AUTOPHAGY IN SKELETAL HOMEOSTASIS: ROLE IN THE OSTEOBLAST LINEAGE UNDER PHYSIOLOGICAL AND STRESS CONDITION

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ABSTRACT

Throughout life bone is constantly renewed to meet the changes deriving from loading forces and metabolic needs, via the process of bone remodelling. An imbalance in bone remodelling, in favor to bone resorption, results in loss of bone mass and strength, leading to osteoporosis. Multiple factors play a causative role in this process, which include extrinsic (losses of sex steroids, excess of exogenous glucocorticoids, lipid oxidation and marrow adipogenesis, decreased growth factors) and intrinsic (oxidative stress) mechanisms of cell dysfunction. Although these intrinsic mechanisms remain mainly unclear, we hypothesized that autophagy, a recycling-lysosome based pathway, may play a critical role in maintaining bone cells function and viability and that a decline in autophagy with age may be part of the pathogenetic mechanism of age-related skeletal involution.

The goals of the study proposed here are to investigate the role of autophagy in bone and to determine whether loss of autophagy in osteocytes increases their susceptibility to stress, such as exogenous glucocorticoids.

For these purposes, we inactivated autophagy in osteocytes by conditional deletion of Atg7, a gene essential for autophagy, and found that osteocyte-specific autophagy deficient mice displayed low bone mass and strength, reduced bone turnover and increased oxidative stress. Importantly, all these changes were similar to those that occur with age in wild type mice, suggesting that a decrease in autophagy may contribute to the degenerating effects of aging on the skeleton.

To establish whether the autophagy pathway helps osteocytes resist stress, mice lacking autophagy in osteocytes were treated with glucocorticoids (Prednisolone) or placebo for 28 day. Our results demonstrate that exogenous glucocorticoids stimulate autophagic flux in osteocytes in vivo but lack of autophagy in osteocytes does not accentuate the negative impact of glucocorticoids on the skeleton, suggesting that autophagy in this cell type does not appear to be an important defence mechanism opposing the negative effects of glucocorticoids on the skeleton. In conclusion we demonstrated that experimental inactivation of autophagy in osteocytes accelerates skeletal changes associated with aging, but does not accentuate the impact of exogenous glucocorticoids on the skeleton. Overall, these findings identify autophagy as a critical determinant of bone homeostasis and as an intrinsic mechanism to bone cells that contributes to the age-related bone loss, providing a new potential therapeutic target in osteoporosis.
CHAPTER 1: Introduction

1.1 Bone biology

1.1.1 Bone structure and cells of the skeleton

Bone is a connective tissue, physiologically mineralized, that undergoes constantly regeneration throughout life by a continual process of bone remodelling. The skeletal system consists of calcified cartilage in the growth plate (in growing skeletons), the bone marrow cavity, and the mineralized and non-mineralized (osteoid) components of the cortical and cancellous bone. The skeleton functions as a body-framework to sustain the organs, preserve skeletal size, shape, and structural integrity and to respond to mechanical forces. Moreover the bone acts as an endocrine organ in order to regulate mineral homeostasis. In an adult body, bone tissue is composed of 50-70% hydroxyapatite crystals [Ca_{10}(PO_4)_6(OH)_2] enabling bone to be a reservoir for these ions and participate in mineral homeostasis; 20-40% organic matrix, made up by collagen and non-collagenous proteins, which confers flexibility; 5-10% fluid water that provides mechanical properties and less than 3% lipids

Bone can be divided into two compartments: trabecular (or cancellous) bone, a sponge-like network consisting of myriads of highly interconnected bony trabeculae, and cortical (or compact) bone, shaped like a cylinder around the trabecular bone (Fig 1a).

Figure 1a. Three dimensional reconstruction of a mouse tibia in a micro-computed tomography (μCT) scan. Epiphysis and diaphysis of long bones are made up of cancellous bones surrounded by a cortex of compact bone.
Although identical in their chemical composition, the abundance of these two compartments varies at different skeletal sites and they can respond differently to metabolic changes, aging and therapeutic treatments.

Bone is continuously forming and being resorbed during growth and in response to mechanical or metabolic signals. Three major cell types perform bone remodelling: bone-resorbing osteoclasts, bone-forming osteoblasts and osteocytes, which are the sensors of bone regulating osteoclast and osteoblast formation.

Osteoclasts are multinucleated cells that resorb bone. They constitute 1-2% of the bone cells and originate from the myeloid/monocyte lineage. Osteoclast formation, differentiation and survival are dependent on the activity of two cytokines: macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). M-CSF binds to its receptor, c-fms, on osteoclast precursors and activates MAP kinases and ERKs cascades during the early phase of differentiation. RANKL binds to its receptor, RANK, on the surface of osteoclast progenitors, activating NF-κB, c-Fos, phospholipase Cγ (PLCγ) and nuclear factor of activated T cells c1 (NFATc1) signaling to induce differentiation into osteoclasts. Once mature osteoclasts are formed, to resorb bone they need to attach to the bone matrix. This process requires a profound rearrangement of the cytoskeleton allowing the formation of an actin ring surrounded by αvβ3 integrins called podosome. Osteoclasts attach to bone matrix via interaction of αvβ3 integrins with Arg-Gly-Asp (RGD) motifs of osteopontin and bone sialoprotein in bone matrix (2-4), generating the sealing zone. Nuclei are moved in the opposite direction of the sealing zone while lysosomes vesicles containing degrading enzymes are transported to the plasma membrane and then here by endocytosis they fuse with the plasma membrane, originating the osteoclast specific membrane, the ruffled border.

Acidification of the resorption lacuna dissolves the mineralized component of bone exposing the organic matrix (4;5). The exocytosed resorption enzymes such as Cathepsin K (CatK) and matrix metallo-proteases work best in acidic conditions and thus degrade the exposed organic matrix in the resorption lacunae (4;6).

A mesenchymal progenitor can become an osteoblast cells via sequential activation of two transcription factors Runx2 and Osterix. Osteoblastic cells are specialized, terminally differentiated cells defined histologically as cuboidal or round-shape cells with enlarged Golgi and extensive endoplasmic reticulum, to sustain a high synthetizing activity. Osteoblasts under basal conditions constitute ~4-6% of all the bone cells. Osteoblasts are recruited in team to the bone surface where they start depositing new bone matrix that
undergoes mineralization. At the end of this process some osteoblast will die by apoptosis, some will enter a resting phase and acquire a flattened morphology becoming lining cells and some will be buried by the mineralized bone matrix and become osteocytes, which are postmitotic cells that make up 90% of the cells in the (7).

During development osteoblast formation is regulated by several signalling pathways and growth factors such as the Indian hedgehog (Ihh), Notch, Wnt, bone morphogenic protein (BMP) and fibroblast growth factor (FGF) pathways (8). Ihh pathway is required for endochondral bone formation during embryogenesis and postnatal development growth. Notch signalling can either inhibit osteoblast differentiation from early progenitors or stimulate late osteoblastic differentiation. Other soluble factors such as IGF-1, FGF23 and prostaglandin E2 also participate in postnatal osteoblastogenesis.

Lining cells have been proposed to play a role in bone remodelling by forming a canopy over the area to be remodelled and recruiting osteoclasts. In addition, recent studies have shown that lining cells can transform back into osteoblasts in response to PTH (9). However, due to lack of knowledge on lining cell specific gene expression and the difficulty in visualizing them in murine bone, knowledge about lining cells and their function is limited (9-12).

Osteocytes are postmitotic cells that differentiate from mature osteoblasts, when become entrapped in the mineralized matrix (Fig 1b). Osteocytes, entombed in a lacuna, account for over 90% of all bone cells. They are dispersed throughout the mineralized bone and establish connections to cells on the bone surfaces and with other osteocytes and to blood vessel through a network of dendritic process. Osteocytes can live as long as the entire body life (13). Because of these peculiar features, osteocytes can be considered as major controller of the bone (14). These cells, for a long time considered static residents of the bone tissue participate actively in bone homeostasis regulation and can be considered secretory cells.

Loss-of-function studies where osteocytes specific genes have been deleted, have revealed the important roles for osteocytes in skeletal biology. They act as mechanosensors, by receiving and responding to mechanical strength and send signals to form and remove bone. Studies in mice lacking the RANKL gene specifically in osteocytes (DMP1-Cre;Ranklf/f) demonstrated that osteocytes, but not matrix-synthesizing osteoblasts, are the major producers of osteoclastogenic cytokine receptor activator of NFκB ligand (RANKL) and regulate cancellous osteoclast formation and therefore bone resorption during physiological conditions (15;16). Moreover they act as an endocrine organ, regulating phosphate homeostasis via the DMP1- FGF23 pathway (17). Moreover, osteocytes produce Sclerostin, the product of the
SOST gene, which inhibits WNT signalling and osteoblast differentiation (18). Dysfunctional osteocytes may be the culprit in several bone disease such as glucocorticoids-induced skeletal fragility, loss of bone during osteoporosis and aging.

Figure 1b: Osteocytes differentiation. Bone matrix synthetizing osteoblasts (1) differentiate into osteoid osteocytes (2). When mineralization of the osteoid is complete, osteocytes become embedded and shift their morphology from cuboidal (3,4) to spindle-like shape (5). Adopted from “Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, Chapter 4: Osteocytes, p. 35, Eight Edition”.

1.1.2 Bone remodelling

Throughout life, bone is constantly renewed at numerous sites of the skeleton to meet the changes deriving from damage, loading forces and metabolic needs. This process of replacement of old bone with new bone is called bone remodelling. Bone remodelling is critical for the maintenance of calcium homeostasis and replacement of old or damaged bone with newly formed bone. Bone remodelling is performed by teams of osteoblasts and osteoclasts organized within transient anatomical structures known as “basic multicellular units” (BMUs) Fig. 1c (11;19-26)
**Figure 1c: Basic Multicellular Unit.** This image depicts a resorbing multinucleated osteoclast, followed by a team of osteoblasts, deposing unmineralized bone matrix (osteoid). Buried into the bone matrix there are the osteocytes, main players in the regulation of bone remodelling. Courtesy of Robert Weinstein

In each BMU, teams of osteoblasts follow the resorption front composed of osteoclasts. Bone remodelling can be described in distinct and sequential phases: activation, resorption, reversal, formation, and termination (Fig. 1d)

The activation phase requires a signal to target the bone surface for activation of the BMU. Several signals can initiate the activity of BMU such as RANKL produced by osteocytes or lining cells in response to various stimuli such as mechanical loading, apoptosis of osteocytes, microcracks or hormonal action (PTH). Lining cells are thought to play a role in the initiation of the remodeling cycle (19). Evidence support the role of lining in the formation of a structure known as canopy, that enclose all the cells and soluble factors needed for bone remodelling, keeping local and discrete the remodelling process. Generation of new capillaries, recruitment of osteoclast precursors, differentiation and attraction of the pre-osteoclasts to exposed mineral surface and finally their differentiation into mature osteoclasts lead to the formation of resorption front of a BMU. The initiation phase is followed by the resorption phase in which mature osteoclasts form tightly sealed resorption compartments on bone, acidify these compartments via their proton pumps and secrete lysosomal enzymes such as Cathepsin K. As a result, the mineral and organic phases of bone matrix are dissolved by
the combined action of low pH and the resorptive enzymes. After resorption, osteoclasts die by apoptosis.

**Figure 1d: Bone remodelling cycle.** The sequence of quiescence, resorption, reversal phase, formation and mineralization are depicted in this illustration. Image produced by Biomedical Tissue Research (University of York, http://www.york.ac.uk/res/bonefromblood/background/boneremodelling.html)

Under physiologic conditions the work performed by the BMU is spatially and temporally regulated. If there is coupling between formation and resorption, the osteoblastic team will appear at the right time and in the right number before osteoclasts have finished to resorb bone. This allows the complete reconstitution of the eroded cavity by the osteoblast team and so the process of bone remodelling to be balanced. TGF-β and IGF I and II, released during bone matrix degradation stimulate recruitment of osteoblast to the resorbed site, thus enabling coupling. Another suggested coupling mechanism is signalling by direct contact between osteoclasts and osteoblasts via ephrins (24). Recently, osteoclast-secreted factors have been discovered to be involved in the regulation of coupling (27). The phase of remodelling in which coupling and osteoblast differentiation occur is called the reversal phase (28). The reversal phase is followed by the formation and mineralization phase in which new bone is formed by osteoblasts and mineralized by the combined actions of osteoblasts and osteocytes (28-30).

Hormonal changes, such as increased PTH or loss of sex steroids, aging not only alter the formation of new BMUs, but also affect the life of the cells within BMUs and can thus result in negative remodelling balance to favour resorption leading to net bone loss.
1.1.3 Skeletal aging

The skeletal tissue is continually regenerated and reshaped to allow the replacement of old or damaged bone with new synthetized bone. With aging, the process of bone remodelling become unbalanced and osteoblasts fail to keep up with the work performed by the osteoclasts; thereby the amount of bone that is deposited at any given time is less than the amount of bone that is removed, resulting in a negative balance of the remodelling process and therefore in bone loss. Moreover, bone quality decreases with age, affecting bone strength. Reduction in bone mass and strength, together with a profound increase in cortical porosity make the skeleton more fragile and define one of most common form of metabolic disease with old age, known as osteoporosis. Histologically, reduced wall width, defective osteoblast numbers and a decline in osteocyte density characterize aged human bone (14).

Osteoblasts and osteoclasts are terminally differentiated with a lifespan of weeks or days, respectively, while mesenchymal stem cells (MSCs) and osteocytes are long-lived cells. Therefore it is reasonable to think that these last cells might be more susceptible than osteoblasts or osteoclasts to the age-related changes. Several studies have shown that osteocyte numbers and viability decline with age in both rodents and humans (31). Because osteocytes control bone resorption by secreting Rankl and osteoprotegerin (15;16;116) and bone formation via production of sclerostin (32), a change in the viability of osteocytes population might contribute to the age related change in bone remodelling. Halloran and colleagues have shown that Rankl and sclerostin levels dropped in bone marrow fluid from aged mice (33). However, if a reduction in Rankl explains the drop in osteoclast numbers, the reduction in sclerostin does not reconcile with the low osteoblast numbers associated with skeletal ageing. Therefore additional mechanisms, perhaps the same mechanisms that occur during others degenerative diseases, such as increased levels of oxidative stress or impaired protein recycling may affect bone turnover, by decreasing not only the number of osteocytes but also their proteostasis.

Oxidative stress increases with age in bone (34-36). Although cell extrinsic mechanisms including loss of estrogens, increased endogenous glucocorticoid levels and higher lipid oxidation are associated with the decreased bone formation seen in old age (37), several clinical and epidemiological evidences, in both humans and murine models, suggest that oxidative stress is a critical determinant for the decrease in bone mass and strength that occur with aging and that loss of sex hormones exacerbates the effects of aging on bone, by reducing the cellular defence against oxidative stress (37).
Reactive oxidative species (ROS) affect the birth and the death of bone cells. ROS are by-products of the oxidative phosphorylation that takes place into mitochondria. To some extent ROS are important for cell metabolism because they act as molecule signal. However, to prevent excessive ROS production, cells express several scavenger enzymes, superoxide dismutases (SODs) and catalase as well as thiol-containing oligopeptides that counteract the negative effect of ROS. Moreover, several transcription factors, including FoxOs and p53, have been identified as important defence mechanism against oxidative stress (38-41). It has been shown that FoxOs shift the pool of active β-catenin from TCF to FoxO-mediated transcription in osteoblasts (42). Consistent with this, FoxOs deletion specifically in osteoblast progenitors increases bone mass, suggesting that FoxOs limit the activity of the Wnt-β-catenin signalling in these cells thereby decreasing bone formation throughout life. Pharmacological and genetic studies support the idea that accumulating ROS represents a pathophysiological mechanism for age-related osteoporosis. Moreover the balance between the amount of intracellular ROS and the antioxidant cellular defence (such as of FoxO transcription factors) is critical for bone homeostasis throughout life. Although the role of oxidative stress plays a critical role in the pathogenesis of age-related bone loss, others intrinsic cellular mechanisms could contribute to skeletal aging. In a recent review nine hallmarks of aging have been described as common pathogenetic mechanisms shared among several degenerative disorders, such as Therefore telomere shortening, abnormal protein folding and impaired autophagy (43). Aggravation of these mechanisms may be all culprits of osteoporosis.
1.2 Glucocorticoid-induced bone disease

Glucocorticoids are commonly used in the clinical practice as immunosuppressive and anti-inflammatory drugs; however, their administration is associated with development of secondary osteoporosis. 30 to 50% of the patients receiving long-term glucocorticoids therapy experience fractures (44).

Bone loss in response to long-term glucocorticoid therapy occurs in 2 phases: an early, transient phase of relatively rapid reduction in bone mineral density (BMD) of 6–12% within the first year, and a second phase of slower annual loss of about 3% (45). However, the risk of fractures is greater within the first 3 months after initiation of glucocorticoid therapy and this often occurs before a significant decline in BMD (46).

Risk factors of glucocorticoid-induced osteoporosis (GIO) include, among others, advancing age, prolonged duration of treatment, and polymorphisms in the glucocorticoid receptor. The risk is probably the same in men and women of all ethnicities. Another factor is the activity of the 11β-hydroxysteroid dehydrogenase (11β-HSD) system, a pre-receptor modulator of corticosteroid action (47).

Steroid hormones bind to nuclear receptor and, by modifying the allosteric conformation of the receptor, induce dimerization of the ligand-receptor complex. The ligand-bound dimeric receptor now interacts with the steroid-responsive element region on the DNA and induces transcriptional changes of steroid-target genes (transactivation).

The activity of steroids hormones can be modulated by HSDs enzymes, which act in a pre-receptor fashion, also known as intracrine modulation. 11β-HSD type 2 functions as a NAD+-dependent 11β oxidase to convert the active alcohol steroid (cortisol) to the inactive ketone glucocorticoid (cortisone). Vice-versa 11β-HSD type 1 acts predominantly as a NADPH-dependent 11-ketosteroid reductase and converts cortisone to cortisol (48). Increased fractures caused by glucocorticoid administration in the elderly may be attributed to the increase in 11β-HSD1 that occurs with aging.

It is known that glucocorticoids exert their negative effects on the skeleton in part via a direct action on bone cells, decreasing the production of osteoblasts and increasing the apoptosis of osteoblasts and osteocytes while prolonging the lifespan of mature osteoclasts (46). This has been proved in a series of studies employing transgenic mice overexpressing the 11β-HSD2 enzyme, which inactivated glucocorticoids, specifically in osteoblast and osteocytes. As results these mice, shielded from the action of glucocorticoids in osteoblast and osteocytes, still experienced loss of BMD (due to the action of glucocorticoids on osteoclast) but were
protected from the glucocorticoids-induced decrease in bone formation, osteoblast and osteocytes apoptosis and loss of strength, suggesting that osteocyte viability independently contributes to bone strength (49). Using the same approach, overexpressing the 11β-HSD2 in osteoclasts resulted in preserved bone mineral density, but did not prevent the prednisolone-induced decrease in osteoblast number, osteoid production, and bone formation (50). These results support the idea that osteoblast, osteocytes and osteoclasts are direct targets of glucocorticoid.

Histomorphometric analyses in patients with GIO reveal reduced osteoblast number with reduced matrix synthetizing activity that leads to decreased osteoid formation, reduced bone formation and decreased wall width. Glucocorticoids limit the proliferation and differentiation of osteoblast, and cause premature apoptosis of the mature, matrix-secreting osteoblasts. Increased osteocyte apoptosis also occurs and is associated with decreased in vascular endothelial growth factor (VEGF), skeletal angiogenesis, bone interstitial fluid, and bone strength. Thus, glucocorticoid-induced osteocyte apoptosis could account for the loss of bone strength that occurs before loss of BMD and the resultant mismatch between bone quantity and quality in patients with GIO. Interesting, glucocorticoids stimulate endocortical resorption, increasing cortical porosity and thinning of the cortex (51-53).

Different mechanisms have been proposed to explain how glucocorticoids limit osteoblasts activity: they can stimulate the expression of the Wnt antagonist DKK1 (54), and suppressing BMP2 and Runx2 (55), both required for osteoblast differentiation. Moreover, glucocorticoids increase production of peroxisome proliferator-activated receptor γ, a transcription factor that induces terminal adipocyte differentiation while suppressing osteoblast differentiation, predisposing mesenchymal stem cells residing in the bone marrow to preferentially differentiate into adipocytes, contributing to increased marrow fat and reduced osteoblasts (56).

Studies performed in vitro using human and murine osteoblastic cell line showed that glucocorticoids stimulate RANKL expression while down regulate OPG levels (57;57-59). However whether this is the case in vivo remains still unknown and requires further investigations. Moreover, glucocorticoids could cause increased osteoclast resorption by reducing the circulating levels of the RANKL decoy ligand, osteoprotegerin. Glucocorticoids limit the differentiation of osteoclast precursors in new mature resorbing cells but, contrary to the effects on osteoblasts, prolong the life span of mature osteoclast by inhibiting their
apoptotic death. Therefore cancellous osteoclast numbers in patients receiving glucocorticoids treatments are within the normal range or slightly above the baseline. Furthermore glucocorticoids have been shown to impair the resorbing activity of osteoclast by inhibiting the M-CSF-induced rearrangement of the cytoskeleton in osteoclasts (60).

1.3 Autophagy

1.3.1 Overview

Autophagy is a survival mechanism that operates at basal level in almost all cell types and, in response to stress signals, is activated to ensure metabolic activity and viability of cells. Three distinct forms of autophagy are commonly described: microautophagy, where cytoplasmic content is directly targeted for the lysosome degradation; chaperone-mediated autophagy (CMA), where selective individual proteins are degraded by lysosomes upon binding with the hsp70-chaperone complex and macroautophagy (hereafter referred to as autophagy) which is the most common form of autophagy (61). Autophagy (auto "self" and phagein "to eat") acts a quality control system, via lysosome-based recycling machinery, highly conserved through evolution, which has to main functions: to remove unnecessary intracellular contents and to build new forms of energy. This is achieved via the sequestration of long-lived proteins, damaged organelles and invasive pathogens in double-membrane vesicles called autophagosomes, which fuse with lysosomes allowing degradation of the cargo content. The products of this breakdown are then recycled back to the cytosol, providing new sources of energy to meet the metabolic demand during starvation and other stressful conditions, such as lack of growth factors, hypoxia and ER stress. In this way autophagy helps cells to maintain their viability, function and homeostasis. This process appears to be especially important in long-lived cells where the clearance of dysfunctional components is crucial for the maintenance of health. Impairment of the autophagic process has been shown to contribute to the pathogenesis of several diseases, including cancer, metabolic and neurological disorders and microbe infection; moreover a decline in autophagy has also been described as one the hallmarks of cellular aging (62-66).
1.3.2 Mechanisms of autophagy

The autophagy pathway proceeds through distinct steps: vesicle nucleation, elongation, docking/fusion of autophagosomes-lysosome, and breakdown of the cargo followed by release of the degradation products back into the cytosol (Fig. 1e).

**Figure 1e: Autophagy pathway.** This cartoon depicts the four phases of autophagy: vesicle nucleation, elongation, docking and fusion and degradation. Misfolded proteins and other cargo content are engulfed by double membrane vesicles and targeted for the lysosomal degradation. From: “Basic Biology of Skeletal Aging: Role of Stress response Pathway” J Gerontol A Biol Sci Med Sci 2013 October; 68(10): 1197–1208.

These steps are orchestrated by different sets of ATG proteins, which constitute the core autophagic machinery, highly conserved in eukaryotes, including mammals. Autophagy activity under basal conditions is maintained at a low level; therefore, an efficient mechanism to induce autophagy is crucial for organisms to adapt to stress and extracellular cues. The (mammalian) target of Rapamycin (mTOR), is a negative regulator of autophagy in organisms from yeast to man. mTOR is inhibited under starvation conditions, and this contributes to starvation-induced autophagy via activation of mTOR target ULKs-Atg13-FIP200, a mammalian protein complex that functions as a scaffold for the recruitment of multiple ATG proteins to initiate autophagosome formation.

The source of autophagosome membrane is still debated. One theory supports the de novo synthesis of autophagosome from a nucleating structure, made of lipids of different origins. Another theory explains that the autophagosome originates from the ER, because these two structures are found to be in close relation using study of three-dimensional electron tomography (67). The nucleation of the phagophore vesicle (autophagosome precursor)
requires the class III phosphoinositide 3-kinase (PI3K) Vps34, which acts in a large macromolecular complex that also contains Atg6 (also called Beclin 1), Atg14, and Vps15 (p150). The elongation of membranes, critical step for the autophagosome biogenesis, involves two ubiquitin-like conjugation pathways, the Atg12 and Atg8/LC3 systems. Atg12 is activated by Atg7 (E1 activating enzyme), transferred to Atg10 (E2 conjugating enzyme) and covalently attached to an internal lysine of the substrate protein Atg5. The Atg12–Atg5 conjugate further interacts with a coiled-coil protein Atg16, which links the Atg12–Atg5–Atg16 complex into a tetramer by self-oligomerization and attaches it to the phagophore (68).

LC3, a mammalian homolog of Atg8, is first processed by a cysteine protease, Atg4, exposing a C-terminal glycine residue. The E1 like enzyme Atg7 activates Atg8/LC3 and transfers it to Atg3 (E2). In this way LC3 form I, mostly cytosolic, is conjugated to the target lipid PE via an amide bond, facilitated by the E3-like Atg12–Atg5 conjugate, to form LC3-II, which now is localized to both sides of the phagophore. Of note, Atg7 is indispensable for this conjugation process and genetic inactivation of Atg7 effectively suppresses autophagy (69).

The lipidation of LC3 is widely used to monitor autophagy induction (70). LC3-positive autophagosomes can interact with some substrates in a selective fashion, by binding p62/SQSTM1, which recruits ubiquitinated proteins and inclusion bodies, via an ubiquitin-binding (UBA) domain, mediating the autophagosome cargos recruitment (71). In autophagy deficient cells, p62/SQSTM1 accumulates because it cannot be degraded, confirming that it is selectively recognized and degraded by autophagy.

When autophagy is initiated multiple LC3-positive autophagosomes form randomly in the cytoplasm, after which they are transported to lysosomes, via microtubules, in a dynein-dependent manner. At this stage, autophagosome vesicles fuse with lysosomes, generating autophagolysosomes. Autophagosome-lysosome fusion appears to be mediated by the SNARE proteins, VAMP8 and Vti1B and requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7. After fusion, degradation of the cargo content inside the inner vesicle is dependent on lysosomal activity. The resulting small molecules, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions.
1.3.3 Physiological and pathological roles of autophagy

The basal role of autophagy is to remove damaged organelles and protein aggregates, limit ER stress and ROS generation, in order to ensure cellular fitness and to preserve homeostasis of post-mitotic cells throughout life. However, a numbers of studies conducted in global or tissue-specific autophagy deficient mice have clearly showed that autophagy participates in various physiological roles.

In mammals, fertilization is a strong inducer of autophagy, which plays an essential role in early embryogenesis (72), and global deletion of some ATG genes is embryonically lethal (69). Autophagy plays a role during the early phases of body development and it has been proposed as an explanation for the phenomenon of maternal inheritance of mitochondrial DNA in C. Elegans and, perhaps in mammals (73;74). Autophagy participates in the differentiation process of lymphocytes and active secretory cells like erythroid cells and adipocytes, which require intense rearrangement of their own cytoskeleton and removal of mitochondria (75-79). Autophagy helps to eliminate intracellular microbes, promotes antigen presentation and balances the beneficial and detrimental effects of immunity and inflammation (80).

Failure of autophagy to perform its specific functions in adulthood may underlie the pathogenesis of certain diseases. Defective autophagy is indeed observed in many human diseases (neurodegenerative disease, chronic bacterial and viral infections, atherosclerosis, and cancer) (64). Impaired autophagy and the consequent accumulation of prone-protein aggregates have been proposed as pathogenic mechanisms in neurodegenerative diseases (81;82). Autophagy plays a more complex role in cancer, because it can have a tumour suppressor mechanism or promote the progression of the tumour, by contributing to cancer cells survival when growth factors are depleted or in response to cancer treatments (83). The role of autophagy has also been related to the development of (cardio)myopathy and metabolic diseases.

Furthermore, the correct functioning of the autophagic process has been implicated in longevity (84). Autophagy efficient seems to decline with age (85) and experimental suppression of autophagy mimics the effect of aging in various tissues (86-89). By avoiding accumulation of toxic proteins, autophagy exerts a beneficial effect on cell viability and function, which may also be responsible for its longevity-promoting effect (90).

Interestingly, several treatments or genetic manoeuvres that extend lifespan (caloric restriction, sirtuin activation, TOR suppression) are also able to promote autophagy.
activation, suggesting that various life-prolonging signalling pathways act through modulation of autophagy (91;92).

**Fig 1d: Physiological and pathological roles of autophagy.** Failure of autophagy to perform its specific functions in adulthood may underlie the pathogenesis of certain diseases. The role of autophagy in bone is unclear. Image adapted from: Mizushima N, Komatsu M 2011 Autophagy: renovation of cells and tissues. Cell 147:728-741

Recent results highlighted a novel and non-canonical role of autophagy, not related to lysosomal degradation of autophagosomal contents, which seems to regulate unconventional protein secretion. Secreted proteins have a signal peptide or leader sequence, and after entering the ER-Golgi route are transported to the plasma membrane via vesicular carriers and then secreted. Several studies performed in the last decade have described proteins which don’t follow this conventional protein secretion pathway and that don’t have a signal peptide. Many unconventional secretion modalities have been described and they can be broadly classified into non-vesicular (FG2, MATa, HIV-1 Tat, annexin A2 and FGF1) and vesicular (IL-1β, Acb1 in yeasts) (93). More recently the participation of core autophagic proteins in the regulation of protein secretion has been established for the stimulated secretion of Von...
Willebrand factor in endothelial cells (94), for the release of insulin in pancreatic cells (95), for the ROS-induced release of mucin in goblet cells (96).

How the autophagy machinery contributes to the unconventional secretory pathway remains largely unknown, nevertheless compelling evidences suggest that in addition to its canonical role in intracellular degradation and recycling, autophagy plays has a critical role in the controlled secretion of some proteins in specific cellular types.
CHAPTER 2: Significance of the study

Osteocytes are the longest-lived mature bone cells and are thus the most likely to sustain the cumulative damage to their organelles that is responsible for aging and associated diseases. It has been shown that osteocyte viability decreases with age in humans and rodents and that this is associated with increased oxidative stress, reduced bone vascular volume, decreased solute transport in the lacunar-canalicular system and a decrease in bone strength. Osteocytes express the cellular machinery for autophagy, a cellular recycling process required for adaptation to stress and involved in the survival of long-lived cells. Because aging is associated with the accumulation of damaged cellular components, many have proposed that autophagy may decrease or become less efficient with age. However, the role of autophagy in bone and whether autophagy is important to help bone cells to resist stress is unknown. It is reasonable to hypothesize that autophagy plays an important role in osteocytes survival and function and that its decline might contribute to the age-dependent deterioration of the skeleton. Specifically, autophagy may help osteocytes defend against stresses such as elevated reactive oxygen species. The goal of the studies proposed here are to determine the role of autophagy in osteocytes under physiological and stress conditions. To clarify the state of autophagy in osteocytes and to determine whether loss of autophagy in osteocytes increases their susceptibility to the negative effects of exogenous glucocorticoids, mice lacking autophagy in Dmp1-Cre expressing cells were generated.

The current study will in part uncover the role of autophagy in bone cells during skeletal homeostasis and stress conditions. This may shed light to novel approaches to maintain osteocyte function and viability during aging, leading to increased skeletal strength.
CHAPTER 3: Autophagy in skeletal homeostasis: role in the osteoblast lineage under physiological and stress condition

3.1 Osteocyte autophagy is required for normal bone mass

“This work is an adaptation of a research originally published in Journal of Biological Chemistry. Melda Onal, Marilina Piemontese, Jinhu Xiong, Yiyiing Wang, Li Han, Shiqiao Ye, Masaaki Komatsu, Martin Selig, Robert S. Weinstein, Haibo Zhao, Robert L. Jilka, Maria Almeida, Stavros C. Manolagas and Charles A. O’Brien. Suppression of autophagy in osteocytes mimics skeletal aging. JBC. 2013; 288:17432-17440 © the American Society for Biochemistry and Molecular Biology.”

3.1.1 Introduction

The adult skeleton is continuously remodelled by teams of osteoclasts, which resorb bone, and teams of osteoblasts, which form new bone (7). Because bone mass declines with age, it is clear that the balance between resorption and formation becomes negative with aging. Reduced production of sex steroids causes a rise in the rate of bone remodelling that subsides with time, but age-associated bone loss occurs even in individuals with normal levels of sex steroids (97). Importantly, rodents do not lose estrogens with age, however they do lose bone mass, suggesting that mechanisms intrinsic to bone cells contribute to skeletal involution with age (34). Although these intrinsic mechanisms remain unclear we hypothesized that autophagy, a recycling-lysosome based pathway, may play a critical role in maintaining osteocytes function and viability and that a decline in autophagy with age may be part of the pathogenetic mechanism of osteoporosis and age-related skeletal involution.
3.1.2 Materials and methods

**Animal studies.** The experimental animals used in most of the studies described here were obtained using a two-step breeding strategy. Hemizygous Dmp1-Cre transgenic mice were crossed with heterozygous Atg7-flox mice to generate heterozygous Atg7-flox offspring with and without a Dmp1-Cre allele. These offspring were then intercrossed to generate the following offspring: wild type (WT) mice, mice hemizygous for the Dmp1-Cre allele, mice homozygous for the Atg7-flox allele, hereafter referred to as Atg7-f/f, and Atg7-f/f mice that were also hemizygous for the Dmp1-Cre allele. For studies requiring larger numbers of mice, Atg7-f/f mice were crossed with Atg7-f/f mice that were also hemizygous for the Dmp1-Cre allele. Offspring were genotyped by PCR using the following primer sequences: Cre-for, 5′-GCGGTCTGGCAGTAAAAACTATC-3′, Cre-rev, 5′-GTGAAACAGCATTGTGCACCTTT-3′, Hind-Fw, 5′-TGCTGTCTACTTCTGCAATGATGT-3′, Atg7-ex14-F, 5′-TCTCCCAAGACAGACAGGGTGAA-3′, Pst-Rv, 5′-CAGGACAGAGCCATCACAGCTCCAC-3′, product size 102 bp; Hind-Fw, 5′-TGCTGTCTACTTCTGCAATGATGT-3′, Atg7-ex14-F, 5′-TCTCCCAAGACAGACAGGGTGAA-3′, Pst-Rv, 5′-CAGGACAGAGCCATCACAGCTCCAC-3′, product size 216 bp (WT) and 500 bp (floxed allele). Both the Dmp1-Cre and Atg7-flox mice were crossed into the C57BL/6 genetic background for more than 10 generations prior to initiating generation of mice for this study. All studies involving mice were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System.

**Oxidative stress and autophagy flux detection.** To measure oxidative stress, quantification of intracellular reactive oxygen species (ROS) in freshly isolated bone marrow cells from tibias was performed using dichlorodihydrofluorescein diacetate dye as previously described (98) and quantification of the phosphorylation state of the p66shc protein was done by immunoblot in protein lysate from L6 (34). To confirm suppression of autophagy, we quantified LC3 conversion and p62 levels in proteins extracted from osteocytes-enriched cortical bone. Briefly, proteins were extracted from osteocyte-enriched cortical bone by freezing bones in liquid nitrogen followed by pulverization in liquid nitrogen. The pulverized bone powder was then incubated in RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 1X SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% Glycerol, 1% β-mercaptoethanol and 0.004% bromophenol blue) for 30 minutes in ice,
followed by 10 minutes incubation at 100 C. The pulverized bone powder was then spun down at 14000 rpm for 10 minutes and the obtained protein supernatant was then resolved in 12% SDS polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% nonfat dry milk in TBS and were then incubated with primary antibodies and an appropriate horseradish peroxidase-linked secondary antibody. The following antibodies were used: anti-LC3 (Cell Signaling Technology, Danvers, MA), anti-p62 (Progen Biotechnik, Heidelberg, Germany), and anti-tubulin (Sigma-Aldrich, St. Louis, MO). Blots were developed using enhanced chemiluminescence and the intensity of the bands was quantified using a ChemDoc XRS-plus system (Bio-Rad, Hercules, CA).

**Skeletal analysis and histomorphometry.** BMD was measured in live mice by dual-energy x-ray absorptiometry with a PIXImus Mouse Densitometer (GE Lunar Corp., Madison, WI) using the manufacturer's software as described previously (99). Soft tissue was removed from femurs or L4 vertebra, which were then store in saline at -20 C until analyzed. Micro-CT analysis of cortical and trabecular architecture was performed in femurs and fourth lumbar spine, as previously described (100), followed respectively by 3-point bending and compression test, to measure biomechanical properties (53). L1–L3 lumbar vertebrae were fixed and embedded undecalcified in methylmethacrylate and static and dynamic histomorphometric examination was done on longitudinal sections with a digitizer tablet (OsteoMetrics, Inc., Decatur, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Thornwood, NY) with a drawing tube attachment, as previously described (49). Terminology recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research was used in this study (101).

**RNA and genomic DNA isolation and TaqMan assay.** For genomic DNA isolation, bone pieces were decalcified in 14% EDTA for 1 week after collagenase digestion. Decalcified osteocyte-enriched bone was then digested with proteinase K (0.5 mg/ml in 10 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA, and 1% SDS) at 55° C overnight. Genomic DNA was then isolated by phenol/chloroform extraction and ethanol precipitation. For soft tissues, approximately 30 mg of tissue was digested with proteinase K and processed in the same way as osteocyte-enriched bone. To quantify Atg7 gene deletion, the following custom Taqman
The custom Atg7 assay was used in combination with a Taqman copy number reference assay, Tfrc (catalog number 4458367). To extract Total RNA, the fifth lumbar spine was homogenized in 1ml of Trizol Reagent (Life technology, Grand Island, NY), according manufacturer’s instruction. Quantitation and 260/280 ratio of the extracted RNA were determined using a Nanodrop instrument. 500 ng of RNA was then used to synthesize first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer’s directions. cDNA was amplified by real-time PCR (RT-PCR) using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA)) as described previously (102). The following TaqMan primer-probe sets from Applied Biosystems were used: Sost, Mm00470479_m1; Mepe, Mm02525159_s1; RANKL, Mm00441908_m1; osteoprotegerin, Mm00435452_m1, tartrate-resistant acid phosphatase, Mm00475698_m1; cathepsin K, Mm00484036_m1; osterix-1, Mm00504574_m1; collagen la1, Mm00801666_g1; osteocalcin, (forward, 5′-GCTGCAGCTCTTGCTCTCTGAA-3′, reverse, 5′-TGCTTGGACATGAAGGCTTTG-3′, probe, 5′-FAM-AAGCCAGCCGGCC-NFQ-3′); and the house-keeping gene ribosomal protein S2, (forward, 5′-CCCAGGATGGCGACGAT-3′, reverse, 5′-CCGAATGCTGTAATGGCGTAT-3′, probe, 5′-FAM-TCCAGAGCAGGATCC-NFQ-3′. Relative mRNA levels were calculated using the ΔCt method (103). To quantify mitochondrial DNA, the following custom Taqman assay for the mitochondrial gene ND2 was utilized: forward, 5′-CATGACAAAAAATTTGGCTCCCTATCAA-3′; reverse, 5′-ATGCCCTATGAAATAGAATTGCT-3′, probe, 5′-FAM-CCCCTACTCAACTCTCT-CFQ-3′. The amount of mitochondrial DNA was calculated using results from the ND2 and Tfrc copy number reference assays and the ΔCt method (103).

**Cell culture.** Bone marrow cells were harvested from long bones and used to quantify colony forming units (CFU)-fibroblast (CFU-F), CFU-osteoblasts (CFU-OB), and osteoclast progenitors as previously described (104;105). Osteoblast differentiation of bone marrow precursors was evaluated by plating bone marrow cells in 12-well plates at 5×106 cells/well and culturing in α-MEM containing 15% fetal bovine serum, 1% penicillin/streptomycin/glutamine, 1% ascorbic acid, and 10 mM β-glycerolphosphate. One-half of the culture medium was changed every 3 days. After 21 days, the cultures were fixed
with 10% Millonigs modified phosphate buffered formalin and then stained with an aqueous solution of 40 mM alizarin red. After photography, the alizarin red was extracted with 10% acetic acid and quantified as previously described (106). Osteoblast-specific gene expression was evaluated in parallel cultures lacking β-glycerolphosphate and harvested after 12 days. The ability of bone marrow cells to support osteoclast differentiation was evaluated by plating bone marrow cells as above and adding vehicle or 10-7 M parathyroid hormone (PTH) for 12 days followed by RNA extraction and quantification of osteoclast-specific genes.

**Apoptosis quantification.** Femurs from 6-month-old female mice were fixed in 10% Millonigs modified phosphate buffered formalin for 24 hours, decalcified in 5% formic acid and dehydrated in 100% ethylene glycol monoethyl ether, prior to paraffin-embedding and sectioning. Apoptotic osteocytes were detected using TACS® 2 TdT DAB (diaminobenzidine) Kit (Trevigen, Gaithersburg, MD).

**Transmission Electron Microscopy.** Decalcified bones were placed into fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.025% calcium chloride in a 0.1M sodium cacodylate buffer, pH 7.4) and allowed to fix for 3 hours at room temperature. The bones were post fixed with osmium tetroxide, en bloc stained with 2.0% uranyl acetate, dehydrated in a graded ethanol series, and embedded in pure epoxy resin. Thin sections were cut using a diamond knife and an LKB 2088 ultramicrotome and placed on copper grids. Sections were stained with lead citrate and examined in a FEI Morgagni transmission electron microscope. Images were captured with an AMT 2K digital CCD camera (Advanced Microscopy Techniques, Danvers MA).

**Statistics.** Data were analysed using SigmaStat (SPSS Science, Chicago, IL). We performed two-way analysis of variance (ANOVA) or Student’s t-test to detect statistically significant treatment effects, after determining that the data were normally distributed (Shapiro-Wilk test) and exhibited equivalent variances. In some cases, we used log transformation to obtain normally-distributed data. Multiple comparisons were evaluated with Bonferroni or Holm-Sidak post hoc tests. *P*-values less than 0.05 were considerate as significant. All values are reported as the mean ± S.D. Data that did not pass the normality test were evaluated using the Mann-Whitney Rank Sum Test.
3.1.3 Results

**Suppression of autophagy in osteocytes decreases bone mass.**

To directly address the role of autophagy in osteocyte, we generated mice lacking autophagy in Dmp1-Cre-expressing cells by crossing Atg7-flox mice with transgenic mice expressing the Cre recombinase under the control of Dmp1 regulatory elements, hereafter designated Dmp1-Cre mice. The Dmp1-Cre transgene results in recombination in osteocytes and some mature osteoblasts (15;107). Atg7, which is an E1-like enzyme, activates an ubiquitin-like protein known as LC3, which becomes conjugated to phosphatidyl ethanolamine and is required for autophagosome production (108). Importantly, Atg7 is essential for autophagy (69). Analysis of genomic DNA extracted from osteocyte-enriched cortical bone revealed a 75% reduction of the Atg7 conditional allele (Fig. 1A). Direct analysis of LC3 expression in protein extracted from bone shafts demonstrated reduced conversion from form I (unlipidated) to form II (lipidated) in the conditional knockout mice (Fig. 1B) as well as accumulation of p62/sqstm1, a protein frequently elevated in cells with reduced autophagy (Fig. 1C). Taken together, these results confirm that autophagy was effectively suppressed in osteocytes by deletion of Atg7 using the Dmp1-Cre transgene.

![Figure 1: Atg7 deletion suppresses autophagy in osteocytes.](image)

(A) Quantitative PCR of loxP-flanked genomic DNA, normalized to a control locus, using genomic DNA isolated from osteocyte-enriched femoral cortical bone or the indicated soft tissues (n = 7-9 male 6-month-old mice per group). (B) Immunoblot of LC3 in protein extracted from osteocyte-enriched femoral cortical bone. The ratio LC3-II to LC3-I based on quantification of the bands in the immunoblot is shown on the right (n = 4-6 male 2-month-old mice per group). (C) Immunoblot of p62 in protein extracted from osteocyte-enriched femoral cortical bone. The intensity of the p62 band normalized to tubulin is shown on the right (n = 4 male 2-month-old mice per group). *P < 0.05 using Student’s t-test.
BMD measurement in 6 months old conditional knockout mice revealed low bone mass at all skeletal sites compared to control littermates (Fig. 2A). Littermates homozygous for the Atg7-conditional allele (Atg7-ff/f) or harbouring only the Dmp1-Cre transgene had bone mass indistinguishable from wild-type littermates demonstrating the specificity of the bone mass phenotype (Fig. 2A). Based on the latter observation, all further analysis was confined to Atg7-ff/f and Dmp1-Cre;Atg7-ff/f littermates.

Analysis of the skeleton by MicroCT revealed decreased cancellous bone volume in the spine and femur (Fig. 2B-D). In addition, cortical thickness was reduced and cortical porosity was increased in the femurs of conditional knockout mice (Fig. 2E-F). Consistent with these changes in bone mass, biomechanical testing revealed that compression strength was reduced in lumbar vertebrae of the conditional knockout mice (Fig. 2G).

**Figure 2:** Low bone mass in Dmp1-Cre;Atg7-ff/f mice. (A) BMD measured by DEXA in the femur, spine, and whole body of wild type (WT), Dmp1-Cre, Atg7-ff/f, and Dmp1-Cre;Atg7-ff/f littermates. (B) High resolution μCT images of the distal femur and L4 vertebra. (C) Bone volume per tissue volume (BV/TV) of cancellous bone in L4 vertebra. (D-F) Cancellous BV/TV in the distal femur, cortical thickness at the femoral diaphysis, and cortical porosity at the femoral diaphysis. (G) Compression strength (stress) of L4 vertebra. All values in figure 2 were determined in 6-month-old male mice (n = 6-9 mice per group). *P < 0.05 using two-way ANOVA (A) or Student’s t-test (C-G).
Bone turnover is reduced in mice lacking autophagy in osteocytes.

To determine whether changes in bone remodelling might explain the low bone mass of the mice lacking autophagy in osteocytes, histomorphometric analysis of lumbar vertebrae was performed. Osteoclast number and the extent of bone surface covered by osteoclasts were decreased by approximately 50% in the conditional knockout mice (Fig. 3A-B), as were osteoblast number and surface (Fig. 3C-D). In line with reduced osteoblast number, the bone formation rate was reduced in the conditional knockout mice due to a reduced amount of mineralizing surface but no change in the mineral apposition rate (Fig. 3E-H). Reduced wall width is a consistent histological finding in aged human and murine bone and reflects the reduced amount of work (new bone matrix) performed by teams of osteoblasts (34;109). Importantly, wall width was significantly lower in the conditional knockout mice compared with control littermates (Fig. 3I). A circulating marker of bone resorption, C-terminal telopeptide of type I collagen (CTX), was reduced in the blood plasma of the conditional knockout mice, although there was no significant change in the bone formation marker aminoterminal propeptide of type I collagen (P1NP) (Fig. 3J-K). Taken together, these results demonstrate that 6-month-old mice lacking autophagy in osteocytes exhibit a low rate of bone remodelling similar to that observed in aged wild type mice.

Figure 3: Dmp1-Cre;Atg7-f/f mice have low bone turnover. (A-I) Histomorphometric analysis of lumbar vertebra 1-3. (A-B) Osteoclast surface per bone surface (Oc.S/BS) and osteoclast number per bone surface (N.Oc/BS). (C-D) Osteoblast surface per bone surface (Ob.S/BS) and osteoblast number per bone surface (N.Ob/BS). (E-F) Bone formation rate per bone surface (BFR/BS) and mineralizing surface per bone surface (MS/BS). (G) Photomicrographs of calcein-labeled surfaces in vertebral cancellous bone. (H-I) Mineral apposition rate (MAR) and wall thickness (W.Th). (J-K) CTX and osteocalcin (OCN) measured in blood plasma. *P < 0.05 using Student’s t-test.
Autophagy plays an important role in the terminal differentiation of some cell types. For example, mice lacking autophagy in erythrocyte progenitors develop severe anemia and die by 14 weeks of age (75). To determine whether osteocyte differentiation from osteoblasts was altered by suppression of autophagy, we quantified osteocyte density and found that it was unchanged in either the cancellous or cortical bone of conditional knockout mice (Fig. 4A). Similarly, the percentage of apoptotic osteocytes was not different between genotypes (Fig. 4B). Consistent with these findings, examination of osteocyte morphology in newly embedded and mature osteocytes by electron microscopy showed no obvious changes (Fig. 4C). Lastly, the expression of osteocyte-specific genes such as Sost and Mepe was not altered in the conditional knockout mice (Fig. 4D-E).

Figure 4: Osteocyte formation in Dmp1-Cre;Atg7-f/f mice. (A) Osteocyte density measured in cancellous (canc.) and cortical (cort.) bone of lumbar vertebra 1-3 of 6-month-old male mice (n = 6 per group). (B) Osteocyte apoptosis measured in cancellous (canc.) and cortical (cort.) bone of the femur of 6-month-old female mice (6 per group). (C) TEM images of newly-embedded osteocytes (left) or mature osteocytes (right) in femoral cortical bone from 2-month-old male mice. bar = 2 μm (D-E) Quantitative RT-PCR of Sost and Mepe mRNA in calvaria and tibia shafts from 3-month-old male mice (n = 6-11 per group).
Figure 5: Osteoclast and osteoblast differentiation. (A) Quantitative RT-PCR of RANKL and OPG mRNA in calvaria and tibia shafts from 3-month-old male mice (n = 6-11 per group). (B) Osteoclast (OC) progenitor number quantified in bone marrow from 12-month-old female mice (n = 3 wells per group). (C) Quantitative RT-PCR of TRAP and cathepsin K (CatK) in bone marrow cultures treated with vehicle or PTH for 11 days to induce osteoclast formation (n = 4 wells per group). (D) CFU-F and CFU-OB in bone marrow cells from 12-month-old female mice (n = 3 wells/group). (E) Alizarin Red staining and quantification of primary bone marrow cells cultured for 21 days in osteoblast differentiation medium (n = 3 wells/group). (F) Quantitative RT-PCR of osterix-1 (Osx1), collagen1a1 (Col1a1), and osteocalcin (Ocn) in 12-day primary bone marrow cultures (n = 3 wells/group). *P < 0.05 using Student’s t-test.

Two studies have recently shown that mice lacking RANKL in osteocytes have reduced cancellous bone remodelling due to a reduction in osteoclast formation (15;16). Thus it is possible that suppression of autophagy in this cell type altered production of RANKL or its soluble decoy receptor osteoprotegerin (OPG) to reduce osteoclast formation in Dmp1-Cre;Atg7-f/f mice. However, measurement of RANKL and OPG mRNAs in calvarial bone or osteocyte-enriched cortical bone did not reveal any changes in expression in conditional knockout mice (Fig.5A). We then examined the osteoclastogenic potential of bone marrow progenitors and found that, rather than being reduced, it was slightly elevated in conditional knockout mice (Fig. 5B). It is also possible that the support of osteoclastogenesis by bone marrow stromal cells may have been altered by deletion of Atg7 in osteocytes. However, osteoclast differentiation in primary bone marrow cultures from conditional knockout mice was similar to cultures from control mice (Fig. 5C). Similar to the osteoclastogenic potential, a small but significant increase in colony forming units that give rise to fibroblasts (CFU-F)
and osteoblasts (CFU-OB) in the bone marrow of conditional knockout mice was observed (Fig. 5D). In contrast, osteoblast differentiation, as measured by mineralizing nodule formation and osteoblast specific gene expression in bone marrow cultures, was not affected by deletion of Atg7 in Dmp1-Cre-expressing cells (Fig. 5E-F). Thus the reduced bone formation and lack of balance between bone resorption and bone formation in the conditional knockout mice is not due to insufficient numbers of osteoblast progenitors or an inability of progenitors to differentiate into mature osteoblasts.

**Suppression of autophagy in osteocytes increases oxidative stress in bone.**

It is known that aging in wild type mice is associated with increased oxidative stress as revealed by elevated ROS production in bone marrow cells and increased phosphorylation of the p66shc adaptor protein in bone tissue (34). Strikingly, p66shc phosphorylation was increased in L6 vertebrae of these mice, compared with control littermates (Fig. 6A). Moreover, ROS levels were significantly higher in the bone marrow of conditional knockout mice (Fig. 6B). Previous studies have demonstrated that suppression of autophagy increases ROS in part by increasing the number of mitochondria (110;111). Consistent with this, mitochondrial DNA content in osteocyte-enriched cortical bone was higher in conditional knockout mice compared with control littermates (Fig. 6C). These results demonstrate that suppression of autophagy in osteocytes is sufficient to increase oxidative stress in the bones of young mice to levels normally seen in aged animals, possibly via suppression of mitochondrial turnover.

![Figure 6: Oxidative stress in bone from Dmp1-Cre;Atg7-f/f mice.](image)

(A) Immunoblot of phospho-p66shc in lumbar vertebra 6 from 3-month-old male mice (n = 5 per group). (B) ROS in bone marrow isolated from tibiae of 3-month-old male mice (n = 5 per group). (C) Ratio of mitochondrial:nuclear DNA determined by Taqman PCR of DNA isolated from osteocyte-enriched femoral cortical bone of 6-month-old male mice (n = 7-9 per group *P < 0.05 using Student’s t-test (A-B) or Mann-Whitney Rank Sum test (C).
3.1.4 Discussion

All the structural, cellular, and biochemical changes that occur in wild type mice with age, were observed in Dmp1-Cre;Atg7-f/f mice at 6 months of age. Thus, experimental aggravation of autophagy in osteocytes is sufficient to mimic many of the skeletal changes that occur with advanced age in young adult mice.

Similar to aged mice, the low bone mass must be due to an imbalance between resorption and formation such that bone is incompletely replaced in each remodelling cycle. The decrease in wall width that occurs in the conditional knockout mice is consistent with this idea. Since the Dmp1-Cre transgene is active in at least some matrix-synthesizing osteoblasts, it is possible that the skeletal phenotype of the conditional knockout mice is due in part to loss of autophagy in these cells. However, osteoblast differentiation and osteoblast gene expression in primary bone marrow cultures from Atg7 in Dmp1-Cre-expressing cells were not affected, suggesting that the skeletal phenotype is most likely due to suppression of autophagy in osteocytes rather than osteoblasts and that suppression of autophagy in osteocytes alters production of factors that control osteoclast and osteoblast number. Consistent with this idea, osteoclast numbers were reduced in the conditional knockout mice even though osteoclasts or their progenitors do not express the Dmp1-Cre transgene so did not undergo Atg7 deletion (107). There are several potential mechanisms by which suppression of autophagy in osteocytes may control bone remodelling. Suppression of autophagy in other long-lived cell types, such as neurons and myocytes, increases the basal rate of apoptosis. However, we did not detect any changes in osteocyte number or apoptosis in the conditional knock mice. Moreover, since osteocyte apoptosis has been associated with (112), or shown to cause (113-115), increased osteoclast formation and bone resorption, an increase in osteocyte apoptosis would not explain the reduced osteoclast formation in mice lacking autophagy in osteocytes. Osteocytes have been shown to control osteoclast formation by producing RANKL and OPG and to suppress osteoblast formation by producing sclerostin (15;16;18;116). However, we did not detect changes in the expression of mRNAs encoding these factors in osteocytes of the conditional knockout mice. Nonetheless, it is possible that autophagy controls production of these factors via a non-transcriptional regulation. In support of this idea, autophagy has been shown to play an important role in the unconventional (not mediated by the classical endoplasmic reticulum/Golgi-dependent pathway) secretion of proteins such as IL-1β and IL-18 (93;117). Thus it will be important to determine whether suppression of autophagy in
osteocytes alters secretion or cell-surface expression of factors that are important for bone remodelling such as RANKL, OPG, and sclerostin. It is also possible that previously unrecognized factors produced by osteocytes contribute to the low remodelling and bone mass caused by deletion of Atg7 in these cells.

An increase in oxidative stress has been functionally associated with the bone loss caused by estrogen-deficiency and has been detected in the bones of aged wild type mice (34;118). Thus, the increase in oxidative stress in the bones of mice lacking autophagy in osteocytes may contribute to the imbalance between bone resorption and formation. The elevated mitochondrial DNA content of the osteocyte-enriched bone suggests that the likely source of the elevated ROS is an accumulation of damaged mitochondria in the osteocytes of the conditional knockout mice. We have shown previously that blockade of glucocorticoid action on osteoblasts and osteocytes blunts the loss of bone mass and strength caused by aging in mice (53). We also noted in those studies that corticosterone levels in the circulation increase with age. Together, these results suggested that an increase in endogenous glucocorticoids is partially responsible for the decrease in bone mass and strength caused by aging. The results of the present study suggest that a decline in osteocyte autophagy may also contribute to the adverse effects of age on the skeleton. Recent studies have provided evidence that glucocorticoids stimulate autophagy in the MLO-Y4 osteocytic cell line (119). Based on these studies, the increased endogenous glucocorticoid levels in aged mice might be expected to increase autophagy. However, it is important to note that autophagy is regulated by numerous signaling pathways and conditions such that the effects of glucocorticoids alone may not predominate in aging mice. Direct comparison of autophagy in osteocytes from young versus old mice will be required to determine whether this is the case. Our preliminary attempts to measure autophagy in old bone have been hampered by the increase in cortical porosity, and thus the presence of other cell types, in osteocyte-enriched bone from old mice (data not shown). It is somewhat surprising that the conversion of matrix-synthesizing osteoblasts to osteocytes was not altered by deletion of Atg7 in Dmp1-Creexpressing cells. Osteoblasts and recently embedded osteocytes, also known as osteoid osteocytes (13), contain abundant endoplasmic reticulum and mitochondria that are progressively reduced as the cells mature within mineralized bone. Since autophagy is required for similar reductions in cellular components during the maturation of other cell types (75), one might have anticipated altered osteocyte morphology or increased cell death when Atg7 was deleted using the Dmp1-Cre transgene. However, since this transgene does not become active until the matrix synthesizing
stage of osteoblast differentiation, it is possible that even after the Atg7 gene was deleted, sufficient Atg7 protein remained to allow autophagy to continue until osteocytes were fully formed. This would of course depend on the half-life of the Atg7 protein, which likely varies in different cell types and conditions. Nonetheless, it remains possible that the process of osteocyte formation may be affected when the Atg7 gene is deleted using Cre driver strains that become active at earlier stages of osteoblast differentiation. In summary, the results presented herein demonstrate that suppression of autophagy in osteocytes causes skeletal changes very similar to those caused by aging and suggest the possibility that reduced autophagy may contribute to the detrimental effects of aging on bone mass and strength.
3.2 Suppression of autophagy in osteocytes does not accentuate the negative impact of glucocorticoids on the skeleton

3.2.1 Introduction

Elevated levels of glucocorticoids have been shown to cause stress in several cell types (120). Moreover, the therapeutic use of glucocorticoids, such as Prednisolone, is associated with the development of secondary osteoporosis, leading to at least one traumatic fracture in 30-50% of patients on steroids therapy (46). Previously studies in mice expressing 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an enzyme that inactivates glucocorticoids, specifically in osteoblasts and osteocytes, showed that the loss of bone mass and strength caused by glucocorticoids is primarily due to a direct effect on bone cells, by reducing osteoblasts proliferation and differentiation and by increasing osteoblasts and osteocytes apoptosis (49). Moreover, mice where osteoclasts were protected from the action of glucocorticoids (TRAP-11βHSD2), displayed reduced cancellous osteoclast numbers and were prevented from the loss of bone mass, indicating that glucocorticoids act directly on osteoclasts, by prolonging their lifespan and inhibiting their apoptosis, to decrease bone mass (50).

Autophagy is a lysosome-based recycling pathway that degrades intracellular components in order to promote cell survival under stressful conditions and to maintain cell homeostasis. Specifically, old organelles or unfolded proteins become engulfed by a double membrane vesicle called an autophagosome that fuses with lysosomes allowing degradation of the contents (121). In this way autophagy provides new sources of energy and helps cells to eliminate damaged organelles such as mitochondria, thereby promoting cell survival, viability and function. This role appears to be especially important in long-lived cells and a decline in autophagy has been proposed as an explanation for the changes that occur in degenerative diseases (64). Important, deletion of genes such as Atg7 completely suppresses the process of autophagy (69) allowing one to examine the significant of this pathway in various cell types. Deletion of the Atg7 gene in mature osteoblast and osteocytes suppresses autophagy and caused changes similar to those that occur with age in wild type mice (34;122;123), suggesting that a decrease in autophagy in osteocytes may contribute to skeletal aging. Moreover it has been shown that glucocorticoids stimulate autophagy in the MLO-Y4 osteocytic cell line, and that inhibition of autophagy in this cell type aggravates the effect of glucocorticoids on cell viability (119;124). Together these observations suggest the possibility that autophagy may oppose the negative actions of glucocorticoids on osteocytes and
therefore, in the absence of autophagy, the impact of glucocorticoids on the skeleton might be increased. However whether this is the case in vivo is unknown, therefore the goal of the present study was to determine whether loss of autophagy in osteocytes in vivo increases the negative effects of exogenous glucocorticoids on the skeleton. Our results demonstrate that although exogenous glucocorticoids stimulate autophagy in osteocytes in vivo, suppression of autophagy in this cell type does not accentuate the negative impact of glucocorticoids on the skeleton.

3.2.2 Materials and methods

Animal studies. Mice lacking autophagy in osteoblasts and osteocytes were generated by deleting the Atg7 gene, which is essential for autophagy, using a Dmp1-Cre transgene, as previously described (89). Six-month-old female conditional knockout mice (Dmp1-Cre;Atg7f/f) and their control littermates (Atg7f/f), were stratified into four groups according to spinal bone mineral density (BMD) and slow-release pellets of placebo or prednisolone (2.1 mg/kg/day) (Innovative Research of America, Sarasota, FL) were implanted subcutaneously. After 28 days, a second BMD measurement was performed and then animals were sacrificed to analyse the impact of exogenous glucocorticoids on the skeleton. All animal experimentations described in the present work were conducted in accord with the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences.

Oxidative stress and autophagy flux detection. For oxidative stress measurements, we performed quantification of intracellular reactive oxygen species (ROS) levels in freshly isolated bone marrow cells from tibias using dichlorodihydrofluorescein diacetate dye (98) and quantification of the phosphorylation state of the p66shc protein by immunoblot from L6 (34). To confirm suppression of autophagy and to determine the effect of prednisolone on autophagy, we quantified LC3 conversion and p62 levels in proteins extracted from osteocytes-enriched cortical bone. Proteins were extracted from osteocyte-enriched cortical bone by freezing in liquid nitrogen followed by pulverization in liquid nitrogen. The pulverized bone powder was then incubated in RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 1X SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8,
2% SDS, 6% Glycerol, 1% β-mercaptoethanol and 0.004% bromophenol blue) for 30 minutes in ice, followed by 10 minutes incubation at 100 C. The pulverized bone powder was then spun down at 14000 rpm for 10 minutes and the obtained protein supernatant was then resolved in 12% SDS polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% nonfat dry milk in TBS and were then incubated with primary antibodies and an appropriate horseradish peroxidase-linked secondary antibody.

The following antibodies were used: anti-LC3 (Cell Signaling Technology, Danvers, MA), anti-p62 (Progen Biotechnik, Heidelberg, Germany), and anti-tubulin (Sigma-Aldrich, St. Louis, MO). Blots were developed using enhanced chemiluminescence and the intensity of the bands was quantified using a ChemDoc XRS-plus system (Bio-Rad, Hercules, CA).

**Micro computed tomography (μCt), biomechanical testing and histomorphometry.** Micro-CT analysis of cortical and trabecular architecture was performed in femurs and fourth lumbar spine, as previously described (100), followed respectively by 3-point bending and compression test, to measure biomechanical properties (53). L1–L3 lumbar vertebrae were fixed and embedded undecalcified in methylmethacrylate and static and dynamic histomorphometric examination was done on longitudinal sections with a digitizer tablet (OsteoMetrics, Inc., Decatur, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Thornwood, NY) with a drawing tube attachment, as previously described (49). Tibias were fixed in 10% Millonig’s formalin for 24 hours, decalcified in 14% EDTA for 1 week, and embedded in paraffin to obtained 5 μm longitudinal sections. After removal of paraffin and rehydration, sections were stained for TRAP activity and counter-stained with fast green and osteoclasts were enumerated on the endocortical surface. Terminology recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research was used in this study (101).

**TaqMan assay.** Osteocytes-enriched bone shafts were prepared by cutting off the epiphysis and flushing out the bone marrow. The bone samples were then scraped several times along the outer and inner surface to remove osteoblasts and other cells in order to obtain an osteocytes-enriched preparation. The cortical bone shafts were then homogenized in 1ml of Trizol Reagent (Life technology, Grand Island, NY) to extract Total RNA, according manufacturer’ instruction. Quantitation and 260/280 ratio of the extracted RNA were
determined using a Nanodrop instrument. 500 ng of RNA was then used to synthesize first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer’s directions. cDNA was amplified by real-time PCR (RT-PCR) using TaqMan Universal PCR Master Mix (Applied Biosystems) as described previously (102). The following TaqMan primer-probe sets from Applied Biosystems were used: calcitonin receptor (Mm00432271_m1); osteoprotegerin (Mm00435452_m1), and the housekeeping gene ribosomal protein S2, (forward, 5′-CCCAGGATGGCGACGAT-3′, reverse, 5′-CCGAATGCTGTAATGGCGTAT-3′, probe 5′-FAM-TCCAGAGCACGGATCC-NFQ-3′). Gene expression was calculated using the delta Ct method (103) and the ribosomal protein S2 (ChoB) was used as internal control gene.

**Statistics.** Data were analysed using SigmaStat (SPSS Science, Chicago, IL). We performed two-way analysis of variance (ANOVA) or Student’s t-test to detect statistically significant treatment effects, after determining that the data were normally distributed (Shapiro-Wilk test) and exhibited equivalent variances. In some cases, we used log transformation to obtain normally-distributed data. Multiple comparisons were evaluated with Bonferroni or Holm-Sidak post hoc tests. *P*-values less than 0.05 were considerate as significant. All values are reported as the mean ± S.D. Data that did not pass the normality test were evaluated using the Mann-Whitney Rank Sum Test.
3.2.3 Results

Glucocorticoids stimulate autophagy in osteocytes in vivo.

To determine whether glucocorticoids control osteocyte autophagy in vivo and whether autophagy helps osteocytes resist the negative effects of glucocorticoids, we compared the impact of glucocorticoid administration on the skeleton of mice with and without functional autophagy in osteocytes. Mice lacking autophagy in osteocytes were generated by crossing mice harbouring a conditional allele of Atg7 with Dmp1-Cre transgenic mice, which express the Cre recombinase primarily in osteocytes. We have shown previously that this approach deletes Atg7 from bone but not soft tissues and that it significantly reduces autophagic flux in osteocytes in vivo (89). In the current study, 6-month-old female Dmp1-Cre;Atg7f/f mice, hereafter referred to as conditional knockout mice, or control littermates (Atg7f/f) were implanted with pellets releasing placebo or prednisolone (2.1 mg/kg/day), maintained for 28 days, and then euthanized for further analysis.

To confirm suppression of autophagy in osteocytes in the present study, we measured the levels of LC3 and p62 by immunoblot analysis. LC3 is a docking protein that is incorporated into growing autophagosome membranes. This incorporation requires lipidation of the protein, which can be monitored on immunoblots by conversion of the unlipidated form I to the lipidated form II. p62 is a scaffolding protein that brings cargos to the autophagosome for degradation and can accumulate when autophagy is suppressed (125). Comparison of conditional knockout and control mice implanted with placebo pellets revealed that deletion of Atg7 from Dmp1-Cre-expressing cells inhibited conversion of LC3 and caused accumulation of p62 (Figure 1A-B) in osteocyte-enriched cortical bone, findings which are consistent with our previous report (89).

We then compared autophagic flux in the four groups of mice and found that prednisolone stimulated LC3 conversion osteocyte-enriched cortical bone of Atg7f/f mice but not in conditional knockout mice (Fig. 1A). Therefore this result supports the idea that glucocorticoids stimulate autophagic flux in osteocytes in vivo. An increase in autophagic flux can also lead to reduced abundance of p62 (126). Therefore, the stimulation of autophagy in Atg7f/f mice by prednisolone might be expected to reduce p62 levels in osteocyte-enriched bone. However, prednisolone did not alter p62 levels in Atg7f/f mice or conditional knockout mice (Fig. 1B).
Figure 1: Deletion of ATG7 in Dmp1-Cre expressing cells suppresses autophagy and Prednisolone stimulates osteocytes autophagy in vivo. Protein lysates extracted from osteocytes-enriched tibia shafts of females conditional knockout mice or control littermates (n=7 animal per group) implanted with placebo or 2.1/mg/day of Prednisolone, were subjected to immunoblot to detect LC3 (A) and p62 (B). The intensity of the LC3-I and LC3-II bands was quantified and plotted as the ratio of LC3-II to LC3-I. P62 expression was quantified and normalized to tubulin. *p <0.05 effect of treatment within genotype; # p <0.05 effect of genotype within treatment.

Suppression of autophagy in osteocytes does not accentuate the negative impact of glucocorticoids on the skeleton.

We have shown previously that suppression of autophagy in osteocytes results in reduced cortical thickness, increased cortical porosity, and low cancellous bone volume and that these changes are associated with reduced bone strength (89). In the present study we observed similar effects of autophagy suppression in osteocytes in the placebo-treated mice, with the exception that cancellous bone volume was not lower in the femurs of conditional knockout mice (Fig. 2A-G). However, despite the presence of these changes in the conditional knockout mice under basal conditions, administration of prednisolone reduced cortical thickness and bone strength and increased cortical porosity to similar extents in the femurs of both Atg7flo,flo controls and conditional knockout mice (Fig 2B-D). Prednisolone also reduced cortical
thickness in L4 vertebra (Fig. 2F) but did not alter bone strength at this site (Fig. 2G). Administration of prednisolone did not alter cancellous bone volume or architecture in either the femur (Fig. 2A) or L4 vertebra (Fig 2E and 3A-D) of either genotype, findings that are consistent with our previous observations.

Figure 2: Suppression of autophagy in osteocytes does not accentuate the negative impact of glucocorticoids on the skeleton. 6 month-old females Dmp-1Cre;Atg7f/f mice (CKO) and control Atg7f/f littermates (Ctrl) (n=9-11 per group) were implanted with placebo or Prednisolone pellets for 28 days and bone architecture was analysed in femoral midshaft and spine by μCT. Bone Volume/Total Volume (BV/TV) of femur (A) and spine (E); Femoral (B) and spinal (F) cortical thickness; femoral strength measured by 3-point bending test in femur (C) and compression test in spine (G) and expressed in Newton. (D) Cortical porosity of femoral midshaft. *p <0.05 effect of treatment within genotype; # p <0.05 effect of genotype within treatment.

Figure 3: Administration of prednisolone did not alter cancellous bone volume or architecture, but suppression of autophagy reduces trabecular bone parameters and strength in fourth lumbar vertebra. Trabecular number (A); trabecular thickness (B); trabecular separation (C) and (D) strength of fourth lumbar vertebra expresses as maximum compressive stress at the breaking point in megapascals (MPa)
We have previously shown that prednisolone administration increases oxidative stress in bone as measured by Reactive Oxygen Species (ROS) levels in the bone marrow and by phosphorylation of the redox sensitive protein p66$^{shc}$ in the bone protein lysates (127). In addition we have shown that deletion of the Atg7 gene in osteocytes increases oxidative stress as measured by these same parameters (89), which we confirmed in the placebo treated mice in this experiment (Fig. 4H and 4I). However, despite the elevated level of oxidative stress in the conditional knockout mice under basal conditions, prednisolone increased oxidative stress by a similar magnitude in both genotypes (Fig. 2H-I). Together these results indicate that suppression of autophagy in osteocytes does not aggravate the changes in cortical structure or the increase in oxidative stress caused by prednisolone.

**Figure 4**: Suppression of autophagy does not further increase oxidative stress in prednisolone treated mice. Quantification of Reactive Oxygen Species in the bone marrow normalized to protein contents (E) and p66 phosphorylation in whole bone lysate extracted from L6 and normalized to β-actin (I). n= 5-6 animals per group. *p <0.05 effect of treatment within genotype; # p <0.05 effect of genotype within treatment.

**Cellular changes induced by Prednisolone were not worse in the absence of autophagy.**

Even though prednisolone did not alter cancellous bone volume (Fig. 2A and 2E), others and we have shown that it consistently reduces osteoblast number and bone formation in this skeletal compartment (49;128). Therefore, we examined the effects of prednisolone administration and suppression of autophagy, both separately and combined, on the histology of cancellous bone of the spine. Similar to what we observed in our previous study, suppression of autophagy in osteocytes reduced the overall rate of bone remodelling. Specifically, osteoblast and osteoclast (number and surface), mineralizing surface and bone
formation rate were all lower in placebo-treated conditional knockout mice compared to placebo-treated Atg7f/f mice, with no change in MAR (Fig. 5A-G). Consistent with our previous findings in wild type mice (33), administration of prednisolone had no impact on osteoclast number or surface in cancellous bone in either genotype (Fig. 5C and 5D). In contrast, prednisolone dramatically reduced osteoblast number, surface and bone formation rate in Atg7f/f mice but did not further reduce the already low osteoblast number and bone formation rate in conditional knockout mice (Fig. 5A-B and 5E and 5G).

Figure 5: Cellular changes induced by Prednisolone were not worse in the absence of autophagy. Static and dynamic histomorphometric analyses were performed in lumbar vertebra 1–3, from 6-month old CKO and control mice (n=5 per group). (A)Osteoblast number per bone perimeter (Ob.N/B.Pm); (B) osteoblast surface per bone surface (OcS/BS); (C) osteoclast number per bone perimeter (OcN/B.Pm) and (D) osteoclast surface per bone surface (OcS/BS). (E) Mineralizing surface per bone surface (MS/BS), (F) Mineral Apposition Rate (MAR) and (G) Bone Formation Rate (BFR). *p <0.05 effect of treatment within genotype; # p <0.05 effect of genotype within treatment.

Because prednisolone reduced cortical thickness and increased cortical porosity, we also examined osteoclast number at the endocortical surface of the tibia. In contrast to cancellous bone, suppression of autophagy in osteocytes did not change osteoclast number at the
endocortical surface. Importantly prednisolone administration significantly increases osteoclast numbers at the endocortical surface in both genotypes, as shown by the quantification of the TRAP stained tibia sections (Fig 6A-B). Consistent with these results, Calcitonin receptor mRNA levels, measured in osteocytes enriched bone shafts, were significantly increased by Prednisolone in both genotypes (Fig. 6C). Interesting, these changes were associated with a similar suppression of osteoprotegerin expression by Prednisolone in osteocytes-enriched bone from both genotypes (Fig. 6D), with no change in RANKL expression (data not shown). Thus, glucocorticoids-induced cortical bone loss due to stimulation of endocortical resorption was associated with suppression of osteoprotegerin RNA levels in osteocytes, and loss of autophagy in this cell type did not worsened this response.

Figure 6: Prednisolone stimulates endocortical resorption and down regulates OPG mRNA levels in osteocytes. (A) Histological sections of tibia were stained for TRAP activity (osteoclasts stained red) and counterstained in fast green (original magnification 20X) and osteoclast number (Oc.N/B.Pm) and surface (Oc.S/BS) were counted on the endocortical surface starting from the region under the primary spongiosa. (B) Calcitonin Receptor (Cal.R.) and Osteoprotegerin (OPG) mRNA levels in osteocytes-enriched tibia shafts were measured by RT-PCR (n = 4–6 animals/group). *p <0.05 effect of treatment within genotype; # p <0.05 effect of genotype within treatment.
3.2.4 Discussion

Autophagy is a stress-activated response that removes and recycles damaged components in order to maintain cellular health and viability. Impaired or dysfunctional autophagy has been related to different conditions, such as neurological and metabolic disorders, cancer and several studies have highlighted the causative effect of autophagy decline on aging (65;85;121). We have recently proposed that a decline of autophagy in osteocytes may contribute to skeletal aging (89). By deleting the Atg7 gene using a Dmp-1Cre promoter, we have efficiently suppressed autophagy in osteocytes. This manoeuvre led to mice with low bone mass, low bone remodelling and increased oxidative stress. A similar phenotype was obtained when a ULK-interacting protein required for autophagosome generation, FIP200, was deleted using different osteoblast Cre drivers, leading to mice with osteopenia (129).

Endogenous glucocorticoids levels increase with age, contributing to the decrease of bone mass and strength (53); elevated levels of glucocorticoids are known to be deleterious for osteoblasts and osteocytes because induce their apoptosis and stimulate the production of oxidative stress (52). Here we show that, based on the quantification of LC3 turnover, cortical osteocytes in control mice are able to activate the autophagic process when exposed to Prednisolone for 28 days. p62 levels depend on autophagy turnover (22) and when autophagy is activated p62 degradation increases. This did not occur in conditional knockout mice under basal conditions. It is possible that the presence, in the cortical bone of Prednisolone treated mice, of elevated number of osteoclastic cells, which have abundant levels of p62 in their cytoplasm, may have masked the decrease in p62, expected with stimulation of autophagy.

Previous studies have shown that Dexamethasone treatment activates autophagy in lymphocytes (130) and in the MLO-Y4 osteocytic cell line. In this last study, the authors reported activation of autophagy and antioxidant related genes by Dexamethasone and that impairment of autophagy aggravates the effect of Dexamethasone on osteocytes viability in vitro (119;130). More recently, autophagy impairment has been shown to enhance the deleterious effects of high glucose in an osteoblastic cell line, by increasing oxidative stress (131). Because osteocytes are post-mitotic cells trapped in the bone matrix (13), misfolded proteins and old organelles cannot be removed through cell division and therefore it is reasonable to think that in this cell type autophagy may help to maintain viability and function, by limiting the metabolic stress that accumulates with age. To test this hypothesis we stressed mice that lacked autophagy in osteocytes with an excess of glucocorticoids and
found that loss of bone mineral density as well as the cortical changes, the decrease in strength and the increase in oxidative stress caused by Prednisolone were not further aggravated compared to mice with an intact autophagic response. Consistent with this, the cellular changes induced by Prednisolone were not affected by loss of autophagy, concluding that autophagy in osteocytes doesn’t play a major protective role opposing the negative effects of glucocorticoids on the skeleton.

We reported that mice lacking autophagy in Dmp-1Cre expressing cells under basal condition do not display an increase in the numbers of apoptotic osteocytes compared to control mice, indicating that cell viability is not compromised (89). Yet, it is still possible that in response to stressful signals, such as in the presence of an excess of glucocorticoids, cells lacking autophagy may become more susceptible and therefore die more. However changes in osteocytes apoptosis would not be relevant, given that none of the skeletal and cellular effects produced by Prednisolone administration was worsened in the absence of autophagy.

Several studies have shown that some of the autophagy-related proteins can function in processes not related to autophagy. For instance proteins without a leader peptide cannot enter the canonical ER-Golgi route for the translocation to the plasma membrane and subsequent secretion; therefore they are secreted through alternative pathways, such as the unconventional protein secretion pathway (93;132;133). Several types of unconventional secretion have been described, that allow cargos to directly pass the plasma membrane (FGF2, Annexin 2, HIV-Tat) or in conjunction with vesicle-like structure (IL-1β, IL-18, yeast acyl–coenzyme A (CoA) binding protein Acb1,nuclear protein HMGB1 and engrailed homeoprotein), which requires autophagy related organelles (134). A non-autophagic function has been described for several Atg proteins and LC3 protein, involved in the regulation of the secretion process in bone resorbing osteoclasts and in the formation of the ruffled border of the same cells (135). Moreover, recently autophagy has been found to be responsible for the stimulated-secretion of the Von Willebrand factor in endothelial cells (94) and that impaired autophagy affects the secretion of insulin from pancreatic beta cells (95;136), supporting a new, non-canonical functional role of autophagy. Given these observations, it is possible that the negative effects on the skeleton caused by Prednisolone are not aggravated in the absence of autophagy because autophagy in osteocytes might have additional functions, such as the regulation of secretion and trafficking of factors important for bone homeostasis and that the increase in autophagy that occurs in osteocytes after Prednisolone administration might be unrelated to its recycling and protective role.
Cortical and trabecular bone compartments have been shown to behave differently in response to aging, hormonal signals and mechanical stimulations (34;137;138). Prednisolone, at the dose and time used in this experiment, profoundly affected the cortical compartment, in either femur or spine, but did not cause cancellous bone loss, as observed in the control mice. Although osteoclast numbers on the trabecular surface of Prednisolone treated mice were in the normal range, they might not have been fully functional. It has been shown that glucocorticoids administration can impair osteoclastic bone resorption, by inhibiting the rearrangement of osteoclast cytoplasm in the presence of M-CSF (60). Therefore the inhibition of bone formation together with the impaired bone resorption could explain why mice didn’t lose cancellous bone after prednisolone treatment.

More interesting for the glucocorticoids-induced bone disease, we found, for the first time to our knowledge, that Prednisolone down regulated transcripts levels of osteoprotegerin in osteocytes-enriched bone in vivo. Our data are consistent with previous in vitro finding showing that glucocorticoids down regulated OPG mRNA levels in human osteoblastic cells (139;140), in the MLO-Y4 osteocytic cell line (141) and that circulating levels of osteoprotegerin are diminished in patients treated with glucocorticoids (142). This result, together with the increase in endocortical resorption, supports the hypothesis that a reduction in osteoprotegerin in osteocytes may be responsible for the glucocorticoid-induced bone disease, by enhancing bone resorption. However we cannot rule out that the observed increase in endocortical resorption was due to a direct effect of glucocorticoids on osteoclasts (7), and further studies are required to establish whether this is a direct or indirect effect and which are the underlying molecular mechanisms. Nevertheless, as observed for all the other skeletal and cellular changes, the reduction in osteoprotegerin mRNA levels that occurs in osteocytes in response to an excess of glucocorticoids is not made worse by suppression of autophagy.

In summary these results demonstrate that exogenous glucocorticoids stimulate autophagy in osteocytes in vivo, suppression of autophagy does not accentuate the negative impact of glucocorticoids on the skeleton and that glucocorticoids-induced bone disease occurs independently of autophagy function. Thus we conclude that although glucocorticoids are able to stimulate autophagy in osteocytes, autophagy in osteocytes does not appear to be an important defence mechanism opposing the negative effects of glucocorticoids on the skeleton.
CHAPTER 4: Conclusion and future work

Although researchers have recently gained new insights into the role of autophagy in skeletal homeostasis (119;129;135), the significance of this pathway in bone tissue remains mainly unclear. In the present study we carried out experiments to investigate the role of autophagy in osteocytes and mature osteoblasts and determine whether loss of autophagy in this cell types increase their susceptibility to the negative effects of exogenous glucocorticoids. To do this, we generated mice lacking autophagy specifically in osteocytes and mature osteoblasts, using the Dmp1-Cre transgene. Suppression of the Atg7 gene, essential for autophagy, in mature osteoblast and osteocytes decreased bone mass and strength and recapitulated the effect of skeletal aging. These data identify autophagy as an important determinant of bone development and reveal a novel role of autophagy in osteoblast and osteocytes function.

We also hypothesize that autophagy may oppose the negative actions of glucocorticoids on osteocytes and therefore, in the absence of autophagy, the impact of glucocorticoids on the skeleton might be increased. However, even though glucocorticoids stimulate autophagic flux in osteocytes in vivo, suppression of autophagy does not aggravate the negative impact of exogenous glucocorticoids on the skeleton.

In conclusion, this study tried to elucidate the role of autophagy in bone, in physiological and stress conditions. We suggest that autophagy is required to maintain normal bone mass and that experimental aggravation of autophagy in osteocytes accelerates skeletal aging. Furthermore, we conclude that autophagy does not appear to be a major mechanism helping osteocytes to resist glucocorticoids-induced stress. Identification of the molecular mechanisms underlying the low and unbalanced bone remodelling caused by suppression of autophagy in osteocytes will further clarify the role of autophagy in bone. We have observed an increase in mitochondrial DNA content and oxidative stress with age and in autophagy-deficient osteocytes. Thereby it is possible that suppression of autophagy may determine a decrease in bone mass via increasing oxidative stress. To test this hypothesis we will overexpress an antioxidant enzyme into the mitochondria of autophagy deficient mice and determine whether this blunts the increase in oxidative stress and the decrease in bone mass. If we obtain these or similar results, we will conclude that autophagy plays an important role in maintaining osteocyte viability, possibly by suppressing oxidative stress.
One other line of evidence suggests that autophagy may be implicated in the secretion of proteins that do not follow the conventional ER-Golgi route (Unconventional protein secretion). It is possible therefore that the low bone mass phenotype may be due to a decreased ability of osteocytes to produce and/or secrete mediators such as RANKL, OPG and sclerostin that control bone remodeling. To further investigate this option, a more extensive and comprehensive analysis of the proteome of conditional knockout mice could indicate whether there is a specific protein that has been profoundly affected by loss of autophagy or whether conditional knockout mice have in general altered secretion ability, in line with data that support a role for autophagy in protein trafficking and secretion.

Recent evidences suggest that autophagy may be important for the long-term health of progenitors. Specifically, deletion of Atg7 from hematopoietic stem cells (HSCs) in mice causes mitochondria accumulation and increased oxidative stress, which was associated with increased proliferation and DNA damage (143). These results suggested that autophagy regulates the maintenance of the HSC compartment in adult mice. Another study has shown that HSC display basal levels of autophagy and that this cell type can mount a robust autophagy response to survive under stressful conditions, such as nutrient deprivation (144). Therefore we will also expand our analysis to other cells types such mesenchymal progenitors and the entire osteoblast lineage. Based on these and our initial findings in mice lacking autophagy in osteocytes, it is possible that autophagy may play a similar role in the maintenance, survival and function of earlier stages of osteoblast-lineage cells and that change in autophagy may underlie changes in progenitor’s behavior with age.

Although the molecular mechanisms underlying such phenotypes are still not fully understood and further mechanistic studies are required, it is clear that an impairment of autophagy process mimics the skeletal changes that occur during age, identifying autophagy as one possible contributor to the age-related bone loss and providing a new potential therapeutic target in osteoporosis.
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