive under certain stress conditions [25]. In addition, mitophagy can remove dysfunctional mitochondria, reduce oxidative stress and prevent the occurrence of bone tumors [26]. Therefore, a better understanding of the biological function of mitophagy in bone regeneration and the associated molecular mechanisms will be beneficial for developing bone healing therapeutics.

The role of autophagy in bone metabolism

Autophagy in osteogenesis of MSCs

MSCs are found in various tissues, including dental pulp, kidney, brain, adipose tissue, and bone marrow. Due to the

high cell numbers and proliferative activity, BMSCs are widely used in preclinical and clinical studies. MSC can be differentiated into multiple mesenchymal lineages, including fat, muscle, cartilage, and bone. It has attracted much attention in scientific research and clinical practice due to its multidirectional differentiation potential, immunomodulatory effect, and promotion of tissue and organ repair [27]. MSC-derived secreted factors include cytokines, chemokines, growth factors, or extracellular vesicles, which are key regulators of regeneration by carrying or transmitting signals to regulate endogenous cell proliferation, migration, differentiation, activation, or immune cell activation/function [28].

It is currently recognized that autophagy and MSC participate in tissue regeneration *via* two mechanisms: i) Autophagy level can determine the regeneration potential and function of MSC; ii) MSC can regulate the autophagy level of other cells in the damaged tissues/organs, especially the inflammatory immune cells, by reducing their proliferation, survival, and function and contributing to inflammation suppression [14]. Previous studies have observed that undifferentiated MSCs contain more autophagosomes than differentiated ones, demonstrating that autophagy plays a crucial role in MSC differentiation. The accumulated autophagosomes can degrade rapidly when MSCs are exposed to osteogenesis stimulation, providing the corresponding energy and metabolic precursors for the morphological, function, and metabolism changes required for cell differentiation [29]. Another study also found that changes in autophagy flux may affect MSC secretion and thus change its function, by activating or inhibiting autophagy in MSC with rapamycin or *Belin1*-silencing, respectively. MSCs pretreated with rapamycin show enhanced wound healing capacity, whereas those with *Becn1*-silencing show the opposite effect [30]. One possible mechanism is that autophagy enhances VEGF secretion from MSC to improve angiogenesis through the phosphorylation of MAPK/ERK since angiogenesis plays a central role in wound healing [30]. Taken together, autophagy plays indispensable roles in MSC-directed tissue regeneration by providing energy and metabolic precursors required for MSC differentiation and regulating MSC secretion of regenerative growth factors.

Autophagy in osteoblast differentiation and function

Osteoblasts, the bone-forming cells of the remodeling unit, secrete the organic matrix of bone and participate in the mineralization process. During bone formation, osteoblasts can further differentiate into osteocytes—which are embedded in their mineralized matrix and exhibit unique morphology and function [7].

The increased level of autophagy in osteoblast differentiation was first published in 2013 [31]. Subsequent studies have also demonstrated that a lack of autophagy reduces osteoblast mineralization and disrupts the balance between osteoblasts and osteoclasts. The autophagic proteins BECN1, ATG5, and ATG7 are required for the mineralization of osteoblast cell lines. The ATG5 deficiency is associated with increased osteoclast number and decreased bone volume [32]. Appropriate levels of autophagy maintain osteoblasts' survival and function (Figure 2). Upon autophagy inhibition, the elevated oxidative stress inhibits the differentiation of osteoblasts and leads to cell apoptosis. Early autophagy initiation alleviates the damage to osteoblasts caused by oxidative stress, which is closely related to the ER stress pathway [8]. Additionally,

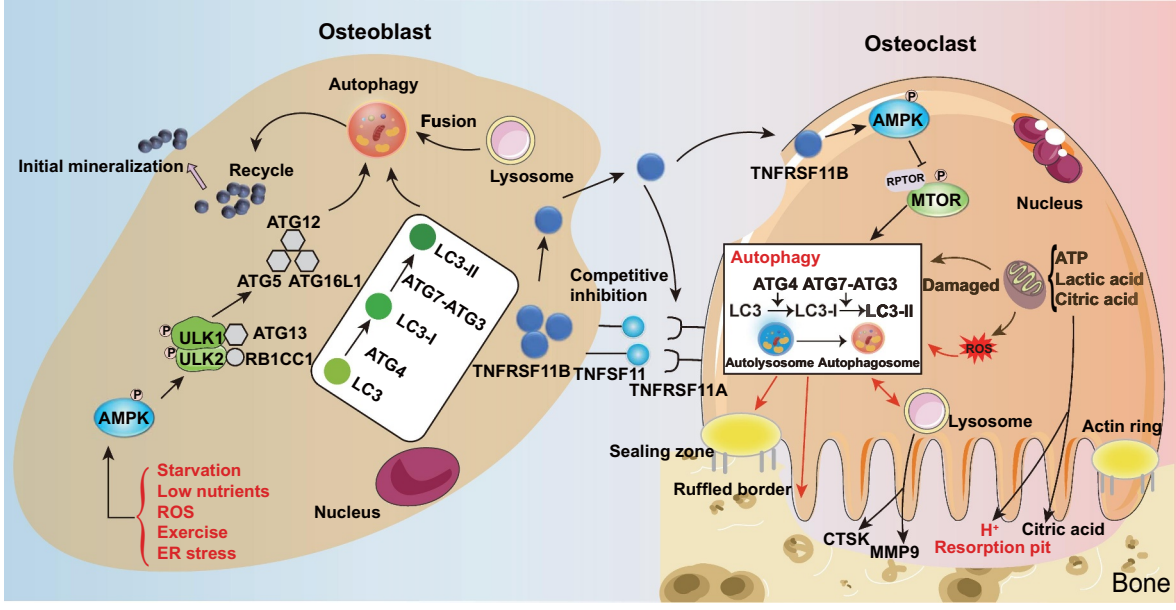

Figure 2. The role of autophagy in osteoblast and osteoclast involved in bone metabolism. Autophagy is activated under stress conditions (oxidative stress, hypoxia, starvation, inflammation); for osteoblast, ROS and ER stress participate in autophagy regulation, promote the reuse of intracellular substances and release vesicles to participate in the initial mineralization process; TNFRSF11B is a decoy receptor of TNFSF11 and competitively inhibits osteoclast differentiation and maturation by blocking the interaction between TNFSF11 and TNFRSF11A. TNFRSF11B inhibits osteoclast and bone resorption by enhancing autophagy through activation of AMPK-MTOR signaling pathway. For osteoclasts, autophagy is involved in forming the ruffled border and actin rings [33], as well as releasing lysosomal proteolytic enzymes, including CTSK and MMP9. Mitochondria can provide ATP and release acidic substances (such as citric acid and lactic acid) to the bone resorption pit. Mitophagy to clear the damaged mitochondria can counter osteoclast-derived bone resorption.

autophagy protects osteoblasts from toxic stimuli (e.g., lead chloride) and stressful environments [34]. Negative pressure wound therapy (NPWT) is widely used in clinical practice and can affect various cell types, including BMSC. NPWT promotes the osteogenic differentiation of BMSCs [35] by activating the AMPK signaling, as detected in the rat cranium defect under NPWT treatment. Subsequently, the AMPK signaling induces the phosphorylation of ULK1 to activate autophagy. Thereby, this study found an axis of AMPK-ULK1-autophagy in response to NPWT stress which promoting osteoblast differentiation and bone regeneration [36]. Moreover, mice with deletion of the autophagy suppressor gene RUBCN/Rubicon show specifically upregulated expression of osteoblasts and degradation of the NOTCH intracellular domain, which promote osteoblast differentiation and effectively prevented OP [37].

Conversely, autophagy inhibition has reduced osteogenesis/induce osteoclastogenesis. Deletion of the *Becn1 or Atg7* gene significantly reduces the mineralization capacity of osteoblasts. Similarly, a targeted *Atg5* inactivation experiences a 50% reduction in trabecular bone mass. In addition, autophagy-deficient osteoblasts show increased oxidative stress and enhanced secretion of TNFSF11/RANKL (TNF superfamily member 11), which was conducive to the formation of osteoclasts [32]. *Atg7* deficiency hampers osteoblasts mineralization and promotes apoptosis partially through the DDIT3/CHOP (DNA damage inducible transcript 3) and MAPK8/JNK1 (mitogen-activated protein kinase 8)-SMAD1-SMAD5-SMAD9/SMAD8 pathway *in vitro*. Notably, the inhibited-osteoblast function (due to *atg7* knockout) and skeletal balance can be eliminated by removing stress [8].

The mechanisms of autophagy-regulated osteoblast differentiation/function could be partially explained by the fact that autophagy actively interacts with the osteogenic signaling pathways. IGF1 (insulin like growth factor 1) promotes osteoblast differentiation and function by activating AMPK and stimulating autophagy [38]. Interestingly, in a study using pluripotent stem cells (cranial neural crest cells) in bone formation, it was found that the enhanced BMP signaling inhibits autophagy and thus blocks the autophagy degradation of CTNNB1/β-catenin, which promotes bone and cartilage tissue formation [39]. In addition, the deficiency of the autophagy gene *Atg7* in chondrocytes leads to the retention of procollagen type II in the ER, which highly reduces the content of type II collagen in the cartilage matrix. Autophagy acts as an FGF-signaling agent in endochondral bone formation mediated by FGF18 activation of BECN1 *via* FGFR4 and MAPK/JNK pathways [40]. Homozygous mutant mice with *nbr1* gene knockout (an autophagy cargo receptor) show increased bone mass by about 50% at 6 months of age. The improved osteoblast differentiation is achieved through the MAPK/p38 (mitogen-activated protein kinase) pathway [41].

The FOXO (forkhead box O) transcription factor family mediates the survival, differentiation, and function of osteoblasts in response to autophagy activation [42]. FOXO binds to the autophagy gene promoter fragment directly to enhance autophagy flux and synergies with AMPK to enhance autophagy and maintain protein homeostasis [43]. During MSC differentiation into osteoblast, FOXO negatively regulates adipogenesis by inhibiting the transcription and expression of PPARG/PPARγ, a major transcription factor for adipogenesis [44]. This suggests that FOXO may partially facilitate MSC differentiation into osteoblast because of the inhibition of MSC adipogenic differentiation. In addition, aging results in decreased MSC activity and reduced osteogenic differentiation, while FOXO1, FOXO3 and FOXO4 overexpression prevents aging-related bone loss. FOXO1 upregulates proteoglycan 4 expression to maintain cartilage surface region integrity, and FOXO1 overexpression also antagonizes IL1B/IL-1β (interleukin 1 beta)-mediated bone damage [45]. Since autophagy maintains cell survival and participates in senescence-related cell physiology, FOXO is speculated to be a crosstalk with autophagy in maintaining bone homeostasis and promoting chondrogenesis and maturation.

ATF4 (activating transcription factor 4) is associated with osteoblasts function and autophagy activity, which is necessary for osteoblast terminal differentiation and bone formation. Similarly, the injection of extracellular vesicles containing ATF4 enhanced the autophagy of chondrocytes to reduce chondrocyte apoptosis in OA [46]. Overexpression of ATF4 in fibroblasts promotes osteocalcin synthesis and expression of osteogenic genes (*Runx2* and *Sp7/Osterix*) [47]. Interestingly, ATF4 is closely related to autophagosome formation, elongation, and function, which binds to the AARE sequence within the *Sqstm1/p62* promoter to trigger different transcriptional responses according to stress intensity. Therefore, ATF4 contributes to the survival and function of osteoblasts under various stress conditions [48].

Taken together, autophagy is activated and plays an indispensable role in osteoblast differentiation and mineralization by interacting with osteogenic signaling pathways and transcriptional factors. Meanwhile, autophagy inhibition can facilitate osteoclastogenesis by inducing TNFSF11 production of osteoblasts, suggesting the critical role of autophagy in determining bone remodeling balance.

Autophagy in osteocyte differentiation and function

Osteocytes are the terminally differentiated osteoblast lineage cells. As the most abundant cell type in bone, osteocytes are embedded in bone matrix and facilitate the maintenance of bone mineral homeostasis. Due to their long lifespan and location, these cells are susceptible to stress stimulations. They are therefore presumed to be highly dependent on autophagy for survival (Figure 2). In fact, autophagy deficiency may lead to osteocyte dysfunction. Osteocytes from murine and human cortical bone tissue express a punctuated LC3 distribution, suggesting autophagy should be maintained at a certain level in osteocytes to make them survive the microenvironment changes such as starvation and hypoxia [49]. Bone mineralization can be divided into primary mineralization (rapid onset) and secondary mineralization (slow progression), the latter of which is also thought to be associated with increased autophagy in osteocytes [50].

Despite maintaining osteocyte physiology, autophagy is involved in the regulation of growth hormones on bone metabolism. Glucocorticoid (GC) treatment increases bone fragility, leading to OP, and the activation of autophagy in

osteocytes may be the primary mechanism in response to high GC. GC treatment results in increased expression of autophagy markers (LC3A, LC3B, ATG2, ATG7), and the accumulation of autophagosomes *in vitro and in vivo*, suggesting the promoted autophagy level in osteocytes [51]. However, the induced autophagy in osteocytes may play a protective role against OP since low doses of GC increase autophagy activity and antioxidant response by about 20–30 times, whereas high doses downregulate antioxidant protein gene expression and the number of autophagic osteocytes [52].

Osteocytes are sensitive to mechanical stimulation. Mechanical loading is a particularly effective stimulus for bone cells to enhance bone strength and prevent bone loss due to aging. Recent studies have determined that fluid shear stress (FSS) stimulates protective autophagy in osteocytes. ATP can be loaded into autophagic vacuoles and released into extracellular space in response to starvation by fusing with the plasma membrane. FSS facilitates the induced release of ATP from the autophagic vacuoles, therefore providing energy to help bone cells to tolerate stress stimuli and promote osteocytes' survival [49]. Thus, autophagy in response to induced mechanical load is considered an adaptive response of osteocytes *via* regulating ATP synthesis and release.

In summary, autophagy plays a central role in maintaining the homeostasis of osteocytes, which ensures osteocytes' viability and function under stress conditions such as starvation and hypoxia. Furthermore, autophagy participates in bone mineralization and plays a protective role in GC treatment to prevent OP. In addition, autophagy fuels osteocytes with more energy (in the form of ATP) under fluidic mechanical stimulation.

Autophagy in osteoclastogenesis

Osteoclasts are giant, multinucleated cells that resorb bone and maintain bone development and remodeling. The activity and function of osteoclasts are closely related to the TNFRSF11A/RANK (TNF receptor superfamily member 11a)-TNFSF11 pathway. Excess osteoclast activity leads to bone loss and OP, whereas inhibition of osteoclast activity leads to osteopetrosis and bone marrow failure [53]. Abnormal osteoblast necrosis stimulates osteoclast-directed bone resorption by macrophage-induced CLEC (C-type lectin) [54]. Therefore, changes in the microenvironment *in vivo* lead to osteoclast inhibition or overactivation, which is detrimental to maintaining bone tissue homeostasis. Here, we mainly discuss the effect of autophagy on osteoclast activity and the associated mechanisms (Figure 2).

Local environmental changes such as hypoxia and pressure facilitate osteoclast autophagy. HIF1A/HIF-1α (hypoxia inducible factor 1 subunit alpha) stimulates the expression of BECN1, ATG5, and LC3, thereby enhancing the expression of NFATC1 (nuclear factor of activated T cells 1), a key marker for osteoclastogenesis, and MMPs (matrix metallopeptidases) to promote the proliferation and differentiation of osteoclasts [55]. Further studies identified the HIF1A-*MIR20A*-ATG16L1 axis as the mechanism for HIF1A-induced autophagy in osteoclast [56]. Scaffold protein GIT1 (GIT ArfGAP 1) facilitates osteoclast differentiation. Knockdown and overexpression of GIT1 showed corresponding autophagy flux, possibly because GIT1 stimulates the phosphorylation of BECN1 and its dissociation from BCL2. Correspondingly, *GIT1* knockout reduces the number of osteoclasts at the fracture site [57]. Another protein that plays a vital role in osteoclastogenesis is SQSTM1/p62, an autophagy receptor protein in TNFSF11-induced osteoclast differentiation, which functions by activating osteoclastogenesis-associated transcription factor NFATC1 and NFKB/NF-κB signaling [58]. It has been shown that TNFSF11 stimulates osteoclast differentiation by TRA6 (TNF receptor associated factor 6) mediated BECN1 (an ATG-related protein) ubiquitination rather than ULK1-mediated phosphorylation. BECN1-deficient mice show abnormal cortical bone thickness because of bone resorption deficiency [59]. Therefore, BECN1 plays an essential role in osteoclasts formation and differentiation.

Osteoclasts can migrate in the bone matrix to better utilize bone resorption capacity. The migration process is modulated by continuous rapid assembly and disassembly of podosome rings from the dot-like core of actin filaments. FERMT3/Kindlin 3 (a crucial cohesion protein in podosomes) interacts with LC3B and undergoes autophagy-mediated protein degradation, promoting podosome disassembly. Inhibition of autophagy enhances FERMT3/kindlin3 expression and its interaction with ITGB3 (integrin subunit beta 3), and overactivated ITGB3 leads to impaired osteoclast migration [60]. Therefore, LC3 is an important target for regulating osteoclasts migration.

Since autophagy plays a vital role in the proliferation, differentiation, migration, and function of osteoclasts, inhibition of autophagy flux in osteoclasts can reduce overall autophagy activation and bone resorption, which is important for maintaining bone homeostasis and promoting bone regeneration. IL17A regulates apoptosis of osteoclast precursor through BECN1-autophagy-TRAF3 signal transduction, thus affecting osteoclast formation [61]. Consistently, ursolic acid [62], 4-phenyl butyric acid [63], and W9 peptide [64] significantly inhibit autophagy and thus hinder osteoclast formation and differentiation, therefore reducing OP. TNFRSF11B/Osteoprotegerin is a classical osteoclastogenesis inhibitor, which binds with TNFSF11 to interrupt the TNFSF11-TNFRSF11A axis. A recent study found that this anti-osteoclastogenic effect is partly achieved via autophagy through the AMPK-MTOR-RPS6KB/p70S6K pathway [65]. MBTPS1/Site-1 protease, a protein in the Golgi apparatus, plays indispensable roles in ER stress, lipid metabolism, and inflammatory response. MBTPS1 is a positive regulator of osteoclastogenesis, and the elimination of the *MBTPS1* gene leads to obvious osteosclerosis due to reduced bone resorption in mice. MBTPS1 reduces the expression of LC3 and autophagy flux, and transfection of LC3 adenovirus (for LC3-silencing) significantly eliminated osteosclerosis in *Mbtps1*-deficient mice. Therefore, the regulation of MBTPS1 on osteoclastogenesis is partially in an LC3- and autophagy-dependent manner [66].

Evidence indicates that astronauts experience severe bone loss during space assignments. Simulated microgravity (μXg) conditions upregulate autophagy-related cell signaling molecules, autophagosome components, and inflammatory

cytokines, which jointly adjust the autophagy of preosteoclasts and regulate osteoclast differentiation. Therefore, targeting osteoclast differentiation can effectively prevent OP caused by μXg. The autophagy inhibitors 3-methyladenine (3-MA) and chloroquine (CQ) have significantly inhibit μXg-induced osteoclast differentiation, as well as apoptosis and cell cycle arrest [67].

Taken together, autophagy plays critical roles in bone formation and resorption due to its indispensable roles in the function of osteocyte and differentiation of osteoblast and osteoclast (Figure 2). Although detailed mechanisms are to be explored, autophagy participates in cell differentiation by providing extra energy and facilitating cell survival to stress conditions such as ROS accumulation and mitochondrial damage. To modulate bone remodeling balance, a cell-specified (osteoblast or osteoclast targeting) autophagy regulation is preferred, as a general induction or reduction of autophagy may simultaneously affect both bone formation and resorption.

The role of mitophagy in bone metabolism

Mitophagy in MSCs

Mitochondrial dysfunction in MSCs affects cell fate and results in chronic inflammatory bone diseases such as periodontitis and OA. In chronic inflammation, mitochondrial calcium overload (excessive Ca^{2+} transferred from the ER to mitochondria) and the accumulation of damaged mitochondria (WNT-CTNNB1 pathway activated to inhibit mitophagy) can impair the function and differentiation of MSCs. Recently designed responsive nanoparticles can capture Ca^{2+} to regulate mitochondrial calcium flux, and serve as vehicles for siRNA delivery to induce a targeted inhibition of the WNT-CTNNB1 pathway to repair and control mitochondrial quality [68]. In another study, carbon black treatment inhibits the osteogenesis of MSCs and mitochondrial biogenesis. Meanwhile, the accumulation of PINK1 and PRKN recruitment are observed in carbon black-treated cells, suggesting mitophagy was increased in response to mitochondrial inhibition to remove the damaged mitochondria [69]. Recent evidence suggests that MSCs can communicate through a bidirectional mitochondrial exchange in the microenvironment. Mitochondria of the damaged cells act as signals that reduce the apoptosis of MSCs. Mitophagy is triggered for mitochondrial recovery when damaged mitochondria are transferred and accumulate in MSCs. Then MSCs donate mitochondria to the damaged cells to enhance their ability to resist oxidative stress damage [70]. Therefore, it is speculated that intercellular mitochondrial transmission and energy recovery can serve as key mechanisms to protect MSC viability and function by preventing the cell from oxidative damage and guaranteeing mitochondrial quality control. This suggests mitophagy can serve as a target for improving stem cell therapy in the future.

Mitophagy in osteoblasts and osteoclasts

Mitochondria are crucial in the differentiation of bone progenitor cells. Mitochondrial biosynthesis, function, and ATP content are significantly increased during osteogenic differentiation [71]. In contrast, osteoclasts have lower ATP levels, and high ATP consumption leads to stress mitochondrial crest distortion and enhanced osteoclast-derived bone resorption [72]. Mitophagy is responsible for intracellular homeostasis under physiological conditions and the degradation of damaged mitochondria under pathological conditions. Mitophagy-directed removal of damaged mitochondria is also considered to prevent osteoblast apoptosis [73]. Other studies have also demonstrated the positive effects of mitophagy on osteoblasts. Estrogen-derived activation on mitophagy GPER1/GPR30-MAPK1/ERK2-MAPK3/ERK1 signaling pathway can promote osteoblast proliferation [74]. Mitophagy mediated by PINK1-PRKN signaling positively regulates osteogenesis and mineralization in type 2 diabetic mice [75]. Mitophagy can counteract the damage of environmental metal poison aluminum, enhance osteoblasts' activity, increase the calcium and phosphorus content, and improve the expression of collagen type I and BGLAP/osteocalcin [76]. Mitophagy can reduce ROS production by damaging mitochondria, limit the energy requirements of ineffective organelles, maintain ATP production during physiological and pathological degradation, and facilitate the recovery of intracellular components to keep tissue homeostasis. In a mice model with autophagy deficiency in osteoblast lineage, an accumulation of ER and mitochondria was observed in osteoblasts, resulting in low bone mass and more fractures [77]. This suggests that the inhibition of mitophagy results in difficulty in eliminating excessive mitochondrial fragments and swollen mitochondria, and functional mitophagy may be crucial for maintaining the survival and function of osteoblasts.

Studies have shown that the accumulation of ROS released by damaged mitochondria leads to the activation of NLRP3 in the inflammasome, producing inflammatory factors to promote osteoclast differentiation and maturation [78]. It is noteworthy that ULK1-induced mitophagy is closely associated with osteolytic diseases. Knockout of the *ULK1* gene resulted in more mature multinucleated osteoclasts, which could be reversed by reconstructing ULK1 expression. This was mainly due to the upregulation of osteoclast function-related genes *Ctsk, Car2, Ttgav*, and *Itga3* in the absence of ULK1. Accordingly, when *ULK1* is knocked out, the total mitochondrial mass and ROS are significantly increased, which is also caused by reduced mitophagy [24]. NAD+-dependent SIRT3 (sirtuin 3) is a mitochondrial protein deacetylase that plays a vital role in mitochondrial biogenesis, mitochondrial dynamics, and mitophagy. Similarly, the weakened mitochondrial respiratory chain of osteoclasts in *sirt3*-KO elderly mice also generates an inhibitory effect on osteoclastogenesis. SIRT3 increases the expression of mitophagy through PINK1, but the levels of autophagy markers LC3 and SQSTM1 are almost unaffected [74]. Therefore, the osteoclast differentiation promoted by Sirt3 may be due to the regulation of mitophagy in osteoclasts through the deacetylation of PINK1.

Taken together, it is speculated that mitophagy can modulate bone remodeling balance by acting in different roles in the differentiation of osteoblast and osteoclast (Figure 3). In

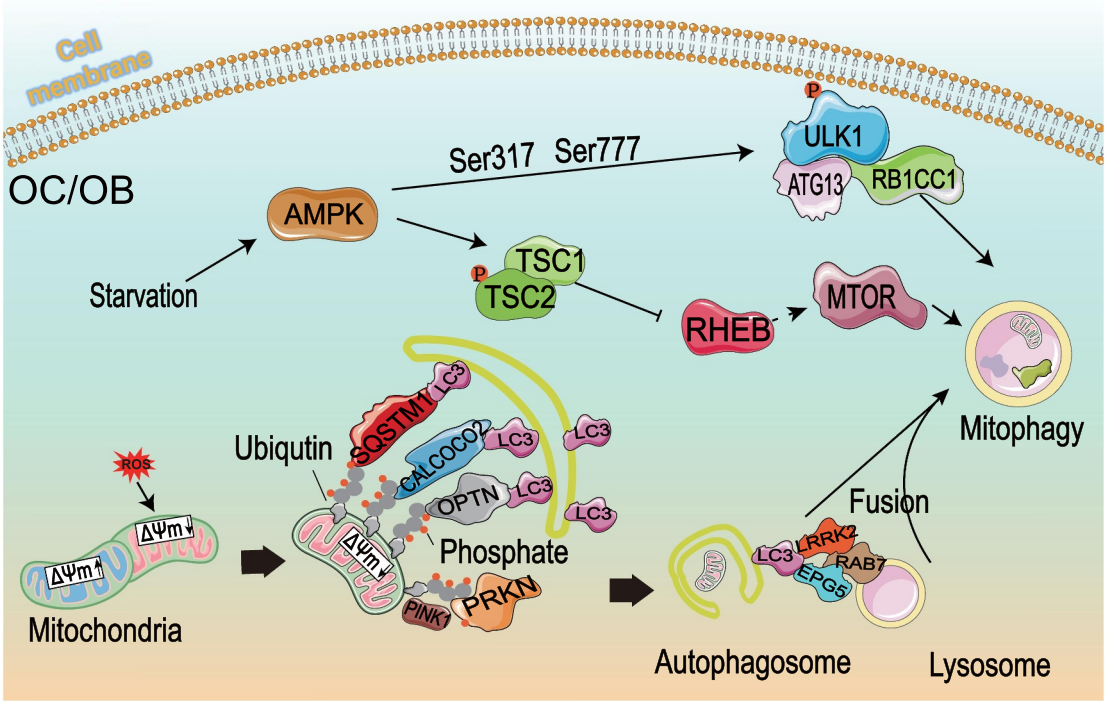

Figure 3. Mitophagy and its role in osteoclastogenesis and osteoblast differentiation. ROS and other stresses (e.g., starvation and DNA damage) can destroy mitochondria and thereby lead to δψm depolarized in osteoclasts and osteoblasts to induce mitophagy in a PRKN-dependent manner: Mitochondria depolarize during stress conditions, phosphorylated PINK1 accumulates and subsequently recruit PRKN to mitochondria. PINK1 then induces phosphorylation of PRKN and ubiquitinated mitochondrial outer membrane proteins, which can bind LC3 directly in autophagosome membranes or indirectly through SQSTM1, OPTN, and CALCOCO2/NDP52. The process of mitophagy is completed through the ligand fusion of LC3-II and EGF5-RAB7-LRRK2 on lysosome. When osteoclasts and osteoblasts are deficient in energy, AMPK is activated and phosphorylates TSC2 to inhibit MTOR. Conversely, phosphorylation of AMPK at Ser317 and Ser777 activates ULK1, and the activated ULK1-ATG13-RB1CC1 protease complex stimulates autophagy and mitophagy.

osteoblast, mitophagy to relieve oxidative stress is required to maintain the differentiation and function of osteoblast, and mitophagy-inhibition can significantly impair bone healing. Conversely, mitophagy plays a negative role in osteoclastogenesis by interrupting osteoclast function; additionally, mitophagy can result in an environment unfavorable for osteoclastogenesis by removing ROS and lowering the release of the inflammasome. Therefore, mitophagy is considered to favor bone formation over resorption.

Mitophagy in hematopoietic stem cells

Hematopoiesis is the highly specialized process of blood formation from hematopoietic stem cells (HSC) in the bone marrow stroma. It fulfills the physiological or stress needs by switching between inactive and active states. The content of mitochondria in HSCs changes with HSC proliferation and differentiation. Still, once its integrity is damaged or its dysfunction leads to mitochondrial accumulation, hematopoiesis will be damaged, and bone healing will be adversely affected. Hematologic pathology associated with mitochondrial dysfunction includes anemia, leukopenia, neutropenia, and platelet deficiency. Reduced oxygen delivery due to decreased hemoglobin [79], insufficient cytokine secretion due to downregulated neutrophils [80], and platelet-poor plasma [81] have been shown to affect the process of bone regeneration negatively. It is foreseeable that by the role of mitophagy in mitochondrial transformation and the regulation of mitochondrial function, the homeostasis of bone marrow hematopoietic microenvironment can be restored to a certain extent, which is also significant for the study of bone regeneration.

Autophagy in the pathogenesis of bone diseases

Degenerative orthopedic diseases

IVDD and OA are the leading causes of chronic low back pain and disability in the elderly, respectively, which seriously affect the patient's life quality and cause a tremendous social burden. The inner NP, a component of the intervertebral disc, contains chondrocyte-like cells. Oxidative stress injury can result in mitochondrial dysfunction and damaged mitochondrial accumulation in NP cells and chondrocytes, which is critical for the progression of IVDD and OA. Under H_2O_2 stimulation (an *in vitro* model of ROS accumulation), mitochondrial dysfunction, mitophagy, and cellular senescence were induced in NP cells. When mitophagy was reduced *via* PINK1-depletion, NP cell senescence was exacerbated, suggesting the critical role of mitophagy to prevent senescence in NP cells [82]. Similarly, inhibition of autophagy flux in the cartilage endplate of the intervertebral disc leads to apoptosis and disc degeneration [83]. Intervertebral discs suffer from mechanical stress, and excessive stress, such as compression, can promote mitophagy, senescence, or damage in NP cells in a time-dependent manner by promoting PINK1-PRKN [84].

In addition, the fusion of autophagosome and lysosome into autolysosome is interrupted by the abnormal lysosome. The compression force of 1.0 MPa initiates mitophagy but hinders mitophagy flux, which is also attributed to mitochondrial lysosomal fusion damage and lysosomal degradation in NP cells. Fortunately, the mitochondrial-targeted antioxidant MitoQ can rescue the compression-induced mitochondrial dysfunction and NP cell apoptosis by maintaining mitophagy flux and mitochondrial kinetic balance [85].

Similar to the role of autophagy in IVDD, more and more evidence also indicates that autophagy plays a vital role in the pathological development of OA. The progression of OA inflammation involves the regulation of autophagy. IL1B, a key OA-related mediator, stimulates chondrocytes to overproduce ROS, which results in the accumulation of impaired mitochondria and mitochondrial dysfunction. Overexpression of PRKN to induce mitophagy facilitates the clearance of dysfunctional mitochondria and reduces ROS and chondrocyte apoptosis [86]. Enhanced autophagy not only improves the pathological progression of OA in rats but also improves chondrocyte apoptosis induced by IL1B stimulation, which is partially mediated by isopsoralen-induced autophagy through the AMPK-MTOR pathway [87]. Similarly, autophagy activation attenuates the secretion of inflammatory cytokines such as IL1B in chondrocytes in OA, which is associated with FOXO expression [45]. Recently, autophagy modulators have been reported to regulate the pathogenesis of OA. For example, spermidine, trehalose, isopsoralen, hydroxytyrosol, and TFEB (transcription factor EB) have been demonstrated to promote autophagosome formation and enhance autophagy flux or induce mitophagy to protect chondrocytes in OA [88]. In addition, ATP metabolite adenosine binds to its receptor (A2AR) to regulate chondrocyte function. A2AR-deficient mice develops spontaneous OA accompanied by increased ROS accumulation in chondrocytes and impaired mitophagy. Accordingly, A2AR agonists can regulate autophagy/mitophagy and improve mitochondrial dynamics and function in the OA mouse model [89]. Taken together, autophagy and mitophagy can be considered therapeutical targets for degenerative orthopedic diseases such as IVDD and OA due to their protective roles in the pathogenesis of these diseases.

Bone metabolic disorders

OP is a disease with bone mass loss and degeneration of bone microstructure, which affects one in three postmenopausal women and one in six men after age 50 [90]. As life expectancy increases globally, OP fractures are expected to rise from 1.7 million in 1990 to 6.3 million in 2050 [91]. The progression of OP in postmenopausal women includes two stages. The first stage is characterized by rapid bone loss, generally lasting 3–5 years. At this stage, bone resorption and bone formation increase simultaneously, but the ability of bone resorption is more vital, and autophagy plays a more critical role in bone resorption [92]. The second stage after menopause is similar to senile bone loss and can last for about 10–20 years. During this stage, cortical and trabecular bone is lost proportionally. Increased resorption cavities lead to increased bone tissue porosity. In addition, osteocyte autophagy decreases with age, which is associated with senile osteoporosis [92] (Figure 4). Estrogen deficiency is a common cause of OP, and a significantly negative correlation between osteocyte autophagy level and bone loss has been found in ovariectomy-induced estrogen deficiency rat models [93]. In contrast, *Atg7*-targeting or systemic delivery of the autophagy inhibitor chloroquine effectively reduced osteoclast activity in ovariectomized mice, while specific-inhibition osteoblast autophagy exacerbated bone loss in aging or estrogen deficiency cases [94]. In addition to the loss of estrogen, increased oxidative stress is also considered a critical factor in the progression of OP. Chronic use of GC, which is widely used as an anti-inflammatory and immunomodulator, can lead to bone fragility and increased fracture. During GCs-induced excessive bone resorption, autophagy is usually required to assist osteoclast formation and bone resorption. The resulting osteocytes apoptosis during this period may also be due to the inhibition of autophagy and antioxidant gene expression in osteocytes by high dose GC. Another cause of bone loss is inflammatory bone resorption, in which lipopolysaccharide (LPS) is a key factor, and autophagy assists LPS in increasing the number and activity of osteoclasts. In general, a decline in autophagy generates an increase in oxidative stress leading to bone loss and OP, and this process can be reversed by increasing the autophagy pathways. Along with autophagy reduction, the oxidative stress increases and then upregulates PI3K-AKT-FOXO signaling, which promotes FOXO to enter the nucleus and bind to TCF/LEF, thereby blocking osteogenic differentiation [95]. Inhibition of ubiquitination and degradation of FOXO3 protects osteoblasts from oxidative stress-induced apoptosis in OP [96]. In addition, acute bone loss and osteoblasts homeostasis were observed after *Atg7* inactivation in HSCs, which may be related to the loss of H vessels (connecting osteocytes and HSCs) [97]. PI3K-AKT-MTOR signaling in osteoclasts is also involved in GC-induced OP, and the treated osteoclasts show enhanced autophagy activity both *in vitro* and *in vivo*, suggesting that targeting the autophagy pathway may be an effective therapeutic target for OP [98].

Osteitis deformans is a chronic focal high-turnover bone disorder affecting one or more bones, mainly in the elderly. Osteitis deformans is usually asymptomatic and associated with OA, pathological fractures, bone malformations, bone pain, etc. Heredity predisposition factors play an essential role in the pathogenesis of this disease, the most important of which is the mutation on *SQSTM1* (along with aging), which results in severe osteitis deformans, as observed in approximately 40% of familial cases and 10% of sporadic cases [99]. Besides its role as an NFKB signaling activator in osteoclastogenesis, SQSTM1 acts as a receptor for autophagy. Ubiquitin-related domain point mutations in the *Sqstm1* gene appear to be sufficient to induce osteitis deformans in mice, and elevated levels of autophagy in osteoclasts were detected [100]. The *OPTN* (optineurin) gene has also been identified as a genetic risk factor for osteitis deformans. Similar to SQSTM1, OPTN, as an autophagy receptor, is also important for autophagy to remove pathogens, damaged mitochondria, and protein aggregates [101]. Interestingly, OPTN deficiency

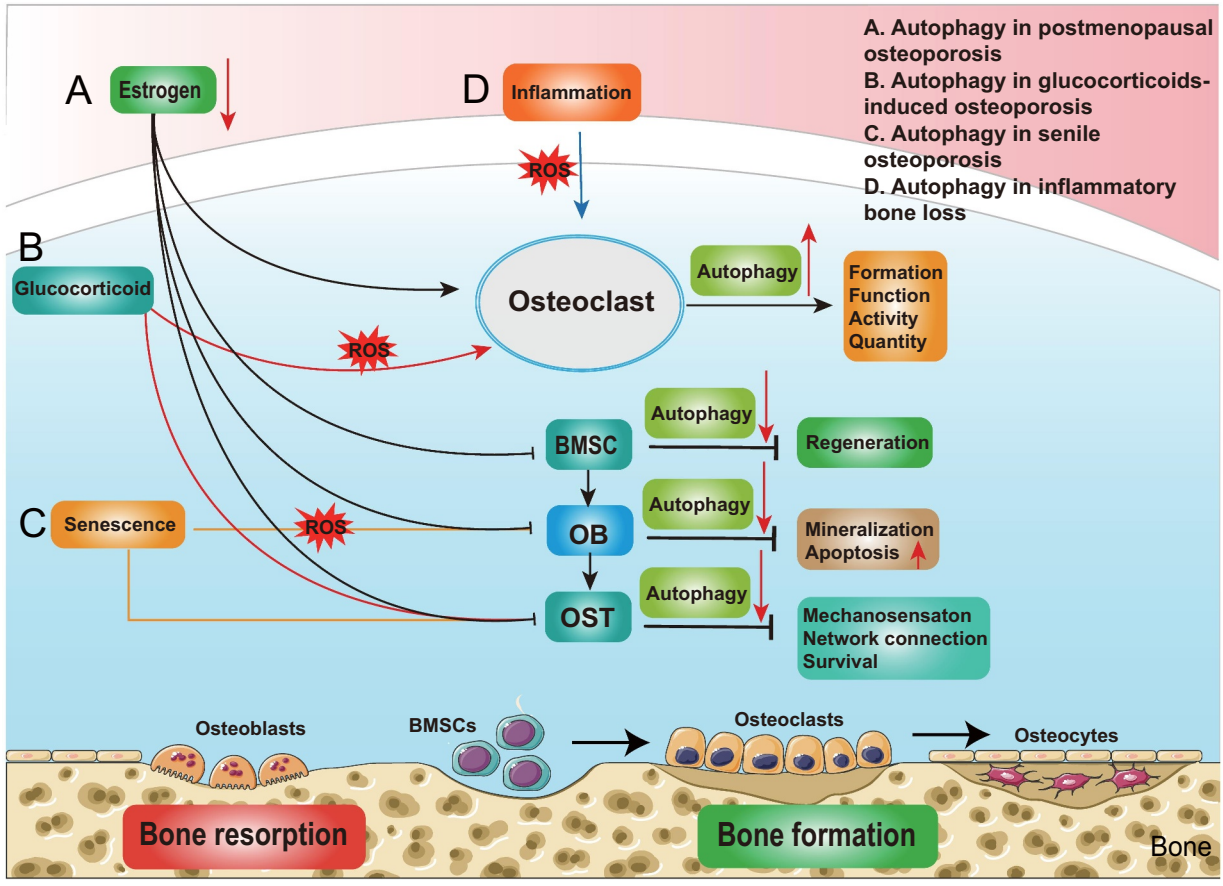

Figure 4. The role of autophagy in the association between different types of cells in OP pathogenesis. A) the early stage of OP is also known as the period of high bone turnover, which can last for 3–5 years. It is characterized as increased bone resorption and formation, but higher bone resorption efficiency leads to net bone loss. B) GC-induced OP with increased bone resorption but decreased bone formation. Autophagy is mainly involved in bone resorption and osteocyte survival. C) Senile OP, characterized by persistent slow bone loss, entails the resorption cavity and porosity increasing progressively, and the osteocytes autophagy is gradually inhibited with aging. D) Inflammation-related OP, enhanced autophagy participates in osteoclast-mediated bone resorption, which is the primary mechanism leading to bone loss.

leads to increased osteoclast activity and bone turnover, which may be due to the impaired conduction of type I interferon (IFN) signaling via IFNAR1 (interferon (alpha and beta) receptor 1); this receptor is part of a negative feedback loop for osteoclast formation and survival [102]. These studies confirm the relationship between autophagy and osteitis deformans disease occurrence and progression and provide new therapeutic targets.

Osteopetrosis is a genetically heterogeneous disease that often leads to an abnormal increase in bone mass, resulting in malformations or changes in bone morphology. Autosomal recessive osteosclerosis (ARO) is a malignant course characterized as osteoclast dysfunction. Osteoclasts in ARO patients are morphologically normal but lose their bone resorption ability due to defective ruffled-border formation. It is critical for osteoclasts to dissolve bone minerals and degrade bone matrix [103]. This process involves the inadequate transport of lysosomes in osteoclasts. Similarly, the formation of autolysosomes is essential to degrade the cargo of autophagy. Therefore, the importance of autophagy in ARO is becoming clear. This idea has also been demonstrated in animal experiments, that ARO mice with homozygous mutation of the *TCIRG1* gene show autophagy deficiency as evidenced by decreased LC3-II and increased SQSTM1 protein levels [104]. In summary, ARO gene mutations, which affect lysosomal transport in osteoclasts, appear to be associated with autophagy regulation.

Therapeutic approaches for bone diseases by modulating autophagy

Autophagy-induction in osteogenesis and bone regeneration

The formation and remodeling of bone tissue is a complex multi-step process, in which autophagy is involved in many factors positively regulating osteogenesis. Previous studies have found that some hormones, which affect bone regeneration and remodeling, exert their effects through autophagy regulation (listed in Table 1). PTH (parathyroid hormone) promotes the autophagy of osteoblasts and chondrocytes, which is beneficial for the remission of OA [105]. Moreover, PTH upregulates the expression of LC3-II and BECN1 in the MLO-Y4 osteocyte cell line [113]. A recent study confirmed that the osteogenic effect of PTH is eliminated in *SQSTM1*-

Table 1. Hormones modulating autophagy and osteogenesis.

Hormones	Cells with autophagy-modulation	Outcome or Mechanism	Ref
PTH	Osteoblasts, chondrocytes, osteocyte	PTH promotes the autophagy of osteoblasts and chondrocytes, which is beneficial for the remission of OA	[105]
Thyroid hormones (T3)	Osteoblasts	T3 promotes osteoblast autophagy and subsequent bone formation	[106]
Glucocorticoid	Osteoclast	Inhibition of autophagy results in reduced osteoclast differentiation and prevents Glucocorticoid-induced bone loss.	[94]
Estrogen	Osteoblast, osteocyte, BMSC	They are inducing SIRT1 to upregulate AMPK and restraining the MTOR pathway to promote autophagy and activate FOXO3 to inhibit osteoblast apoptosis, Inducing autophagy of osteocytes to combat apoptosis.	[107,108]
β-ecdysone	Osteoblast	Inducing autophagy to ameliorate the inhibition of bone regeneration and osteoblast apoptosis induced by prednisolone treatment	[109]
Melatonin	Nucleus pulposus cell	Activating autophagy via the NFKB signaling pathway to prevent extracellular matrix degeneration in the intervertebral disc.	[110]
Leptin	BMSC	LEP promotes the osteogenic differentiation of BMSCs and protects BMSCs from apoptosis by inducing autophagy.	[111]
Insulin	BMSC	Insulin enhances bone loss in T2DM patients by inhibiting autophagy and promoting premature aging of BMSCs.	[112]

deficient mice, suggesting that SQSTM1 and autophagy play vital roles in PTH-stimulating bone regeneration [114].

Similarly, estrogen reduces osteoblast apoptosis by promoting autophagy and upregulated RAB GTPase activating proteins (RABGAPs) to increase autophagy flux, to ensure the longevity and mineralization of osteoblasts [115]. Another study showed that estrogen could induce SIRT1 (sirtuin 1) to upregulate AMPK and restrain the MTOR pathway to promote autophagy and activate FOXO3 to inhibit osteoblast apoptosis [107]. Accordingly, estrogen deficiency can lead to osteocyte apoptosis, suggesting that estrogen-induced anti-apoptosis effects may be achieved *via* autophagy regulation in osteocytes [108].

Despite PTH and estrogen, growth hormone stimulates the production of IGF-1, which induces AMPK to promote osteoblast differentiation, a process associated with autophagy, since Inhibition of AMPK activity resulted in decreased activity of BECN1, LC3-II, and phosphorylation of ULK1 [38]. β-ecdysone (β-ECD), a phytoecdysteroid that can improve osteogenesis *in vitro*, ameliorates the inhibition of bone regeneration and osteoblast apoptosis induced by prednisolone treatment *via* β-ECD-induced autophagy [109]. Melatonin is produced by the pineal gland and is an active substance secreted by the body. It has been reported that melatonin therapy effectively increases autophagy flux. In contrast, the partial elimination of autophagy flux reduces the effect of melatonin on preventing cell apoptosis and dysfunction [116], suggesting that the protective effect of melatonin is partially achieved through autophagy. Melatonin-promoted autophagy prevents IVDD in ECM both *in vivo and in vitro*, indicating its therapeutic potential for controlling IVDD [110]. Another circulating hormone that has a positive effect on bone growth is LEP (leptin). LEP promotes the osteogenic differentiation of BMSCs and protects BMSCs from apoptosis by inducing autophagy which may be related to AMPK and MTOR pathways [111].

Vitamin D is necessary for bone mineralization, and its deficiency can lead to orthopedic diseases such as rickets. Vitamin D3 enhances autophagy activity by activating CAMKK2-PRKAA signaling and inhibiting MTORC1 activity by increasing calcium levels in the cytoplasm [117]. Similarly, vitamin D3 activates the activity of the p-AMPK-AMPK signaling pathway and decreases the p-MTOR-MTOR pathway to increase chondrocyte autophagy, thereby reducing OA inflammation [118]. Excessive autophagy induced by high glucose in diabetic patients is not conducive to the proliferation and differentiation of osteoblasts. PI3K-AKT signaling activated by vitamin D3 can help inhibit FOXO1 expression to reduce bone loss caused by excessive autophagy [119].

Metal ions can activate autophagy to generate positive effects on bone homeostasis. Calcium ions (Ca^{2+}) are the most abundant metal ions in the human body, and extracellular Ca^{2+} is important for maintaining the potential difference in protein and bone formation. It has been proved that calcium is involved in autophagy signaling pathways (including MTOR and AMPK). Cytosolic calcium affects not only the initial stage of autophagy but also the proximal and distal steps during the autophagy process. Calcium and phosphorus are essential ionic components for forming hydroxyapatite during the mineralization of bone extracellular matrix, and the deficiency of autophagy may reduce the mineralization capacity. It also reported that autophagic vacuoles could be used as a carrier for the secretion of apatite crystals by osteoblasts [32]. Magnesium ions are natural agonists of calcium. The upregulation of two magnesium transporters (TRPM7 and MAGT1) is involved in osteoblast differentiation, and the silencing of either activates autophagy to accelerate osteoblast differentiation [120]. Besides, inhibition of autophagy impairs strontium-induced osteogenic differentiation [121]. Gold nanoparticles also stimulate the expression of stress-response genes and autophagy in human periodontal progenitor cells as cellular defense mechanisms against stress [122]. It should be noticed that ion concentrations play determinant roles in the effects of ions on autophagy. For example, a high concentration of magnesium inhibits MAPK/ERK phosphorylation and reduces chondrocyte autophagy expression and mineralization to protect articular cartilage [123]. Under physiological conditions, mitochondrial uptake of Ca^{2+} is required to prevent AMPK-activated autophagy and maintain cellular biological energy [124].

In conclusion, autophagy can be regulated by various factors, including numerous hormones, growth factors, or metal

ions, which provides a good reference for understanding the correlation between osteoblast activities (e.g., growth, proliferation, and differentiation) and autophagy. In addition, since osteoblasts and osteocytes are mechanically sensitive cells, more attention can be focused on the effects of mechanical pressure, shear force, elastic force, stiffness, and even bone fatigue strength on autophagy, as well as the underlying mechanisms.

Autophagy in stem cell-based therapy

BMSC can repair tissue damage caused by disease, trauma, surgery, and chemotherapy. However, cell loss and low survival rate after transplantation are the main reasons for their low therapeutic efficiency. Heat shock pretreatment (HSP) can improve the activity and proliferation and reduce apoptosis of BMSC. The MAPK/p38 is an inducer of autophagy, and it can be activated in response to exogenous stimuli (including hypoxia, starvation, and heat shock). Its activity is regulated by HSP to improve cell survival [125]. In addition, it has been reported that HSP-induced autophagy of BMSC is essential to their survival when exposed to H_2O_2, which may be due to the activation of the CXCL12/SDF1-CXCR4 axis [126]. Consequently, autophagy protects BMSC from adverse factors. For example, autophagy blocks TNF-induced apoptosis of BMSCs to improve their survival in an inflammatory environment. Similarly, the activation of autophagy effectively protects BMSCs from ionizing radiation [127]. Incidentally, it should not be ignored that the resistance of malignancies to radiotherapy may also be due to the protection of cells generated by autophagy activation [128].

Senescence is a complex and inevitable physiological process, in which the senescence of stem cells results in the pathophysiological condition. Aging of BMSC leads to reduced self-renewal function and transformation from osteogenic to lipid differentiation, which is closely related to the pathogenesis of OP. The decline in autophagy is associated with a decreased osteogenesis capacity of senescent BMSCs in OP. Due to the imbalance of lysosome pH value during cell senescence, a stimulation of lysosome acidification is beneficial to inhibit cell senescence. After acidification treatment, the proliferation and autophagy levels of BMSC were promoted, and the senescence of BMSCs was effectively prevented [129]. In addition, cholesterol can regulate the cell cycle, autophagy level, and ROS-TP53/p53-CDKN1A/p21/Cip1/Waf1 pathway to delay BMSC aging [130].

Interestingly, exposing MSC to hypoxia can promote autophagy and protect cells from apoptosis. Hypoxia preconditioning (rapamycin treatment can generate similar results) increases the expression of APLNR/APJ, APLN/apelin, BECN1, and LC3-II:LC3-I ratio, along with an induced autophagosome formation in BMSCs, and the proliferation of BMSC is enhanced in a time-dependent manner. At the same time, inhibition of BECN1 and LC3-II/LC3-I eliminates the process, suggesting the APLN/APJ autophagy pathway may be involved in the proliferation of hypoxia-induced BMSC [131]. Similarly, *HIF1A*-modification (infected with *HIF1A* GFP lentiviral vector) enhances cell viability and inhibits apoptosis in BMSCs, and to some extent restrains the production of pro-inflammatory factors [132]. Furthermore, GC administration can impair the proliferation of BMSCs, and the proliferation ability of BMSCs further decreases, and the apoptosis increases after the addition of autophagy inhibitor 3-methyladenine [133].

MTOR kinase is a key inhibitory molecule in autophagy. Pathways that activate MTOR, such as AKT and MAPK, inhibit autophagy, while pathways that inactivate MTOR, such as AMPK and TP53, promote autophagy. Leonurine promotes BMSC proliferation and osteoblast differentiation by activating autophagy and possibly inhibiting the PI3K-AKT-MTOR pathway [134]. In another study, osteogenic differentiation of BMSC is associated with the time-dependent regulation of autophagy as well as with the AKT-MTOR [121]. Increased bone marrow adipose tissue is often accompanied by low bone mass, and this inverse relationship has also been reported in mouse models [135]. The osteoblast or lipoblast lineage of BMSC is directly related to bone development and homeostasis regulation. TSC1 (TSC complex subunit 1) and TSC2 are upstream inhibitors of MTORC1, and *tsc2* gene deletion in osteoblasts and *tsc1* gene deletion in osteoblast progenitors both increase bone mass. The reduction of trabecular bone in $tsc1^{-/-}$ mice may be associated with increased adipose tissue and the downregulation of WNT-CTNNB1 signaling. This suggests that TSC1-MTORC1 signaling may be a regulator of osteoblast adipocyte differentiation in bone marrow [135]. Therefore, it is suggested that MTOR and autophagy could be regulatory targets for bone regeneration.

Accordingly, autophagy induction can promote the osteogenic differentiation of BMSC. Some orthopedic biomaterials improve bone regeneration via an autophagy-dependent manner, including dicalcium silicate nanoparticles [136], strontium-doped titanium nanoparticles [137], and lithium chloride [138], which can upregulate autophagy to promote the expression of osteogenic genes and proteins. Due to the absence of cytological characteristics of proliferation and differentiation, extracellular vesicles secreted by BMSC (BMSC-EVs) have more stable biological features and reduced risk. BMSC-EVs also alter the process of bone regeneration partially by regulating the level of autophagy, although the exact mechanism remains unclear. Fortunately, BMSC-EVs have been shown to inhibit nucleus pulposus (NP) cell apoptosis, ameliorate IVDD [139], and improve chondrocyte survival [140] by enhancing autophagy flux. These studies provide new insights into how BMSC-EVs can improve cellular function, cartilage reconstruction, and disease, further indicating the importance of BMSC autophagy regulation in bone regeneration.

In short, autophagy modulation can change the therapeutic properties of BMSC *via* the following mechanisms. First, autophagy is closely related to the differentiation potential of BMSCs, because there are more autophagosomes in the undifferentiated BMSCs. Second, some authors think that the immunosuppressive effect of BMSC is related to the autophagy level, which may provide insights into the field of bone tissue engineering. Autophagy regulation in BMSC may also cause the secretion of growth factors such as VEGF. Conversely, autophagy blockage can significantly impede the therapeutic effect

Table 2. Phytochemicals modulating autophagy and osteogenesis.

Phytochemical	Model	Autophagy affects cells	Outcome or Mechanism	Ref
Allicin	Osteosarcoma model	Osteosarcoma cells	Inactivate the MALAT1-*MIR376A*-WNT-CTNNB1 signal to promote oxidative stress and autophagy, thereby reducing changes in osteosarcoma cells	[141]
Anthocyanin	Murine tumor xenograft model	Osteosarcoma cells and osteoblasts	Inhibition of NFKB signal transduction in osteosarcoma cells inhibited cell proliferation and induced apoptosis.	[142]
Berberine	Insulin resistance mice model	Macrophage	May prevent inflammation by stimulating macrophage autophagy through AMPK signaling.	[143]
Curcumin	Human disc nucleus pulposus cell model	Intervertebral disc cell	AKT- and autophagy-dependent to prevent intervertebral disc cell apoptosis, inflammation, senescence, and matrix catabolism	[144]
Genistein	Mice periodontitis model	Osteoclasts and macrophage	Inhibition of osteoclast differentiation induced by NFKB ligand or LPS-stimulated macrophages	[145]
Ginsenoside	Rat Ovariectomy model	Osteoblasts	Enhance AMPK signaling or inhibit MTOR to promote autophagy, osteogenesis differentiation, or mineralization	[146]
Quercetin	Rat Ovariectomy model	Osteoblasts, osteoclasts, osteocytes	Treat OP by regulating the total amount of bone cells, autophagy, and reducing apoptosis.	[147]
Resveratrol	Rat tibial defect model	BMSCs, Osteoblasts	Stimulation of autophagy drives BMSC survival, osteogenesis, and angiogenesis.	[148]
Shikonin	OA cell model	Chondrocytes	Promote chondrocyte anabolism	[149]
Sulforaphane	Mouse calvarial model	Osteoclasts	Reduce the number of autophagosomes and inhibit autophagy activation of osteoclasts from reducing osteoclast formation	[150]
Ursolic acid	Rat Ovariectomy model	Osteoclasts	Block osteoclast autophagy and reduce the expression of FOS and NFATC1	[62]

of BMSC. The evidence reported above strongly suggests that autophagy-induction could be a strategy to improve the osteogenic differentiation of BMSCs, which ultimately contributes to the therapeutic effects. In addition, future studies should be performed to explore the autophagy modulators of BMSCs, the roles of these modulators in BMSC-based therapy, and the associated molecular mechanisms.

Current therapeutical autophagy regulators

Given the critical role of autophagy in skeletal physiology and pathology, several known autophagy modulators, including MTOR inhibitors, AMPK activators, and phytochemicals (Table 2), have been reported to be beneficial to bone health.

Sirolimus (also known as rapamycin) and its derivatives/analogs are MTOR inhibitors, therefore, are classical autophagy inducers. It can inhibit osteoclast formation and activity *in vitro*, thus prevent oophorectomy bone loss by 60% *in vivo* by reducing osteoclast-driven bone resorption [151]. Bone metastasis in cancer is a complex vicious cycle involving cancer cells, osteoblasts, and osteoclasts, which occurs in up to 70% of patients who die of prostate or breast cancer. Interestingly, rapamycin is excellent at reducing osteolytic lesions caused by cancer bone metastasis, primarily by lowering osteoclast count and osteolysis [152]. Additionally, raddeanin A, the main bioactive ingredient of sea anemone, inhibits osteoclast formation, metastatic bone dissolution, and AKT-MTOR signaling to prevent the growth and invasion of breast cancer cells [153].

Metformin, an antidiabetic drug, improves diabetes-associated bone complications, such as lower bone mineral density and increased fracture risk. Diabetes can dampen the bone quality in patients with diabetes due to hyperglycemia, the toxicity of advanced glycation end products to bone tissue, and the destruction of the microvascular bone system. The antidiabetic drug metformin accelerates calcium and AMP accumulation to activate AMPK and consequently induces autophagy and has been shown to improve bone mass and reduce fracture risk in people with diabetes [154]. Metformin can protect nucleus pulposus cells from senescence and apoptosis through autophagy, and promote the expression of anabolic genes *Col2a1* and *Acan* while inhibiting the catabolic genes *Mmp3* and *Adamts5*, thus ameliorating the process of IVDD [155]. Excitingly, metformin can also enhance extracellular vesicle production of MSCs through the autophagy pathway, which holds great potential for treating musculoskeletal degenerative diseases [156].

Simvastatin, another AMPK activator, has been shown to inhibit ROS-mediated signaling and osteoclast formation [157]. Similarly, simvastatin remarkably increased BMSCs autophagy and osteogenic differentiation and reduced ROS production under adverse conditions. In hypoxia-induced mitochondrial dysfunction, simvastatin also inhibits overactivated mitophagy-resulted osteoblast apoptosis and alleviates bone resorption [158].

Traditional Chinese medicine is a philosophical and medical system that combines the theories and practices of thousands of years of outstanding Chinese medical scientists. There are phytochemicals that humans ingest daily basis have been tested by modern medicine as valuable sources of autophagy modulators. Berberine is a benzyl isoquinoline alkaloid in the bark and root of berberis species. Besides antibacterial, anti-inflammatory, antioxidant, and anti-apoptotic activities, it can activate autophagy to play IVDD therapeutic effects, including inhibition of NP cells apoptosis and extracellular matrix degeneration [159]. Curcumin, a yellow phenolic pigment extracted from turmeric, enhances autophagy flux in an AMPK-MTOR-ULK1 signaling pathway-dependent manner. It can inhibit excessive ROS production and mitochondrial dysfunction and promote TFEB nuclear translocation to reduce inflammation [160,161]. Quercetin, an anti-inflammatory antioxidative and anticancer dietary flavonoid, has been observed to upregulate autophagy, reverse oxidative stress, and improve mitochondrial and lysosomal functions after treatment. It has also been shown to regulate osteocyte autophagy to improve OP in rat oophorectomy models [147]. In addition, various phytochemicals can

effectively maintain bone mass by activating Sirt1, Nrf2 transcription factors, and GUCY1 (guanylate cyclase 1 soluble). For example, resveratrol, ursolic acid, ferulic acid, melatonin, glucosamine, and thymoquinone can promote Sirt1 activation; lipoic acid, Vitamin D, melatonin, thymoquinone, astaxanthin, and sulforaphane can boost the activity of NFE2L2/Nrf2; Biotin can directly stimulate sGC [162]. These autophagy modulators also show extraordinary potential for protecting bone health, including enhancing MSC proliferation and osteoblast differentiation [163], inhibiting ROS and reducing osteoclast formation [160], alleviating osteoblast apoptosis or increasing bone mineralization [164], and promoting angiogenesis through autophagy pathway regulation [165].

Autophagy-modulatory therapeutical biomaterials

Despite the small-molecular drugs, recent advances in Material Science have proposed the importance of biomaterials as drug vehicles. Biomaterials, especially nanoparticles, can facilitate drug delivery in a controlled manner, ensuring a long-term effect and avoiding drug side effects. There is growing evidence suggesting the importance of autophagy in a wide range of physiological and pathological processes, and researchers have focused on the internalization of nanomaterials to trigger autophagy. First, nanomaterials/cells can be functionalized by osteoconduction/induction molecules or drugs (attached to the material surface), including nano-silicon, chitosan, nano-titanium, alumina, silver nanoparticles, and polydopamine-templated hydroxyapatite to trigger autophagy for osteogenic differentiation, angiogenesis, and bone loss inhibition. Another option is to modify the material surface/structure to make it more suitable for cell adhesion and growth, such as material shape, surface roughness, affinity, biocompatibility, and chirality. The rough surface is prone to induce the development of small granular cells to form cell clusters, which are crucial for the formation and mineralization of bone nodules. This process is closely related to autophagy [166]. The early contact with the tube structure (days 1–3) temporarily promoted autophagy compared with the flat material surface, which may be related to the stretching of the membrane structure [167]. Three-dimensional nano scaffolds (polycaprolactone) provide a favorable extracellular matrix microenvironment for cell growth, differentiation, and mineralization. Autophagy is also found to play essential roles in the early stage of osteogenic differentiation in this model [167]. Noteworthily, the chirality of nanomaterials represents a unified structural measure of the biological and non-biological forms of materials. Achiral and left – and right-handed biomimetic gold nanoparticles have been shown to exhibit different immune responses *in vivo* and *in vitro* [168]. Chiral nanomaterials also enhanced intracellular oxidative stress and accumulation, showing significant chirality-dependent autophagy induction. Currently, chiral gold nanoparticles show better performance in cell internalization, autophagy regulation, osteogenic differentiation, and tissue regeneration in rat models [169].

The critical role of autophagy has provided a therapeutic basis to design biomaterials for cancer-associated bone diseases, especially osteosarcoma, including inducing an antitumor immune response (depletion of T lymphocytes due to elevated programmed death ligand 1 on the surface of tumor cells) [170]. Chemotherapy for osteosarcoma is the primary treatment for the advanced stage. Autophagy is crucial to chemotherapy resistance of osteosarcoma by inhibiting apoptosis and thus increasing resistance to chemotherapy [171]. Bio-functional materials, notably, are being developed (e.g., zinc oxide nanoparticles) to induce autophagosome accumulation and lysosomal function to promote autophagy, which in turn promotes zinc ion release targeting mitochondrial destruction resulting in ROS-triggered osteosarcoma cell death. The early inhibition of autophagy restores cell viability by eliminating apoptosis, while the late inhibition enhances apoptosis [172]. While materials exhibit good mechanical properties and biocompatibility, the inertness of some materials, such as titanium, is not conducive to drug binding. Dopamine coating or heparin can be an excellent solution to this problem, but we still need to overcome the problems of low drug loading efficiency and drug release kinetics.

Additionally, based on the mechanisms of autophagy and various cells of bone, autophagy can be modulated to regulate the formation and remodeling of bone tissue. Some drugs commonly used to prevent and treat OP, such as vitamin D3 [164], sirolimus [173], bisphosphonates [174], and teriparatide [175], have been shown to affect autophagy and promote bone health, resulting in the improvement of autophagy in MSCs, osteoblasts, osteocytes, and osteoclasts. Furthermore, biomaterials designed to target autophagy for bone tissue bioengineering can more accurately achieve therapeutic properties.

Further studies should be performed to dissect the role of autophagy in hemostasis-osteogenesis interplay, a recently identified vital part of bone regeneration. It is noteworthy that research on blood clots has gradually emerged in the field of bone regeneration, especially in terms of the kinetics of the contribution of fibrin, tissue factor, and platelets [176]. Consequently, future studies may need to focus on the relationship between autophagy and the structure/function of clots, whether an interaction between autophagy and the progress of inflammation or the function of cells or platelets, which affect the differences in the structure of thrombi and kinetics of formation as a consequence of bone injury.

Conclusion

In conclusion, the exploration of bone and autophagy has gradually revealed the relationship between autophagy and bone metabolism in recent years. In short, autophagy is an intracellular self-feeding and self-editing mechanism which is conducive to the differentiation and function of bone metabolism-related cells, including MSC, osteoblast, osteocyte, and osteoclast; and therefore, is one of the fundamental mechanisms for maintaining the physiological homeostasis of bone. A dysregulation on cellular autophagy can disrupt bone remodeling balance and therefore result in bone disorders. Especially under aging conditions, cellular senescence is accompanied by a decline in autophagy, which dampens the capacity of cells (osteoblasts) to endure stress. In addition, impeded mitophagy results in the accumulation of damaged mitochondria and ROS, thus reducing osteogenesis while inducing osteoclastogenesis. Excessive bone loss and reduced bone production cause bone loss and lead to

various clinical bone metabolic diseases such as OP, PDB, etc. Therefore, autophagy plays a vital and complex role in bone metabolism. This provides novel targets, strategies, and methods for bone disorders prevention and treatment, by modulating autophagy/mitophagy (in certain type of cells) via pharmaceutical and especially biomaterial-based approaches—which can facilitate a local, sustainable, and cell-targeting therapeutic delivery to lower drug dose and avoid affecting other cell/tissue/organ. It should be noted that autophagy induced by different stages, cells, species, and even different molecules may play different roles, so the clinical use of autophagy regulators needs to make different judgments according to individual differences.

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