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Boning Up on Autophagy: The Role of Autophagy in Skeletal Biology

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Abbreviations

ADAMTS, a disintegrin and metalloproteinase with a thrombospondin type 1 motif; ALFY, Autophagy-linked FYVE protein; ALS, amyotrophic lateral sclerosis; AMPK, AMP activated protein kinase; ATF4, Activating transcription factor 4; ATG, autophagy related proteins/genes; BAF, Bafilomycin; BID, BH3 interacting-domain death agonist; BMP, bone morphogenetic protein; BNIP3, BCL2/Adenovirus E1B 19kDa Interacting Protein 3; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; CXCL12, Chemokine (C-X-C motif) ligand 12; Dvl2, Dishevelled; FOXO, (family of) forkhead transcription factors; HIF, hypoxia-inducible factor; GAG, glycosaminoglycans; GWAS, Genome-wide association study; HIF, hypoxia inducible factor; KIR, KEAP1-interacting region; LAMP2, lysosome associated membrane protein2; LC3, microtubule-associated protein 1 light chain3; LIR, LC3-interacting regionLSD, Lysosomal Storage Disorders; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; MPS, Mucopolysaccharidoses; MSD, Multiple Sulfatase Deficiency; MTOR, mechanistic target of rapamycin; NBR1, neighbor of breast cancer susceptibility gene 1; NDP52, nuclear dot protein 52; NF1,neurofibromin; NF-κB, nuclear factor kappa B; NRF2, NF-E2-related factor 2 ; OA, osteoarthritis; OPTN, optineurin; PB1, Phox and Bem1p domain; PDB, Paget's disease of bone; PHD, prolyl-4-hydroxylase domain; PI3P, phosphatidylinositol-3-phosphate; PTH and PTHrp, parathyroid hormone and parathyroid hormone-related protein; RANKL, Receptor activator of nuclear factor kappa B ligand; ROS, reactive oxygen species; RUNX2, runt-related transcription factor 2; S1P, sphingosine 1-phosphate; AKT, serine/threonine protein kinase; SNARE, soluble NSF attachment protein receptors; SQSTM1, Sequestosome-1; SUMF1, Sulfatase Modifying Factor 1; TGF-β, transforming growth factor-β; TAB, TGFβ-activated kinase 1 (TAK1)-binding proteinTLR4, Toll-like receptor 4; TRAF6, TNF receptor associated factor 6; TSC2, Tuberous sclerosis protein 2; UBA, ubiquitin-associated (domain); UPS, ubiquitin proteasome system; VCP, valosin containing protein; VEGFA, Vascular endothelial growth factor A.

1. Introduction to Skeletal Tissues

The fossil records and phylogenetic systematics indicate that mineralized tissues have existed in a myriad of chemical forms and in numerous configurations for millions of years. Close examination of fossilized long bones and vertebrae indicates that both osseous and cartilage-like tissues appeared early in evolution, indeed, it was thought that cartilage, a poorly organized tissue, may have predated bone. However, closer examination indicates that while a cartilaginous skeleton is an optimal structure for organisms living in aqueous environments, it was likely preceded by the denser and mechanically stronger mineralized bone. Romer noted in his paper to the NY Academy of Sciences that:

> "the early vertebrates had a considerable degree of ossification which was followed in a majority of cases by a slump toward a cartilaginous condition. Bone is an ancient, rather than a relatively new, skeletal material in the history of vertebrates" [1]

We are just now beginning to understand the complex interplay between all of these mineralized tissues and other tissues in the body. For example, Karsenty's laboratory has shown that regulation of bone mass is coordinated with both energy metabolism and even fertility. [2]

Not surprisingly, bone mass, structure and function is tightly regulated. In the adult, this is achieved by two active and coupled processes, bone resorption and bone formation. Prime regulators of these processes include hormones, especially estrogen, parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) and vitamin D metabolites. Bone marrow-derived osteoblasts are responsible for new bone formation while macrophage-like osteoclasts mediate the resorptive process. The activities of these cell types are linked through a bone remodeling cycle (Fig 1). Osteoclasts activate the cycle by inducing the resorption of old bone; this activity is followed by a formative phase during which osteoblasts synthesize new bone to replace the resorbed tissue. Those osteoblasts that become engulfed in the mineralized bone, osteocytes, play a pivotal role in functionally adapting the tissue to applied mechanical forces As will be highlighted in this review, autophagy is relevant for the survival and function of each of these cell types within their highly specialized matrices.

When the equilibrium between these two processes is disturbed, and there is excessive bone formation this can lead to over mineralization of bone or osteopetrosis. More frequently, there is increased bone loss leading to a porotic state i.e. osteopenia or osteoporosis. Osteoporosis, or more commonly post-menopausal osteoporosis, is a condition which increases bone fracture risk thereby challenging the quality of life and longevity of elderly women and men.[3]. The relationship between the autophagic pathway and osteoporosis, was highlighted in a Genome-wide Association Study (GWAS) of wrist bone mineral density; the pathway-based analysis showed significant

associations with regulation-of-autophagy genes including *ATG* (autophagy related gene)*12, PIK3C3, , PRKAA2, ATG5 GABARAPL1, BECN1, IFNA13* and *ATG7*[4]. The authors related variants in these genes to the pathogenesis of osteoporosis in terms of modulating factors required for bone formation and/or remodeling.

While a great deal of new information is accruing concerning skeletal cell homeostasis, there is growing awareness that autophagy enables osteoblasts, osteoclasts and chondrocytes to survive within a hypoxic, and even hypertonic environment. In this way, cells can overcome stressor challenges and nutrient deficiencies and, for osteocytes and articular chondrocytes fulfill their fates as exceedingly long-lived terminally differentiated cells. The focus of this review is to examine the significance of the autophagic process in terms of the functional demands of the skeleton in growth and homeostasis, and to provide evidence that disregulation of autophagy is involved in the pathogenesis of diseases of bone (Paget's disease of bone, PDB) and cartilage (osteoarthritis, OA, and the mucopolysaccharidoses, MPS). (see Table 1)

2. Osteoblast and Osteocyte Function and Autophagy

Signaling Pathways Regulating Osteoblast Activity and Autophagy: Osteoblasts secrete the organic matrix of bone (osteoid) and participate in its mineralization. During its encasement in bone, the osteoblast becomes fully differentiated and assumes the morphology and function of an osteocyte (see later and Fig. 1). It is likely that the physiological stimulus for new bone formation is linked to intermittent shifts in PTH levels and the resorptive activities of osteoclasts which release growth factors such transforming growth factor-β (TGF-β), insulin-like growth factors and bone morphogenetic proteins (BMPs), from the extracellular matrix of the bone. In addition, a neuronal involvement cannot be excluded since bone mass is responsive to the sympathetic tone of the nervous system, through modulation of the β2-adrenergic receptor/cAMP signaling system.[5] During resorption, release of these buried agents from the resorbing tissue promotes osteoblast migration, activation and even new bone formation.

The BMPs are clinically powerful bone forming agents that bind to a common cognate receptor on osteoblasts, and transduce signals through the Smad signaling system. The functional activity of the BMP ligands are regulated by extracellular protein antagonists that include noggin, chordin and sclerostin.[6] That BMPs may regulate autophagy is suggested by the studies of Cao et al.[7] who showed that noggin reduced LC (microtubule-associated protein 1 light chain)3-II levels, albeit in acute pancreatitis cells; this was overcome by administration of BMP-2 which increased the levels of both BECLIN I and LAMP2. Indeed, a mutation in the BMP type I activin receptor-like kinase 2 causes a rare and very disabling disease, Fibrodysplasia Ossificans Progressiva.

Whether disease progression is related to expression of an autophagic phenotype awaits further study.

Activities of proteins of the Wnt/β-catenin pathway are major regulators of chondrocyte and osteoblast function. Wnt signaling plays a major role in regulating stem cell commitment to the osteoblast lineage and osteoblast differentiation. In tumor and hepatic cells, Wnt signaling has been negatively linked to autophagy. This is mediated through the interaction of Dishevelled (Dvl2) which binds to the autophagy receptor sequestosome 1 (SQSTM1)/p62 (see later) which in turn facilitates LC3-mediated autophagosome recruitment, ubiquitylation and degradation.[8] Likewise, activation of the Wnt/β-catenin pathway is central to the pathogenesis of both rheumatoid arthritis and OA. While it is tempting to speculate that activation of this pathway suppresses autophagy and enhances osteoblast or chondrocyte death, this relationship has not as yet been fully established.

The functional role of autophagy in the osteoblast is still largely unexplored, particularly *in vivo*. However, several autophagy-related proteins profoundly impact osteoblast biology. The autophagy receptor Neighbor of Breast Cancer Susceptibility 1 gene (NBR1) is involved with targeting ubiquitinated cargos to the autophagosome. [9] It fulfills this function by interacting with ATG8 (LC3) protein family members through its LC3-interacting region (LIR) and with target proteins through the ubiquitin-associated (UBA) domain. A knock-in mouse model with deletion of both LIR and UBA domains in the *NBR1* locus results in increased osteoblast differentiation and activity. [10] This aberrant osteoblastic activity appears to be dependent on p38 MAPK hyperactivation. The moderate increase in the autophagy receptor SQSTM1 in osteoblasts isolated from *NBR1* knock-in mice can impair proteasomal function and activate the stress responsive transcription factor NF-E2 related factor (NRF2), which results in the induction of many cytoprotective genes.

At least two families of transcription factors with known roles in autophagy control osteoblast survival and function. Of these, family of forkhead transcription factors (FOXO) serves key roles in cell growth, cell proliferation, DNA repair, cell cycle arrest, reactive oxygen species (ROS) generation, energy homeostasis and glucose metabolism.[11] FOXO1, 3, 4 and 6 are downstream of early signaling events in the insulin pathway and negatively regulate serine/threonine protein kinase (AKT) signaling.[12] FOXO activation potently induces autophagy by directly binding to the promoter regions of target genes. Genetic deletion of *FOXOs* in osteoblasts induces oxidative stress and increased apoptosis mimicking the aging process. Conversely, *FOXO3* overexpression prevents bone loss associated with aging.[13] Since autophagy has an important cytoprotective role against oxidative stress and other aging related phenotypes, and with glucosamine promotes autophagic flux in chondrocytes, [14] it is

tempting to consider that the role of FOXO in maintaining bone homeostasis is at least, in part, mediated by the induction of autophagy.

Activating transcription factor 4 (ATF4), a member of the CREB family of B ZIP proteins, is required to maintain osteoblast function and promote terminal differentiation; it also protects cells from amino acid starvation and enhances amino acid import into cells. Changes in ATF4 activity have been linked to skeletal manifestations of two human genetic diseases, Coffin-Lowry syndrome and neurofibromatosis type I. It was shown that this transcription factor induces osteoblast-specific gene expression in fibroblasts together with osteocalcin synthesis, thus predisposing these cells to aberrant mineral deposition.[15] Interestingly ATF4 is thought to promote cell survival through the transcription of several autophagy genes including *MAP1LC3B* and *ATG5*. Elefteriou *et al*. [16] noted that the increased bone-mass phenotype resulting from neurofibromin (*NF1*) deficiency can be rescued through nutritional restriction of protein intake, a finding that would strongly suggest that a link exists between expression of this protein and autophagic flux.

Autophagy and the Osteoblast–Osteocyte Transition: In contrast to the short half lives (days/weeks) of osteoblasts, osteocytes are very long-lived cells that exhibit an architecture closer to neurons than cartilage or bone cells. The basic features of the osteocyte is a cell subsumed into mineralized bone lacunae with a large number of long dendritic processes contained in bone canals (canaliculi) many of which interconnect with other osteocytes to form a syncytium as well as interacting with osteoblasts and bone lining cells (Fig. 1). It is likely that their primary role is mechanotransduction i.e. converting mechanical forces on the bone into biological signals that serve to promote the remodeling process. While supporting little biological activity, there is evidence that like osteoblasts, these cells can express the cytokine Receptor activator of nuclear factor kappa B ligand (RANKL) (see later) and thereby influence osteoclastogenesis and resorption. Osteocytes can also secrete sclerostin which inhibits signaling and possibly enhances osteocyte apoptosis through the Wnt signallng pathway. Not surprisingly, studies by Zahm *et al.*[17] clearly showed that *in situ* a considerable number of osteonal osteocytes display a punctuate distribution of LC3-II protein indicative of a basal level of autophagy (Fig. 1).

In addition in culture, using pre-osteocyte-like murine (MLO-A5) cells, autophagy was found to be upregulated following nutrient deprivation and hypoxic culture, stress conditions that osteocytes encounter *in vivo* (see Fig.1, Table 1). Furthermore, in response to calcium stress, the transcription factor hypoxia inducible factor (HIF)-1 regulated MLO-A5 autophagy, indicating that low pO_2 may serve as a positive regulator of autophagy in this cell type. Lastly, it should be mentioned that low-dose glucocorticoid therapy profoundly influences osteocyte function and increases autophagic activity and anti-oxidative responsiveness 30-fold. High doses reduce both

the expression of genes encoding anti-oxidant proteins and the number of autophagic osteocytes.[18] Bone formation, measured by serum osteocalcin and surface-based histomorphometry, was greatly reduced by chronic or high dose glucocorticoid treatment. Xia *et al*. [19] proposed that modification of the oxidative and autophagic pathways may provide promising new targets for maintaining bone formation in the presence of glucocorticoids while preserving bone mass.

3. Osteoclastogenesis, Osteoclast Function and Autophagy

Regulation of Osteoclastogenesis: At remodeling sites, hematopoietic mononuclear myeloid stem cells, mostly resident in the bone marrow, commit to the osteoclast phenotype and migrate to the tissue surface. When activated, the terminally differentiated osteoclast becomes tightly adherent to the bone surface. Attachment is mediated through one or more specialized structures termed podosomes. The podosome contains bands of actin filaments, as well as F-actin and actin monomers. At the bone surface, circumferentially bounded by the podosome, secretory lysosomes fuse with specialized domains of the plasma membrane to form a ruffled border. This undulating membrane is the site for secretion and externalization of proteases and hydrochloric acid; the acid dissolves the mineral phase of bone, while key proteases such as matrix metalloproteinase (MMP)9 and13 and cathepsin K, hydrolyse the collagen-rich organic bone matrix.[20]

Differentiation of the adherent cells into an active osteoclast is dependent on macrophage colony-stimulating factor (M-CSF) and RANKL. [21] Following fusion with other mononuclear precursors of the macrophage–monocyte lineage, they form multinucleated giant cells. [22] Recruitment is chemokine-dependent, especially chemokine (C-X-C motif) ligand (CXCL) 12 which regulates cell migration. Recently, a lysophospholipid-derived from sphingomyelin, sphingosine 1-phosphate (S1P), has been shown to be an osteoclast chemoattractant. [23] Osteoclasts express the S1P receptor, and by modulating the activity of mechanistic target of rapamycin (MTOR), S1P counteracts autophagy and promotes apoptosis. [24] Lee *et al.* [25]have shown that S1P levels were higher in postmenopausal women and possibly due to increased bone resorption, its action is associated with low bone mineral density.

While differentiating osteoclasts populate the surface and interior of bone trabeculae, the local environment is probably hypoxic. Arnett *et al*. [26] showed that a low pO_2 promoted increased expression of BCL2/adenovirus E1B 19kDa interacting protein (BNIP)3. BNIP3, increased autophagic flux and LC3 recruitment to autophagosomes and osteoclast differentiation. These observations fueled the speculation that a HIF-1α--BNIP3 signaling pathway promoted osteoclastogenesis and differentiation. DeSelm and colleagues showed that ATG5, ATG7 and ATG4B and LC3 are required for osteoclastogenesis and activation of bone resorption. [28] ATG5 and ATG7 promoted

bone resorptive activities in vivo and in vitro and serve to target lysosomes to the actin ring of the functioning osteoclast. However, neither protein influenced osteoclastogenesis, the numbers of nuclei in the osteoclasts, the presence of secretory lysosomes or even the expression of actin ring proteins. ATG4B modulation of LC3, blocked both resorptive activity and expression of cathepsin K. These findings lend support to the notion that lysosomal targeting is regulated by Atg5, Atg7, LC3, and Atg4B, while control of bone resorption is independent of autophagic activity. [28] This concept was further developed by Chung et al who knocked down LC3 and confirmed that it did not influence multinucleation, although it inhibited actin ring formation, and cathepsin K release. [29] Using the ATG5 knockdown, these workers showed that the lowered amount of LC3-II protein levels caused a loss in Cdc42 activity and actin ring disruption. This was especially marked at the periphery of the actin ring where it co-localized with LC3. Based on these findings, the possibility exists that LC3 can regulate bone-resorbing activity *via* Cdc42-dependent actin ring formation and ruffled border organization. [29] Overall these observations confirm an important, non-canonical role for autophagy in the regulation of osteoclast formation and function.

Osteoclast Function and Autophagy: There is a considerable amount of new evidence that points to a role for the autophagic process in the clearance of cytotoxic protein aggregates – these accumulate in disease states due to impairment of the ubiquitin proteasome system (UPS) system. Aberrant, misfolded proteins, along with chaperones, are commonly found in p62 and ubiquitin-positive aggregates (sequestosomes) which are precursors to the inclusion bodies seen in many age-related neurodegenerative and liver diseases. 30 Several proteins that have been linked to autophagic regulation of protein aggregates include autophagy-linked FYVE protein (ALFY), SQSTM1/p62, NBR1, nuclear dot protein (NDP)52, optineurin (OPTN) and valosin containing protein (VCP). Currently, it is not clear if their involvement is limited to selective autophagy or bulk autophagy in response to starvation. However, as noted previously some of these proteins act as autophagy cargo receptors (e.g. NBR1 and SQSTM1), whilst others are adaptors (e.g. ALFY) which facilitate autophagosome membrane formation around the cargo to be degraded.31In osteoclasts, ALFY interacts directly with SQSTM1 (via its PH-BEACH domain), ATG5 (via its WD40 repeat domain) and phosphatidylinositol-3-phosphate (PI3P) (via its FYVE domains) and forms large cytoplasmic aggregates (see section 4). 32

Using an ALFY siRNA knock Filimonenco et al. 33 showed that while there was no direct effect on starvation-induced autophagic clearance of Htt poly Q protein, ALFY nuclear localization and shuttling may be the rate limiting step for aggregate clearance in HeLa and N2a cells. When compared to mononuclear precursors, amino acid starvation of mature multinucleated cells resulted in ALFY rapidly relocating to the cytoplasm and interacting and co-localizing with SQSTM1 in cytoplasmic aggregates.

Importantly, ALFY together with the proteins mentioned above can be linked to skeletal homeostasis; SQSTM1 and VCP mutations as well as OPTN genetic variants are linked to human disorders associated with a skeletal phenotype (see section 4) while NBR1 regulates osteoblast function (see section 2). 10,34 The important role of autophagy in mature osteoclast function and survival is probably best illustrated by consideration of the osteoclast-specific effect of SQSTM1 mutations observed in patients with Paget's disease of bone (PDB).

4. Autophagy and the Pathogenesis of Paget's Disease of Bone

Pathogenesis of PDB: PDB is a common age-dependent skeletal disorder characterized by focal areas of increased and disorganized bone turnover. Clinically, if left unchecked, PDB can cause bone deformity and fracture. [35] PDB is principally a disorder of the osteoclast, characterized by an increase in their number, size and activity within the bone lesion. PDB osteoclasts exhibit increased sensitivity to RANKL *in vitro* and appear to be more resistant to apoptosis.[36] The underlying cause(s) of the abnormal osteoclast activity and function in PDB is unclear, although evidence for a secondary involvement of osteoblasts in lesion development, is now emerging.[35]

The etiology of PDB involves a complex interplay between genetic and environmental factors [37-39] with some studies implicating paramyxovirus infection.[40] Interestingly, many viruses exert their effects by subverting autophagy, although to date no studied have directly determined the impact of viral infection on osteoclast autophagy. As many as 40% of patients have a positive family history of disease and PDB is 7–10 times more common in first-degree relatives of affected individuals.[41] Current thinking is that PDB is caused by a combination of rare, high-penetrance variants in small number of genes, together with common variants in other genes which together increase the risk of developing the disorder.

The only gene to date identified as being causally related to PDB is *SQSTM1*, which encodes the autophagy receptor SQSTM1/p62 protein.[42] However, *SQSTM1* mutations are found only in 5-20% of PDB patients.[43] The functional domains of SQSTM1 include a UBA domain, an N-terminal PB1 domain and two internal regions representing an LIR and KIR (KEAP1-interacting region). It co-localizes with LC3 in cellular 'protein bodies', including those containing aggregated mutant proteins, a process dependent on both the UBA and Phox and Bem1p (PB1) domains.[30] The direct interaction (*via* the LIR) of SQSTM1 with LC3 facilitates the autophagic degradation of ubiquitin-modified cytosolic protein aggregates and organelles.[44,45] However, the precise complement of autophagic substrates of SQSTM1 *in vivo* remains to be fully clarified.

Consistent with its role as a cargo receptor for the autophagic degradation of ubiquitin-modified targets, SQSTM1 is up-regulated by various stressors including starvation, proteasome inhibition, and NF-κB and NRF2 activation. Functionally, it cooperates with the autophagy receptor NBR1 *via* PB1 domain-mediated interactions, and as was noted earlier in osteoclast-like cells with ALFY to facilitate degradation of misfolded proteins.[32,45] Further, interaction of SQSTM1 *via* its KIR region regulates the levels of this adapter protein. [,47, 48] KEAP1 in turn controls levels and activity of the NRF2 transcription factor which regulates the expression of cytoprotective genes, thus contributing to the cell's capacity to defend itself against chemical and oxidative stress and controls proliferation and differentiation of osteoblasts. [49] Interestingly, KEAP1 is also reported to down-regulate TNFα-induced NF-κB activation through autophagic degradation of IKKβ [50,51] Recent work shows a direct and functional interaction between SQSTM1 and components of mTORC1, establishing it as key regulator of nutrient sensing.[52] In terms of regulation of SQSTM1-mediated autophagic function at the molecular level,, phosphorylation of the UBA domain at Ser403 was found to serve as a signal to promote autophagy.[53] Ser403 phosphorylation appears to regulate the ubiquitin-binding ability of p62 and a phosphomimetic mutant (S403E) was shown to promote the formation of 'sequestosomes', presumed to be precursors of autophagosomes.[53] Genetic inactivation of *SQSTM1* in mice results in impaired osteoclastogenesis *in vitro* and *in vivo.*[52] and importantly, in a mouse carrying a P394L-p62 missense mutation (equivalent to the most common PDB-associated P392L human mutation), a PDB-like disorder was seen with altered osteoclast autophagy (see later).

Disease Mechanisms: At the protein level, most of the disease-associated mutations affect the UBA domain of SQSTM1 and cause a loss of ubiquitin-binding activity.[54-55] While the PDB-mutant SQSTM1 causes an increase in osteoclast activity involving NF-κB signaling, [56,57] little is known of the impact of the mutation on autophagy. As there is crosstalk between the two systems,[58] changes in NF-κB activity and autophagic function could be expected. For example, induction of autophagy in macrophages in response to TLR4 is associated with TRAF6-mediated ubiquitination of BECLIN-1 [59] and TRAF6 may be degraded by a form of autophagy that is dependent on NDP52 (although not associated with the canonical conversion of LC3-I to LC3-II). [60] Further, the IKK complex, which is an essential mediator of the RANK-NF-κB pathway, contributes to the induction of autophagy and is activated by multiple autophagy inducers, without affecting NF-κB nuclear translocation.[61] Conversely, levels of IκB, the inhibitor of NF-κB, appear to be regulated by autophagy.[51] Further emphasizing common pathways, under resting conditions BECLIN-1 forms a complex with the TGFβ-activated kinase 1 (TAK1)-binding proteins (TAB) 2 and 3; during autophagy, BECLIN-1 activate IKKβ and induces autophagy. [62,63] NF-κB activation can also limit autophagy activators (BNIP3,

JNK1, and ROS) and increase expression of autophagy inhibitors (A20 and BCL-2/XL) (see review by Salminen *et al* [64]).

PDB-Associated Mutations of SQSTM1 and Autophagy: Indirect evidence supports the notion that alterations in autophagy are linked to the pathogenesis of PDB: disease-causing mutations increase osteoclast activity; autophagy positively regulates osteoclast activity[28] and SQSTM1, which is commonly mutated in PDB, is an autophagy receptor. Further, PDB-associated mutations map to regions of SQSTM1 which are relevant for its autophagy-dependent function: principally the UBA domain,[42, 65,] but also the LIR (D335E)[66] and the KIR (S349T) domains. [67]

Earlier observations that SQSTM1 is over-expressed in PDB patient samples, regardless of *SQSTM1* mutation status, were the first indication that autophagy may be altered in the pagetic state (Table 1). [68] However, perhaps the best evidence of alterations in autophagic function comes from studies of the P394L-p62 mouse, which, as noted earlier, develops a PDB-like bone disorder with focal bone lesions. [60] Osteoclast precursors from the mutant animals, exhibit increased expression of *SQSTM1, ATG5* and *LC3* along with increased LC3-II protein levels in the presence of Bafilomycin (BAF) suggesting a possible increase in autophagic flux (and consistent with the known relationship between autophagic and osteoclastic activity). [28] In a cell model of PDB with *SQSTM1* mutations, a preliminary report describes alterations in autophagic flux. [68]

Other Related Disorders and Autophagy: Curiously, *SQSTM1* mutations, including some UBA domain mutations which are associated with PDB, have also been reported in patients with amyotrophic lateral sclerosis and frontotemporal lobar degeneration.[69,70] Interestingly, several of the recently identified new genes for ALS represent autophagy receptors including *OPTN* (also linked to PDB), *UBQLN2* and *VCP.*[69] The latter is perhaps the most relevant, given that *VCP* mutations are a cause of the multi-system disorder inclusion body myopathy associated with PDB and frontotemporal dementia. Although its precise role in autophagy is unclear, in muscle cells the mutant VCP appears to be linked to alterations in autophagy.[71] Indeed, its expression was associated with accumulation of non-degradative autophagosomes and a failure to degrade aggregated proteins.[72] In knock-out animals with mutant VCP there was increased levels of LC3B-II in muscle cells, osteoclast precursors exhibited increased sensitivity to RANKL, and there were focal bone lesions. [73] Finally, *OPTN*, encodes the autophagy receptor, OPTN/optineurin which been implicated in PDB[74] with preliminary studies suggesting that it negatively regulates osteoclast activity. [75]

5. Autophagy in the Growth Cartilage

Function of the Growth Plate: The epiphyseal growth plate is a transient form of cartilage located at sites of long bone growth and composed of chondrocytes embedded in a matrix containing collagen II and proteoglycan. In comparison with articular cartilage (see below), chondrocytes in the growth plate have a very short half-life (days, not years) [76]. During growth, a number of well-defined zones can be delineated; most obvious is a columnar cell zone that contains proliferating cells. As these cells mature, they become terminally differentiated hypertrophic chondrocytes. Mineralization of the mature cartilage begins in the deep hypertrophic zone in cell-derived particles, matrix vesicles. Enzymes in the matrix vesicle, remove local inhibitors of mineralization, enhance calcium transport and localization and promote mineral deposition.[77] During the growth period, there is evidence of chondrocyte autophagy and indeed treatment of rats with the autophagy activator rapamycin impairs longitudinal growth.[78]

Autophagy and Chondrocyte Function: Regulation of the cellular changes described above is complex and involves aninterplay between agents generated by chondrocytes, (PTHrp and Indian Hedgehog), cytokines and systemic factors including hormones of the hypothalamus-pituitary axis. [79, 80] Once mineralization has begun, there are autophagic changes in hypertrophic chondrocytes: reorganization of LC3-II and BECLIN-1 proteins into punctate granules. In addition, transmission electron microscopy studies indicate the presence of double membrane vacuoles. Not surprisingly, suppression of MTOR causes a marked increase in chondrocyte autophagy. [81] Following autophagy, chondrocytes are deleted from the plate by the initiation of programmed cell death. [80]These terminal changes and the control of growth through Wnt signaling pathway would suggest that autophagy serves not just to regulate the final stages of the chondrocyte life cycle, but also the rate at which chondrocytes enter the maturation process.

Metabolic Control of Chondrocyte Autophagy: Chondrocytes generate metabolic energy through anaerobic glycolysis, an environmental adaptation that permits them to survive in the restricted vascular supply of cartilage. HIF-1 is expressed at high levels in hypertrophic chondrocytes. Conditional inactivation of the *HIF-1α* gene caused a reduction in the number of maturing hypertrophic cells, an elevation in numbers of apoptotic cells and a disorganized layer of subchondral (metaphyseal) bone. [82] To further explore this activity and relate it to autophagy, a chondrocyte line was developed (N1511) that mimics many of the phenotypic changes expressed by epiphyseal chondrocytes.[83] When serum-stressed, these cells robustly expressed punctate LC3 protein and BECLIN-1.[83]

Not surprisingly, at a low pO_2, the cultured chondrocytes are refractory to an apoptotic challenge. It was noted that HIF-1 suppressed BECLIN-1, leading to enhanced cell death. The observation that BECLIN-1 suppression resulted in increased BH3 interacting-domain death agonist (BID), cleavage and caspase-8 activation clearly links

apoptosis with autophagy. Thus, BECLIN-1 served to maintain chondrocyte survival activity, possibly by regulating the activities of pro-apoptotic genes.[83] Another HIF target is AMPK , a protein that responds to the energy status of the cell, and activated in a HIF-1-dependent manner. As might be expected this protein is robustly expressed in the glycolytic growth plate.[884] A number of studies have shown a connection between AMPK (AMP activated protein kinase) and MTOR. Once activated, AMPK phosphorylates tuberous sclerosis protein 2 (TSC2) which then suppresses MTOR and hence promotes autophagy. By down-regulating the phosphorylation of targets S6K1 and 4E-BP1, MTOR integrates multiple signals including those from nutrients, as well as metabolic signals from glycolysis and ATP.[85]

From a physiological viewpoint, in the growth plate, autophagy is controlled by two environmental sensors: AMP kinase and MTOR and probably HIF *via* the prolyl-4-hydroxylase domain (PHD) oxygen sensors [84]. The hypoxic plate would foster the expression of HIF-1 and the high glycolytic activity would elevate AMP levels and suppress MTOR. Once autophagy is activated, it would serve to maintain the lifespan of the hypertrophic cell, thus allowing the cells to reach their final maturation stage. Eventually, extended autophagic activity would lead to sensitization of terminally differentiated chondrocytes to local and intrinsic signals, resulting in apoptosis, deletion of cells from the growth plate and bone growth.[86]

6. Autophagy and the Pathogenesis of Lysosomal Storage Disorders (LSDs): The Mucopolysaccharidoses (MPSs)

Lysosomal Storage Disorders: LSD are recognized as a cohort of nearly 60 different inherited disorders, each with a genetic defect that renders the lysosomal system dysfunctional and unable to degrade specific molecules. As a consequence, many tissues and organ systems are affected, including bone and cartilage.[87] The MPSs comprise a group of LSDs caused by deficiency in the enzymes catalyzing the degradation of glycosaminoglycans (GAGs), which are long, repeating chains of complex sugar molecules, normally degraded in the lysosome.[88] Multiple Sulfatase Deficiency (MSD) is a very severe form of MPS due to mutations in the Sulfatase Modifying Factor 1 (*SUMF1*) gene. [89] The SUMF1 protein is responsible for an essential post-translational modification of the sulfatase enzymes, a class of hydrolases that remove sulfate groups from different molecules including GAGs. Many sulfatases are lysosomal, and thus one of the major consequences of the lack of sulfatase activity is the accumulation of multiple sulfated substrates in the lysosomes. [90] The $Sumf1^{-/-}$ mouse recapitulates most of the features of the human MSD disease and in particular displays a remarkable skeletal dysplasia, characterized by severe shortening of the axial and appendicular skeleton.[91] Multiple measurements of autophagy in $Sumf1^{-/-}$chondrocytes revealed severe lysosomal vacuolization and an increased number of autophagosomes compared to wild-type chondrocytes. Since cells treated

with an inhibitor of lysosomal acidification (BAF) accumulate autophagic vacuoles and present with low ATP content, it is likely that the decreased survival of the Sumf1$^{-/-}$chondrocytes is directly linked to the defective digestion of the autophagic cargo. [92]

Pathogenesis of the MPS: While the pathogenic mechanisms are still unclear, defective autophagy is a generalized phenomenon occurring in many LSD; cells and tissues isolated from patients and mouse models of LSD display higher number of autophagosomes compared to controls, most likely the result of a defective lysosome-autophagosome fusion (Table 1). As a consequence, autophagic substrates, such as polyubiquitinated proteins and dysfunctional mitochondria, are significantly elevated in LSD cell and tissue samples. [93,94] Providing a direct insight into the biochemical defect, Sumf1$^{-/-}$ mice exhibited a significant accumulation of cholesterol in lysosomal membranes. As a consequence, there is impaired distribution of the SNARE (soluble NSF attachment protein receptors) that are key components of the cellular membrane fusion machinery. [95] Interestingly, wild-type cells "loaded" with cholesterol *in vitro* mirror the fusion defects observed in LSD cells. Conversely, lowering cholesterol levels restores normal lysosomal function. From this perspective, the lysosome-autophagosome fusion defect in LSD reflects the abnormal lysosomal membrane lipid composition. [95]

7. Autophagy and the Pathogenesis of Osteoarthritis (OA)

Pathogenesis of Osteoarthritis: OA is among the most prevalent aging-related diseases and the most prevalent joint disease. [96] The two main risk factors for OA are aging and mechanical load. Excessive mechanical loading can occur in the acute setting of joint injury or chronically due to abnormal joint shape, malalignment or as a result of occupational and recreational activities. [97] The earliest changes in cartilage are enzymatic degradation of GAGs and cartilage proteins, and loss of chondrocytes in the superficial zone; this region is exposed to shear and compressive forces during movement. [98] With depletion of many of the original chondrocytes, there is emergence of clusters of densely packed cells, which are phenotypically distinct from the original cartilage cells. Cell activation in OA cartilage has been interpreted as an unsuccessful attempt at tissue repair and as the condition progresses, it leads to further cartilage defects.[99]

Changes in autophagy protein expression and activation in aging and OA: Conceptually, autophagy in normal adult articular cartilage is an important mechanism for cellular homeostasis. Thus, cells in the superficial zone display a robust expression of autophagy proteins BECLIN-1, ATG5 and LC3. [100] When LC3 was tagged with GFP,

the highest GFP signal was observed in cells present in the superficial and middle zones of the knee articular cartilage (Table 1). Few cells in the deep cartilage zone exhibited detectable levels of GFP-LC3 signal. As with other tissues, starvation increased the number of autophagosomes in chondrocytes.[14]

During the aging process in mouse and human knee articular cartilage, there is a decrease in ULK1, LC3, and BECLIN-1 protein expression. The reduction of these key regulators of autophagy is accompanied by increased apoptosis.[14] Using a rapidly progressing experimental mouse of OA, a time-dependent reduction in these autophagy proteins was noted.[100] Since this reduction was observed in relatively young mice it is apparently not a consequence of aging-related events. However, for both surgical OA and mechanically injured cartilage, the increase in cell death suggests that autophagy may contribute to survival mechanisms.

In contrast to the reduction in autophagic proteins in non-proliferating chondrocytes, the cell clusters in OA cartilage express high levels of these proteins[100] thereby confirming an earlier study that suggested that chondrocytes in OA cartilage displayed numerous autophagic LC3 puncta.[101] Levels of LC3-II were also increased in the superficial and middle zones in a rat model of OA.[14] When full thickness cartilage explants were subjected to high impact mechanical compression, there were immediate matrix changes and a low level of cell death, accompanied by a short and transient increase in the levels of LC3-II, and a marked reduction in ULK1, LC3, and BECLIN-1.[100] Thus, during the development of OA, increased autophagy may reflect an adaptive stress response. Further, failure to mount an autophagic response may lead to further degeneration.

Consequences of dysregulated autophagy in OA: The reduction in autophagy protein levels and activity lends strong support to the hypothesis that basal autophagic activity decreases with age, thus contributing to the accumulation of damaged organelles and macromolecules and susceptibility to aging-related diseases.[102] Indeed, prior studies demonstrated mitochondrial dysfunction in various animal models and in human OA.[103] In addition, mitochondrial DNA mutations are known to increase in OA chondrocytes.[104] Damaged mitochondria, producing high levels of ROS, promote pro-inflammatory signaling, as they initiate formation of inflammasomes and activation of other inflammatory pathways.[105] In knee chondrocytes, IL-1- or NO - dependent increase in expression of LC3 and BECLIN-1 activates autophagy.[106] Further, autophagy activation prevents IL-1-mediated suppression of cartilage matrix degradation while reducing the levels of MMP-13, ADAMTS, (a disintegrin and metalloproteinase with a thrombospondin type 1 motif) 5 and ROS. Given that one of the cytoprotective functions of autophagy is removal of damaged mitochondria[107], the IL-1–induced OA-like gene expression changes might possibly occur through reduction in the intracellular ROS level *via* elimination of damaged mitochondria.

As discussed earlier, HIF proteins have recently been linked to the regulation of autophagy in chondrocytes. [88] With respect to HIF-1, in the superficial cartilage zone, there is moderate level of protein expression; both expression and autophagy is increased in OA cells. [101] It is likely that HIF-1 upregulation due to the hypoxic state of the tissue serves to promote chondrocyte autophagy. With respect to HIF-2α, in young animals, the highest expression level is in the superficial zone. In this case, upregulation of HIF-2α lowers intracellular ROS levels by promoting the activities of the dismutating proteins, catalase and superoxide dismutase; from this perspective, it can be regarded as cytoprotective. In OA cartilage, HIF-2α expression is induced in the earlier stage of OA, and it is down-regulated at later stages.[108,109]

A second aspect of HIF-2α is that it increases expression of RUNX2 (runt-related transcription factor 2), a transcription factor, which induces expression of proteins associated with chondrocyte hypertrophy (collagen X, MMP13 and vascular endothelial growth factor A, (VEGFA).[110] As RUNX2 and its target genes are overexpressed in OA cartilage, it has been suggested that chondrocytes express a differentiation program that is more characteristic of a hypertrophic state[111]. On this basis, increased HIF-2α-induced RUNX2 activation and hypertrophic differentiation may promote OA. HIF-2α over-expression promotes cartilage destruction and conversely, the severity of experimental OA is reduced in HIF-2α mutant mice.[109, 112] Indeed, when HIF-2 is silenced, IL-1β-induced expression of ADAMTS4, MMP1, MMP3, MMP9, MMP12 and MMP13 is significantly decreased. Thus, the two HIF isoforms have overlapping but differing roles: while HIF-2α appears to be cytoprotective, HIF-1 both promotes autophagy while sensitizing cells to local apoptogens, Put another way, HIF-2α regulates the extent of the autophagic response and can be viewed as acting as a brake to the accelerator function of HIF. Finally it should be noted that stress or cytokine induced activation of HIF-2α, could outweigh the homeostatic effects of HIF-1α, and promote chondrocyte hypertrophy, cell death and matrix degradation.[101] The marked inhibition of autophagy would negatively impact chondrocyte survival and differentiation.

8. Therapeutic targets for skeletal disease

This review highlighted the import of the autophagic response in relationship to the pathogenesis of diseases affecting bone and cartilage. The authors suspect that aside from disorders discussed here in some detail, there are hints that autophagic dysfunction may influence other diseases of the skeletal tissues. An incomplete list would include growth disorders, disregulated endocrine function, osteoporosis, fibrous dysplasia, rheumatoid arthritis and degenerative disc disease.

A priori, before delineating therapies directed at removing dysfunctional organelles and accumulated aggregated proteins, the underlying autophagic ‘defects’ needs to be defined with care. From what is already known of the autophagic process, it is clear that unless the nature of the defect is established, promotion of autophagic flux may well

trigger the induction of apoptosis or even senescence. In this case, for many skeletal tissues where cell numbers are normally low and longevity is extended, this type of treatment would exacerbate the disease state. In contrast, when the site of aberrant dysfunction is known, modulation of the autophagy response may be a rational approach for treatment of conditions like the MPS.

While caution is urged, there have been some attempts to modify skeletal cell function through modulators of the autophagic response. Of the drugs examined, in OA tissues, the autophagy activator rapamycin has been shown to prevent cell death and GAG loss, maintain cartilage cellularity and decrease the expression of ADAMTS-5, an important enzyme in cartilage extracellular matrix degradation. [113] Likewise, on the basis of PMTOR and or p-p70S6K expression, there is evidence indicating that over 50% of chordomas, tumors of the intervertebral disc are responsive to MTOR inhibitors. [114]Since this kinase is the nexus for signals from the AKT/PI3K, MAPK/ERK1/2 and P53 signaling pathway, as well as nutrient (amino acid) levels and sensors of the oxygen and energy status, there is likely to be multiple sites for control of these skeletal tumors. Undoubtedly, molecular delineation of the autophagic process in skeletal tissues will provide unique functional insights, while at the same time uncover new approaches to treating the host of diseases that affect the axial and appendicular skeleton.

FIGURE 1. (LEGEND)

Autophagy in bone, articular cartilage, the growth plate and articular cartilage. A. Schematic showing cellular control of bone remodeling. A basic multicellular unit consists of osteoblasts (OB) osteoclasts (OC) and bone lining cells (LC). Pro-osteoclasts (PrOC) and pro-osteoblasts (prOB) enter the unit through capillaries (CAP) and home to the bone surface where they undergo differentiation into OB and OC respectively. A number of osteoblasts will undergo terminal differentiation and become osteocytes (O'CYTES). Under the influence of local factors including those secreted by osteoblasts (MCF, RANKL), pro-osteoclasts differentiate and resorb bone liberating Ca^{2+} and amino acids (AA) from the bone matrix. Sclerostin an inhibitor of osteoblast activity is also released from the bone matrix. At these sites, a resorption lacuna can be seen (RL). Another secreted factor, osteoprotegrin (OPG), inhibits osteoclast mediated bone resorption by serving as a physiological inhibitor of RANKL. A number of factors are released from the resorbing bone that include BMP and TGFβ. These proteins promote osteoblast maturation and osteoid synthesis and mineralization. B Schematic of a long bone showing articular cartilage (AC), growth plates (GP) and bone marrow (BM). C. Section through a demineralized osteon showing the presence of autophagic osteocytes (inset, stained with an anti LC3 antibody). From Zahm *et al.,* Cells Tissues Organs. 2011;194(2-4):274-8D, and with permission from S. Karger AG. D. Section through the rat growth plate showing the presence of autophagic pre-hypertrophic chondrocytes (arrows) stained with an antibody to LC3. From Srinivas *et al.,* Cells Tissues Organs. 2009;189(1-4):88-92 and with kind permission from S. Karger AG. E. Chondrocyte autophagy in knee cartilage of GFP-LC3 transgenic mice. Confocal microscopy rendered reconstruction using 3D IMARIS (Bitplane Inc.) indicating that the highest levels of GFP-LC3 signal were observed in chondrocytes in the superficial and upper middle zone of the articular cartilage. In contrast, only few cells in the deep zone contained detectable levels of GFP-LC3 signal. Mag x 63.

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