The pivotal role of autophagy in bone cells: bone-related cell activity and bone metabolism

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Abstract

BACKGROUND: The importance of autophagy for maintaining cellular homeostasis and stress response has long been recognized. As a way for cells to selectively clear their damaged organelles to achieve the recycling of cellular components, autophagy has a pivotal role in bone metabolism.

OBJECTIVE: To review the role and possible mechanisms of autophagy in regulating bone-related cell activity and function among bone marrow mesenchymal stem cells, osteoblasts, osteocytes, and osteoclasts.

METHODS: PubMed was searched for studies related to autophagy using the keywords of "autophagy; bone marrow mesenchymal stem cells; osteoblasts; osteocytes; osteoclasts."

RESULTS AND CONCLUSION: We finally included 84 papers. Autophagy plays an important role in bone metabolism. Autophagy is involved in maintaining the balance between mineralization and absorption, and then maintaining bone homeostasis. An appropriate autophagy inducer may also benefit bone remodeling. Abnormal autophagy can lead to disorders of bone balance, leading to diseases such as osteoporosis. We may prevent or treat bone-related diseases by regulating the level of autophagy as its function in maintaining the balance of mineralization and resorption in bone homeostasis.

Key words: autophagy; bone marrow mesenchymal stem cell; osteoblast; osteocyte; osteoclast; bone homeostasis; bone mineralization; bone resorption

Introduction

Life is in constant dynamic activities, constantly communicating with the outside world, generating and consuming energy to maintain normal activities. In addition to obtaining energy and substances from the outside world, researchers in recent years have found that when cells face external environmental pressures, such as lack of nutrients, hypoxia, injury, or under pathological conditions, they can use lysosomal-dependent pathways to damaged organelles, organic matter, which are recycled to meet their own needs. This phenomenon is called autophagy[1].

Autophagy level can be up-regulated when cells are exposed to external stresses, which is critical for maintaining the cell viability and normal metabolic functions in living bodies. Current evidence shows that autophagy is involved in the development of many common diseases, such as stroke, cancer, obesity, atherosclerosis, myocardial infarction, diabetes, inflammation, infectious diseases, aging and neurodegenerative diseases[2]. Furthermore, autophagy participates in the process of many bone-related diseases, such as osteoporosis, fracture healing, rheumatoid arthritis, osteonecrosis, Paget's disease and osteosarcoma[3-5].

Skeleton is an important part of the motor system. Skeletal muscle is attached to it to provide support and protection for the body and to complete various human actions in cooperation. In order to maintain the strength and function of bone, bone tissue has been constantly reshaped to meet the needs of the body's mechanical properties. Bone remodeling is mainly involved in osteoblasts, the cells responsible for bone formation, osteoclasts, the cells specialized for bone resorption, and osteocytes, the multifunctional mechanosensing cells embedded in the bone matrix, which involves the removal of damaged bone and the possession of new regeneration of mechanically stressed new bone[6, 7]. Bone is in the dynamic balance of osteocytes, osteoblasts, and osteoclasts. The autophagy activities have been shown to clear the waste materials generated during the bone metabolism process, so as to prevent a series of orthopedic diseases.

With the in-depth study of cell autophagy in recent years, increasing evidence has shown that autophagy is related to bone cells and regulates the activity and function of these three kinds of cells. The purpose of this review is to update some recent understanding of the role of autophagy in bone metabolism and how autophagy regulates the activity and function of bone cells.

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DATA AND METHODS

Data sources

PubMed database was searched for relevant articles using the key-words of "autophagy; bone marrow mesenchymal stem cells; osteo-blast; osteocytel osteoclasts."

Inclusion and exclusion criteria

Inclusion criteria: (1) Articles related to autophagy and the activity and function of bone marrow mesenchymal stem cells (BMSCs), osteoblasts, osteocytes, and osteoclasts; (2) preferentially select high-scoring articles with a high degree of correlation in the past 10 years. Exclusion criteria: Articles that do not match the objective of the review.

Quality assessment and data extraction

References that meet the selection criteria were included, and repetitive references that are irrelevant to the objective of the review were excluded. A total of 278 articles published from 2005 to 2020 were retrieved, and 84 articles were finally included according to the inclusion criteria (**Figure 1**).
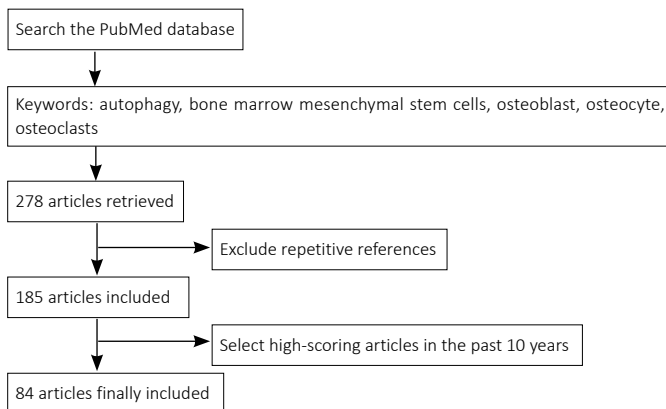

Figure 1 | **Literature screening diagram**

RESULTS

Autophagy and its formation

Autophagy first observed by Ashford and Porter[8] under an electron microscope in 1962 is an essential cellular homeostatic mechanism that maintains homeostasis and stress sensitivity by removing dysfunctional organelles or macromolecules. There are various types of autophagy, including microautophagy, macroautophagy and chaperone-mediated autophagy. Each of them remains its unique mechanisms and functions[9,10]. Both microautophagy and macroautophagy have the capacity to engulf large structures through both selective and non-selective mechanisms, whereas chaperone-mediated autophagy degrades only soluble proteins in a selective manner. In microautophagy, lysosomes directly engulf the surrounding cytoplasm by invading or protruding their membranes. The maintenance of organelle cell, membrane homeostasis, and cell survival under nitrogen restriction are the main functions of macroautophagy[11], while macroautophagy will form double-layered membrane autophagosomes to wrap damaged cell components. Chaperone-mediated autophagy selectively transfers substrates to lysosomal membranes by binding to pentapeptides similar to the KFERQ sequence[12]. Macroautophagy is the main type of autophagy and has been identified to be regulated by more than 40 autophagy-

related genes (ATG). Chaperone proteins will selectively bind to the target protein and directly enter the lysosome to complete the cycle. The autophagy can be divided into the following four stages[13,14] (**Figure 2**).
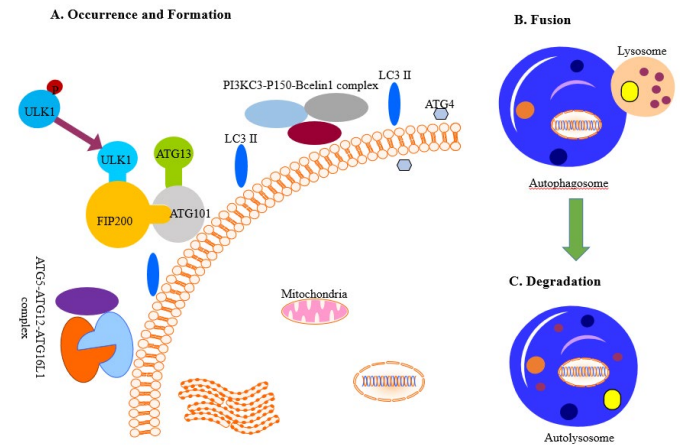
Note: (A) Production and extension of autophagy membrane. (B) Fusion of autophagy. (C) Degradation of autophagosome. ATG: autophagy-related genes; FIP200: focal adhesion kinase family interacting protein of 200 000; LC3: microtubule-associated protein 1A/1B-light chain 3; PI3KC3: class III phosphatidylinositol 3-kinase; ULK1: unc-51 like autophagy activating kinase 1.

Figure 2 | **The main process of autophagy**

I. The occurrence of autophagy membrane: The cells accept the autophagy-induced signal and transfer it to the mammalian rapamycin target protein (mTOR); after inducing the dephosphorylation of the unc-51 like autophagy activating kinase 1 (ULK1), it promotes ATG13, ATG101, FIP200 and ULK1 to form the ATG13- ATG101-ULK1-FIP200 complex thus to induce the extension of the autophagy membrane[15].

II. The formation of autophagosomes: The extension of the autophagy membrane is mainly regulated by two ubiquitin-like systems, class III phosphatidylinositol 3-kinase (PI3KC3), p150, and Beclin1 combine to form a complex, and then induce the autophagy membrane continues to extend. At the same time, ATG7 activates ATG12, and is successively combined with ATG10 and ATG5, and then interacts with ATG16L1 and ATG5 to form the ATG5-ATG12-ATG16L1 poly-complex, together to promote the growth of autophagosomes[16,17]. Subsequently, microtubule-associated protein 1A/1B-light chain 3 (LC3) was processed into LC3-I by ATG4[18]; meanwhile, LC3-I combined with phosphatidylethanolamine to form LC3-II, thereby promoting the formation of autophagosomes. The latter is the second ubiquitin-like system.

III. The transport of autophagosomes: After autophagy membrane extension and expansion is completed, autophagosome and lysosome fused to the formation of autolysosomes, This process may involve related SNARE proteins such as syntaxin 17 on the outer membrane of autophagy[19,20].

IV. The degradation of autophagosomes: The mTOR pathway is the key to the process of autophagy. After the lysosome degrades the substance in the cytoplasm, the degraded product is transported out of the lysosome for reuse by the cells. The release of lysosomal

nutrients will activate the mTOR signaling pathway, forming a negative feedback mechanism to avoid excessive cytoplasmic degradation. Activation will cause the lysosomes to extend out of the tubules. These tubules are often called "protolysosomes" and can continue to develop into mature lysosomes[21].

On one hand, the basal levels of autophagy are important for maintaining normal cellular homeostasis. On the other hand, even though the capacity for large-scale degradation is important for the function of autophagy, but it carries a certain risk because unregulated degradation of the cytoplasm is likely to be lethal[22].

Autophagy regulates bone homeostasis

Autophagy is of great significance to bone homeostasis. Bone is a dynamic organ, the activities and functions of osteocytes, osteoclasts and osteoblasts will directly affect the changes in bone mass and bone strength. Osteoblasts have mainly synthesized bone matrix while osteoclasts have a bone resorption effect, under normal circumstances, bone resorption and bone formation maintain a relative balance. Once the balance is broken, it will lead to osteoporosis, osteonecrosis or Paget's disease, etc.[23] (**Figure 3**).
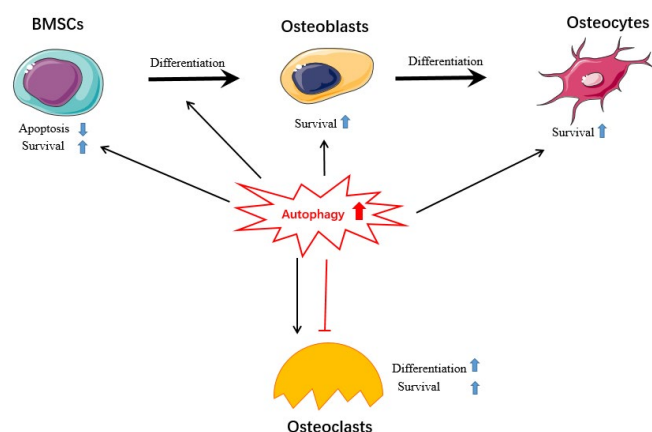
Note: Bone is mainly composed of osteoblasts, osteocytes, and osteoclasts, the dynamic balance of the three maintains bone homeostasis. The increase of autophagy can promote the differentiation of osteoblasts and osteoclasts. When the cells face adverse conditions such as oxidative stress, the increase of autophagy can promote the survival of BMSCs, osteoblasts, osteoclasts and osteocytes. BMSCs: Bone marrow mesenchymal stem cells.

Figure 3 | Autophagy regulator bone homeostasis

When the bone cells were in the condition of nutritional deficiency, hypoxia or infection and other adverse stimuli, a higher level of autophagy would be induced, to maintain the homeostasis of the bone system and prolong life. Therefore, a comprehensive understanding of the role of autophagy in bone and the mechanisms of autophagy in bone-related diseases will benefit for the exploration of the treatment therapies.

Autophagy and BMSCs

BMSCs have the potential for self-renewal and multidirectional differentiation including adipose, bone, cartilage, and muscle[24], The osteoblast precursor cells required for bone tissue development, bone metabolism, and bone repair are all differentiated from BMSCs. Current data demonstrate that the autophagy activity of BMSCs affects their differentiation process, which in turn affects bone mass and even secondary bone. Autophagy regulates

BMSC regeneration and controls osteoporosis development[25]. Older BMSCs are more likely to differentiate into adipocytes than to osteoblasts, and their autophagy activity is reduced, the use of autophagy inhibitor 3-methyladenine can reduce the differentiation and proliferation of BMSCs into osteoblasts while activator rapamycin reverses this process[26, 27]. Recently, it was shown that primary human BMSCs exhibit a high level of constitutive autophagy, with decreased BMSCs' differentiation into osteoblasts[28]. In agreement with this observation, scientists have found that there is a high level of green fluorescent protein LC3 (GFP-LC3) puncta in primary BMSCs isolated from GFP-LC3 transgenic mice, to the authors' surprise; these GFP-LC3 puncta disappear after these cells are differentiated into osteoblast-like cells[29]. These suggest that the level of autophagy in BMSCs is higher in osteoblasts. However, the role of the constitutive autophagy in BMSCs remains unclear.

Researchers have also found that when cells are hypoxic, peripheral reactive oxygen species is produced, high glucose cause apoptosis, and MSCs can produce exosomes through multiple pathways such as AMPK/mTOR, Akt/mTOR to enhance and enhance autophagy, and protect BMSC survival[30-32]. Induced mitochondria[33], vitamin C transporter 2[34] and insulin-like growth factor 1[35] may play key roles in BMSCs' antioxidative stress-related autophagy and apoptosis.

Another discovery is that there are massive undegraded autophagic vesicles in undifferentiated mesenchymal stem cells, and the number of aggregated autophagic vesicles is reduced during the differentiation process of mesenchymal stem cells into osteoblasts[36]. This reminds us that the autophagic vesicles can supply the energy for the promotion of the differentiation of BMSCs.

Autophagy is inseparable from BMSC; however, the detailed biological functions of autophagy in BMSC such as maintenance, self-renewal, and differentiation are largely unknown. The detailed cellular and molecular mechanisms for the regulation of BMSCs during differentiation process demand a future investigation.

Autophagy and osteoblasts

Osteoblasts are mesenchymal-derived cells responsible for synthesis and secretion of bone matrix, and its subsequent mineralization[6]. The development and growth of bone are closely related to osteoblasts. Osteoblasts activate osteogenesis by secreting a large number of bone collagen matrix and some important cytokines, and through this series of factors coupled with the regulation of osteoclast to control the osteoclastogenesis maturation and activation[6]. In the process of bone formation, a portion of osteoblasts are differentiated into osteocytes and the other part is apoptotic[37].

Existing research supports the important role of autophagy in the differentiation and mineralization of osteoblasts. Studies have reported that there is a significantly increased autophagy in the early differentiation and the mineralization of the skull osteoblast, indicating that the autophagy and the differentiation and mineralization of osteoblast are closely related[38]. Liu et al.[39] found autophagic vesicles contain apatite-like structure in BMSCs after he observed the transmission process under electron microscopy,

indicating that autophagic vesicles may be a mean of transportation during bone mineralization, autophagic vacuoles could be used as vehicles in osteoblasts to secrete apatite crystals. The deletion of FIP200 led to an abnormal increase in p62 expression and insufficient conversion of LC3-II, resulting in a defect in osteoblast function[39]. Besides, AMPK activation induced autophagy, as determined by the upregulation of LC3, increased autophagosome density and downregulation of p62[40]. Mice lacking the stress-inducing nuclear protein 1 showed an increase in bone mass. In nuclear protein 1-deficient osteoblasts, the expression of ATG, autophagosome formation, and cell survival was up-regulated[41].

Properly increased autophagy levels cannot only protect BMSCs from oxidative stress damage but also promote the survival of osteoblasts. Oxidative stress destroys many cellular components of osteoblasts and is considered to be a key pathogenic factor for bone loss[42,43]. Silencing ATG5 can inhibit the proliferation and differentiation of osteoblasts, making them more vulnerable to oxidative stress[44]. Autophagy also can regulate the mechanical stimulation required for osteoblast differentiation[45] and reduce the inhibitory and apoptotic effects of glucocorticoids on osteoblasts[46]. The presence of estrogen can promote the differentiation and autophagy of osteoblasts by up-regulating the expression of autophagy-related protein RAB3GAP1, thereby improving its survival rate and mineralization[47], probably through the ER-ERK-mTOR signaling pathway[48]. Besides, autophagy-deficient osteoblasts can cause increased levels of oxidative stress and are potent in the production of osteoclasts[49].

It has been reported that the knockout of the UBA domain and the LIR site in the selective autophagy receptor (*NBR1*) gene of the ubiquitination substrate could inhibit the MAPK-p38 pathway, promoting the differentiation of BMSCs to osteoblast and significantly increasing bone mass[50]. It has been also reported that the expression of autophagy and bone mineral density were significantly increased when the receptor *NBR1* gene was knocked out in an animal model[51]. In an *in vitro* study, scientists further found the osteoblast activity and the differentiation ability were significantly enhanced after the *NBR1* gene was deleted. Furthermore, studies have found that P62 activity has increased when the *NBR1* gene was knocked out. All of these studies demonstrated the *NBR1* gene has a positive effect on the autophagy process and also regulates p38 mitogen-activated protein kinase MAPK in the osteoblast in a negative way[51]. Therefore, knocking out the *NBR1* gene will inhibit autophagy and change the mechanism of osteoblast[52]. Similarly, *ATG7* and *BECN1* genes of a knockout rat can also significantly reduce osteogenic efficiency[49]. Knockout of *ATG7* can cause bone loss in mice and cause stress in the endoplasmic reticulum, and phenylbutyrate acid released through the body can alleviate the decrease in bone formation capacity caused by endoplasmic reticulum stress[53,54].

Indeed, the specific mechanism of autophagy in the regulation differentiation of osteoblasts and its effect remain to be further confirmed.

Autophagy and osteocytes

Osteocytes are the main cells in bone tissue, which are the terminally differentiated cells buried in the bone matrix[55]. The upregulation of autophagy may accompany the transition from osteoblast to osteocyte to recycle organelles and preserve nutrients. Osteocytes make up over 95% of the bone cells in the adult skeleton[56]. Hocking found that autophagy totally exists in the differentiation of osteoblasts to osteocytes. Hocking et al.[57] found that the higher the differentiation degree of the osteocytes, the higher the level of autophagy.

Osteocytes are mechanically sensitive cells that can make adaptive changes to external forces. Appropriate mechanical loading can improve bone strength and inhibit bone loss. King et al.[58] found that mammalian cells are highly sensitive to mechanical stress and can induce non-dependent mTOR pathways that cause temporary autophagy. Autophagic vesicles are observed in osteoblast-like MLO-Y4 cells after fluid shear stress, which causes an increase in LC3-II and degradation of p62, indicating that fluid shear stress can induce protective autophagy of osteocytes, while mechanically induced autophagy is related to ATP metabolism and bone cell survival, suggesting that the use of drugs that regulate autophagy status in the clinic may enhance the survival of skeletal cells, providing new ideas for clinical prevention of bone aging-related diseases[59].

Autophagy of osteocytes is inversely related to oxidative stress and bone loss. Ovariectomy results in increased expression of Atg5, LC3, and Beclin1 in rats, while decreased expression of p62, and also reduces total antioxidant capacity, superoxide dismutase activity[60]. Autophagy can protect cells from the effects of oxidative stress, and give cells a certain ability to resist oxidative stress. Increased oxidative stress can also reduce the basic autophagy activity of bone cells, and vice versa. After moderate-level (1.4 mg/kg) pretreatment with glucocorticoids, osteocyte autophagy levels increased, and the ability to resist oxidative stress injury also increased, possibly by activating the MAPK/ERK signaling pathway[61].

Besides, EphrinB2 in bone cells will limit the accumulation of minerals. Bone cells lacking EphrinB2 can see more autophagosomes *in vitro* and *in vivo*. EphrinB2 may inhibit autophagy through the RhoA-ROCK signal[62]. EphrinB2 can also affect the mineralization function of osteoblasts[63].

Interestingly, ATG7 seems to change the appearance of bone cells. The loss of Atg7 disrupts the formation or maintenance of mouse bone cell networks. The bone cells of knockout mice are large, but the nucleus is small. The shape of the bone cell nucleus looks more round and eccentric. This may be the delay or prevention of Atg7 deletion. The number of cytoplasmic components associated with bone cell maturation[54]. Another study pointed out that periodic mechanical stretching will reduce the size and ovality of bone cells and increase the expression of LC3b and ATG7, indicating that autophagy upregulation will affect the spherical changes of bone cells[64].

Autophagy and osteoclasts

Osteoclasts lie in small cavities called Howship's lacunae. By the way of secret a variety of proteolytic enzymes to form absorption lacunae, the osteoclast finally functions as bone resorption[65]. Osteoclasts secrete β3 integrin and produce actin rings, which are adsorbed on the surface of the bone, causing the surface of the bone to form a wrinkled edge, and secrete proteins such as tartrate-resistant acid phosphatase and cathepsin K to acidify and absorb

bone. The complex folding of this edge is through the lysosome and periosteal fusion. The fusion process of secreted lysosomes and wrinkle edges is closely related to autophagy. Atg5, Atg7, Atg4B, and LC3 are important for the formation of wrinkle edges and osteoclasts for osteoclasts, and for bone resorption *in vivo* and *in vitro* important. kindlin3, which is an necessary adaptor protein in the podosome, will interact with LC3B and undergo autophagy-mediated protein degradation to regulating osteoclast migration[66]. Besides, Rab7, which is required for osteoclast function, is localized to the edges of the folds in a manner dependent on Atg5[67,68].

When osteoclasts are exposed to hypoxic stress, it will cause an increase in autophagy flux and increase the expression of ATG to reduce cellular stress[69]. Beclin-1 is required for RANKL-induced osteoclast differentiation[70], RANKL can induce Bcl-2 phosphorylation and dissociate Beclin1 from the Bcl-2-Beclin1 complex, JNK1 can block this process[71]. During in vitro osteoclast differentiation, autophagy is activated and Beclin1 is enhanced. *In vivo* experiments found that mice lacking Beclin1 showed impaired osteoclast function and increased cortical bone thickness[72]. It may be related to KLF2 (kruppel-like factor 2) regulating Beclin1-mediated autophagy during osteoclast formation[73]. Under starvation conditions, GPCR kinase 2-interacting protein 1 induces disruption of Beclin1 and Bcl2 binding and contributes to osteoclasts autophagy[74]. It was found that the transport receptor p62, which had an important regulatory effect on the formation of autophagosomes, mutated in Paget's bone disease, promoting osteoclast proliferation as well as cell activity[75]. The p62 protein is involved in the mTOR nutrient-sensing signal transduction pathway in influencing the differentiation and function of OCs[76]. During RANKL-induced osteoclast differentiation, p62 is significantly down-regulated in the initial stage of osteoclast formation, and then gradually increases over time. The expression of p62 is related to the ratio of LC3-II/LC3-I, This phenomenon may be caused by autophagy activation and plays an important role in F-actin loop formation[77].

In addition to Beclin1 and p62 being closely related to osteoclasts, IL-17A stimulates osteoclast differentiation and bone resorption and may promote autophagy activity by activating the RANKL-JNK pathway during osteoclast formation[78,79]. JNK1-mediated autophagy could promote RANKL-induced osteoclastogenesis via enhancing TRAF3 degradation, and JNK1 also could prevent osteoclast precursors apoptosis through autophagy-TRAF3 signaling[80]. Pro-inflammatory cytokine tumor necrosis factor activates osteoclasts in rheumatoid arthritis to initiate autophagy, and then promotes osteoclasts to perform bone resorption in the body. Chloroquine and hydroxychloroquine can increase The pH value that can inhibit autophagy and damage protein degradation in autolysosomes, which may explain the mechanism of chloroquine and hydroxychloroquine in preventing bone erosion[81]. It may be related to lysosomal pH regulating mTOR activity in osteoclasts[82].

However, some studies have reached the opposite conclusion. For example, OPG can enhance autophagy and inhibit osteoclast differentiation and bone resorption through the AMPK/mTOR/p70S6K signaling pathway *in vitro*[83]. *In vitro* culture of osteoclasts has found that the use of rapamycin can inhibit the reduction of cells and inhibit bone loss[84].

CONCLUSION

Autophagy is a common phenomenon of life, widely involved in the physiological and pathological process of a variety of organs. Autophagy is closely related to regulating the function of mesenchymal stem cells, osteoblasts, osteoclasts, and osteocytes. It maintains the balance between mineralization and resorption which keeps the bone homeostasis. Appropriate autophagic inducers could also benefit the bone remodeling. Abnormal autophagy will lead to disruption of bone balance and will lead to diseases such as osteoporosis. This also suggests that we can prevent or treat bone-related diseases by regulating the level of autophagy, and by exploring the deeper mechanism of autophagy in bone metabolism, more possible treatments for the bone-related disease will become a reality soon.

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自噬在骨细胞中重要作用的最新研究进展：骨相关细胞活性和骨代谢

孙友强 [1]，马 超 [2]，梁萌梦 [3]，辛鹏飞 [2]，张 华 [1]，向孝兵 [1]
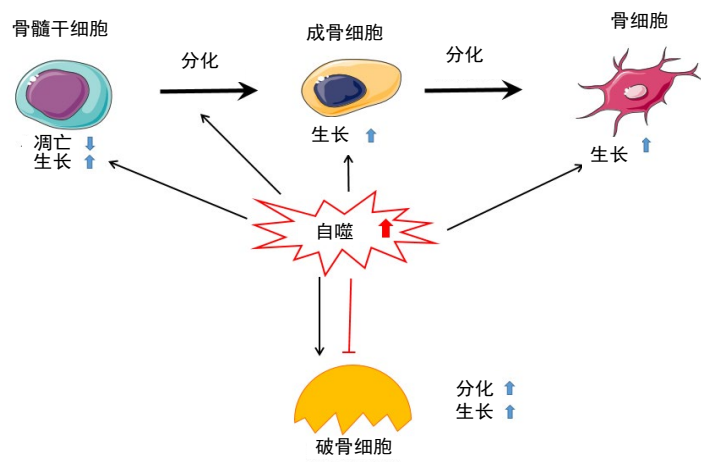
文章快速阅读：

文章特点—

△自噬作为一种选择性清除受损细胞器以实现细胞成分循环的方式，对维持细胞稳态和应激具有重要的作用；

△自噬与骨细胞和骨代谢密切相关。文章重点关注自噬在调节骨髓间充质干细胞、成骨细胞以及成骨细胞和破骨细胞之间的骨相关细胞活性和功能中的作用和可能机制。

文题释义：

自噬：当细胞面临外部环境压力(例如缺乏营养、缺氧、损伤等)时，它们可以使用溶酶体依赖性途径来破坏细胞，回收细胞器、有机物以满足自己的需求，这种现象称为自噬。

骨组织：骨组织的细胞成分主要包括骨髓间充质干细胞、成骨细胞、骨细胞和破骨细胞。成骨细胞主要合成骨基质，破骨细胞具有骨吸收功能，生理状态下，骨组织的生成和吸收处于动态平衡中。

摘要

背景：自噬对维持细胞稳态和应激敏感性有重要作用，作为细胞清除受损细胞器并实现细胞成分再循环的一种重要方式，在骨代谢中发挥着不可或缺的作用。

目的：对自噬对骨髓间充质干细胞、成骨细胞、骨细胞以及破骨细胞的作用机制进行综述，以明确自噬在调节和维持骨组织细胞活性和功能等方面的意义。

方法：以关键词"autophagy；bone marrow mesenchymal stem cells；osteoblasts；osteocytes；osteoclasts"计算机检索PubMed数据库与骨细胞自噬相关的研究。

结果与结论：最终纳入84篇文献。发现自噬在骨代谢具有重要作用。自噬参与保持矿化和吸收的平衡，继而维持骨稳态。适当的自噬诱导剂也可能有益于骨骼重塑。自噬异常会导致骨骼平衡紊乱，导致骨质疏松等疾病。通过调节自噬维持骨稳态代谢平衡，可有助于预防或治疗骨相关疾病。

关键词：自噬；骨髓间充质干细胞；成骨细胞；骨细胞；破骨细胞；骨稳态；骨矿化；骨吸收

文章观点和对数据结果的报道，不存在利益冲突。

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