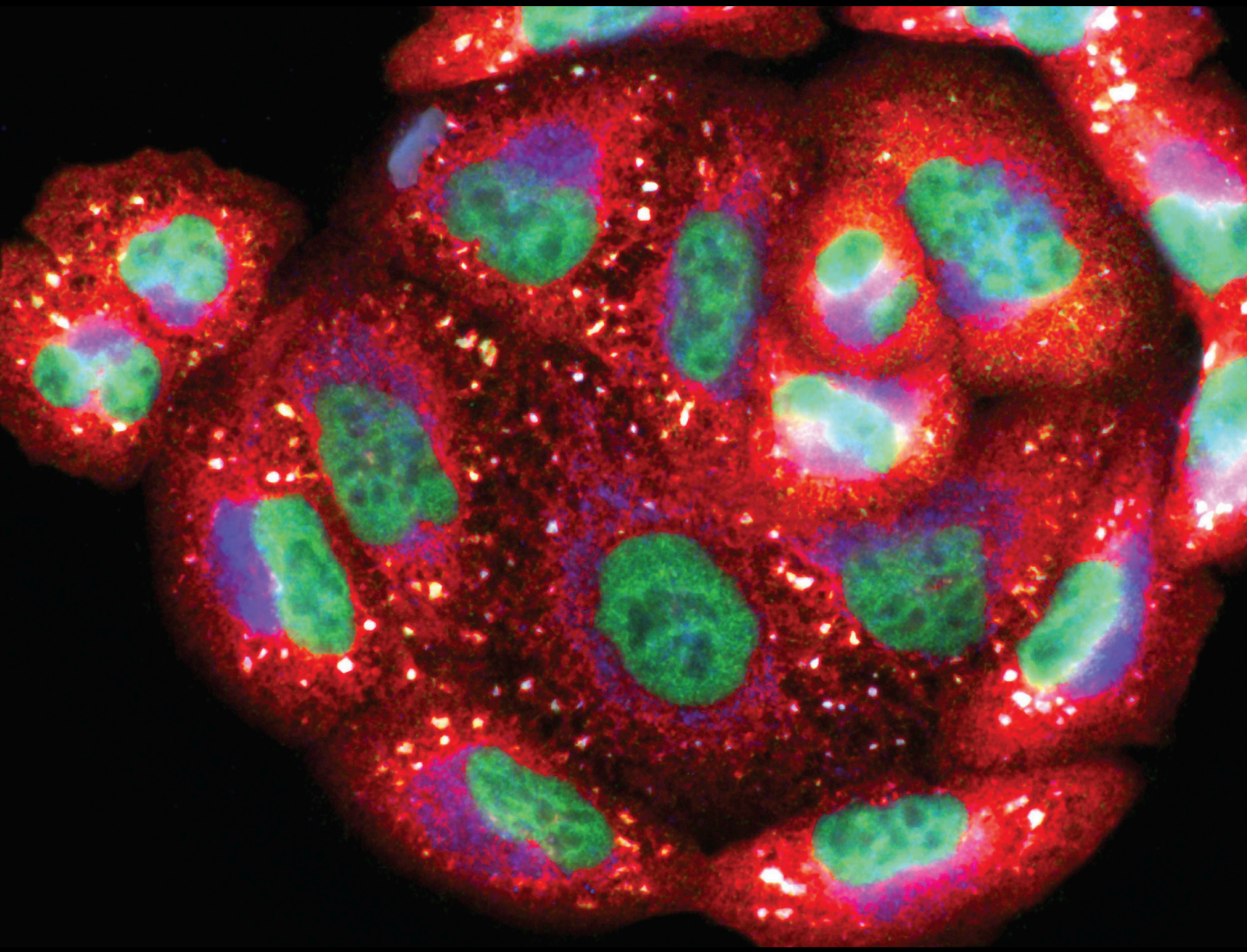
Oxidative Medicine and Cellular Longevity

Transautophagy: Research and Translation of Autophagy Knowledge 2020

Lead Guest Editor: Maria C. Albertini
Guest Editors: Tassula Proikas-Cezanne, Nikolai Engedal, Eva Žerovnik, and Jon D. Lane



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Editorial

Transautophagy: Research and Translation of Autophagy Knowledge 2020

Nikolai Engedal,[1] **Tassula Proikas-Cezanne**,[2,3] **Maria C. Albertini**,[4] **Eva Žerovnik**,[5] **and Jon D. Lane**[6]

[1]*Oslo University Hospital, Oslo, Norway*
[2]*Eberhard Karls University Tübingen, Tübingen, Germany*
[3]*International Max Planck Research School "from Molecules to Organisms", Tübingen, Germany*
[4]*University of Urbino Carlo Bo, Urbino, Italy*
[5]*Jožef Stefan Institute, Ljubljana, Slovenia*
[6]*University of Bristol, Bristol, UK*

Correspondence should be addressed to Nikolai Engedal; k.n.engedal@ncmm.uio.no

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Autophagy is an evolutionary conserved mechanism for degradation and recycling of cellular material, whose altered activity is associated with aging and a growing list of human pathologies. As a selected annual special issue, the "Transautophagy: Research and Translation of Autophagy Knowledge" series—initiated as part of the objectives of the COST Action Transautophagy (CA15138) aims to collect high-quality papers that disseminate novel findings and new knowledge concerning the molecular mechanisms regulating autophagy and the role of autophagy in human pathologies, in particular those related to oxidative stress and aging.

The current special issue, "Transautophagy: Research and Translation of Autophagy Knowledge 2020", comprises 8 cutting-edge publications relevant to the above-described themes. Together, the 4 original research papers and 4 review papers convey important new insights into (i) novel mechanisms of signal control of autophagy by hypoxia, ataxia telangiectasia mutated protein kinase (ATM), the ER stress sensor inositol-requiring enzyme 1 (IRE1), and the process of O-GlcNAcylation; (ii) the role of autophagy in various age-related diseases such as cardiac fibrosis, heart failure, Parkinson's disease, intervertebral disc degeneration (IVDD), and age-related macular degeneration (AMD); and (iii) the role of autophagy in cancer, with a particular focus on resistance mechanisms to anticancer therapy and biomarkers of chaperone-mediated autophagy (Figure 1).

ATM is best known as a mediator of DNA damage-induced signaling. However, recent evidence point towards a more versatile role of ATM in sensing different kinds of stress signaling, including that arising from oxidative stress. M. Blignaut et al. discuss the crosstalk between oxidative stress as an activator of ATM and its potential role as a master regulator of general autophagy as well as of various types of selective autophagy (mitophagy, pexophagy, and aggrephagy). The review includes a special focus on terminally differentiated cells like neurons and cardiomyocytes. In terms of the latter, autophagy has been implicated in cardioprotective responses. J. Qu et al. examined the role of Sigma-1 receptor (Sig1R), a chaperone in the endoplasmic reticulum (ER) membrane, in cardiac fibroblast activation in a mouse model, as well as in neonatal rat cardiac fibroblasts. Their results indicate that Sig1R plays a protective role in the activation of cardiac fibroblasts by inhibiting the IRE1 pathway and restoring autophagic flux. Sig1R may therefore represent a therapeutic target for cardiac fibrosis. The cellular effects of O-GlcNAcylation have received increased attention over the recent years. H. Yu et al. employed an O-linked β-N-acetylglucosamine transferase (OGT) cardiomyocyte-specific knockout mouse model for the first time. They provided

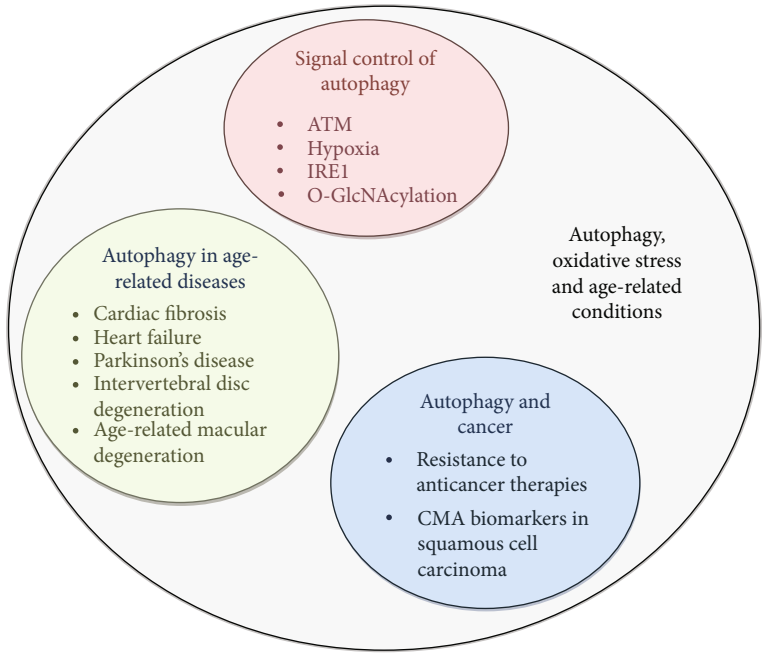

Figure 1: Summary of topics that have been discussed in review articles or have been addressed in original studies in this special issue. Abbreviations: ATM: ataxia telangiectasia mutated protein kinase; IRE1: inositol-requiring enzyme 1; CMA: chaperone-mediated autophagy.

data to indicate that O-GlcNAcylation is required for autophagy in cardiomyocytes and that O-GlcNAcylation of the central autophagy-initiating kinase ULK1 may stimulate its activity towards promoting autophagy. Together, these three papers provide novel insight into signal control of autophagy and molecular mechanisms underlying the association between stress responses, autophagy, and heart disease.

Subsequent papers in this special issue have explored the relationship between autophagy and other age-related conditions. N. Jimenez-Moreno and J. D. Lane reviewed the molecular machinery of the autophagic pathway as a background to analyse the crosstalk between autophagy and redox homeostasis and their role in neurodegenerative diseases, with a particular emphasis on the pathogenesis of Parkinson's disease. They describe how dysfunctional autophagy typically correlates with neurodegenerative diseases, and that mitochondrial dysfunction, with associated increases in oxidative stress, and declining proteostasis control are likely to be key contributors to Parkinson's. Intervertebral disc degeneration (IVDD) is a common cause of lower back pain. H.-J. Kim et al. hypothesized that the nucleus pulposus cells that make up the center of the intervertebral disc can be affected by aging and environmental oxygen concentration, thus affecting the development of IVDD. Their results from analyses of isolated nucleus pulposus cells from rat lumbar discs suggest that nucleus pulposus cells modulate the expression of chondrogenesis-, autophagy-, and apoptosis-related genes under hypoxic conditions. The study also provided hints to changes that occur in nucleus pulposus cells during aging. Age-related macular degeneration (AMD) is a major cause of visual loss and irreversible blindness in the elderly population worldwide. Z.-Y. Zhang et al. describe how age-related, cumulative oxidative stress contributes to the pathogenesis of AMD. Furthermore, they discuss how autophagy can prevent oxidative damage in AMD by protecting retinal pigmental epithelial cells and photoreceptor cells from degeneration and death. Finally, they review potential neuroprotective strategies for therapeutic interventions and provide an overview of such neuroprotective mechanisms.

Cancer is another pathology that is frequently associated with aging and oxidative stress. A. K. Verma et al. provide an overview of the autophagic pathway and its regulation as a framework to discuss the dual role of autophagy in cancer, followed by a focused review of the role of autophagy in resistance mechanisms to anticancer therapy and strategies to modulate autophagy in order to overcome resistance. Different types of autophagy exist, and while the process of autophagosome-associated autophagy (macroautophagy and autophagosome-associated selective autophagy) has been most extensively studied, much less is known about the chaperone-mediated autophagy (CMA) pathway. CMA is characterized by the direct import of cytosolic proteins into degradative lysosomes, in a process where LAMP2A and HSC70 are essential players. T. Losmanová et al. examined the expression LAMP2A and HSC70 in pulmonary squamous cell carcinoma (pSQCC) tissue from 336 patients and compared the expression to clinical data. They reported that high LAMP2A or HSC70 expression was associated with worse outcome, including overall survival. The authors suggested that elevated levels of LAMP2A or HSC70 could be indicative of increased CMA activity and that these two proteins might be useful potential biomarkers for future CMA-inhibiting therapies.

In summary, this annual special issue has disseminated important novel developments in the field of autophagy and oxidative stress research and provides more understanding of molecular and cellular mechanisms of different diseases with emphasis on translation of knowledge to treatment, including topics of autophagy regulation and oxidative stress in aging, age-related diseases, neurodegenerative diseases, and cancer.

Conflicts of Interest

The Guest Editors declare that there are no conflicts of interest regarding the publication of this special issue.

Acknowledgments

We wish to thank all the authors, editors, and reviewers who participated in this issue. We acknowledge the COST Action (CA15138) "Transautophagy" for providing financial support. Above all, we would like to thank the first chair of the Transautophagy COST Action (CA15138) Professor Caty Casas, who sadly passed away on June 30, 2020. Caty worked as an Associate Professor at the Universitat Autònoma de Barcelona at the Institute of Neuroscience. Friends and colleagues remember Caty as an amazing and bright scientist, full of energy and kindness. Unfortunately, because of the illness, she could not safely lead the entire period of the COST Action, but we do not forget that the "Transautophagy" network could not have been created without her.

Nikolai Engedal
Tassula Proikas-Cezanne
Maria C. Albertini
Eva Žerovnik
Jon D. Lane

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Corrigendum

Corrigendum to "Chaperone-Mediated Autophagy Markers LAMP2A and HSC70 Are Independent Adverse Prognostic Markers in Primary Resected Squamous Cell Carcinomas of the Lung"

Tereza Losmanová,[1] Félice A. Janser,[1] Magali Humbert,[1] Igor Tokarchuk,[1,2] Anna M. Schläfli,[1] Christina Neppl,[1] Ralph A. Schmid,[3,4] Mario P. Tschan,[1,2] Rupert Langer,[1,5] and Sabina Berezowska[1,6]

[1]*Institute of Pathology, University of Bern, Bern 3008, Switzerland*
[2]*Graduate School for Cellular and Biomedical Sciences, Bern 3012, Switzerland*
[3]*Division of General Thoracic Surgery, Inselspital University Hospital Bern, Bern 3010, Switzerland*
[4]*Department of Biomedical Research (DBMR), University of Bern, Bern 3008, Switzerland*
[5]*Institute of Pathology and Molecular Pathology, Kepler University Hospital, Johannes Kepler University Linz, 4021 Linz, 4040 Linz, Austria*
[6]*Institut de Pathologie, Centre HospitalierUniversitaire Vaudois et Université de Lausanne, Lausanne 1011, Switzerland*

Correspondence should be addressed to Sabina Berezowska; sabina.berezowska@chuv.ch

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In the article titled "Chaperone-Mediated Autophagy Markers LAMP2A and HSC70 Are Independent Adverse Prognostic Markers in Primary Resected Squamous Cell Carcinomas of the Lung" [1], the authors identified an error in the name of the manufacturer of the antibody mentioned in Materials and Methods. The following should be corrected from

"LAMP2A (Novus Biologicals, Zug, Switzerland, rabbit polyclonal, #NB600-1384)

HSC70 (LabForcembl, Nunningen, Switzerland, rabbit polyclonal, #PM0045)"

to

"LAMP2A (Abcam, Cambridge, UK, rabbit polyclonal, ab18528)

HSC70 (Thermo Fisher Scientific, Waltham, MA, USA, mouse monoclonal, MA3-014)"

References

[1] T. Losmanová, F. A. Janser, M. Humbert et al., "Chaperone-Mediated Autophagy Markers LAMP2A and HSC70 Are Independent Adverse Prognostic Markers in Primary Resected Squamous Cell Carcinomas of the Lung," *Oxidative Medicine and Cellular Longevity*, vol. 2020, Article ID 8506572, 12 pages, 2020.

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Review Article

Ataxia Telangiectasia Mutated Protein Kinase: A Potential Master Puppeteer of Oxidative Stress-Induced Metabolic Recycling

Marguerite Blignaut, Sarah Harries, Amanda Lochner, and Barbara Huisamen

Centre for Cardio-Metabolic Research in Africa (CARMA), Division of Medical Physiology, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa

Correspondence should be addressed to Marguerite Blignaut; mblignaut@sun.ac.za

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Ataxia Telangiectasia Mutated protein kinase (ATM) has recently come to the fore as a regulatory protein fulfilling many roles in the fine balancing act of metabolic homeostasis. Best known for its role as a transducer of DNA damage repair, the activity of ATM in the cytosol is enjoying increasing attention, where it plays a central role in general cellular recycling (macroautophagy) as well as the targeted clearance (selective autophagy) of damaged mitochondria and peroxisomes in response to oxidative stress, independently of the DNA damage response. The importance of ATM activation by oxidative stress has also recently been highlighted in the clearance of protein aggregates, where the expression of a functional ATM construct that cannot be activated by oxidative stress resulted in widespread accumulation of protein aggregates. This review will discuss the role of ATM in general autophagy, mitophagy, and pexophagy as well as aggrephagy and crosstalk between oxidative stress as an activator of ATM and its potential role as a master regulator of these processes.

1. Introduction

Ataxia Telangiectasia Mutated protein kinase (ATM) derives its name from the severe, recessive autosomal disease Ataxia-Telangiectasia (A-T). Although this neurodegenerative disease was initially identified in 1926 [1] and described as a clinical entity in 1958 [2], the gene and protein responsible for the disease were only characterized in the early 90's [3–5]. Null mutations in the *Atm* gene that cause the loss of functional ATM, a 370 kDa protein, results in severe characteristic cerebral ataxia and dilated blood vessels present in the conjunctivae of the eyes, also known as telangiectasia [6]. Moreover, nonfunctional ATM has been associated with an increased risk for cancer, radiation sensitivity, endocrine disruption, progressive neurodegeneration, premature ageing, and chromosomal instability (most recently reviewed by Shiloh [7]). The degree of disease severity is dependent on the type of mutation in the *Atm* gene (single or bi-allelic) and heterozygous patients, which make up as much as 1.4-2% of the general population, also exhibit a high incidence of ischaemic heart disease and insulin resistance [8, 9].

Constant oxidative stress is a common denominator in many of the A-T clinical and cellular phenotypes [10]. The loss of functional ATM results in prolonged activation of stress response pathways in the cerebellum but not in the cerebrum or liver [11]. More importantly, this suggests a cytoplasmic role for ATM. The protein resides predominantly in the nucleus of dividing cells [12], where it acts as a transducer in the DNA damage response pathway (DDR), but ATM is mainly found in the cytoplasm of nondividing neuronal cells where it maintains basal metabolic flux [13]. In these cell types, ATM maintains autophagy, a catabolic process that delivers cytoplasmic components for degradation to the lysosome, as well as redox homeostasis, rather than genomic stability and apoptosis. Moreover, it has been suggested that these divergent pathways could be a result of ATM's subcellular localization, as well as different mechanisms of activation and cell survival outcomes [13]. The seminal study of Guo et al. [14] demonstrated for the first time that ATM can be activated in the cytosol in response to exogenous hydrogen peroxide (H_2O_2) independently of DNA damage response, through the formation of a reversible

disulphide bond at the only cysteine site within the protein kinase domain, Cys^{2991}. Low levels of ROS are sufficient to activate ATM at this residue, independently of the DNA damage response pathway [15], and these distinct activation mechanisms allow ATM to respond to different stresses as well as control different cytoplasmic pathways [16]. More recently, studies showed that ATM can be activated by endogenous ROS including peroxisomal reactive oxygen species (ROS) induced by clofibrate treatment [17] and mitochondrial superoxide induced by low doses of the redox-cycling chemical, menadione [18]. Both peroxisomal and mitochondrial ROS activation of ATM increase autophagy through the activation of AMPK that results in mTOR suppression in the cytosol [19]. Taken together, this suggests that ATM can directly modulate the rate of autophagy in a ROS dependent manner [20] and will be discussed in further detail.

ATM acts as an important sensor of oxidative stress in cells and regulates defences against redox stress [14] by rerouting of glycolysis to the pentose phosphate pathway (PPP) [21] (reviewed more extensively by Blignaut [22]). ATM also regulates mitochondrial biogenesis and DNA content [23] and can lead to mitochondrial dysfunction when absent [24, 25]. Antioxidative treatment that targets the mitochondria in the absence of ATM can decrease the metabolic syndrome, which supports the notion that A-T might be a mitochondrial disease [26, 27]. Importantly, ATM also contributes to glucose homeostasis [28] and is required for the phosphorylation of the insulin-dependent protein kinase, Akt [29, 30].

This review will focus on crosstalk between ROS as an activator of ATM and autophagy as a regulatory mechanism of protein aggregation and oxidative stress in the context of nondividing cells.

2. ATM and Oxidative Stress

ATM is a relatively large protein of 370 kDA, consists of approximately 3056 residues and is part of the PI-3 kinase-like protein kinase (PIKKs) family [31]. The catalytic function of ATM identifies with the mechanisms mostly found in serine-threonine proteins that phosphorylate downstream proteins on the hydroxyl group of the serine or threonine residues [32].

The most common function of ATM is to respond to double strand DNA breaks in the nucleus, where the protein is autophosphorylated at Ser^{1996}, followed by monomerization of the dimer, and activated in response to DNA damage [33–35]. Upon activation, ATM is responsible for the phosphorylation and activation of downstream proteins, including the Mre11, Rad50, and Nbs1 complex (MRN complex), which aid in DNA repair [35].

Alternatively, ATM can be activated in response to oxidative stress and hypoxic conditions [36, 37], but the question remained whether this can be achieved independently of the DDR pathway. This was answered in a groundbreaking study that reported the direct activation of ATM by hydrogen peroxide (H_2O_2) as an inducer of oxidative stress [14]. This study investigated ATM activation under oxidative stress conditions generated with H_2O_2 and double strand DNA breaks (DSBs) with bleomycin, a well-known genotoxic agent, in human fibroblasts. Although p53 was phosphorylated at Ser^{15} and Thr^{68} in response to H_2O_2 and bleomycin, in an ATM-specific manner, the histone variant, H2AX, as a marker of DNA repair, was only phosphorylated in response to the latter treatment. Inhibition of ATM ablated the phosphorylation of the DNA damage-specific proteins p53, ATM, and Chk2 in the presence of H_2O_2, whilst activation of ATM by H_2O_2 was inhibited in the presence of the strong hydroxyl scavenger, N-acetylcysteine (NAC). They reported that oxidation resulted in a conformational change in ATM but not the monomerization observed in response to DNA damage. The study found that ATM forms a reversible disulphide bond at the cysteine site, Cys^{2991}, and mutation of this site from Cys^{2991} to Ala^{2991}, resulted in a construct that can be activated in the presence of DSBs but not oxidative stress. Although ATM contains several disulphide bonds, it is the covalent disulphide bond at Cys^{2991} through which ROS modulates its effects.

However, it should be noted that the interplay between oxidized ATM and DSB-activated ATM is complicated: Guo et al. [14] suggested that oxidative stress disrupts DNA binding at the complex responsible for ATM recruitment to the damaged site and can therefore inhibit ATM activation by DSBs, resulting in the oxidation of ATM under high ROS conditions. A more recent study showed that excess endogenous ROS represses ATM-dependent homologous DNA repair in cells obtained from ataxia patients with oculomotor apraxia type 3 (AOA3 cells) which has implications for both neurodegeneration and tumorigenesis [38]. Irrespective of the lack of consensus with regard to the oxidation of ATM under either high or low ROS conditions, many of the ATM substrates identified with proteomic analyses, implicate ATM in metabolic signalling pathways [39].

Under normal physiological conditions, ROS act as signalling intermediates in many cellular processes to induce redox homeostasis. On the other hand, elevated ROS levels, aptly described as oxidative stress, have been linked with over 150 diseases, most notably atherosclerosis, diabetes, and cancer [19]. It has therefore been suggested that A-T might, in essence, be an oxidative stress disorder [40]. In order to understand how ATM contributes towards the maintenance of basal metabolic flux and redox homeostasis, a short overview of oxidants and their cellular targets is required.

Briefly, ROS derive from the reduction of molecular oxygen which most notably includes oxygen ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2\bullet$), and alkoxyl ($RO\bullet$), as well as certain nonradicals that are either oxidizing agents or can be converted into radicals such as hypochlorous acid (HOCl), ozone (O_3), single oxygen ($^1O_2$), and H_2O_2 [41]. Metabolism of nitric oxide (NO) results in the formation of reactive nitrogen species (RNS) that can either contribute to oxidation, nitrosation, or nitration [42]. The enzymatic action of nitric oxide synthase (NOS) results in the formation of nitric oxide (NO) but can also produce $O_2^{\bullet-}$ under the right circumstances. A rapid reaction between NO and $O_2^{\bullet-}$ results in the formation of peroxynitrite (ONOOH) which is involved in oxidation, nitrosation, and nitration. In the case of nitration, nitrotyrosine can be formed and alter cell signalling

pathways. For example, nitrite together with HOCl has been detected in diseased human vascular tissue and drives the formation of artherogenic LDL which is implicated in atherosclerosis [43].

There are numerous sources of endogenous ROS including the cytoplasm, where $O_2^{\bullet-}$, generated by either mitochondria or the NOX-family (nicotinamide adenine dinucleotide phosphate (NADPH) oxidases), is converted to H_2O_2, as well as the production of H_2O_2 by the endoplasmic reticulum (ER) as a byproduct of protein oxidation and as an end product in several peroxisomal oxidation pathways including β-oxidation of long-chain fatty acids [44, 45]. NOX1, -2, -4, and -5 transport electrons across biological membranes in order to reduce oxygen to superoxide and are expressed throughout the cardiovascular system, brain, and cerebrovascular tissue (extensively reviewed by [41]). This protein family is one of the best known sources of cytoplasmic ROS, which in itself has been described as the cornerstone of cellular signalling and disease pathophysiology [46–48]. The broad impact of ROS is made possible by the large number of molecules that ROS can interact with, including small organic molecules, proteins, lipids, carbohydrates, and nucleic acids. These interactions can either destroy or irreversibly change the function of the target molecule and accordingly contribute towards pathogenesis [41].

Most redox reactions, however, occur through the reversible reduction and oxidation of crucial reactive cysteine residues that form thiolate anions at a physiological pH [49]. Oxidation of this residue, as is the case for ATM at Cys^{2991}, results in a sulfenic residue (SOH), which is further modified to form an intramolecular disulphide bond. As mentioned previously, the addition of exogenous H_2O_2*in vitro* forms an active ATM dimer of two covalently-linked monomers. Possible *in vivo* sources of oxidants, that can reduce thiol and oxidizing disulphide bonds, are generated by the membrane bound NOX-family of NADPH oxidases. These enzymes produce anions that can be dismutated into H_2O_2 which selectively re-enters the cell through aquaporin channels [44].

NOX-4, which is located in close proximity of the nucleus in a wide range of human cells, produces ROS innately and is elevated in A-T cells [50]. Specific inhibition of both NOX-4 and NOX-2 alleviates increased cancer risk in A-T null mice, whilst the inhibition of ATM increased NOX-4 expression in normal cells. NOX-4 is thus potentially a critical mediator of ROS and in the development of A-T.

Most recently, Zhang et al. [18] reported that ATM acts as a redox-sensor in response to endogenous mitochondrial ROS (H_2O_2) and serves as a critical juncture in the regulation of carbohydrate metabolism. The study showed that glutathione production, which is also an endogenous antioxidant, is increased in cells expressing an ATM Cys^{2291}Ala mutant construct and suggests that this is an attempt to compensate for a lower glucose flux through the PPP, thus decreasing the availability of NADPH.

Taken together, ATM can be activated in response to exogenous (H_2O_2) and endogenous ROS (mitochondrial) as well as through NADPH-oxidases, allowing it to respond as a redox-sensor for the PPP [16]. However, ATM has also been shown to mediate autophagy in response to oxidative stress, which will be discussed in the following section.

3. ATM-Mediated Autophagy

Autophagy is a highly regulated catabolic process that literally translates to "self-eating"; this general term describes the delivery of cytoplasmic components, including parts of the cytosol and large protein complexes, within a double membrane vesicle (autophagosome) to the lysosome for degradation [51]. Basal physiological autophagy ensures cellular homeostasis and protein recycling within all eukaryotic cells [52] but can also be stimulated in response to cellular stress, including but not limited to, oxidative stress, hypoxia, nutrient starvation, DNA damage, and protein aggregation [53]. The ubiquitin-proteosome system (UPS) targets only individual, short lived, or misfolded proteins for degradation, whilst autophagy recycles larger components such as damaged organelles, excessive, or toxic byproducts and larger protein complexes and aggregates [54]. This diverse but specific degradation response is enabled by three types of autophagy, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy, which differ with regard to their targeted substrate and sequestration mechanism [55]. This review will focus on the role of ATM in ROS-induced macroautophagy.

The initiation of macroautophagy occurs in the cytoplasm via the activation of adenosine 5′-monophosphate-(AMP-) activated protein kinase (AMPK) in response to nutrient starvation and hypoxia [56]. Moreover, AMPK activation results in the inhibition of lipid and glycogen synthesis, whilst concurrently activating free fatty acid oxidation and glycolysis [56]. Moreover, the activation of AMPK phosphorylates and activates TSC2 (tuberous sclerosis complex 2) resulting in the repression of mTOR complex 1 (mechanistic target of rapamycin complex 1), which is a negative regulator of autophagy [57]. AMPK activation can also phosphorylate the mTOR-binding partner, raptor, and induces 14-3-3 binding to raptor, which is required for the inhibition of mTORC1 [58], as well as phosphorylate mTORC1 directly at Thr^{2446} [59]. Once mTORC1 is repressed, unc-51-like kinase (ULK1) is dephosphorylated and consequently activated. Under starvation conditions, AMPK promotes autophagy through the direct phosphorylation of ULK1 at Ser^{317} and Ser^{777}, whereas sufficient nutrients promote mTOR activity and prevents ULK1 activation through phosphorylation at Ser^{757}, consequently disrupting the interaction between ULK1 and AMPK [60]. ULK1, together with Atg1, form one of at least five core molecular components that is required for the formation of the autophagosome membrane [61]. The other core molecular components include the Beclin1/class III PI3K complex; the transmembrane proteins, Atg9 and vacuole membrane protein 1 (VMP1); and two ubiquitin-like protein conjugation systems, Atg12 and Atg8/LC3 [62]. Once the formation of a phagophore is initiated, the vesicle expands sequentially and engulfs the cytosolic cargo, in either a selective or nonselective manner, to form the autophagosome [63]. The formation of the autophagosome is driven by the Atg (AuTophagy related) proteins and has been reviewed extensively [63, 64].

Selectivity of the targeted cargo is conferred by receptors that recognize and interact with lipidated ATG8 family proteins, which are located on the concave side of the developing autophagosome [65]. This interaction is enabled by LC3 interacting regions (LIR) that bind to the LIR docking sites of ATG8 family proteins [65]. The ATG8 family consists of the LC3/GABARAP protein family and includes the microtubule-associated protein 1 light chain 3 (MAP1LC3A-B and C) or γ-aminobutyric acid (GABA) type A receptor–associated protein (GABARAP and GABARAP-like 1 and -2) [66]. Of these proteins, the best-studied protein is LC3B, which confers selectivity, together with the GABARAP proteins, for pexophagy and mitophagy through interaction with adapter proteins [66]. LC3B associates with the forming autophagosomal membrane through the formation of a covalent bond to phosphatidylethanol (PE) enabled by a ubiquitination-like sequence of enzymatic events where ATG7 acts as the LC3 activating enzyme and ATG 3 as the conjugating enzyme that transfer LC3 to PE to form lipidated LC3-PE/LC3-II [67, 68].

Autophagy adapter proteins interact directly with the ATG8 proteins and share the ability to interact simultaneously with the autophagosome through interaction of their LIR motif with LC3 as well as the cargo substrate, which is often ubiquitylated [67]. These receptors include, amongst others, p62/SQSTM1, BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein), FUNDC1 (Fun14 domain containing 1), NBR1 (neighbour of BRCA1), NDP52 (nuclear dot protein of 52 kDa), and optineuron, of which many have a ubiquitin-binding domain (UBD) that can interact with different ubiquitin chain linkages associated with the targeted cargo and in doing so, provide selectivity [67]. Once the cargo is tethered to the forming autophagosome, LC3B and the GABARAP subfamily promote the elongation and fusion (closure) of the autophagosome [66], which can then fuse with the lysosome to form an autolysosome and results in pH changes to occur in the lumen of the lysosome [69]. The change in lysosomal pH is essential for successful protein degradation as the hydrolyses responsible for cargo breakdown are activated in an acidic environment [70]. The process of autophagosome maturation, trafficking, and lysosomal fusion as well as the proteins involved in this process has recently been reviewed extensively [71].

In view of the oxidative stress induced activation of ATM, as well as the pathophysiology associated with elevated ROS in ATM-deficient cells, Alexander et al. [19] reported that the activation of oxidized ATM increases autophagy through the activation of TSC2 via the liver kinase B1 (LKB1)/AMPK pathway, resulting in the repression of mTORC1. Moreover, inhibition of mTORC1 with rapamycin results in the concomitant improvement of ROS levels in $ATM^{-/-}$ mice. The authors found that low concentrations of H_2O_2 rapidly induced mTORC1 repression that could, in turn, be rescued by the addition of NAC or pretreatment with catalase. Of relevance as well, is that chemical mitochondrial uncoupling which depletes the antioxidant, glutathione, also repressed mTORC1 signalling, indicating that both exogenous and endogenous ROS activation of ATM can induce mTORC1 repression.

The same research group found that nitrosative stress (nitric oxide (NO)) also activates ATM and results in the phosphorylation of AMPK through LKB1, activation of the TSC2 complex and consequent repression of mTORC1 [72]. ATM-mediated repression of mTORC1 decreased phosphorylation of direct target proteins of mTORC1 such as 4E-BP1 (4E-binding protein 1), S6K (ribosomal S6 kinase), and ULK1 (Unc-51 like autophagy activating kinase). Consequently, nitrosative stress-mediated activation of ATM can increase autophagy by decreasing mTORC1 mediated phosphorylation of ULK1 at Ser^{757} and increasing ULK1-phosphorylation at Ser^{317} by AMPK. However, the precise mechanism through which NO activates ATM is still unknown. Induction of autophagy by NO also resulted in decreased cell viability, which suggests a cytotoxic response.

LKB1 can be phosphorylated directly at Thr^{366} by active ATM in response to ionising radiation (IR) [73] as well as through oxidative stress as discussed above, consequently activating AMPK directly to modulate apoptosis [74] or autophagy in the event of energetic stress.

On the other hand, one of the key roles of AMPK in cardiac tissue is the response to hypoxia/ischaemia, which is also under the direct control of LKB1; in the absence of LKB1, mouse hearts show increased mTORC1 signalling and protein synthesis that can lead to hypertrophy [75]. Interestingly, Emerling et al. [76] showed that the hypoxic activation of AMPK in mouse fibroblasts is dependent on mitochondrial oxidative stress that is generated by the ETC and not the cytosolic adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio. Although they did not evaluate the role of ATM in their study, it supports the notion that the oxidative activation of ATM, due to increased mitochondrial dysfunction, can potentially mediate the activation of AMPK in response to hypoxic stress.

In a nutshell, autophagy is a catabolic process responsible for the degradation and recycling of damaged organelles and is central to the maintenance of cellular homeostasis. The activation of ATM through ROS and NO places ATM directly upstream of AMPK, which in turn, drives the inhibition of mTORC1 and upregulation of autophagy through ULK1. This allows cells to eliminate damaged organelles that can drive increased oxidative stress and recycle these components to maintain nutrient and energy homeostasis, but this process can also be mediated independently of ATM. ROS-induced autophagy can be induced by either $O_2^{\bullet-}$ [77] or H_2O_2 [78] that is produced in response to either glucose or nutrient starvation and can cause mitochondrial energetic stress due to decreased ATP availability [52]. Both redox balance and ROS formation can be regulated by changes in the autophagy rate and consequently either directly regulate mitochondrial homeostasis or indirectly regulate mitochondrial function [79].

Key to effective autophagy, which is also responsible for the degradation of ATM [80], is the fusion of the autophagosome to lysosomes, in order to form an autolysosome where the targeted content is degraded. Recent observations in $ATM^{-/-}$ neurons showed upregulated autophagic flux of lysosomes with a more acidic pH and led to the finding that the ATPase, H^+ transporting lysosomal V1 subunit A

(ATP6V1A proton pump) is a target of ATM [80]. The absence of ATM results in the peri-nuclear accumulation of lysosomes which suggests that this could be due to a physical interaction between ATM and the retrograde transport motor protein, dynein. Lysosomal dynein accumulates in ATM $^{-/-}$ mouse brains indicating that ATM inhibits axonal transport through dynein motor proteins. Similarly, the study found that the loss of ATM resulted in the impaired glucose uptake due to the inhibition of the translocation of the SLC2A4/GLUT4 (solute carrier family 4 (facilitated glucose transporter) 4) to the plasma membrane, and increased trafficking to lysosomes instead [80]. This observation further supports previous reports that decreased ATM activity is associated with metabolic syndrome [81] and insulin resistance [82]. The importance of ATM in autophagy is highlighted by the accumulation of lysosomes, as well as increased oxidative stress in the cerebellum of ATM-null mice [83].

Increased oxidative stress and a weakened antioxidant defence due to dysfunctional autophagy can induce cellular damage and result in neuronal cell death, which is the major causative factor in the development of Parkinson's disease, which predominantly affects aged individuals above 60 [71]. Increasing age results in a decline in autophagy as well as an increase in protein misfolding and oxidative stress [67] and can lead to the disruption of cellular homeostasis [68]. Similarly, age-associated decreases in ATM protein levels [84] may result in the development of metabolic syndrome, lysosomal accumulation, and protein aggregation that are associated with age-related neuronal diseases [85] and the development of cardiac dysfunction including fibrosis and hypertrophy [86].

4. ATM and Aggrephagy

Protein aggregates develop when proteins are misfolded due to mutations, incomplete translation, inappropriate protein modifications, oxidative stress, and ineffective assembly of protein complexes [87]. The accumulation of misfolded and dysfunctional protein aggregates, often due to oxidative stress [88, 89] or downregulated or disrupted autophagy [90], can be toxic to the cell and cause a disruption of cellular homeostasis that is detrimental to cellular survival in many diseases, and in particular, neurodegenerative diseases [91]. Aggregation is driven by exposed hydrophobic patches in misfolded proteins that sequester other proteins. Misfolding can be repaired by molecular chaperones, but if the damage is too great, the misfolded proteins are guided by chaperone complexes for degradation by either the ubiquitin-proteosome system (UPS) or the lysosome through chaperone mediated autophagy or aggrephagy [87]. The latter process specifically refers to the selective sequestration of protein aggregates by macroautophagy and will be the discussed further.

Protein aggregation is classically associated with neurodegeneration but has been observed in nearly every cardiometabolic disease [92]. The accumulation of proteins and dysfunctional organelles contributes to the development of pathology in almost all tissues and thus requires a very fine balance between apoptosis and autophagy [93]. There seems to be synergistic roles for ATM and p53 with regard to the regulation of autophagy, where ATM regulates mitochondrial homeostasis and oxidative stress in order to prevent cells from undergoing apoptosis in response to nongenotoxic p53 activation [94]. Genetic or pharmacological loss of ATM kinase activity blocks autophagy and increases ROS, which is sufficient to commit cells to apoptosis in response to Nutlin 3 treatment, an inhibitor of the p53 E3 ubiquitin ligase MDM2 that activates p53 [94].

Most recently, it has been shown that the loss of function mutation that blocks ATM activation by oxidative stress, but not genotoxic stress, results in widespread protein aggregation, especially when cells are exposed to low levels of ROS, and includes polypeptides mainly implicated in DNA metabolism and gene expression [95]. This implicates a role for ATM in protein homeostasis. Moreover, protein aggregation is very relevant to neurodegeneration, especially with regard to the loss of function of Purkinje neuronal cells, which is a hallmark of A-T [96].

Proteasomal degradation is also required for the maintenance of autophagy at physiological levels as is the case with ULK1; it is specifically ubiquitinated by the E3 ligase NEDD44 that marks it for proteasomal degradation, whilst still being actively translated and transcribed [75]. The transcription of ULK1 is in turn inhibited by mTOR during prolonged autophagy and allows for the maintenance of ULK1 protein at basal levels within the cell.

It is also possible that ATM can play a more active role in ULK1 phosphorylation through p32. Although p32 was first recognized as a novel substrate of ATM in cardiac DNA damage [97], it has recently been identified as a regulator of ULK1 stability [98]. In a study that investigated the cardiotoxicity and genotoxic effect of chemotherapeutic agents that induce cell death through the ATM-mediated phosphorylation of p53, the protein, p32 (CIQBP/HABP1), was identified as an endogenous substrate in mouse hearts [97]. The protein is phosphorylated at Ser^{148} by ATM in response to genotoxic stress, but the authors did not comment on the physiological effect thereof [97]. However, p32 has been found to be essential for maintaining the activity and stability of ULK1 [98]. The study found that the ablation of p32 results in increased proteolysis of ULK1, that consequently impaired starvation-induced autophagic flux as well as the clearance of damaged (uncoupled) mitochondria, and highlights the importance of p32 for ULK1 activity. The phosphorylation of ULK1 by AMPK also regulates the translocation of ULK1 to mitochondria in response to hypoxia [99] where it phosphorylates the autophagy cargo receptor, FUNDC1 [100], and regulates mitophagy [101]. Although ATM was not investigated in this context, it is tempting to hypothesize that ATM could influence ULK1 potentially through the phosphorylation of p32 in the heart.

5. ATM Mediates Selective Autophagy

Constitutive autophagy plays a protective role in mitochondrial rich cardiomyocytes, where accumulation of abnormal proteins and organelles, especially mitochondria,

may directly cause cardiac dysfunction [102]. Mitophagy, a specialized mechanism of autophagy that specifically aims to degrade and maintain mitochondrial quality, is central to maintaining cellular integrity and cellular homeostasis [103]. The process of mitophagy is known to decrease during ageing, thus resulting in mitochondrial dysfunction [104].

Classically, depolarized mitochondria initiate the accumulation of (PTEN-) induced kinase 1 (PINK1) on the outer mitochondrial membrane [105]. PINK1 is degraded in healthy mitochondria but accumulate on the outer mitochondrial membrane of damaged mitochondria that, in turn, drives the recruitment and translocation of Parkin [85] to the mitochondria. Parkin is an E3 ubiquitin ligase which is phosphorylated by PINK1, stimulating its translocation to the mitochondria where it ubiquinates several outer mitochondrial membrane proteins. This promotes further PINK1 phosphorylation and the formation of ubiquitin chains that localizes mitophagy receptors that contain UBDs to Parkin-ubiquitylated mitochondria, including p62 (SQSTM1), NBR1, and optineurin [106] that can attach to autophagosomal membranes and envelop the damaged mitochondria (reviewed by Nguyen et al. [107]) for degradation [108].

PINK1 also phosphorylates the fusion protein, mitofusin 2 (Mfn2), which can serve as a mitochondrial receptor for Parkin, promoting its ubiquitination [88]. The loss of Mfn2 prevents the translocation of Parkin in depolarized mitochondria and suppresses mitophagy, which drives the accumulation of dysfunctional mitochondria and decreased mitochondrial respiration in mouse cardiomyocytes [88]. Alternatively, mitophagy can be mediated by mitophagy receptors. Mitochondrial receptor-mediated autophagy is mediated by the pro-apoptotic proteins, BNIP3 and NIX, that localize to the outer mitochondrial membrane and act as receptors for targeting autophagosomes through direct interaction of conserved LC3-interacting regions (LIRs) with LC3 on the autophagosome, often in response to hypoxia [109] and in the absence of mitochondrial membrane permeabilization [110]. FUNDCI is an outer mitochondrial membrane protein that has been implicated in hypoxia-mediated mitophagy in mammalian cells [111]. Similar to BNIP3 and NIX, FUNDC1 acts as a receptor for the autophagosomal membrane and interacts directly with LC3 through LIR. The serine/threonine protein phosphatase, PGAM5, dephosphorylates FUNDC1 during hypoxia or mitochondrial membrane depolarization and promotes interaction with LC3 with consequent mitophagy [112].

More recently, a direct link between ATM and PINK1/-Parkin recruitment was shown, as ATM was able to initiate the accumulation of PINK1 and translocation of Parkin in the presence of spermidine and lead (Pb) initiating mitophagy [113, 114]. Spermidine is a natural polyamine involved in several biological processes including cell proliferation and apoptosis and tends to decline with age [115]. Spermidine also elicits mitochondrial depolarization that causes the formation of mitophagosomes and mitochondrial targeted lysosomes, which has been suggested to occur via ATM-dependent activation of the PINK1/Parkin mitophagy pathway [114]. Spermidine-induced mitochondrial depolarization is abrogated in the presence of the chemical ATM inhibitor, KU55933. Moreover, spermidine promotes the colocalization of phosphorylated ATM and PINK1 on the outer mitochondrial membrane, which, together with the translocation of Parkin, can be blocked by the ATM inhibitor. The authors suggest a model whereby activated ATM drives PINK1 accumulation as well as Parkin translocation with consequent mitophagy in response to spermidine treatment (Figure 1).

ATM may therefore be central to mitophagy by directly activating the pathway or by indirectly activating autophagy in response to oxidative stress. Thus, if pathological ATM signalling occurs, mitophagy could be affected, predisposing the cell to mitochondrial oxidative stress [22]. ATM is also activated by nitrosative stress and contribute to sustained mitophagy of damaged mitochondria through the newly characterized ATM-denitrosylase S-nitrosoglutathione reductase (GSNOR) axis [116].

The chronic oxidative stress observed in A-T has led to the suggestion that A-T might be a mitochondrial disease [40] and has also been linked with intrinsic mitochondrial dysfunction [24]. The latter study found that lymphoblastoid cells from A-T patients contain an increased population of mitochondria with a decreased membrane potential, when compared to control cells. Proteins with specific roles in mitochondrial DNA damage and/or ROS scavenging, including mnSOD, peroxiredoxin 3, and mitochondrial topoisomerase, were also elevated in these cells. Indeed, the decreased membrane potential translated into decreased respiratory activity in the A-T cells compared to the wild type controls. Concomitantly, the authors showed that the *in vivo* loss of *ATM* in mice resulted in mitochondrial dysfunction in thymocytes that was accompanied by increased mitochondrial content and mitochondrial ROS due to a decrease in mitophagy. Interestingly, they observed a significant decrease in complex I activity as well as ATP production and an increase in oxygen consumption. The study also found that autophagy was not affected by the absence of ATM and suggested that changes in mitochondrial dynamics such as fission and fusion could contribute towards defective mitophagy. The authors concluded that the observed defects in the absence of ATM suggest that ATM might localize directly to mitochondria. Fractionation studies in cells revealed that the mitochondrial fraction of HepG2 cells was enriched with ATM and activated ATM in response to H_2O_2 treatment [117]. In contrast to previous observations that ATM associated with the peroxisomal fraction [118], Morita et al. [117] detected almost no ATM in this fraction. This reverberates with the suggestion [119] that both the cell type and culture conditions of immortalized A-T cells can affect mitochondrial homeostasis and autophagic responses which explain the differences in mitochondrial content reported in A-T deficient cell lines.

Mitochondrial respiration inhibition can also lead to increased mitochondrial ROS production. Treatment of HeLa cells with either rotenone or Antimycin C failed to increase mitochondrial hydrogen peroxide production although it did increase mitochondrial superoxide production [18]. Superoxide itself failed to drive ATM dimerization and suggested that mitochondrial superoxide must be

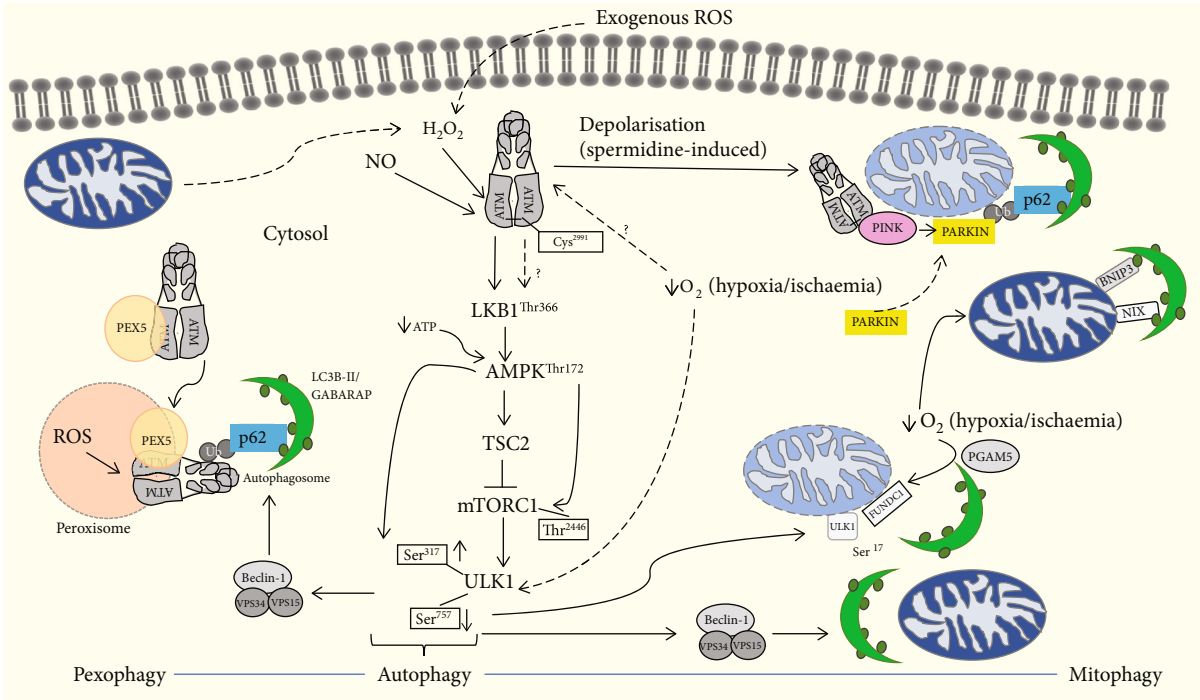

Figure 1: ROS can activate cytosolic ATM. ATM is activated in response to both endogenous and exogenous ROS, as well as NO at Cys^{2991}, where it forms a disulphide bond. Once activated, it phosphorylates LKB1 at Thr^{366} which phosphorylates AMPK and drives the inhibition of mTORC1 through TSC2. The inhibition of mTORC1 phosphorylates ULK1 at Ser^{757}, whilst AMPK phosphorylates ULK1 at Ser^{313}. This initiates autophagy and the formation of an autophagosome that targets peroxisomes specifically for degradation through the ATM-mediated ubiquitination of PEX5. It is currently unknown whether ATM is involved in either the activation of AMPK or suppression of mTOR in response to ROS to induce mitophagy. ATM mediates PINK/Parkin mitophagy pathway in response to spermidine treatment, which induces ROS and consequently activate ATM, that is then recruited to the permeabilized mitochondrial membrane where it colocalize with PINK and drives the recruitment of Parkin which is ubiquitinated. The ubiquitin chain binds to LC3 (green balls) on the autophagosome, which then engulfs damaged mitochondria for lysosomal degradation (not shown). Hypoxia or mitochondrial uncoupling can also activate ULK1, driving its translocation to the damaged mitochondrion membrane where it phosphorylates FUNDC1, which enhances its binding to LC3, whereas the dephosphorylation of FUNDC1 by PGAM5 also allows FUNDC1 to directly interact with LC3. BNIP and NIX can act as mitochondrial receptors in response to hypoxia when the mitochondrial membrane is not permeabilized and bind to LC3 on the autophagosome. Damaged mitochondria produce less ATP that activates AMPK, which in turn phosphorylates ULK1 and activates the Beclin1-VSP34-VSP15 complex and drives the formation of an autophagosome. Damaged mitochondria can also produce ROS which inhibits mTOR and leads to the activation of autophagy.

converted to H_2O_2 in order to activate ATM in either the cytosol or nucleus of HeLa cells.

Our group reported that ATM is directly associated with the inner mitochondrial membrane of cardiac mitochondria, and the inhibition thereof decreases oxidative phosphorylation and the ATP synthesis rate in a complex I-mediated manner [120]. Similarly, $ATM^{-/-}$ thymocytes exhibit decreased complex I activity [25], whereas the chemical inhibition of ATM resulted in a posttranslational decrease of COX-IV [121]. This is interesting because the inhibition of COX-IV has been associated with increased ROS production at complex I, albeit in the mitochondrial matrix [122]. Depletion of ATP in neuronal Purkinje cells results in increased ROS production that can activate ATM, consequently leading the phosphorylation of Nrf1 that specifically upregulates the expression of nuclear-encoded mitochondrial genes and improves electron transport chain capacity and restores mitochondrial function [123]. Similarly, Fang et al. [124] reported increased mitochondrial content in ATM-knockdown (ATM-KD) rat neurons coupled to increased ROS production. The authors suggested that this could reflect decreased ATP production and either inadequate or inefficient mitophagy. Moreover, the study showed that mitophagy is suppressed in ATM-KD HeLA cells and rat neurons but that the phenotype could be rescued by replenishing cellular NAD^+ which significantly improved life-span in $ATM^{-/-}$ mice [124].

Interestingly, Beclin-1 heterozygosity in $ATM^{-/-}$ mice reduces mitochondrial ROS and complex I abnormalities in thymocytes [25]. Beclin-1 forms part of the complex required for the induction of autophagy [125] but is also required for the recruitment of Parkin to the mitochondrial membrane where it induces ubiquitination and proteasomal degradation of proteins on the outer mitochondrial membrane [126]. This leads to the inhibition of fusion and the trafficking of dysfunctional mitochondria [126]. It is still unclear why the allelic loss of Beclin1 would promote improvement of mitochondrial dysfunction in $ATM^{-/-}$ mice, but it has led to the suggestion that Beclin-1 might have additional functions besides its role in autophagy [25].

Terminally differentiated cells such as cardiomyocytes and neuronal cells are dependent on the efficient removal and replacement of dysfunctional mitochondria to ensure cell survival and to maintain cellular homeostasis [111, 127]. A decrease in ATP production and increased ROS production as indicators of mitochondrial dysfunction can result in either the release of apoptotic proteins or the selective clearance of the damaged mitochondria. Mitophagy thus serves as an early cardioprotective response through the removal of damaged mitochondria, and if this fails, apoptosis can be induced in response to excessive oxidative stress [128]. Moreover, reduced autophagy, together with the accumulation of dysfunctional mitochondria, has been associated with heart failure and aging [111].

Pexophagy is the targeted selective degradation of peroxisomes [129] and is another example of selective autophagy [130]. Peroxisomes utilise β-oxidation to reduce long-chain fatty acids into medium length fatty acids that can be shuttled to the mitochondria. These highly metabolic organelles generate ROS during β-oxidation and require homeostatic maintenance to prevent oxidative stress. ATM binds to the peroxisome importer receptor, PEX5, in response to excessive ROS and mediates peroxisome-specific autophagy (pexophagy) by phosphorylating PEX5 at Ser^{141} and promoting mono-ubiquitylation at Lys^{209}, whilst simultaneously inducing autophagy through the activation and phosphorylation of TSC2 and ULK1 [17, 131, 132]. Ubiquitylation of PEX5 is mediated by the complex PEX2-PEX10-PEX12 and is then recognized by the autophagy adapter proteins, p62 and NBR1, which directs the autophagosome to the peroxisomes for pexophagy [129].

Loss of function mutations in ATM, such as the ability to sense oxidative stress, can result in a reduction in mitochondrial antioxidant defences, lead to the accumulation of ROS and oxidative damage to mitochondria and other cellular components [18], as well as protein aggregation [95]. Selective autophagy seems to be mainly mediated by ubiquitination which is essential for conferring selectivity [133], as is the case of ATM-mediated pexophagy. As previously discussed, this also implies a potential role for ATM in aggrephagy (degradation of damaged or misfolded proteins) which is dependent on p62 ubiquitination [92].

6. Conclusion

This broad overview describes the apical protein, ATM protein kinase, at the nexus of oxidative stress-induced autophagy [14, 18] as well as nitrosative stress-induced autophagy [72, 116], mitophagy [113, 114], and pexophagy [17, 132] mainly in the context of nondividing cells such as cardiomyocytes and neurons. Site-specific mutations that renders ATM insensitive to oxidative stress increase protein aggregation [95], whilst loss of function increases perinuclear lysosomal accumulation [80] as well as mitochondrial oxidative stress [25] and dysfunction [24, 25, 120]. Cytoplasmic ATM thus plays a central role in redox homeostasis and ROS-mediated autophagy.

As a master regulator of DNA repair, activation of ATM by exogenous and endogenous oxidative stress, independently of DNA strand breaks, only recently came to light [14, 18]. This finding paved the way to understanding the severe neurodegeneration and associated protein aggregation observed in A-T patients that is largely due to disrupted ATM protein kinase functioning leading to disrupted autophagy, mitophagy, and pexophagy [80, 95]. Additionally, the regulation of ATM levels by autophagy [80] and the role of ATM in oxidative stress-mediated autophagy in an AMPK/mTORC1 dependent manner were discovered [13, 19, 132]. Similarly, ROS-induced pexophagy is modulated by ATM through the TSC2/AMPK/mTORC1 pathway in which the disruption of this signalling pathway leads to interrupted cellular homeostasis causing pathologies linked to neurodegeneration [17, 131, 134].

It has been suggested that the pathogenesis of A-T could be ascribed to excessive ROS and that A-T might therefore be an oxidative stress disease [40]. Several studies have investigated the effect of the absence or inhibition of ATM on mitochondrial function and found that ATM is innately associated with the inner mitochondrial membrane and oxidative phosphorylation of cardiac mitochondria [120]. In addition, the absence of functional ATM in the mitochondria of ATM-null thymocytes and fibroblasts was associated with decreased ATP production, increased ROS production [24, 25], and a decrease in mitophagy [119, 123].

Therefore, activation of ATM by oxidative stress and the consequent maintenance of redox homeostasis through autophagy, pexophagy, aggrephagy, and mitophagy place ATM at the centre of cross-talk between ROS and autophagy signalling.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

M.B conceptualized and wrote the manuscript and created Figure 1. SH contributed to the writing of the manuscript. B. H and A. L reviewed and modified the manuscript. All authors approved the final version of the manuscript.

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Review Article

Autophagy Paradox of Cancer: Role, Regulation, and Duality

Amit Kumar Verma,[1] Prahalad Singh Bharti,[2] Sahar Rafat,[1] Deepti Bhatt,[1] Yamini Goyal,[1] Kamlesh Kumar Pandey,[3] Sanjeev Ranjan,[4] Saleh A. Almatroodi,[5] Mohammed A. Alsahli,[5] Arshad Husain Rahmani,[5] Ahmad Almatroudi,[5] and Kapil Dev[1]

[1]*Department of Biotechnology, Jamia Millia Islamia, New Delhi, India*
[2]*Department of Biophysics, All India Institutes of Medical Sciences, New Delhi, India*
[3]*Department of Anatomy, All India Institutes of Medical Sciences, New Delhi, India*
[4]*Institute of Biomedicine, Cell and Tissue Imaging Unit, Finland*
[5]*Department of Medical Laboratories, College of Applied Medical Science, Qassim University, Buraidah, Saudi Arabia*

Correspondence should be addressed to Kapil Dev; kdev@jmi.ac.in

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Autophagy, a catabolic process, degrades damaged and defective cellular materials through lysosomes, thus working as a recycling mechanism of the cell. It is an evolutionarily conserved and highly regulated process that plays an important role in maintaining cellular homeostasis. Autophagy is constitutively active at the basal level; however, it gets enhanced to meet cellular needs in various stress conditions. The process involves various autophagy-related genes that ultimately lead to the degradation of targeted cytosolic substrates. Many factors modulate both upstream and downstream autophagy pathways like nutritional status, energy level, growth factors, hypoxic conditions, and localization of p53. Any problem in executing autophagy can lead to various pathological conditions including neurodegeneration, aging, and cancer. In cancer, autophagy plays a contradictory role; it inhibits the formation of tumors, whereas, during advanced stages, autophagy promotes tumor progression. Besides, autophagy protects the tumor from various therapies by providing recycled nutrition and energy to the tumor cells. Autophagy is stimulated by tumor suppressor proteins, whereas it gets inhibited by oncogenes. Due to its dynamic and dual role in the pathogenesis of cancer, autophagy provides promising opportunities in developing novel and effective cancer therapies along with managing chemoresistant cancers. In this article, we summarize different strategies that can modulate autophagy in cancer to overcome the major obstacle, i.e., resistance developed in cancer to anticancer therapies.

1. Introduction

The term "autophagy" comes from "auto" and "phagy", which means, respectively, "self" and "eating" in Greek. It is an evolutionary conserved catabolic process, critically required for the maintenance of cellular homeostasis, metabolism, and growth regulation. It is a self-degrading system in which autophagic substrates like damaged organelles, misfolded/aggregated proteins, and enzyme complexes are degraded. Autophagy acts as a quality control mechanism in cells. Physiologically, it is a strategy of survival under stress conditions by the renewal of the by-products such as amino acids, fatty acids, nucleotides, and carbohydrates. It is constitutively active at the basal level in healthy cells, under the tight regulation of the mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK). The level gets significantly increased under stress conditions [1–3]. Apart from the degrading nature, it additionally takes part in biosynthetic and secretory processes [4]. It also has roles in development, differentiation [5, 6] organellar remodelling, quality control of organelles and proteins, genotoxic stress prevention, suppression of tumor, elimination of pathogen, immunity and inflammation regulation, maternal DNA inheritance, and programmed cell death [7, 8]. Malfunctioned autophagy is related to a variety of human maladies such as neurodegeneration, aging, and

cancer [9]. Autophagy is also known as type II cell death mechanism. It has been found in some circumstances that autophagy can lead to cell death; for instance, autophagy shows association in degenerating neurons of Parkinson's and Alzheimer's patients, in MCF-7 cancer cell lines treated with 4-hydroxyxytamoxifen (selective estrogen receptor modulator), in the regression of corpus luteum [10], deterioration of Mullerian duct structure during male genital development [11], and involution of mammary and prostate glands [12–15]. But recent *in vivo* and *in vitro* studies suggested that autophagy acts as a major survival mechanism in response to various stresses like nutrient depletion, hypoxia, damaged organelles, accumulation of anomalous proteins, activated oncogenes, and deactivated tumor suppressor genes [16]. This review discusses the role, regulation of autophagy with a focus on cancer, and how autophagy is responsible in causing resistance to various cancer therapies. We summarized different strategies like pharmacological, RNA-based therapies and combinational drug therapies; conventional drugs with new formulations (nanoformulation) are inhibiting the autophagy and overcoming the autophagy-mediated tumor resistance against therapies. Besides, we also discussed about activators of autophagy as anticancer agents. Different drugs, natural products, or extracts along with siRNA combination enhance autophagy-mediated tumor cell death both in vivo and in vitro studies.

2. Autophagy Pathway

Autophagy, also referred to as macroautophagy, is an effective degradation mechanism. Substrates that have to be degraded are targeted to the double-membrane vesicles called autophagosomes which ultimately fuse with lysosomes. Autophagosome is a hallmark feature of autophagy. Autophagy is mediated by evolutionarily conserved genes called autophagy-related genes (Atg) and their encoded proteins (ATGs). Various Atg and their functions have been studied in yeast at different stages of autophagy [17]. There are more than 30 ATGs that have been discovered and about 17-20 ATGs take part in forming conjugation complexes that are necessary for the initiation of autophagy and autophagosome formation [18].

Autophagy process includes six steps—initiation, nucleation, elongation, maturation, fusion, and degradation (Figure 1) that are dependent on various protein complexes like Unc-51-like kinase 1 (ULK1 complex), phosphoinositide 3-kinase (class III- PI3K) complex, ATG9 complex, ATG2-ATG18 complex, ATG8/LC3 complex, and ATG12 conjugation complex (Figure 2).

2.1. ULK1 Complex. Induction of autophagy is dependent on the kinase activity of ULK1 [19, 20]. It is found in the inactive form if nutrients are readily available as it is dephosphorylated by the mammalian target of rapamycin (mTOR). During stress, a decrease in intracellular energy or an increase in the AMP/ATP ratio under low energy level activates adenosine monophosphate kinase (AMPK) which acts as a metabolic sensor by regulating glucose and lipid metabolism. [19, 21, 22]. AMPK-mediated deactivation of mTOR and phosphorylation of ULK1 lead to the activation of the ULK1 complex which consists of ULK1/ATG1, ATG13, FIP200/ATG17 (FAK-family kinase-interacting protein), and ATG101. ATG13 gets dephosphorylated (highly phosphorylated under nutrient-rich conditions), which enables the binding of ULK1 and increases its kinase activity.

2.2. Class III-PI3K (Phosphoinositide 3-Kinase) Complex. The PI3K-III complex containing Beclin-1/ATG6, ATG14, VPS34, and VPS15 (vacuolar protein sorting 34 and 15) is activated by ULK1 complex which is also important in the formation of isolation membrane or phagophore. VPS34 and VPS15 are class III-PI3 kinases, and VPS34 acts as a catalytic subunit, whereas VPS15 acts as a regulatory subunit in this complex. This complex phosphorylates PIP2 (phosphatidylinositol diphosphate) to PIP3 (phosphatidylinositol triphosphate) which is a prerequisite for the initiation and nucleation processes.

2.2.1. ATG9 and ATG2-ATG18 Complexes. Increase in the PIP3 concentration leads to the recruitment of other proteins such as DFCP1 (double FYVE domain-containing protein 1), WIPI/ATG18 (WD-repeat domain phosphoinositide-interacting protein 1), ATG2, and ATG9 at the phagophore formation site [23]. During phagophore formation, endoplasmic reticulum (in the form of omegasomes) works as a membrane source in the presence of DFCP1 and ATG2-WIPI/ATG18 complex [24–26], while ATG9 mediates the use of membrane vesicles from the endoplasmic reticulum, plasma membrane, and mitochondria. In plasma membrane, ATG16L and heavy chain clathrin interaction is required for the formation of autophagosome precursor [27, 28], whereas, in mitochondria, ATG5 and LC3 localization to the outer membrane of mitochondria is required, which serves as a cornerstone for phagophore formation [29].

2.3. ATG8/LC3 and ATG12 Conjugation Complexes. Another protein that is important for preautophagosome elongation and maturation is MAP1LC3 (microtubule-associated proteins 1A/1B light chain 3) or LC3/Atg8. LC3 is found in cells in the inactive form, i.e., proLC3 which is converted to LC3-I by ATG4 (a cysteine protease). Along with LC3, ATG12 conjugation complex (ATG12-ATG5-ATG16) (E3-like protein) plays an important role in elongation and maturation of autophagosomal membrane as it helps in recruitment and conversion of LC3-I to LC3-II by adding phosphatidylethanolamine (PE) with the help of ATG7 (E1-like protein) and ATG3 (E2-like protein). This LC-II is recruited to the autophagosomal membrane through its lipid moiety and is not freely available in the cytoplasm like LC-I. When LC3-I is converted to LC3-II, autophagosome is elongated (marker to monitor autophagy) [30] and it becomes a vesicle that is called a mature autophagosome [31]. After completion, LC3 remains bound to the lumina. During nucleation and elongation, some adaptor proteins play important roles in cargo selection which is LC3-dependent. Ubiquitin-binding protein p62/sequestosome-1 (p62/SQSTM1), neighbor of BRCA1 (NBR1), nuclear dot protein 52 kD (NDP52), and optineurin transport their cargos to nucleation site by their

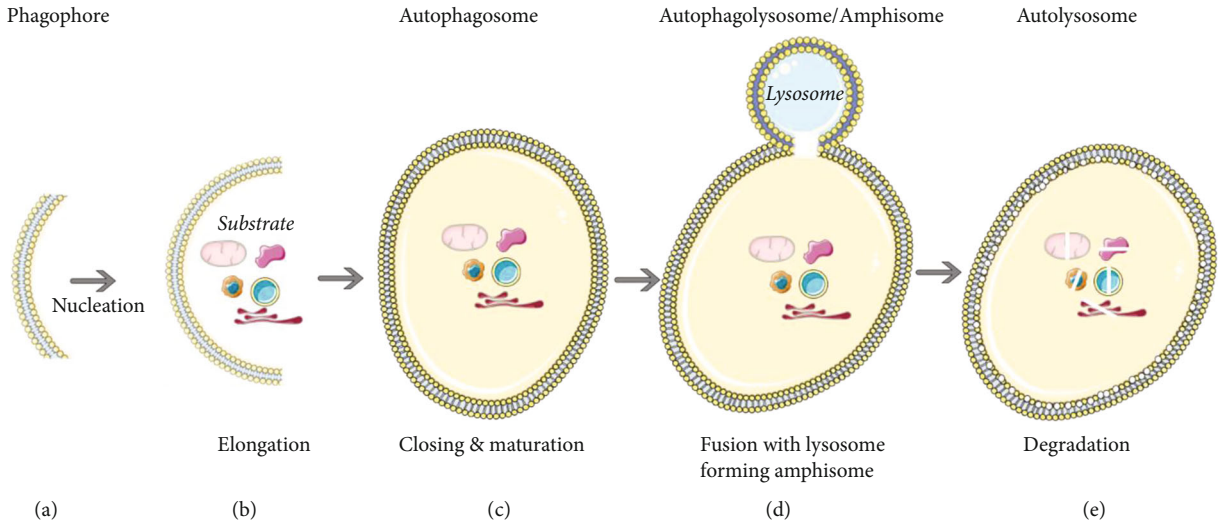


Figure 1: Stages in autophagy: (a) initiation—formation of double-membrane isolation membrane or phagophore; (b) nucleation and elongation—targeted autophagic substrate sequestration and elongation of the phagophore; (c) maturation—formation of autophagosome after the closure of phagophore with the entrapped substrate; (d) fusion—fusion of the mature autophagosome with lysosome forming autophagolysosome; (e) degradation—degradation of the substrate and inner membrane of autophagosome by lysosomal enzymes resulting in autolysosomes.

LC3-interacting region (LIRs) or ATG8-interacting motifs (AIMs) which facilitate cargo selection as well as selective autophagy. Once the protein recruitment and formation of autophagosome are completed, the mature autophagosome with its contents fuses with lysosomes via sets of protein families: soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), including syntaxin-17 (STX17), synaptosomal-associated protein 29 (SNAP29), and vesicle-associated membrane protein 8 (VAMP8) [32]; Ras-related protein Rab-7 (Rab7); and HOPS (the homotypic fusion and protein sorting-tethering complex) [33]. After the fusion of the autophagosome with the lysosome, it becomes autolysosome. The autophagosome releases its content into the lysosome, and those substrates are degraded in the acidic environment of the lysosome by particular proteases (cathepsins B and L). "Alternative macroautophagy" has been introduced which is independent of LC-II, ATG5, and ATG7, but is critically dependent on ULK1 and Beclin1. It is debated whether it is a different form of autophagy [34]. At first, autophagy was believed to be a nonspecific degradative process. But now, many selective pathways have been demonstrated and are named according to the particular substrate that is degraded. For instance, for mitochondria, it is called mitophagy, for ferritin, it is called ferritinophagy, for ER, it is reticulophagy, for bacteria, it is xenophagy, etc. [35, 36].

3. Regulation of Autophagy

Nutritional status, energy level, growth factors, insulin ER stress, SOS (response when cells are exposed to stress causing DNA damage and cell cycle arrest), and other signals modulate the autophagy process by various target proteins. Of all target proteins, mTOR, also known as FRAP1, is a master regulator that negatively regulates autophagy [37, 38]. It consists of two complexes—mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2)—out of which, mTORC1 is sensitive to rapamycin. Rapamycin (sirolimus) is a mTOR inhibitor extracted from bacterium *Streptomyces hygroscopicus*.

In nutrient-rich conditions, mTORC1 hinders autophagosome formation and thus suppresses autophagy [39–42]. Upstream of mTORC1, growth factor receptors, and tyrosine kinase receptors get phosphorylated and activated which leads to the activation of PIP3K either directly or indirectly via adapters such as GAB2 [growth factor receptor-bound 2- (GRB2-) associated-binding protein 2] and insulin receptor substrate 1 (IRS1) [43, 44]. Activated PI3K converts PIP2 to PIP3 and recruits protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) and RAC-alpha serine/-threonine-protein kinase1 (AKT1). AKT1 gets activated by PDK1 which inhibits heterodimerization of tuberous sclerosis 1 (TSC1) and TSC2, resulting in inhibiting Ras homolog enriched in brain (Rheb) and hence activation of its GTPase activity. GTP-Rheb activates mTOR which leads to autophagy suppression. Phosphatase and tensin homolog (PTEN) is a tumor suppressor which dephosphorylates PIP3 to PIP2 and counterbalances the whole cascade (Figure 3) [45]. Loss of tumor suppressors like liver kinase B1 (LKB1), promyelocytic leukemia protein (PML), PTEN, and TSC1/2 or gain in function mutations can activate mTOR, thus inhibiting autophagy, whereas cellular stress and mTOR inhibitors like rapamycin, Torin 1, and Tamox downregulate or inhibit mTOR and induce autophagy [46, 47]. Another mTOR complex mTORC2 is involved in the activation and phosphorylation of AKT1 [8, 48, 49]. 6-TG (6-thioguanine) induces autophagy and requires activation of mTOR [50]. So, it is not universal that mTOR activity is inversely proportional to autophagy.

Hypoxia (low oxygen status which can be present in cells in poorly vascularized regions) activates autophagy through

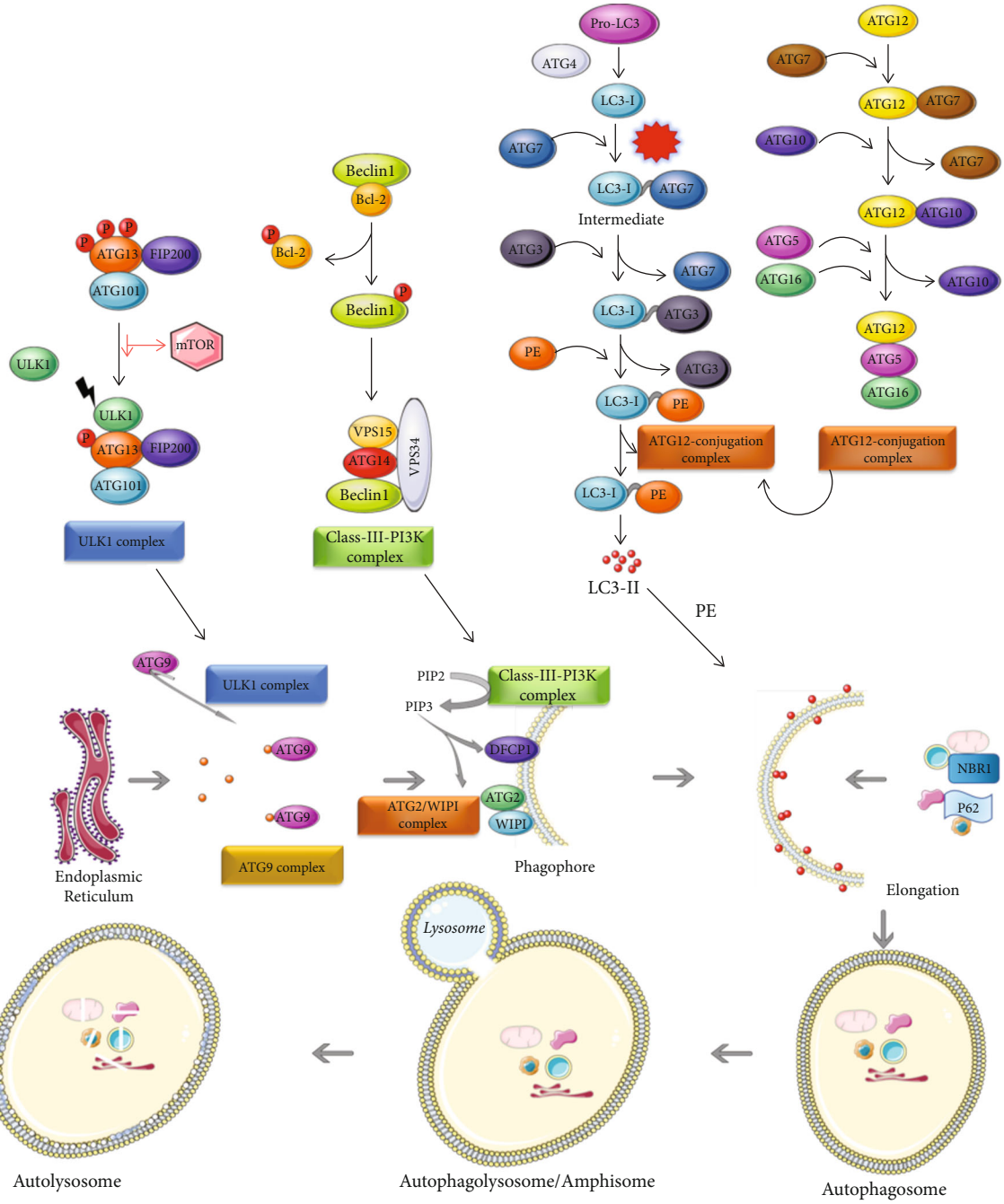


Figure 2: Autophagy pathway in mammalian cells: initiation of autophagy is dependent on 6 protein complexes: ULK1 complex, class III-PI3K (phosphoinositide 3-kinase) complex, ATG9 complex, ATG2-ATG18 complex, ATG8/LC3 complex, and ATG12 conjugation complex.

hypoxia-inducible factor- (HIF-) dependent and hypoxia-inducible factor- (HIF-) independent pathways [16, 51, 52]. The HIF-dependent pathway involves selective autophagy mediated by HIF1 and its target Bcl-2 nineteen-kilodalton-interacting protein 3 (BNIP3) [53, 54]. They are affected by the concentration of oxygen and some growth factors which lead to modulation in the regulation of erythropoiesis, angiogenesis, metabolism, pH regulation, cell migration, and tumor invasion [53] by increases in the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and nitric oxide synthase (NOS). BNIP3 activates Beclin1 by inhibiting Bcl-2 [52]. So, both HIF and BNIP3 are required for prosurvival hypoxia-induced autophagy in normal as well as in cancer cells [53–55]. Also, it has been seen that misfolded proteins can also regulate autophagy by binding itself to ER chaperone-binding immunoglobulin protein (BiP)/GRP78 and release three ER membrane-associated proteins: PKR-like eIF2a kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme 1 (IRE1). PERK and ATF6 induce autophagy, whereas IRE1 inhibits [56].

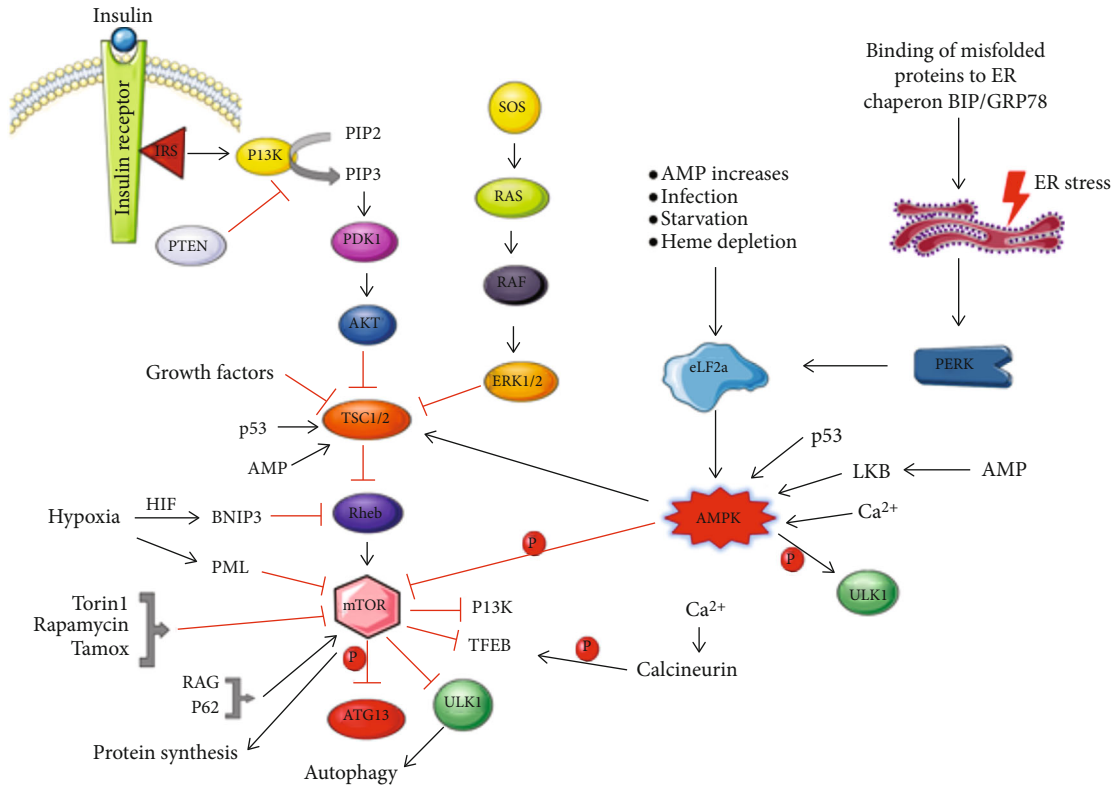


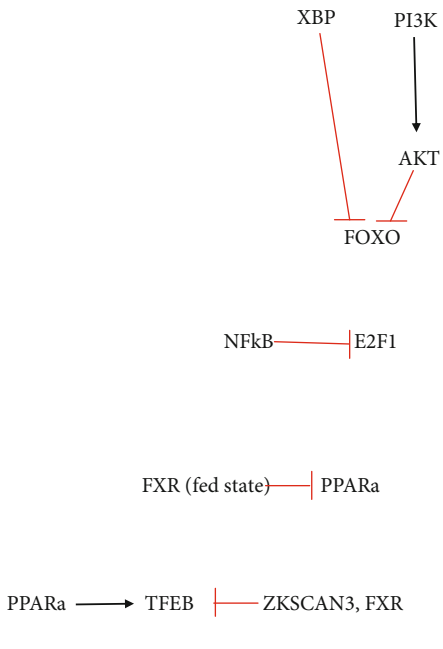
FIGURE 3: Regulation of autophagy: binding of growth factors to its receptor (tyrosine kinase) gets activated leads to the activation of PIP3K which phosphorylates PIP2 to PIP3 and recruits protein kinases PDK1 and AKT to inactivate TSC and RHEB. Activated GTPase Rheb activates mTOR which inhibits by phosphorylation of downstream targets of autophagy pathway like Beclin1 and Atg13 and transcription factors like TFEB by mTORC1 in nutrient-rich condition and inhibits autophagy. Other signals like SOS, AMP increase, starvation, and ER stress activate their signal cascade and regulate the PI3K-mTOR axis components.

3.1. Transcriptional Regulation of Autophagy. There are some transcriptional factors which act as upregulators or downregulators of autophagy. For example, transcription factor EB (TFEB), E2F transcription factor 1 (E2F1), activating transcription factor 4 (ATF4), Forkhead box O3 (FOXO3), nuclear factor erythroid-derived 2-like 2 (NRF2/NFE2L2), hypoxia-induced factor (HIF), p53, and peroxisome proliferator-activated receptor α (PPARα) are upregulators of autophagy, whereas zinc finger protein with KRAB and SCAN domains 3 (ZKSCAN3), heat shock factor (HSF), transcription factor 4 (TCF4), X-box binding protein 1 (XBP1), farnesoid X receptor (FXR), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are suppressors of autophagy (Figure 4).

TFEB is the prime regulator of lysosomal biogenesis and gets activated by dephosphorylation during starvation which allows it to enter the nucleus to induce transcription of its targets such as Atg4, Atg16, LC3, p62, WIPI proteins, ULK1, and some cathepsins. [57–59]. E2F1 is involved in stress-related mechanisms and is inhibited by NF-κB; it upregulates BNIP3, ULK1, LC3, and Atg5 and helps in the preautophagosome initiation [60–63]. ATF4 is upregulated by itself and activated by severe hypoxia; it enters into the nucleus and upregulates autophagy machinery components ULK1, LC3, and Atg5 [62, 64, 65]. FOXO3 is inhibited by AKT (which is activated by PI3K). Activated FOXO (suppressed AKT and PI3K) enters the nucleus and activates Atg4, Atg2, Atg5, Atg12, Beclin1, LC3, and ULK1 [62, 66, 67]. NRF2 is regulated by amino acid content and nutrient signaling and upregulates p62. HIF is activated by mild hypoxia, whereas ATF4 is activated during severe hypoxic conditions [53, 54]. p53 comes into picture during DNA damage; it upregulates Atg4, Atg7, and ULK1 [62, 68, 69]. PPARα is activated during starvation, whereas, in the fed state, it is suppressed by FXR. PPARα induces Atg3, Atg5, Atg7, Beclin1, LC3, TFEB, and ULK1 [70–72].

ZKSCAN3 is a negative and major regulator of TFEB. In a fed state, it enters the nucleus and binds to autophagy machinery target genes such as ULK1, LC3, and WIPI proteins [62, 73]. HSF is opposite of NFR2 as it suppresses p62 [74]. TCF4 is regulated by β-catenin. TCF4 suppresses p62 when β-catenin binds to TCF4. But when LC3 binds to β-catenin, it leads to proteasomal degradation. So, β-catenin suppresses TCF4. XBP1 is activated by ER stress and enters the nucleus. It has a dual function by upregulating Beclin1 and suppressing FOXO3 which in turn suppresses Atg4, Atg5, Atg12, LC3, and ULK1 [62, 75]. FXR is a negative transcriptional repressor of PPARα and it inhibits genes of Atg3, Atg5, Atg7, Beclin1, L3, ULK1, and TFEB. NF-κB has dual effects; it enters the nucleus and upregulates Beclin1 and p62; on the other hand, it also inhibits E2F1, i.e., inhibits Atg5, LC3, and ULK1 [22, 62, 63, 76–78].

During starvation, calcium ions are released into the cytoplasm from lysosome and activate a phosphatase



Transcriptional upregulators (green) and suppressors (pink) of autophagy components

TFEB, NRF2, NFkB	P62	HSF, TCF4
TFEB, E2F1, ATF4, FOXO, p53.PPARa	ULK1	ZKSCAN3, NBP, FXR, NFkB
TFEB, E2F1, ATF4, FOXO, PPARa	LC3	ZKSCAN3, TCF4, XBP, FXR, NFkB
FOXO, PPARa, XBP, NFkB	BECLIN1	FXR
TFEB	WIPI	ZKSCAN3
E2F1, FOXO, HIF	BNIP3	——
PPARa	ATG3	FXR
TFEB, p53.PPARa	ATG4	XBP
E2F1, ATF4, FOXO PPARa	ATG5	XBP, FXR, NFkB
p53, PPARa	ATG7	FXR
FOXO	ATG12	XBP
TFEB	ATG16	-

Figure 4: Transcriptional factors regulating autophagy [62].

calcineurin which removes phosphate from TFEB (phosphorylated by mTOR in a fed state) and gets activated. During starvation, ZKSCAN and FXR are kicked out of the nucleus which allows TFEB and PPARα to enter into the nucleus, bind to its targets, and induce autophagy by induction of many autophagy proteins. During starvation, c-Jun also gets translocated into the nucleus which induces expression of ANXA2 (Annexin A2) which is important for increased secure autophagic trafficking [62, 79].

3.2. Regulation of Autophagy by p53. p53 is the "guardian" of cell integrity. It is a tumor suppressor protein and is mutated in more than 50% of the cancers [80]. It is reported in almost every type of cancer ranging from 10% in hematopoietic malignancy [81], 20-40% or more in breast cancer, colorectal cancer [82], and other type of cancers [83], and 50-60% in esophageal carcinoma to approx. 100% in high-grade serous carcinoma of ovary [84]. Depending upon the stimuli and subcellular localization, it can function as a positive as well as a negative regulator of autophagy. Nuclear p53 acts as an activator of autophagy in a transcription-dependent or transcription-independent manner. AMPK gets activated by stress-activated p53 which upregulates autophagy by downregulating the mTOR pathway. p53 also induces activation of the autophagy pathway by upregulating damage-regulated autophagy modulators (DRAMs), Sestrin 1 and 2, and death-associated protein kinase 1 (DAPK1). DRAM (a lysosomal protein) stimulates autophagy at various stages of the autophagic process. DAPK-1 stimulates autophagy by inhibiting antiautophagic microtubule-associated protein 1B (MAP1B) protein (an LC3 interactor). AMPK increases level of NAD^+- and NAD^+-dependent activity deacetylase Sirt1, which plays an important role in stimulation of autophagy by numerous ATG activations and deacetylations [85]. Cordani et al. reported that mutant p53 substantially counteracts the autophagic vesicle formation and fusion of these vesicles with lysosomes through the suppression of several important autophagy-related enzymes and proteins such as DRAM1, BECN1, and AMPK [86]. Nuclear p53 also releases Beclin1 via phosphorylation from the sequestration of Bcl-2/Bcl-xL/Mcl-1 [87]. It is also observed that deficiency of Beclin 1 is associated with deficiency in tumor suppressor p53, causing malignancies in BECN1-deficient mice [87–90]. Furthermore, p53 can also induce autophagy via transactivation of the p14ARF tumor suppressor (or p19ARF in mouse, or also known simply as ARF), which can stabilize p53 by inhibiting Mdm2 (E3 ubiquitin ligase) and subsequently inhibiting the proteasome-mediated degradation of p53 [91–94].

However, cytoplasmic p53 acts as an inhibitor of autophagy. It targets TP53-induced glycolysis and apoptosis regulator (TIGAR) gene that downregulates glycolysis and suppresses ROS, rather than via the mTOR pathway (Figure 5) [90, 95]. Tasdemir et al. and Sui et al. have reported that cytoplasmic p53 directly interacts with FIP200, thus regulating the crucial initiation step of autophagy [80, 90]. To induce autophagy, it is required to degrade cytoplasmic p53. $p53^{-/-}$ cells do not show any autophagic response when transfected with p53 protein in the cytoplasm. It is due to the lack of nuclear p53 [80]. This dual role is not exclusive to p53; potent oncogene Ras also has been reported to portray this dual role [96–98].

Ataxia telangiectasia mutated (ATM) (a sensor of cellular or DNA damage during the cell cycle) and high mobility

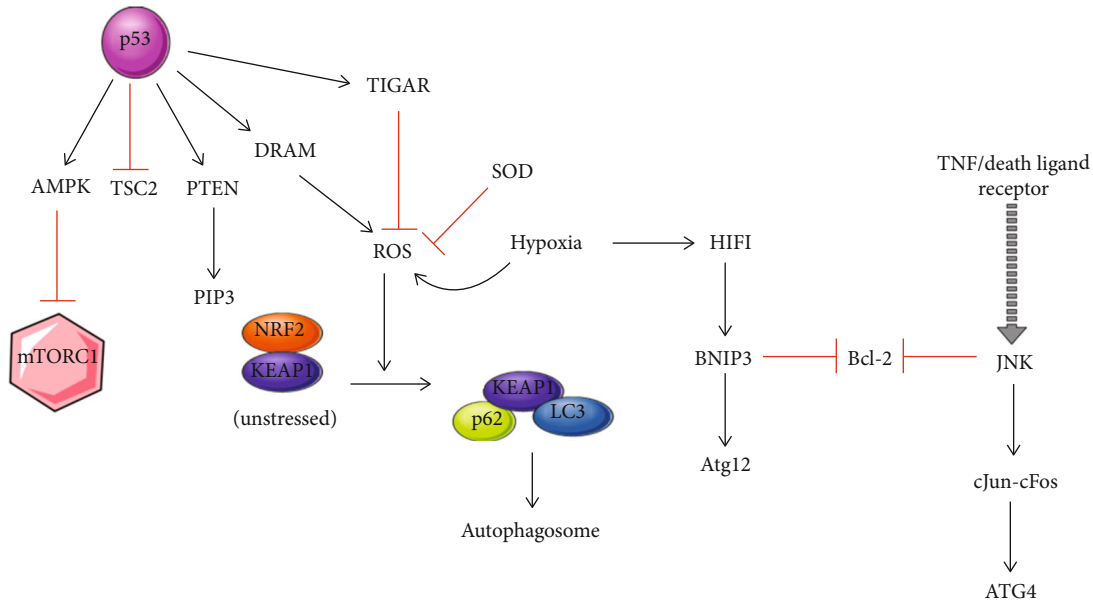


Figure 5: Regulation of autophagy mediated by p53: depending upon p53 subcellular localization, it plays a dual role in the process of autophagy. Nuclear p53 activates AMPK, inhibits mTOR, and induces autophagy. It was also induced by proautophagic modulators DRAM and PTEN. The inhibition of Bcl-2 leads to the release of Beclin 1 and activates autophagy, whereas in cytoplasmic p53, autophagy inhibition is associated with TIGAR.

group box 1 (HMGB1) (an immune modulator) also have been discovered as controllers of autophagy by regulating TSC2/mTORC1 signaling axis and interaction with Beclin1, respectively [99, 100].

4. Autophagy in Cancer

Cancer is a multifactorial disease characterized by uncontrolled cell division and can develop an obtrusive phenotype that is caused by genetic mutations, epigenetic alterations, and environmental factors that change the expression or function of the encoded products and behavioural factors [16]. GLOBOCAN (2018) estimated about 9.55 million deaths every year [101]. In India, about 0.7 to 0.81 million cancer-related deaths are estimated [102]. Cancer uses a variety of sources to meet the needs for unrestricted proliferation. It shifts towards anabolic metabolism and increases the Warburg effect (aerobic glycolysis) [103]. There are increasing evidence that autophagy regulates cancer cell's life. Cancer is one of the major maladies that is linked to dysregulation of autophagy. Autophagy deficiency prompts oncogenic mutations, damaged organelles, and macromolecule accumulation hence inducing oxidative stress, chromatin instability, DNA damage, and tumor susceptibility [104–106].

Tumor cells have more reliance on autophagy for survival as compared to normal cells due to their rapid proliferative ability in the nutrient-deprived environment caused by them. Through the autophagy recycling process, cancer cells overcome metabolic stress during its rapid proliferation. Dysregulated autophagy has implications in both well-being and sickness, specifically in cancer. Autophagy plays a dichotomous job in cancer by restraining tumor commencement (as a tumor suppressor); on the other hand, it is supporting tumor progression [107]. A few key autophagic regulators and their related pathways which play important roles in the regulation of autophagy in cancer include the Beclin1 interactome, Ras-Raf-MAPK pathway, PIR-Akt-mTOR pathway, and TP53 signaling. Several signaling pathways and molecules may modulate the autophagy in cancer, and these agents may act as potential therapeutic targets in treatment of cancer [108].

4.1. Duality of Autophagy in Cancer. A key question is how autophagy plays a dual role in the regulation of cancer. It prevents the development of the cancerous cells; on the other hand, it behaves as a key mechanism for the survival of the cancer cells. It was initially thought that autophagy has a tumor-suppressive role in normal cells by constraining inflammation, tissue damage, and prevention from senescence induced by an oncogene, scavenging damaged organelles and macromolecules, preventing the accumulation of radicles which are toxic and cause instability of the genome, and restricting the metastasis of cancer cells [8]. Also, by limiting tumor necrosis and oncogene-induced increase in senescence during an early stage of metastasis of cancer can act as a metastasis suppressor.

However, autophagy has also been implicated in benefits of cancer cells by either of the two mechanisms: one is the mutation which leads to activation of oncogenes (e.g., PIK3CA Ras, RHEB, and AKT—autophagy inhibitors) and the second being mutations that result in inactivation of tumor suppressor genes (e.g., PTEN AMPK, LKB1, and TSC1/2—autophagy inducers) [9]. The initiation of neoplasia in various genetically engineered mouse models (GEMMs) has also been reported by deleting the autophagy genes for studying autophagy deficiency in cancer. Paradoxically, autophagy can supply nutrients to cancers and, in turn, enhances its survival. In the advanced stage of metastasis of

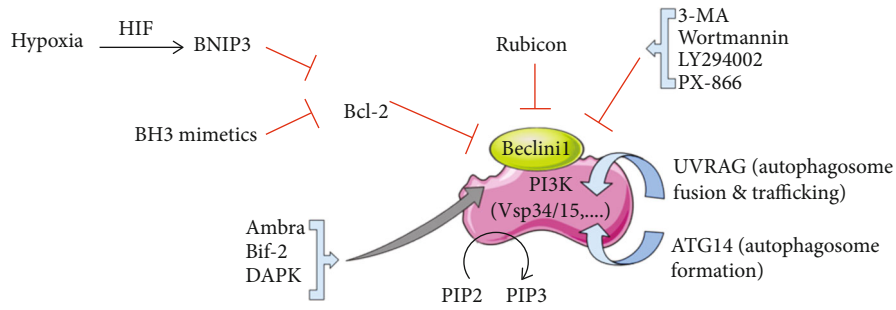


Figure 6: Regulation of Beclin1 and its associated protein activity. Beclin1 phosphorylates PIP2 to PIP3. Interaction of Beclin1 with Bcl-2 inhibits its activity. Ambra1, Bif-2, and DAPK induce autophagy by disrupting the interaction of Beclin1 to Bcl-2. Interaction of Atg14 helps in formation of autophagosome, whereas UVRAG regulates the maturation, fusion, and trafficking stages of autophagy. Binding of Rubicon along with UVRAG inhibits the fusion and trafficking step.

cancer, it promotes ECM disengagement of a metastatic cancer cell to a distinct site and also helps to enter the cell into dormancy if the cell fails to set up a contact with ECM [109]. Additionally, as a response to therapy or to confer resistance to radiations and chemotherapy, autophagy gets frequently upregulated and protects cancer cells from apoptosis [8, 16, 110, 111].

4.1.1. Autophagy as a Tumor Suppressor. Autophagy is frequently downregulated in various types of tumors suggesting its tumor-suppressive role as discussed earlier [112–114]. Constitutive activation of PI3K-Akt-mTOR axis is known to suppress autophagy [115] and is a characteristic of cancer, by promoting the proliferation, growth, and survival of the tumor cells [116]. There are various proteins like Bcl-2-interacting Beclin1 (BECN1/ATG6), Atg4c [117], Bax-interacting factor-1 (Bif-1) [118], BH3-only proteins [7], DAP kinase [119], ultraviolet radiation resistance-associated gene (UVRAG) [120], and PTEN [121] involved in autophagy that shows its role in tumor suppression (Figure 6). There are also some tumor suppressors like LKB1 [8, 122], TSC [123], nuclear p53 [56], and AMPK [124] which regulate the mTORC pathway. Additionally, all ATGs show this tumor suppressor effect recommending that proteins of autophagy acting at various strides of the pathway have a common tumor suppressor property. Several studies reported that the initiation of autophagy causes radiation sensitization in radioresistant and malignant glioma cells. Studies have also shown that certain chemotherapeutic drugs may destroy tumor cells through intrinsic pathway of apoptosis initiated by autophagy [125, 126].

(1) Beclin1 as a Tumor Suppressor. With the studies on Beclin1, it was first recognised that defective autophagy plays a role in cancer. Beclin1 is an ortholog of yeast Atg6/Vsp30 gene and is required for autophagy and vacuolar protein sorting processes [127]. It maps on the centromeric region of BRCA1 on 17q21 chromosome which is responsible for 75%, 50%, and 40% deletion in many cancers like ovarian, breast, and prostate cancers, respectively [128, 129]. Deletion in both BRCA1 and Beclin1 or only BRCA1 was found, but no proof of Beclin1 mutation or loss detected in any cancer which questions its tumor silencing activity in human cancer. But it is identified as a haploinsufficient tumor suppressor that is deleted monoallelically in cancer and decreases in autophagy [114]. It was the first direct link established between autophagy and cancer. Beclin1$^{+/-}$ immortalized baby mouse kidney (iBMK) cells [106, 110] and immortalized mouse mammary epithelial cells (iMMECs) show compromised autophagy and more tumorigenesis when transplanted *in vivo* in nude mouse allografts [124]. Tissue specificity is seen in the Beclin1 tumor-suppressive function. In contradiction, colorectal and gastric cancer shows higher expression of Beclin1 about 95% and 83%, respectively, compared to the normal stomach and colon mucosa with very low and undetectable levels. The Beclin1 protein contains Bcl-2 homology-3 (BH3) domain which interacts with BH3 receptor domain of antiapoptotic proteins like Bcl-2 and Bcl-xL and inhibits Beclin1 autophagic activity as well as its tumor suppressor activity. BH3-only proteins and DAPK under nutrient deprivation induce autophagy by disrupting Beclin1 interaction to Bcl-2/Bcl-xL competitively [7, 130, 131].

(2) UVRAG as a Tumor Suppressor. Reactive oxygen species (ROS), dysfunctional mitochondria, and accumulation of toxic protein aggregates cause aneuploidy and oncogenic transformation in autophagy-deficient cells. Through UVRAG which was isolated in a screen for gene complementing UV sensitivity in xeroderma pigmentosum cells, autophagy can protect against the instability of genome and confer centrosome protection. UVRAG depletion leads to errors in chromosome segregation, malfunctioning of the spindle apparatus [8]. It is represented as a hub for regulating autophagy and cancer as it is a part of Beclin1 and Vps34 multifunctioning protein which behaves as type III PI3K and stimulates autophagy. Low levels of UVRAG cause impaired induction of autophagy. It does not only act early in the initiation of autophagy but also regulates later-on stages like maturation and fusion. Its phosphorylation by mTORC1 blocks the late stages of autophagy. It disrupts Beclin1, dimer stabilized by Bcl-2-like protein to induce autophagy in both *in vivo* and *in vitro*. Similar to Beclin1, it is deleted monoallelically. In colon and

gastric cancers, autophagy decreases and it is done by targeting adenine track of UVRAG gene (A10 in exon 8) for frameshift mutation [128, 129].

(3) Bif-1 as a Tumor Suppressor. Bif-1/endophilin B1, also known as SH3GLB1, is discovered originally as a Bax-binding protein. It is a third protein associated with the class III PI 3-kinase complex [132, 133]. It participates in vesicle formation and membrane dynamics with membranes of organelles of Golgi apparatus, mitochondria. [118]. During nutrient deprivation, Bif-1 along with LC3/ATG8, ATG5, and ATG9 autophagic effectors gets accumulated in the cytoplasmic puncta. It stimulates PI3K complex activity and regulates autophagy by interacting with Beclin1 through UVRAG, although it does not appear to be a constitutive subunit of the complex. Bif-1 is established as a suppressor, as $bif1^{-/-}$ mice, and suppresses autophagosome formation and enhances the lymphomas and tumor development [7].

(4) An ATG Protein as a Tumor Suppressor. It was reported that $Beclin1^{-/-}$ mice die early in embryogenesis and aging; mice with $Beclin1^{+/-}$ are tumor-prone [134, 135], whereas mice with $Atg5^{-/-}$ and $Atg7^{-/-}$ are born normally, but die soon due to low nutritional level and suckling defects in neonates [136, 137]. As compared to $Atg5^{+/-}$ iBMK cells, $Atg5^{-/-}$ iBMK cells are more tumorigenic in nude mouse allografts [106]. Also, in Atg7-deficient liver, neither cell proliferation nor tumorigenesis was observed [136]. Functions of ATG5 and Beclin1 act as "guardians" of the cellular genome. During ischaemic stress parallel with tumorigenesis, their loss displays gene amplification, aneuploidy, and DNA damage. Also, as compared to ATG5 and ATG7, Beclin1-dependent and Beclin1-independent properties play an important role during embryonic development and tumor suppression. Further autophagy defects playing a role in tumorigenesis are confirmed by $Atg4C^{-/-}$ mice (autophagin-3 cysteine protease involved in the processing of LC3), show decreased autophagy which is starvation induced, and also develop fibrosarcoma by chemical carcinogen induction. Cysteine-specific residues in active sites of protein cause ROS-mediated oxidation due to which Atg4C appears as ROS sensor in autophagy [117, 138]. From the discussion, it is found that autophagy acts as a tumor suppressor and its reduced function is a hallmark of cancer.

4.1.2. Autophagy in Tumor Progression. Autophagy is frequently downregulated in various tumors, but this is not always the case; they are more dependent on autophagy for survival than normal cells/tissues. Several evidences indicate that autophagy maintains tumor cell survival and confers stress tolerance in cancer cell after exposure to various chemo- and radiotherapies and hypoxic and hypothermic conditions [100]. In pancreatic cancer cell lines and tumor specimens, increased basal level of autophagy was detected while inhibition of autophagy leads to tumor regression.

Due to the high proliferation rate of cancer cells, they have high metabolic demand (nutrient, oxygen supplies, etc.). Autophagy provides metabolic substrate to maintain tumor metabolism and helps in the survival of tumor cells under unfavourable conditions. It is reported that knocking down of essential autophagic genes results in impaired survival under metabolic stress which ultimately leads to death in *in vivo* models and various tumor cells [100, 139, 140]. Inhibiting autophagy by genetic or pharmacological means in cancer cells has shown tumor regression. Studies showing mouse survival with ATG7 deficiency in prostrate tumor and intestinal cancer suggest that autophagy helps in tumor progression. Also, in mouse models of Kras-driven glioblastoma, ULK1, Atg7, and Atg13 knockdowns demonstrate that autophagy is crucial for the initiation and sustained growth of glioma. Studies establish that in comparison to ATG7 intact, ATG7 deficiency reduces Kras-driven lung tumor cell proliferation and burden in mice [15]. Poor blood supply or limited nutrient supply to the metastasizing cells to organs has prevalent metabolic stress [141]. In metastasis, autophagy is represented as a mechanism of survival which gets upregulated [142]. For example, deficiency of Atg17/FIP200 inhibits the growth of mammary cancer in mice, suggesting that autophagy has a role in promoting tumorigenesis [143, 144]. In ATG7-deficient mouse, melanoma development, initiation, and proliferation are prevented which prolong survival of mouse. Compared to ATG7-deficient liver, combined p62 and autophagy loss in $Atg7^{-/-}$ liver abrogate inclusion body formation, alleviate hepatic injury, and retard tumor progression [140, 145]. Not only this, p62 accumulation is a hallmark of impaired autophagy. Its overexpression and accumulation in defective autophagy tumor cells is sufficient for ROS and DNA damage response induction under metabolic stress. This causes genetic instability and cell division abnormalities which at the end lead to the progression of tumor [146]. In a study, drug-induced stress in HEp-2 cells relays on autophagy to survive and confer chemoresistance to carcinoma cells by coping with p62-related proteotoxicity [147]. Thus, autophagy is regarded as a mechanism of tumor cell survival and also shows a promising therapeutic target for the treatment of different cancers.

4.2. Autophagy: Role in Resistance to Therapy. Autophagy is activated in response to a variety of cancer therapies and may induce cancer cell survival or chemoresistance as an adaptive cellular response [100, 139, 140, 148]. Accumulation of autophagosome has been observed in cancer cells after they were exposed to various chemotherapeutics like temozolomide DNA alkylating agent [149], tamoxifen an estrogen receptor antagonist [150], resveratrol [151], vitamin D_3, and anthocyanins [152]. Along with these chemotherapeutics, hypoxia, hyperthermia, and radiotherapy activate and upregulate autophagy, thus enabling a dormancy state in the cancerous cells which can lead to tumor reoccurrence and progression [118, 130]. Evidence showed enhanced efficiency of anticancer drugs on tumor cells with inhibited autophagy. Chloroquine inhibits autophagy and also enhances the efficacy of p53 or DNA alkylating agent to induce tumor regression or cell death in c-Myc-induced lymphomas in mice [131]. Through pharmacological means or by knocking down Atgs like Atg5, Atg6, and Atg7 is a major therapeutic means for sensitizing cells to anticancer therapies. Studies have reported that Akt inhibitors like triciribine and

perifosine can initiate protective autophagy among tumor cells. Perifosine can also increase the rapamycin cytotoxicity in multiple myeloma by suppressing the PI3K/Akt/mTOR pathway [108]. Furthermore, studies have shown that metformin can suppress progression of tumor by activating AMPK in melanoma and cervical cancer and thus by causing autophagy [153, 154]. Cancer cells can be sensitized to radio- and chemotherapies by siRNA-mediated depletion of ATG proteins, e.g., miRNA-22 is shown to influence chemotherapy-resistant colon cancer cells by inhibiting autophagy and promoting apoptosis [111, 155]. Overexpression of miR-22 in colon cancer cells increased sensitivity to 5-FU which is one of the main chemotherapeutic agents used in the treatment of colorectal cancer [156]. miR-409-3p inhibits autophagy by targeting Beclin-1 resulting in enhanced sensitivity to oxaliplatin. miRNA-210 induces autophagy and reduces radiosensitivity in colon cancer [58]. In a study, miR137 chemosensitize pancreatic cancer cells and inhibit autophagy by targeting Atg5 [157]. It has also been seen that long noncoding RNAs (lncRNAs) and CAIF (cardiac autophagy inhibitory factor) modulate autophagy and prevent defective autophagy-mediated loss of cardiac myocytes. It could be a potential therapeutic tool for the treatment of myocardial infarction and heart failure [158]. Other noncoding RNAs like circular RNA (Hsa-circ0023404) have a role in cervical cancer progression, metastasis, and chemoresistance through autophagy by sponging miR5047 [159]. Thus, it is clear that miRNA and any other noncoding RNAs regulate autophagy under various stress conditions. Various strategies are available to deliver the knockout materials (miRNAs or siRNA) to the target cancer cells such as nanoparticles, lipid vectors, nonlipid vectors, and multistage vector (MSV) delivery system. Lipid vectors such as liposomes and stable nucleic acid lipid particles (SNALPs) and nonlipid vectors such as chitosan, poly(amidoamine) dendrimers, and polyethylenimines are used [160]. Cyclodextrin nanoparticles can be used as an effective tumor targeting, as the surface is decorated with transferrin protein targeting ligand since the transferrin receptors are overexpressed on the surface of cancer cells [161]. Radiosensitization of malignant glioma cells can also be caused by using autophagy inhibitors like 3-methyladenine and bafilomycin A1 [162].

4.2.1. Pharmacological Modulation of Autophagy to Counteract Cancer. Activators of autophagy are considered effective in neurodegenerative diseases, whereas inhibitors of autophagic flux such as chloroquine are considered effective in cancer therapy. It is a fact that autophagy in normal cells is beneficial and the use of its inhibitors as cancer therapy is a major obstacle [8]. So, a drug that targets the autophagic pathway in cancer cells without affecting normal cells could be an ideal drug. This novel paradigm in cancer therapy has been validated in several preclinical studies and is now under investigation in I/II phase of clinical trials involving autophagy inhibition. Antimalarial drug hydroxychloroquine, which blocks lysosomal degradation of the autophagy products by affecting lysosomal pH, in combination with anticancerous drugs involved in standard chemotherapy to achieve better outcomes. Table 1 shows a few of the clinical trials based on the antimalarial drugs in combination with different anticancer drugs used for the inhibition of autophagy. They support that targeting autophagy provides therapeutic benefits in models of chemotherapy resistance and shows very promising results in combinatorial cancer treatment.

Apart from inhibition of autophagy as a tumor-suppressive mechanism in the early stages of tumor formation, there may be a benefit in identifying activators of autophagy as anticancer agents as well. Significant attempts are being devoted to the development of autophagy modulator agents with enhanced pharmacological specificity. Activators such as IFNγ, melatonin, and trehalose and inhibitors such as LY294002 are in clinical trials, whereas activators such as A-769662, BECN1-derived peptide, and BRD5631 and inhibitors such as compound C (also known as dorsomorphin), Mdivi-1, Lys05, SAR405, VPS34-IN1, SBI-0206965, MRT67307, NSC185058, and MRT68921 are in preclinical development stage [163]. There is little evidence that induction of autophagic cell deaths can also be used as a therapeutic strategy for removing cells with high apoptotic threshold or compromised apoptosis cancerous cells lacking BAK, BAX, and caspases. For instance, thalidezine in various cancer cell lines eliminates apoptotic resistance via autophagic cell death [164]; four dauricine derivatives induce autophagy-dependent cell death in HeLa cells [165]. Some natural products in in vivo studies like polyphyllin (PPI) extracted from rhizome *Paris polyphylla* activate AMPK directly and suppress the growth of non-small-cell lung cancer (NSLC) [166]. Ethoxysanguinarine, an alkaloid extracted from *Macleaya cordata*, induces autophagy cell death in breast cancer cells [167]. Various rapamycin derivatives such as CCI-799, RAD001, and AP23573 can also be used as effective therapeutic agents against cancer [168]. In combination with chemotherapeutic agents, autophagic cell death is also found in *in vitro* cancer cells. In MCF-7, the silencing of Bcl-2 by siRNA leads to autophagic cell death. Further siRNA in combination with a low dose of doxorubicin enhances autophagy which inhibits tumor growth and leads to autophagic cell death [169].

Various potential drugs and their nanoformulations such as Doxil (liposomal formulation of doxorubicin) which is a first approved nanodrug used in breast cancer therapy and Abraxane (albumin-bound paclitaxel formulation) can be used in combinational strategies to improve therapeutic effect and reduce the toxicity and side effects [160]. But after all this, future studies are needed to prove that manipulation of autophagy can be useful in clinics.

4.3. Autophagy and Immune System. Autophagy is an important catabolic pathway which plays several roles in different kinds of cells [170]. Autophagy is also necessary for complete macrophage differentiation of mice and human monocytes driven by granulocyte-macrophage colony-stimulating factor (GM-CSF) or colony-stimulating factor-1 (CSF-1) [171]. The initiation of autophagy is crucial for differentiation and survival of monocytes. The signal of differentiation discharges Bcl-2 interacting coiled coil protein-1 (Beclin-1) from Bcl-2 by c-Jun N-terminal kinase (JNK) activation and preventing cleavage of ATG5 which are important for induction of

Table 1: Antimalarial drug combinations are used for cancer treatment in various preclinical studies [173–175].

Compound (autophagy inhibitor)	Indication	Phase	Trial ref # at ClinicalTrials.gov
Chloroquine (CQ)	Small-cell lung cancer	I	NCT00969306
	Breast cancer	II	NCT02333890
Chloroquine + tamoxifen	Breast ductal carcinoma	II	NCT01023477
Velcade and cyclophosphamide with CQ	Multiple myeloma	II	NCT01438177
Cisplatin and etoposide + CQ	Stage 4 small-cell lung cancer	I	NCT00969306
	Non-small-cell lung cancer	II	NCT00933803
Hydroxychloroquine (combination treatment)	Colorectal cancer	II	NCT01006369
	Advanced cancer	I	NCT01266057
	Rectal cancer, colon cancer, metastasis, adenocarcinoma	II	NCT01206530
	Melanoma (skin)	I	NCT00962845
Hydroxychloroquine	Renal cell carcinoma	I	NCT01144169
	Unspecified adult solid tumor	I	NCT00909831
Hydroxychloroquine + ixabepilone	Breast cancer	II	NCT00765765
Hydroxychloroquine + docetaxel	Prostate cancer	II	NCT00786682
Hydroxychloroquine + vorinostat	Advanced solid tumor	I	NCT01023737
Hydroxychloroquine (combination treatment)	Lung cancer	II	NCT00728845
Hydroxychloroquine + bortezomib	Multiple myeloma and plasma cell neoplasm	II	NCT00568880
Hydroxychloroquine + gemcitabine	Pancreatic cancer	II	NCT01128296
Gemcitabine and Abraxane with or without HCQ		II	NCT01978184
		II	NCT01506973

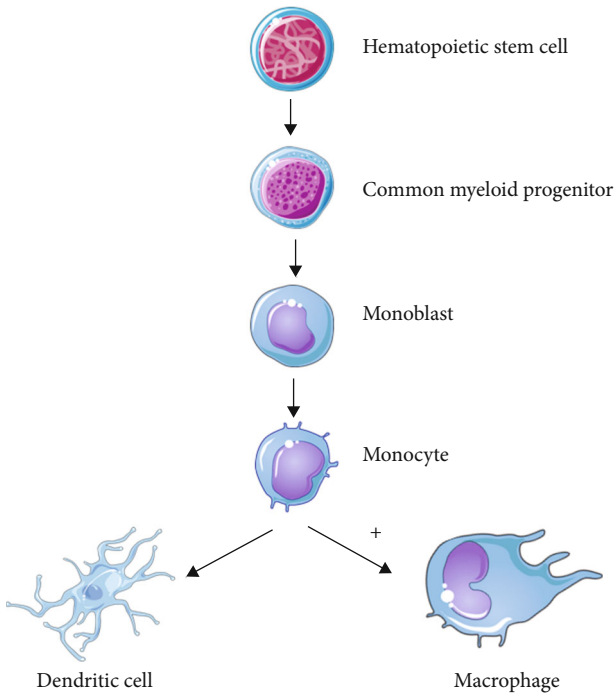


Figure 7: Autophagic activity in differentiation from monocytes to macrophages. Immune cells like dendritic cells and macrophages arise from hematopoietic cell (HSC) which has dedicated to common myeloid progenitor. Monoblast then generates a monocytic cell line which leads to the development of dendritic cells and macrophages. Autophagy involves in the differentiation process of monocytes to macrophages. "+": enhanced function.

autophagy [172]. Prevention of autophagy initiation hinders production of cytokines and differentiation. Hence, autophagy is vital in the conversion from apoptosis of monocyte to differentiation (Figure 7) [172]. In innate immune system, autophagy also modifies cell-specific functions like phagocytosis and antigen presentation. Autophagy can also play a role in microbial defence like antimicrobial peptide production and antigen presentation as pathogens/microbes can be engulfed directly in autophagolysosomes [170].

5. Conclusion and Future Perspective

Autophagy is a cell's recycling machinery, degrades cytosolic substrates in lysosomes, and provides by-products (biomolecules) which are used in anabolic processes and help cells survive in stress conditions. It is under tight regulation of mTOR, various transcriptional factors, p53 localization, etc. Thus, autophagy plays an important role not only in normal cells but also in cancer cell progression. It acts as a double-edged sword; on the one hand, it promotes tumor survival by providing energy and maintains homeostasis, whereas on the other hand, its defects elevate oxidative stress, damage, and mutations which are linked to tumor initiation and progression. It acts as a tumor suppressor during initial stages of cancer but becomes a tumor progressor in advanced stages. It is also responsible for providing resistance against various cancer therapies. Blocking autophagy is an approach to treat established, aggressive cancer, providing a promising hope for clinical applications. Inhibition of autophagy by pharmacological means or by ATG knockdown can promote cancer

cell death and could be therapeutically advantageous but only without affecting normal cells. Other than autophagy regression, its contradictory role, i.e., tumor suppression at the early stages of cancer development, should also be considered. More effort has to be put on the development of effective cancer-specific delivery systems and drugs to make tumor more sensitized to therapies. In addition, a detailed deciphering of the crucial role of noncoding RNAs in autophagy has profound clinical implications. Therefore, along with combinational therapies with the conventional one, more effort should be put on the miRNA-based therapies for therapy-resistant cancers.

The role of autophagy in the development and maintenance of cancer is thoroughly studied, but still there are many aspects of this that need to be addressed. The cancer cells maintain higher basal level autophagy which supports their survival even under various hypoxic conditions and its regulation mechanism is still not fully understood. Also, enlightenment on the mechanism of cargo selection for this heightened basal level autophagy in cancer cells has not been elucidated. Many autophagy-related genes are studied which play an important part in protection and development of therapeutic resistance in various cancer cells, but their regulation and molecular mechanisms are yet to be deciphered fully. The scope of understanding tumor microenvironment is getting attention since tumors can develop and proliferate in various stressful hypoxic and hypoglycemic conditions. This characteristic of cancer is mainly attributed to increased autophagy in cancerous cells. Thus, understanding the interaction between tumor microenvironment and autophagy is of utmost importance which involves various *in vivo* as well as organotypic culture studies. These aspects of autophagy can be highly advantageous in developing various therapeutic regimens which can impede tumor development and progression as well as countering therapeutic resistance. Last but not the least, a lot of effort is still needed to understand the types of various cancers that would be controlled using autophagy inhibition which will depend on the development of newer and better biomarkers of autophagy in cancer.

Conflicts of Interest

The authors declare that they have no conflict of interest associated with this manuscript.

Authors' Contributions

KD, AKV, PSB, and SR conceived the idea. SR, AKV, and PSB performed the literature search and wrote the manuscript. PSB, AKV, and SR made vital contribution to write-up and diagrams. AKV, DB, YG, KKP, SR, SAA, MAA, AHR, and AA provided inputs for the strategy and final edition of the article. All authors read and approved the final manuscript. Amit Kumar Verma, Prahalad Singh Bharti, and Sahar Rafat contributed equally to this work.

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Research Article

Stimulation of Sigma-1 Receptor Protects against Cardiac Fibrosis by Alleviating IRE1 Pathway and Autophagy Impairment

Jing Qu,[1] Miaoling Li,[2] Dongxu Li,[3] Yanguo Xin,[4] Junli Li,[1] Song Lei,[5] Wenchao Wu,[1] and Xiaojing Liu[1,4]

[1]*Laboratory of Cardiovascular Diseases, Regenerative Medicine Research Center, West China Hospital, Sichuan University, Chengdu 610041, China*
[2]*Key Laboratory of Medical Electrophysiology of Ministry of Education, Institute of Cardiovascular Research, Southwest Medical University, Luzhou 646000, China*
[3]*Department of Cardiovascular Surgery, West China Hospital, Sichuan University, Chengdu 610041, China*
[4]*Department of Cardiology, West China Hospital, Sichuan University, Chengdu 610041, China*
[5]*Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, China*

Correspondence should be addressed to Xiaojing Liu; liuxq@scu.edu.cn

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Sigma-1 receptor (Sig1R), a chaperone in the endoplasmic reticulum (ER) membrane, has been implicated in cardiac hypertrophy; however, its role in cardiac fibroblast activation has not been established. This study investigated the possible association between Sig1R and this activation by subjecting mice to sham, transverse aortic constriction (TAC), and TAC plus fluvoxamine (an agonist of Sig1R) treatments. Cardiac function and fibrosis were evaluated four weeks later by echocardiography and histological staining. In an *in vitro* study, neonatal rat cardiac fibroblasts were treated with fluvoxamine or NE-100 (an antagonist of Sig1R) in the presence or absence of transforming growth factor beta1 (TGF-β1). Fibrotic markers, ER stress pathways, and autophagy were then investigated by qPCR, western blotting, immunofluorescence, confocal microscopy, and transmission electron microscopy. Fluvoxamine treatment reduced cardiac fibrosis, preserved cardiac function, and attenuated cardiac fibroblast activation. Inhibition of the IRE1/XBP1 pathway, a branch of ER stress, by a specific inhibitor of IRE1 endonuclease activity, attenuated the pathological process. Fluvoxamine stimulation of Sig1R restored autophagic flux in cardiac fibroblasts, indicating that Sig1R appears to play a protective role in the activation of cardiac fibroblasts by inhibiting the IRE1 pathway and restoring autophagic flux. Sig1R may therefore represent a therapeutic target for cardiac fibrosis.

1. Introduction

Cardiac fibrosis is characterized by the cardiac fibroblast activation, excessive proliferation, and transition into myofibroblast, which lead to excessive deposition and abnormal distribution of extracellular matrix [1–3]. Cardiac fibrosis usually happens postmyocardial infarction and myocardial hypertrophy, causing chronic heart failure finally [4, 5]. Cardiac fibrosis is a common pathological process in the development of various cardiovascular diseases and a risk for sudden cardiac death [6]. It is known that various cellular signaling pathways, such as the renin-angiotensin system, inflammatory factors, and oxidative stress are involved in the process of cardiac fibrosis, whereas the underling mechanisms, especially myocardial fibroblast activation, is not fully understood [7, 8]. Therefore, further exploring the pathophysiologic mechanism of cardiac fibrosis may provide new insights and be helpful for clinical treatment.

ER stress has gained attention as a cellular mechanism for maintaining homeostasis. It is elicited by the disruption of ER homeostasis and the accumulation of unfolded or misfolded proteins, followed by the activation of three sensors that subsequently activate downstream signaling pathways: Protein Kinase R-like ER kinase (PERK), Inositol Requiring Enzyme

1α (IRE1α), and Activating Transcription Factor 6 (ATF6) [9, 10]. Previous studies have confirmed the involvement of ER stress in the pathogenesis of cardiac hypertrophy [11, 12].

Other evidence has also shown that autophagy is critical for the development of cardiovascular diseases, such as cardiac hypertrophy and heart failure [13, 14]. Lysosome-mediated autophagy degrades and recycles cellular wastes, including proteins, lipids, and dysfunctional organelles. ATG-mediated autophagosomes/autolysosomes formation and autophagosome content degradation are key processes involved in autophagy [15].

A variety of autophagy proteins are localized at the endoplasmic reticulum (ER) [16], and autophagy originates from mitochondrial-associated endoplasmic reticulum membrane (MAM), the interface between ER and mitochondria [17, 18]. Sigma-1 receptor (Sig1R), a 223-amino acid ER chaperone at MAM, is related to autophagy and ER stress [19–21].

Sig1R modulates ER stress, autophagy, and apoptosis and has been confirmed to participate in neurodegenerative diseases and cardiac hypertrophy [22–25]. Fluvoxamine, a selective serotonin reuptake inhibitor with high affinity for the Sig1R, ameliorates cardiac hypertrophy and dysfunction deriving from Sig1R activation [26–29]. While this finding introduces the role of Sig1R in modulating cardiovascular disease, it raises many questions regarding the underline mechanisms, especially in cardiac fibrosis.

Therefore, in this study, we determined how Sig1R regulates cardiac fibrosis and cardiac fibroblasts activation, as well as its roles in ER stress, autophagy.

2. Methods and Materials

2.1. TAC Surgery. In this study, all mice received humane care, and our study was approved by the animal ethics committee of West China Hospital of Sichuan University. TAC was performed to induce pressure overload in the mice's heart [30]. Briefly, male mice (6-8 weeks old, 20-25 g body weight, Beijing Vital River Laboratory Animal Co. Ltd. China) were anesthetized with 2% isoflurane inhalation. The animals were then placed in a supine position, intubated orally with a 20-gauge tube, and ventilated (Harvard Apparatus Rodent Ventilator, MiniVent) at 120 breaths per minute (0.1 ml tidal volume). A stitched 27-gauge needle was sutured on the aortic arch between the cephalic artery and left carotid artery to form a reproducible aortic valve stenosis. Control mice were sham-operated. In this study, at the beginning of the experiment, we used 25 C57BL/6 mice for TAC surgery and 6 mice for sham surgery. Five died during the operation of TAC surgery, and a total of 20 TAC mice survived after the operation. We randomly divided the mice after TAC surgery into the TAC group and the fluvoxamine intraperitoneal injection group (FLV group), 10 mice per group. Some mice have died during the feeding process after the end of TAC operation and during the intraperitoneal injection administration, so the statistics number of mice finally included in this study was $n = 6$ in each group.

2.2. Echocardiography. At 4 weeks after the TAC surgery, mice were lightly anesthetized with 1% isoflurane inhalation and subjected to echocardiography using a Vevo3100 instrument (Visual Sonics). Images were captured in the short axis of the left ventricle to calculate internal wall dimensions during systole and diastole. From M-mode images, the thickness and dimensions of the left ventricle (LV) chamber were obtained. LV systolic function was determined by calculating ejection fraction (EF) and fractional shortening (FS). Echocardiography was performed on all mice.

2.3. Histological Staining. Heart tissue was rinsed with ice-cold saline perfusion and then with 0.1 ml of 10% KCl to cause diastolic arrest. The heart tissue was then fixed in 4% paraformaldehyde for 1 week at 4°C, then paraffin-embedded and sectioned at 5 μm. The heart sections were dewaxed and hydrated through a graded ethanol series (100, 95, 75, and 50%) and then stained either with Sirius red and Masson trichrome to observe fibrosis or hematoxylin and eosin (HE) to show the heart structure.

2.4. Quantitative Real-Time PCR (qRT-PCR). The classic TRIzol (Invitrogen, USA) method was performed to extract total RNA from tissues or cells. Next, the RNA was used as a template to synthesize cDNA with a reverse transcription. The reaction system (ReverTra Ace qPCR RT Kit, FSQ-101, TOYOBO) is displayed in Table 1. The q-PCR was conducted using the SYBR Green Supermix kit (Bio-Rad, USA) on BIO-RADCFX96™ Real-Time PCR Detection System and β-actin served as the reference gene. The primers used are displayed in Tables 2 and 3. Relative fold expression values were determined by applying the $^{\triangle\triangle}$CT threshold (Ct) method.

2.5. Protein Isolation and Western Blotting. Protein lysates were collected from mice heart tissue or cardiac fibroblasts and prepared for western blots as previously reported [31]. Briefly, 20-30 μg protein was separated on 10% or 15% SDS-PAGE gels and transferred to PVDF membranes. After blocking by 5% skim milks, blots were incubated with primary antibodies. After incubation with corresponding anti-mouse/rabbit secondary antibodies (1 : 3000; ZSGB-BIO), immunoblots were developed using Chemiscope 6000 (CLINX, China). The relative protein expressions were analyzed by ImageJ software. GAPDH or β-Actin was served as an internal reference.

2.6. Cell Culture and Pharmaceutical Treatments. Neonatal rat cardiac fibroblasts were isolated from the hearts of decapitated Sprague-Dawley rats according to the methods described previously [32]. The CFs were grown in a culture flask with DMEM mixed with 10% FBS and 100 U/ml of both streptomycin and penicillin. CFs at the second passaging were added into 6-well plates and cultivated to reach 50% confluence. The CFs were incubated for 4-6 hours with serum-free DMEM and then later treated with TGF-β1 (a known stimulator of CF, 10 ng/ml, Sino Biological Inc.) for 24 h to induce fibroblasts activation. A subset of cells was treated with fluvoxamine (5 μM) or NE-100 (5 μM) before 2 h exposure to TGF-β1.

Table 1: The reaction system of reverse transcript.

Reagent	Volume (μL)
5 × RT buffer	4.0
Primer mixture	1.0
ReverTra Ace	1.0
1.5 μg RNA	X
RNAase-free ddH2O	(14-X)
Total volume:	20 μL

Table 2: Rat primer sequences and amplicon sizes for RT-PCR.

Genes	Primer sequence (5′-3′)
COL-1	F: 5′ACGTCCTGGTGAAGTTGGTC3′ R: 5′TCCAGCAATACCCTGAGGTC3′
CTGF	F: 5′CAGGGAGTAAGGGACACGA3′ R: 5′ACAGCAGTTAGGAACCCAGAT3′
β-Actin	F: 5′CCUCUCCUUUGGACUGUAU3′ R: 5′ATGCCACAGGATTCCATACCC3′
TGF-β	F: 5′TGAGTGGCTGTCTTTTGACG3′ R: 5′ACTGAAGCGAAAGCCCTGTA3′
Sig1R	F: 5′ATTTCTCTACTCGCTGGGACTC3′ R: 5′GAGCTGTGTCTGGATGTATGTG3′

Table 3: Mouse primer sequences and amplicon sizes for RT-PCR.

Genes	Primer sequence (5′-3′)
POSTN	F: 5′TGGTATCAAGGTGCTATCTGCG3′ R: 5′AATGCCCAGCGTGCCATAA3′
CTGF	F: 5′GGACACCTAAAATCGCCAAGC3′ R: 5′ACTTAGCCCTGTATGTCTTCACA3′
COL-1	F: 5′TAAGGGTCCCCAATGGTGAGA3′ R: 5′GGGTCCCTCGACTCCTACAT3′
TGF-β	F: 5′CTTCAATACGTCAGACATTCGGG3′ R: 5′GTAACGCCAGGAATTGTTGCTA3′
Sig1R	F: 5′GGCACCACGAAAAGTGAGGT3′ R: 5′AGAACAGGGTAGACGGAATAACA3′
β-Actin	F: 5′GTGACGTTGACATCCGTAAAGA3′ R: 5′GCCGGACTCATCGTACTCC3′

2.7. Immunofluorescence Staining and Confocal Microscopy. When the confluence of cardiac fibroblasts reached 90%, the cells were digested with trypsin, centrifuged, and counted. After inoculating cardiac fibroblasts into a 24-well glass culture plate and cultivating it to a density of 50%, according to the purpose of the experiment, the corresponding drugs such as TGF-β1 and fluvoxamine were stimulated for 24 h, then the medium was discarded, and precooled PBS rinse 3 times; 4% paraformaldehyde-fixed for 30 min; 0.5% Triton X-100 permeates the cells. Incubate primary antibody α-SMA (1 : 200) or Sig1R (1 : 200) overnight; the next day, incubate secondary antibody: secondary antibody (anti-rabbit Alexa Fluor 488-conjugated secondary antibody, Invitrogen, USA), diluted 1 : 1000 in PBS, incubated at room temperature in the dark for 2 hours. Nuclei staining: cells were incubated with DAPI (1 : 1000), diluted in PBS, and incubated at room temperature for 5 min. Images were collected using a Laser Scanning Confocal Microscopy (FluoView™ FV1000, OLYMPUS, Japan) and analyzed with ImageJ software.

2.8. EdU Assay to Detect Cell Proliferation. Proliferation was detected using EdU Assay Kit (RibobilTM, China) in the cardiac fibroblast as described [33]. The proportion of EdU-incorporated cells was defined as the proliferation rate. The proliferation rate was calculated by normalizing the number of EdU positive cells to the DAPI-stained cells under the fluorescence microscope (Nikon, Japan). Each assay was performed at least three times. Cell proliferation rate = number of EdU − incorporated cells/total number of cells.

2.9. Wound Healing Assay. Wound-healing assays were used to measure the migration of CFs according to our previous report [30]. When the cell confluence in the 6-well plate reaches 50%, treat it before scratching: use a 200 μl sterile tip to scratch and take a picture at the bottom of the well plate, and take another picture at the same position after the stimulation for comparison. 6 fields of vision were collected in each group, and ImageJ software was used to determine the scratch area. Cell migration rate = (0 h scratch area − 24 h scratch area)/0 h scratch area.

2.10. Small Interfering RNA (siRNA) Transfection. Cells grown to 40-50% confluence were transferred to 6-well plates. They were transfected using transfection reagent riboFECT™ CP (RiboBio™, China). siRNA targeting Sig1R (si-Sig1R) was transfected into NRCFs for 24 hours then treated with TGF-β1 for 48 hours. Individual siRNAs (100 nM, RiboBio™, China), ribo-FECT™ CP reagent and buffer, and DMEM were combined and then incubated for 15 minutes at room temperature. The experiment was divided into four groups: siNC group, siNC+T group, si-Sig1R group, and si-Sig1R+T group.

2.11. mRFP-GFP-LC3 Adenovirus Transfection. Autophagy was detected by mRFP-GFP-LC3 adenovirus transfection [34]. Cells were transfected with mRFP-GFP-LC3 adenovirus (Hanbio Biotech, Shanghai, China) for 24 h and then pretreated with fluvoxamine or NE-100, prior to TGF-β1 administration. Treated cells were fixed with 4% paraformaldehyde in PBS, and images were obtained using a laser scanning confocal microscope. Merged fluorescence from RFP and GFP was analyzed with Pearson's correlation coefficient, and 15 cells were used for quantification in each group.

2.12. Transmission Electron Microscopy Assay. TEM assay was performed as our previous study [30]. Cardiac fibroblasts were washed in precold PBS and then fixed in cold 2.5% glutaraldehyde for 2 h at 4°C; cells were washed with PBS (0.2 mol/L, pH 7.4) for 2 h, fixed with 1% osmic acid for 2 h,

and then washed six times with PBS for 10 min per wash. The samples were dehydrated with ethanol and cleaned with epoxypropane. They were embedded in EPON812 overnight at room temperature. Ultrathin sections (40–60 nm) were cut (EM UC61rt, Leica) and stained with uranyl acetate/lead citrate. Autophagosomes and autolysosomes were observed using a transmission electron microscope from Hitachi (H-7650).

2.13. Antibodies and Reagents. Antibodies used in this study included anti-Sig1R (Abcam, ab53852), anti-POSTN (Abcam, ab14041), anti-α-SMA (Abcam, ab32575), anti-CTGF (Abcam, ab6992), anti-TGF-β (Abcam, ab92486), anti-ATF4 (Cell Signaling Technology, 11815), anti-p-PERK (Cell Signaling Technology, 3173), anti-IRE1α (Cell Signaling Technology, 3294), anti-Xbp1s (Cell Signaling Technology, 82914), anti-LC3B (Abcam, ab92486), anti-ATG7 (Cell Signaling Technology, 8558), anti-GAPDH (Cell Signaling Technology, 5174), anti-P62 (Cell Signaling Technology, 88588), and anti-c-ATF6 (Abcam, ab62576), all obtained from rabbits, and anti-β-Actin (Cell Signaling Technology, 3700) from mice. The following reagents were used: TGF- β1 (10 ng/ml, Sino Biology), fluvoxamine (MCE, HY-B0103A), NE-100 (Sigma-Aldrich, SML0631), 4μ8C (MCE, HY-19707), thapsigargin (MCE, HY-13433), and 4-PBA (Sigma-Aldrich, SML0309).

2.14. Statistics. The data was shown as the average of at least 3 independent experiments (mean ± SEM). Student's t-test was used to compare two data sets and analyze the variance of multiple data sets. Significance is defined as $p < 0.05$. The statistical software used is Prism v.7.

3. Results

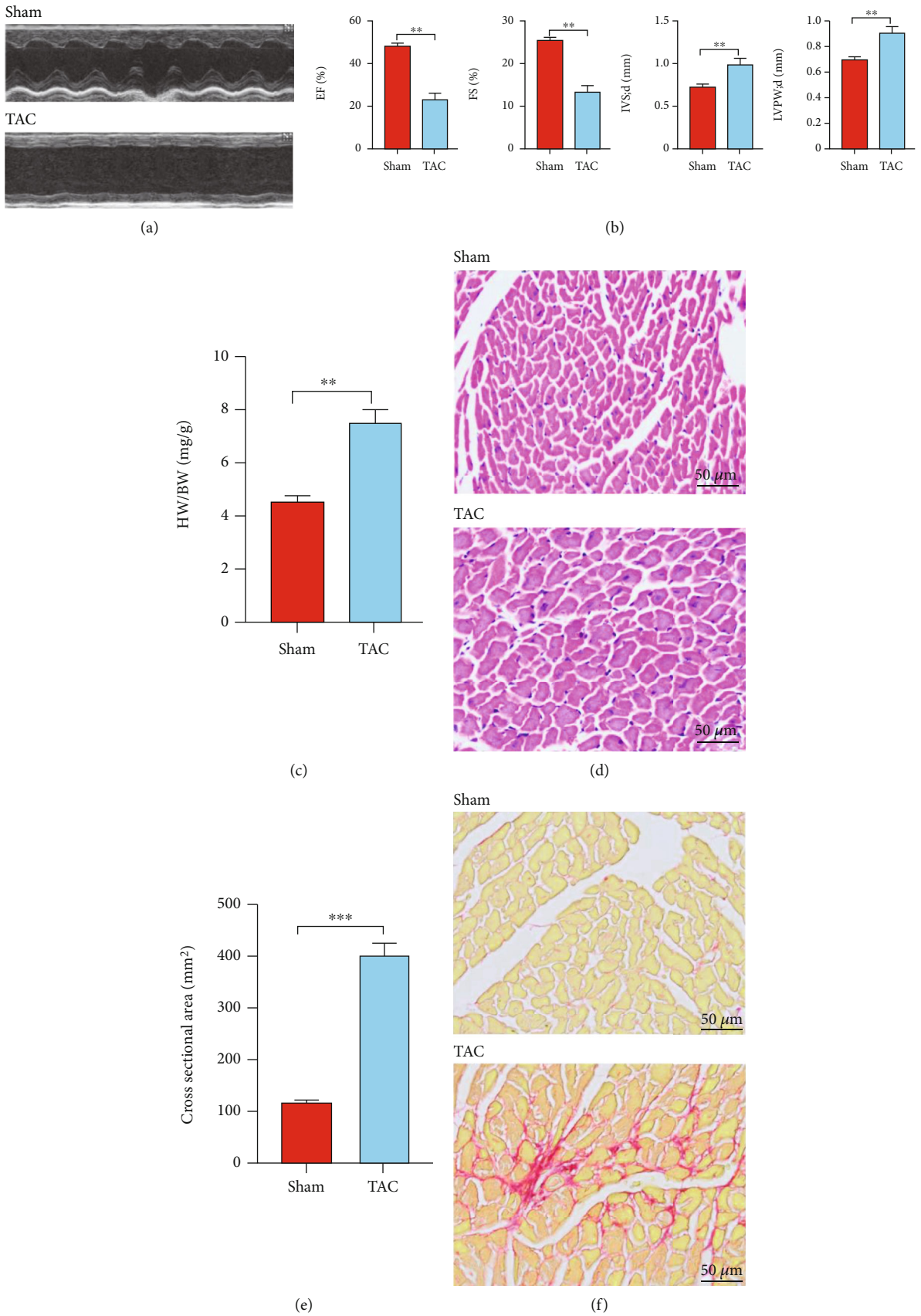
3.1. The Expression of Sig1R Is Decreased in Fibrotic Heart Tissues of TAC Mice and in Activated Cardiac Fibroblasts. To understand the expression of Sig 1R in pathological myocardium, we established a cardiac hypertrophy model with TAC surgery. The TAC mice versus sham-operated mice revealed obvious cardiac function decline (Figures 1(a) and 1(b)) and a higher heart weight to body weight ratio (Figure 1(c)). Cardiac function of TAC mice was significantly decreased as shown by lower fractional shortening (FS), ejection fraction (EF), diastolic interventricular septum (IVS), and left ventricular posterior wall (LVPW) thickness (Figure 1(b)). Hematoxylin/Eosin (HE) staining showed the TAC mice model exhibited significant cardiac hypertrophy (Figures 1(d) and 1(e)). Sirius red (Figures 1(f) and 1(g)) and Masson trichrome (Figures 1(h) and 1(i)) staining showed that cardiac fibrosis was successfully induced in our TAC model. The mRNA expression of cardiac fibrosis markers, collagen I (COL-1), periostin (POSTN), connective tissue growth factor (CTGF), and transforming growth factor-β (TGF-β) was significantly increased by 1.5-, 5.8-, 2.9-, and 1.5-fold, respectively, when compared with expression in the Sham group (Figure 1(j)). The protein expressions of POSTN, α-SMA, CTGF, and TGF-β were increased by 5.6-, 2.5-, 2.4-, and 2.2-fold, respectively, in the TAC group compared with the Sham group (Figures 1(k) and 1(l)). Simultaneously, the mRNA and protein expressions of Sig1R were decreased by 30% and 55%, respectively, compared to expressions in the Sham group (Figures 1(m) and 1(o)).

The fibrotic markers in the activation of cardiac fibroblasts induced by fibrotic agonist TGF-β1 for 24 h are shown in Figure 2. Compared with the control group, the TGF-β group mRNA expressions of POSTN, COL-1, CTGF, and TGF-β were increased by 10.0-, 1.5-, 1.6-, and 1.5-fold (Figure 2(a)), respectively, and the TGF-β group protein expressions of POSTN, CTGF, and TGF-β increased by 15.0-, 1.4-, and 1.6-fold (Figures 2(b) and 2(c)), respectively. Additionally, immunofluorescence staining showed the upregulation of α-SMA induced by TGF-β1 in cardiac fibroblasts (Figure 2(d)). The proliferation (Figures 2(e) and 2(g)) and migration (Figures 2(f) and 2(h)) capacities were also increased in the TGF-β1-stimulated cardiac fibroblasts. Under the stimulated condition, the mRNA and protein expressions of Sig1R in the activation of cardiac fibroblasts were decreased by 58% and 30%, respectively, compared with the control group (Figures 2(i)–2(k)). Moreover, immunofluorescence staining confirmed the decrease of Sig1R in the activation of cardiac fibroblasts (Figure 2(l)). These results indicated that the expression of Sig1R was decreased during the process of cardiac fibrosis.

3.2. Stimulation of Sig1R Attenuates the Activation of Cardiac Fibroblasts In Vitro. Pretreatment of cardiac fibroblasts with fluvoxamine for 2 h before TGF-β1-stimuli decreased the expressions of the fibrosis marker POSTN, CTGF, and TGF-β by 50%, 23%, and 22%, respectively (Figures 3(a) and 3(b)). Furthermore, immunofluorescence staining confirmed a significant reduction in α-SMA expression (Figure 3(c)). Evaluation of cardiac fibroblast proliferation by the EdU incorporation assay revealed a significant reduction in cell proliferation by fluvoxamine pretreatment in activated cardiac fibroblasts (Figures 3(d) and 3(f)). In addition, we found that fluvoxamine did not affect cell proliferation in cardiac fibroblasts, which is not treated with TGF-β1.

Fluvoxamine-pretreated cells also displayed a significant reduction in migration in the scratch-wound healing assay (Figures 3(e) and 3(g)). Taken together, these findings suggested that the stimulation of Sig1R has a potential role in diminishing myofibroblast proliferation and reducing cell migration, as well as ameliorating the activated myofibroblast phenotype.

The role for Sig1R in this pathological condition was further verified, as cardiac fibroblasts pretreated with NE-100, a Sig1R antagonist, prior to TGF-β1 administration showed a more active phenotype than cells treated only with TGF-β1. Western blot revealed the upregulation of protein expressions of POSTN, CTGF, and TGF-β by 1.5-, 1.6-, and 1.6-fold, respectively, in the N+TGF-β group (Figures 4(a) and 4(b)), and immunofluorescence staining also showed the increased protein expression of α-SMA in the N+TGF-β group compared with the control group (Figure 4(c)). NE-100 further promoted the proliferation (Figures 4(d) and 4(f)) and migration (Figures 4(e) and 4(g)) of activated cardiac fibroblasts, supporting a promotion of cardiac fibroblast activation by blocking of Sig1R activity.



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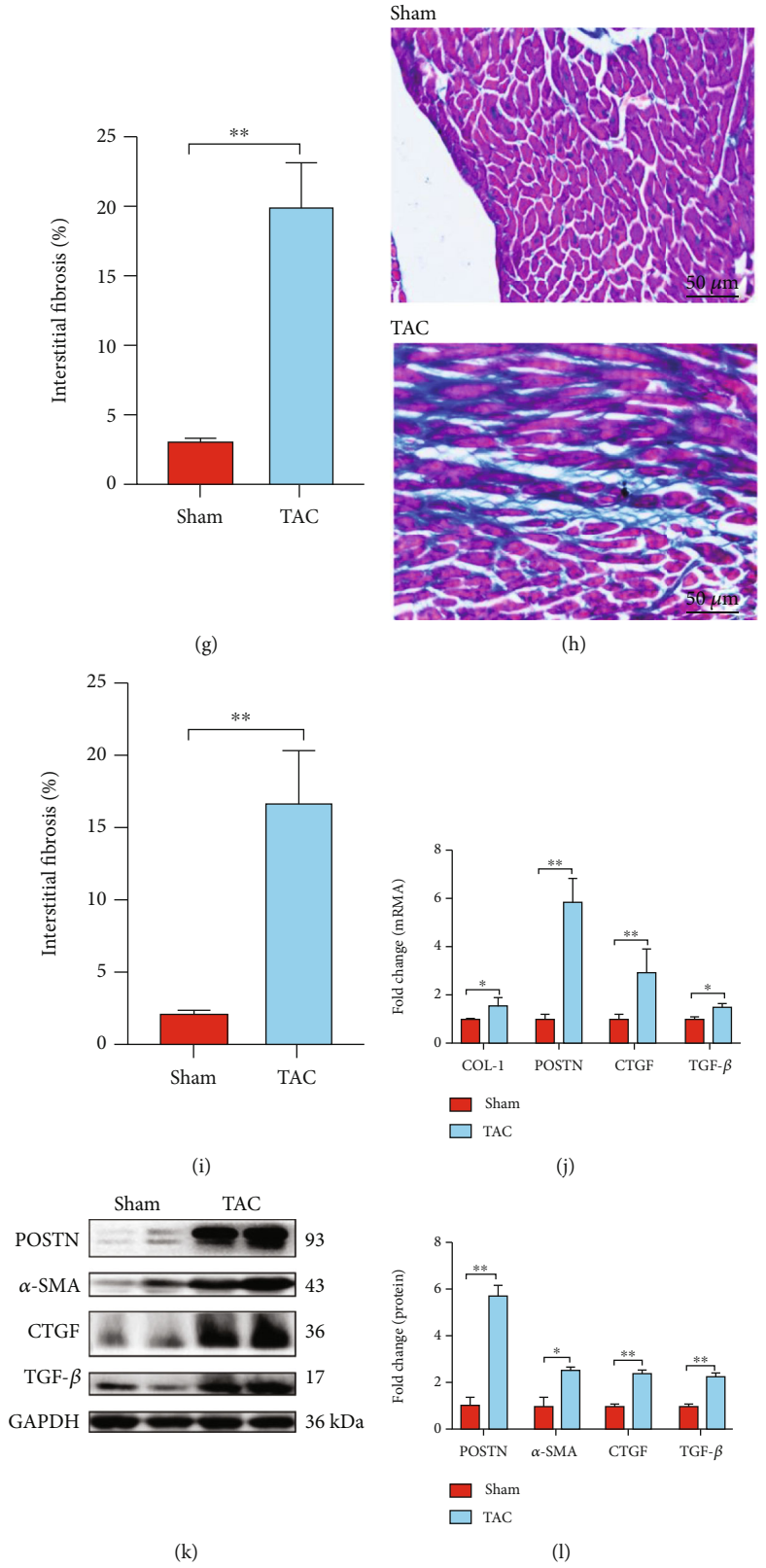

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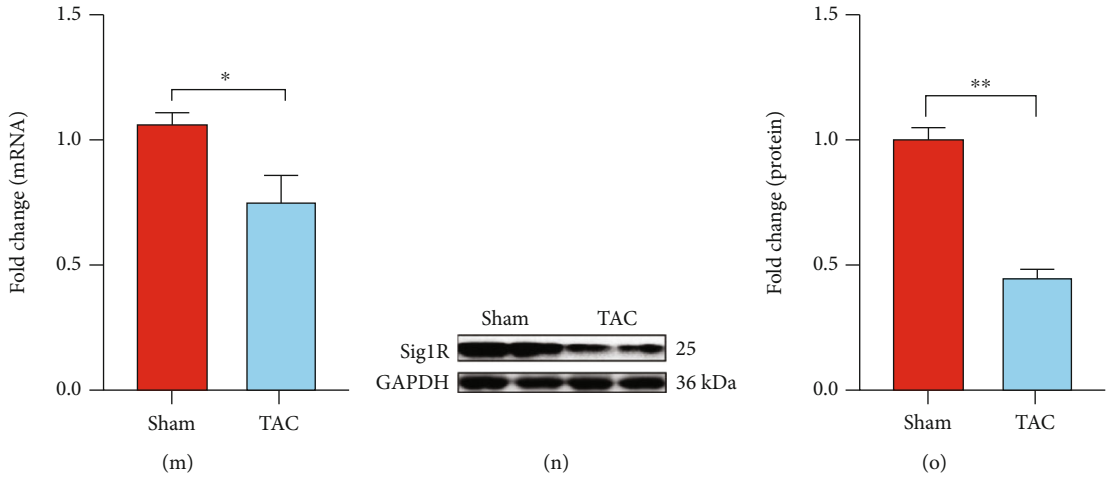


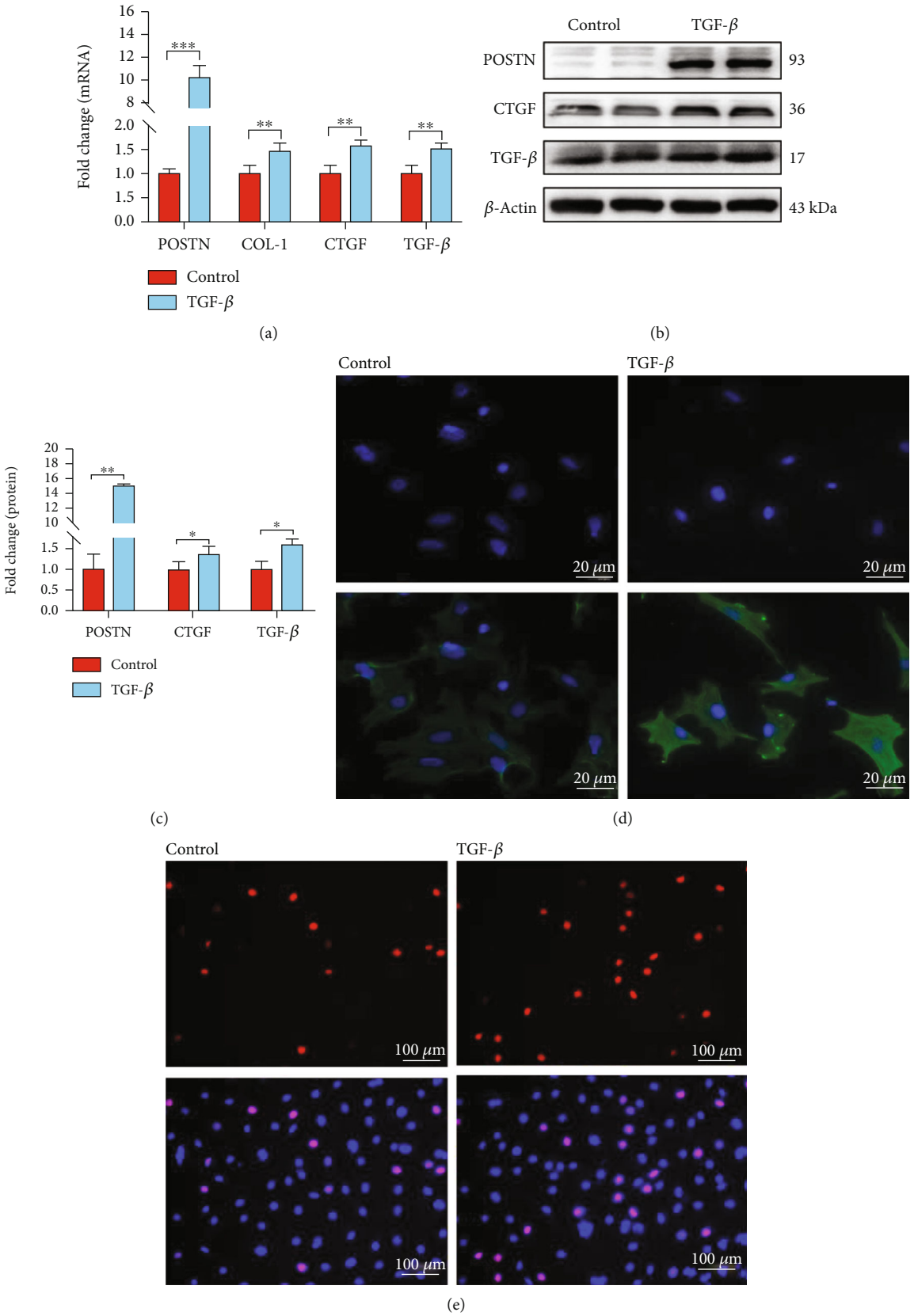
Figure 1: Sig1R is downregulated in mice heart tissue following transverse aortic constriction (TAC) surgery. Mice were randomly divided into two groups: Sham operation and TAC. (a, b) Cardiac function decline and cardiac hypertrophy were evaluated by echocardiography shown by percent EF (ejection fraction) and FS (fractional shortening); diastole IVS (Interventricular Septal) and LVPW (left ventricular posterior wall) thickness. $n = 6$; (c) Cardiac hypertrophy index, HW/BW (heart weight to body weight ratio). $n = 6$; (d, e) Representative cross-sectional images of hematoxylin/eosin-stained cardiomyocytes. Scale bar = 50 μm. $n = 6$. (f–i) Heart sections were stained with Sirius red and Masson trichrome to visualize fibrosis (red and blue). Scale bar = 50 μm. $n = 6$. (j) The mRNA levels of COL-1(collagen I), POSTN (periostin), α-SMA (α-Smooth Muscle Actin), CTGF (connective tissue growth factor), and TGF-β (transforming growth factor-β) in mice heart tissue. $n = 6$; (k, l) The protein levels of POSTN, α-SMA (α-Smooth Muscle Actin), CTGF, and TGF-β in mice heart tissue. $n = 6$; (m–o) The mRNA and protein levels of Sig1R (Sigma-1 receptor) in mice heart tissue. $n = 6$. Shown are representative pictures; statistical significance was determined by unpaired t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.

To further determine the role of Sig1R in the activation of cardiac fibroblast and exclude the several off-target effects of small molecule inhibitors, we used Sig1R siRNA to specifically silence the expression of Sig1R under basic conditions or stimulated by TGF-β1. As shown in Figures 5(a) and 5(b), compared with the negative control group (siNC+T group) treated with TGF-β1, after silencing Sig1R and then TGF-β1 stimulating for 24 h (si-Sig1R + T group). Cardiac fibroblast activation protein marker POSTN, CTGF, and TGF-β were increased by 1.4-, 1.3-, and 1.3-fold, respectively. The results indicated that the silencing of Sig1R expression by siRNA further promoted the activation of cardiac fibroblast. This result is consistent with the effect of small molecule inhibitors.

Collectively, these data demonstrated that the stimulation of Sig1R might be a therapeutic candidate for cardiac fibroblast activation.

3.3. Treatment with Sig1R Agonist Reduces Mice Cardiac Fibrosis and Preserves Cardiac Function. Mice injected intraperitoneally with fluvoxamine (1 mg/kg) once daily [35] for 4 weeks consecutive days after TAC operation showed changes in cardiac function and dimensions detectable by echocardiography (Figures 5(c)–5(f)). Cardiac dysfunction was observed in TAC mice in the form of reduced FS and EF and increased IVS and LVPW (Figure 5(d)) when compared with the sham group. Interestingly, fluvoxamine-treated animals exhibited an attenuation of cardiac function decline in terms of pathologic hypertrophy (Figures 5(d)–5(g)). This observed attenuation of cardiac hypertrophy was confirmed by a reduction in the cell size in heart tissue after fluvoxamine administration (Figures 5(e)–5(g) and 5(j)). Fluvoxamine treatment also decreased collagen deposition, as shown by Sirius red (Figures 5(h) and 5(k)) and Masson (Figures 5(i) and 5(l)) staining. Q-PCR (Figure 5(m)), and western blotting (Figures 5(n) and 5(o)) confirmed a reduction in the levels of fibrotic markers in the LV at 4 weeks after TAC. Taken together, these data indicated that Sig1R treatment helped to preserve cardiac function and attenuated cardiac fibrosis after TAC.

3.4. Sig1R Regulates ER Stress through Inhibition of the IRE1α Signaling in Activated Cardiac Fibroblasts. The decreased expression of Sig1R in TGF-β1-activated cardiac fibroblasts (Figures 6(a)–6(d)) and fibrotic hearts (Figures 6(e) and 6(f)) was associated with ER stress in the present study. As shown in Figures 6(a)–6(d), the administration of TGF-β1 upregulated the levels of the ER stress markers phosphorylated IRE1α (p-IRE1α), spliced Xbp1 (Xbp1s), phosphorylated PERK (p-PERK), ATF4, and cleaved ATF6 (c-ATF6). By contrast, pretreatment with fluvoxamine reversed these inductions (Figures 6(a) and 6(b)), whereas NE-100 pretreatment exacerbated the inductions (Figures 6(c) and 6(d)). ER stress was also activated in the mice model of pressure overload-induced cardiac fibrosis, as shown in Figures 6(d) and 6(f). After TAC surgery, fluvoxamine was injected intraperitoneally for 4 consecutive weeks (FLV group). The protein expression levels of p-PERK, p-IRE1α, ATF4, XBP1s, and c-ATF6 were reduced by 30%, 22%, 25%, 18%, and 15%, respectively (Figures 6(e) and 6(f)). Treatment with fluvoxamine followed by treatment with the ER stress activator thapsigargin decreased the protein expression levels of POSTN and CTGF when compared with thapsigargin treatment only (Figures 6(g) and 6(i)). Treatment with NE-100,



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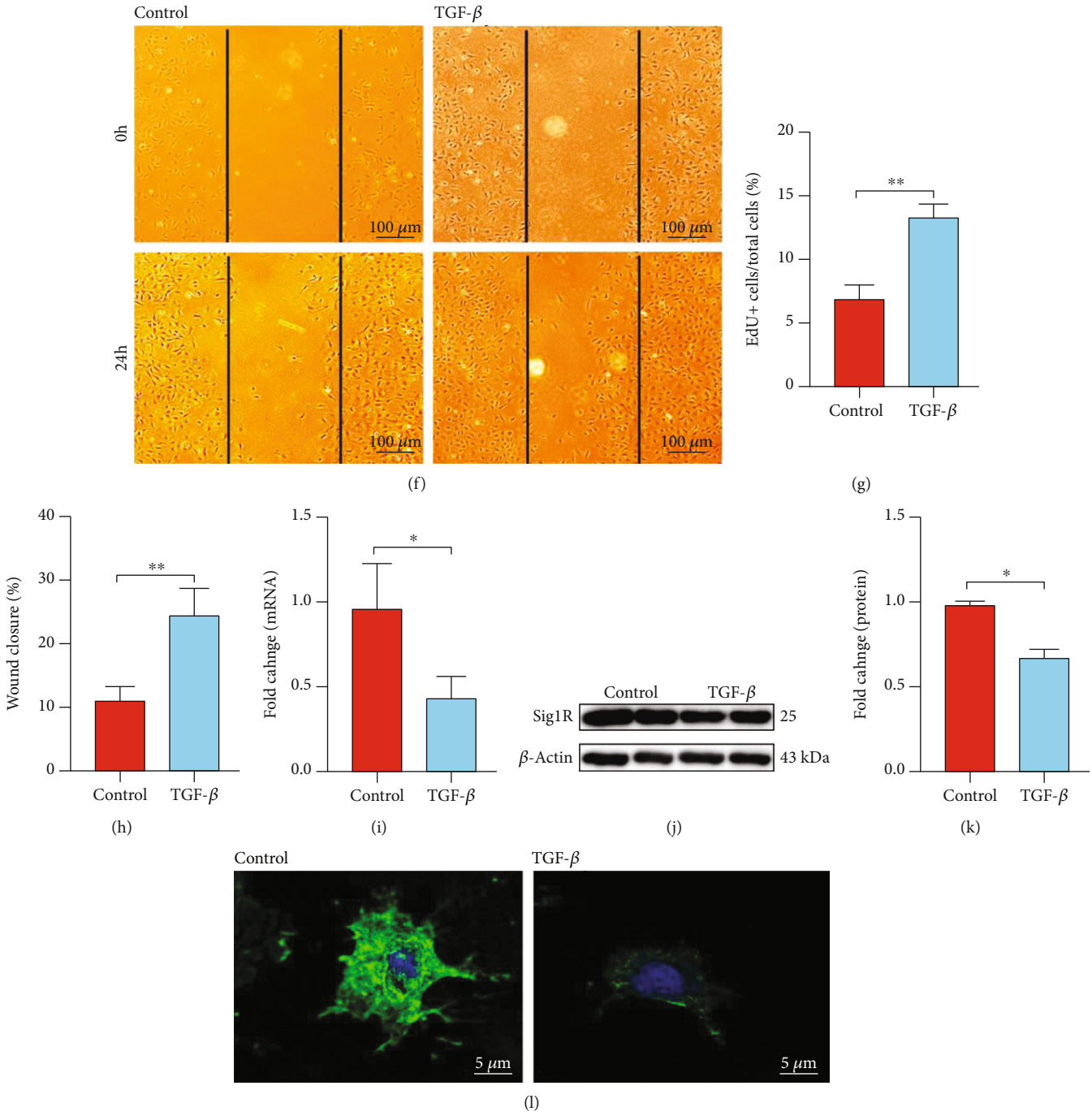
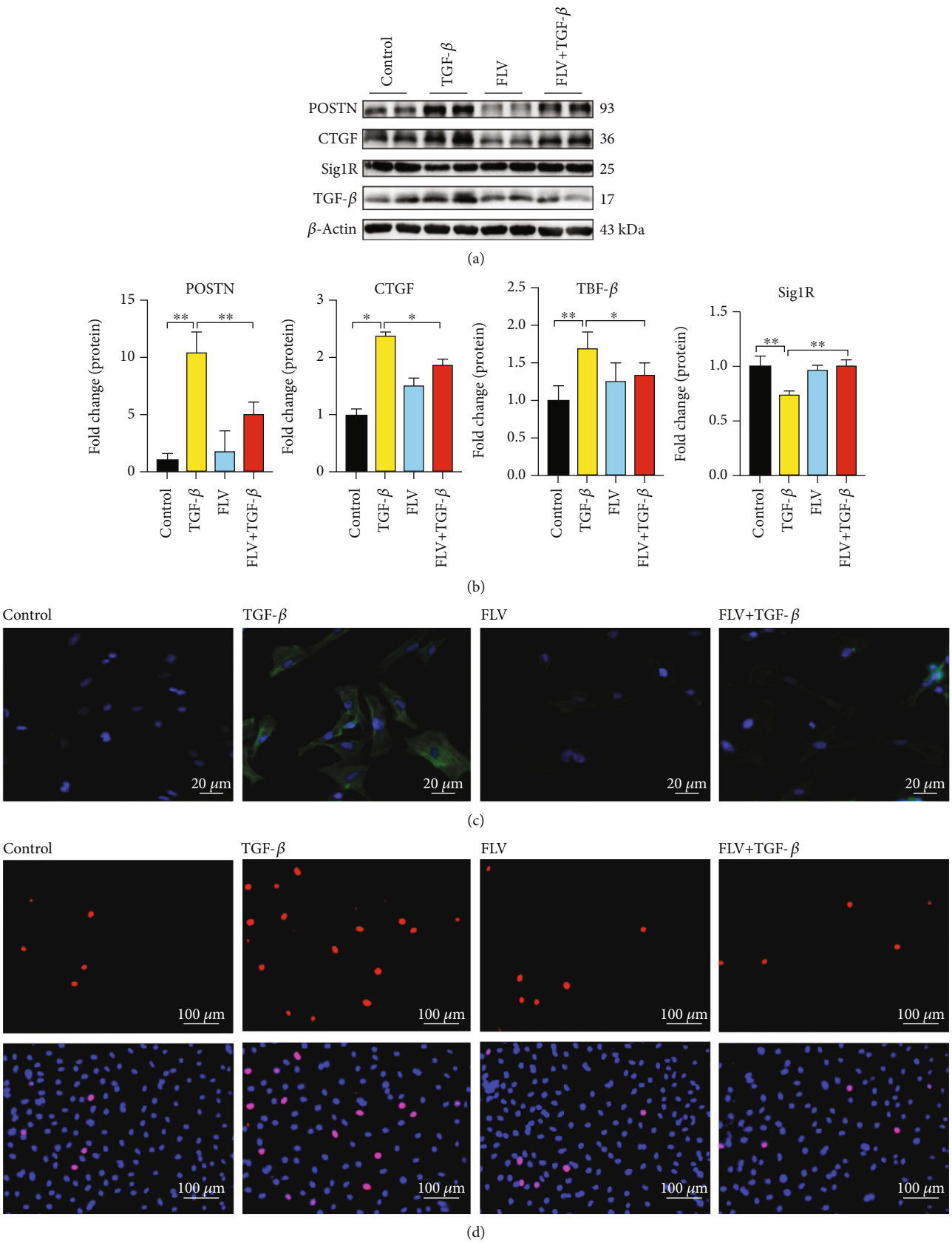


Figure 2: Sig1R is downregulated in the activation of cardiac fibroblasts induced by TGF-β1. Cardiac fibroblasts were randomly divided into two groups. (a) The q-PCR results of COL-1, POSTN, CTGF, and TGF-β in cardiac fibroblasts from control and TGF-β1 treatment (TGF-β) groups. $n = 4$; (b, c) The representative western blot results of POSTN, CTGF, and TGF-β in cardiac fibroblasts from control and TGF-β1 treatment (TGF-β) groups. $n = 3$; (d) Representative images of α-SMA fluorescence of cardiac fibroblasts were shown (the green fluorescence indicates α-SMA and the blue fluorescence indicates the nucleus stained by DAPI). Scale bar = 20 μm, $n = 100$; (e, g) The proliferation rate of cardiac fibroblasts was assessed by EdU assay (the red fluorescence indicates cells that incorporated EdU and the blue fluorescence indicates the nucleus stained by Hoechst 33342). Scale bar = 100 μm, $n = 200$; (f, h) Scratch wound-healing assay showing cardiac fibroblast migration; images were taken at 0 and 24 h postscratch. Black lines denote the wound borders. Scale bar = 100 μm. $n = 6$; (i–k) The mRNA levels of Sig1R were assessed by q-PCR. $n = 4$; (j, k) The representative western blot result of Sig1R in cardiac fibroblasts from Control and TGF-β1 treatment (TGF-β) groups. $n = 3$; (l) Representative images of Sig1R fluorescence of cardiac fibroblasts were shown (the green fluorescence indicates Sig1R expression and the blue fluorescence indicates the nucleus stained by DAPI). Scale bar = 5 μm. $n = 50$. Shown are representative pictures, p was determined by unpaired t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.



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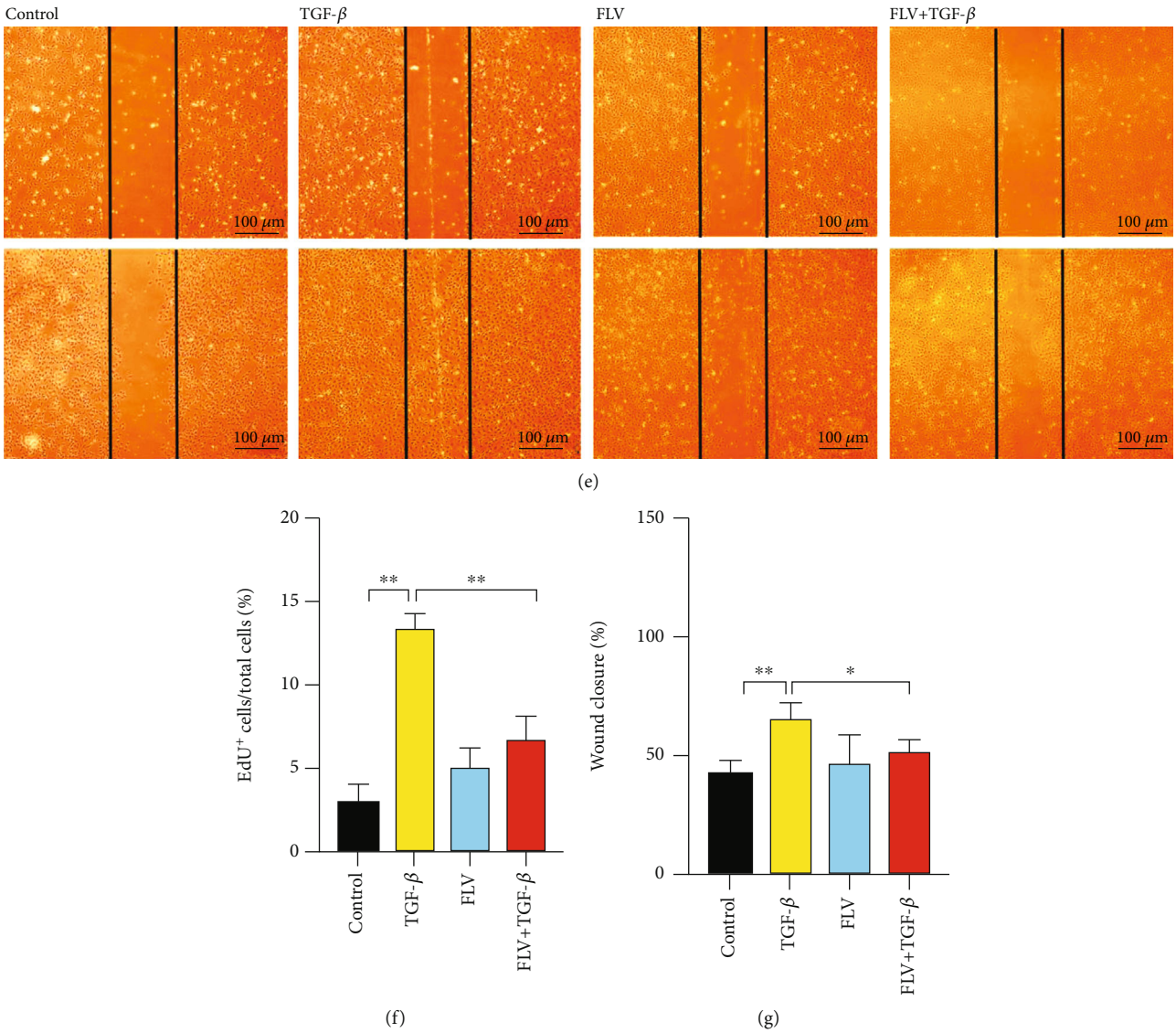
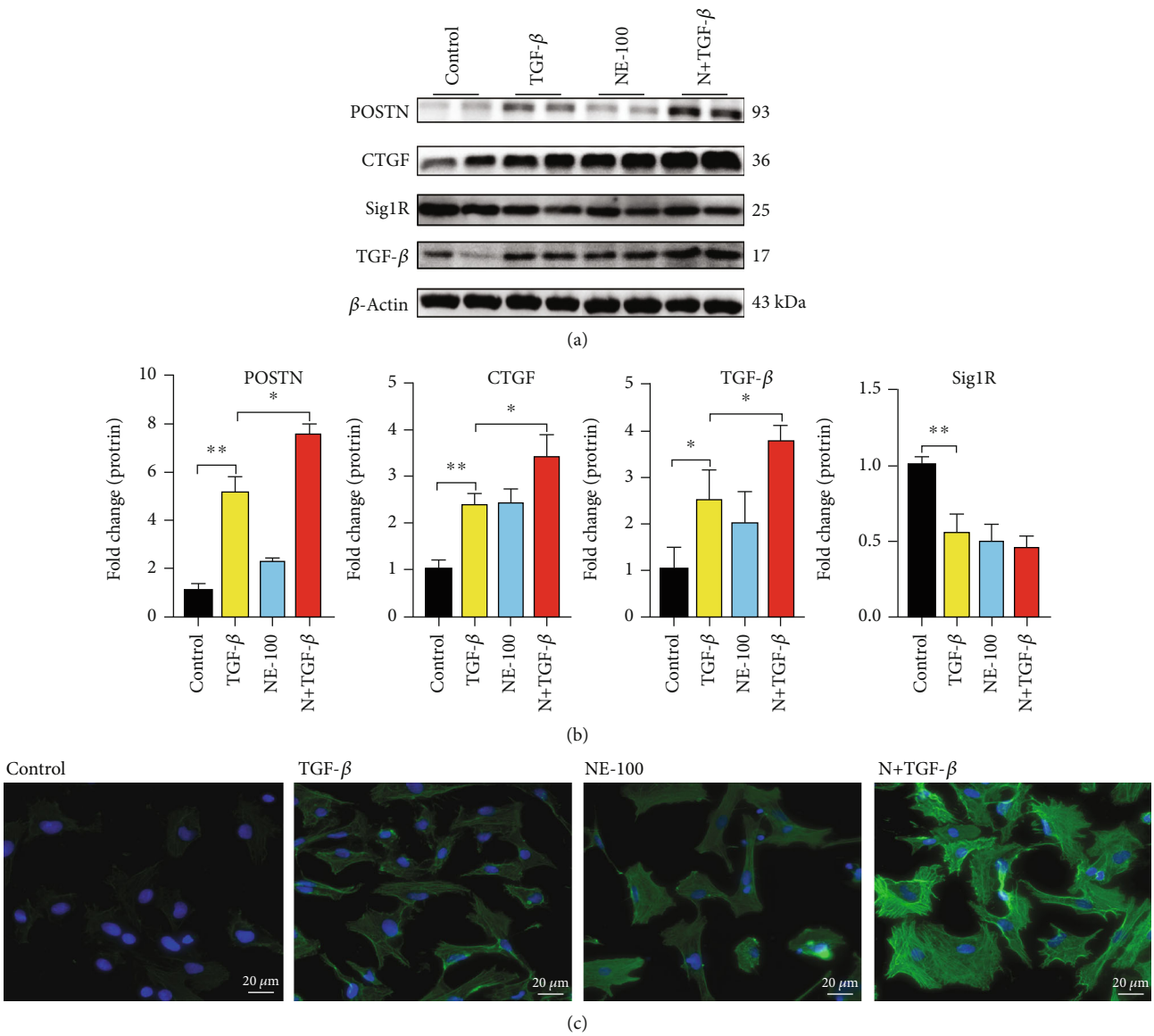


Figure 3: Stimulation of Sig1R attenuates cardiac fibroblast activation. Cardiac fibroblasts were randomly divided into four groups: control, TGF-β, FLV, and FLV+TGF-β. (a, b) The representative western blot results of POSTN, CTGF, and TGF-β in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, fluvoxamine treatment (FLV), or fluvoxamine combined with TGF-β1 treatment (FLV+TGF-β) groups. $n = 3$; (c) Representative images of α-SMA fluorescence in cardiac fibroblasts from different groups. Scale bar = 20 μm. $n = 100$; (d, f) The proliferation rate of cardiac fibroblasts from different groups was assessed by EdU assay. Scale bar = 100 μm, $n = 200$; (e, g) Scratch wound-healing assay in cardiac fibroblasts from different groups; images were taken at 0 and 24 h postscratch. Black lines denote the wound borders. Scale bar = 100 μm. $n = 6$; Shown are representative pictures, p was assessed by one-way ANOVA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.

followed by treatment with the ER stress inhibitor 4-phenylbutyric acid (4-PBA), also decreased the protein expressions of POSTN and CTGF when compared with NE-100 administration only (Figures 6(h) and 6(j)). Notably, TGF-β1 administration increased the expression levels of p-PERK, p-IRE1α, and ATF4, respectively (Figures 6(a)–6(d)), but the expression of c-ATF6 was not significantly altered. IRE1α appeared to be a downstream mediator of Sig1R action in the activation of cardiac fibroblasts, as cells treated with TGF-β1 and NE-100 in the presence of the IRE1α-specific inhibitor 4μ8C showed reduced expression of POSTN and CTGF when compared with the cells without 4μ8C treatment (Figures 6(k) and 6(l)). These findings supported a role for ER stress, and especially the IRE1α signaling, in the decreased expression of Sig1R in activated cardiac fibroblasts.

3.5. Stimulation of Sig1R Ameliorates the Autophagic Flux Impairment in Activated Cardiac Fibroblasts. Sig1R modulates some critical steps in the process of autophagy, and measurement of the LC3-II/LC3-I ratio and P62 expression confirmed that autophagic flux was impaired in activated cardiac fibroblasts and fibrosis heart tissue (Figures 7(a)–7(f)). However, the LC3-II/LC3-I ratio was higher in the



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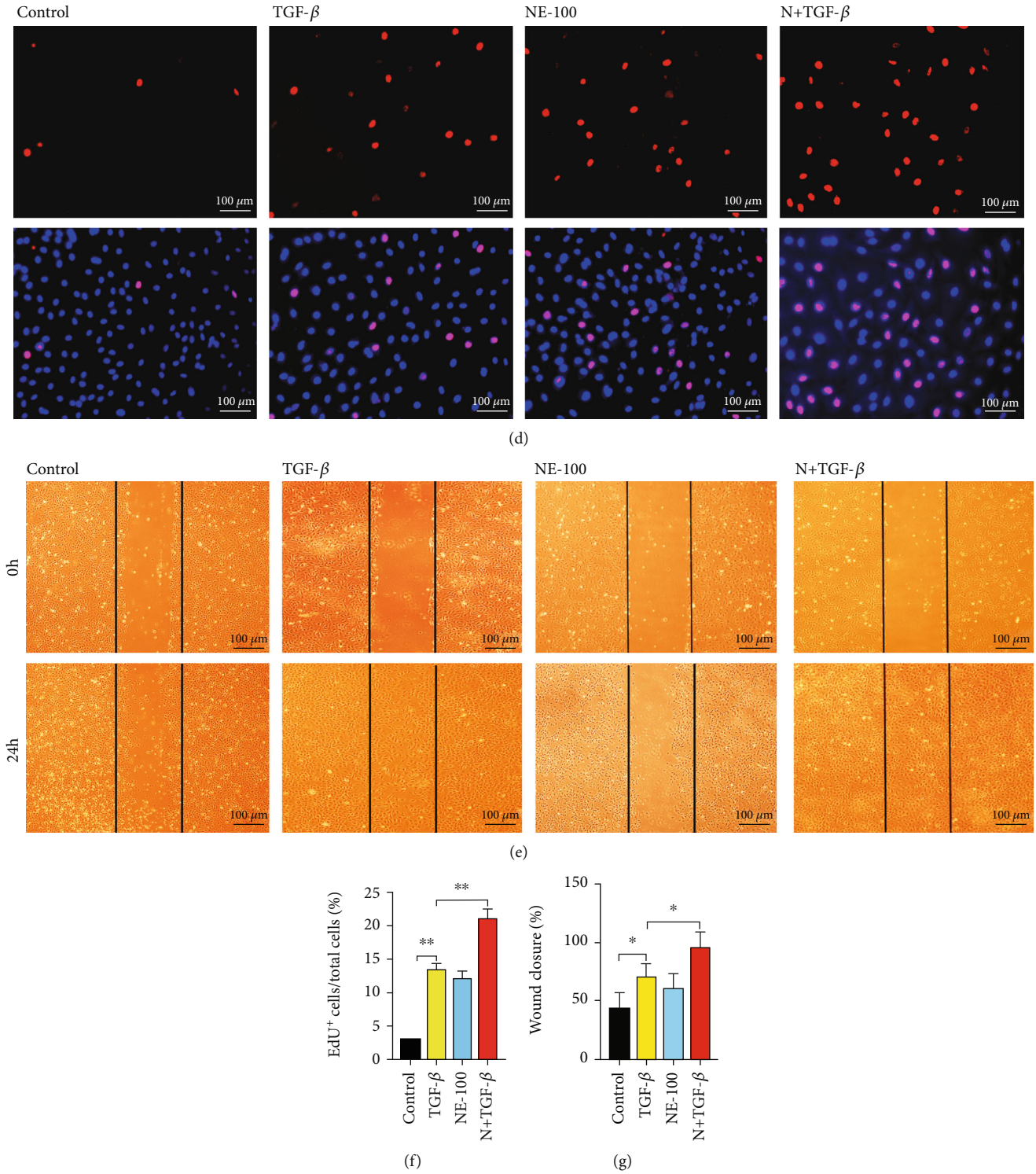
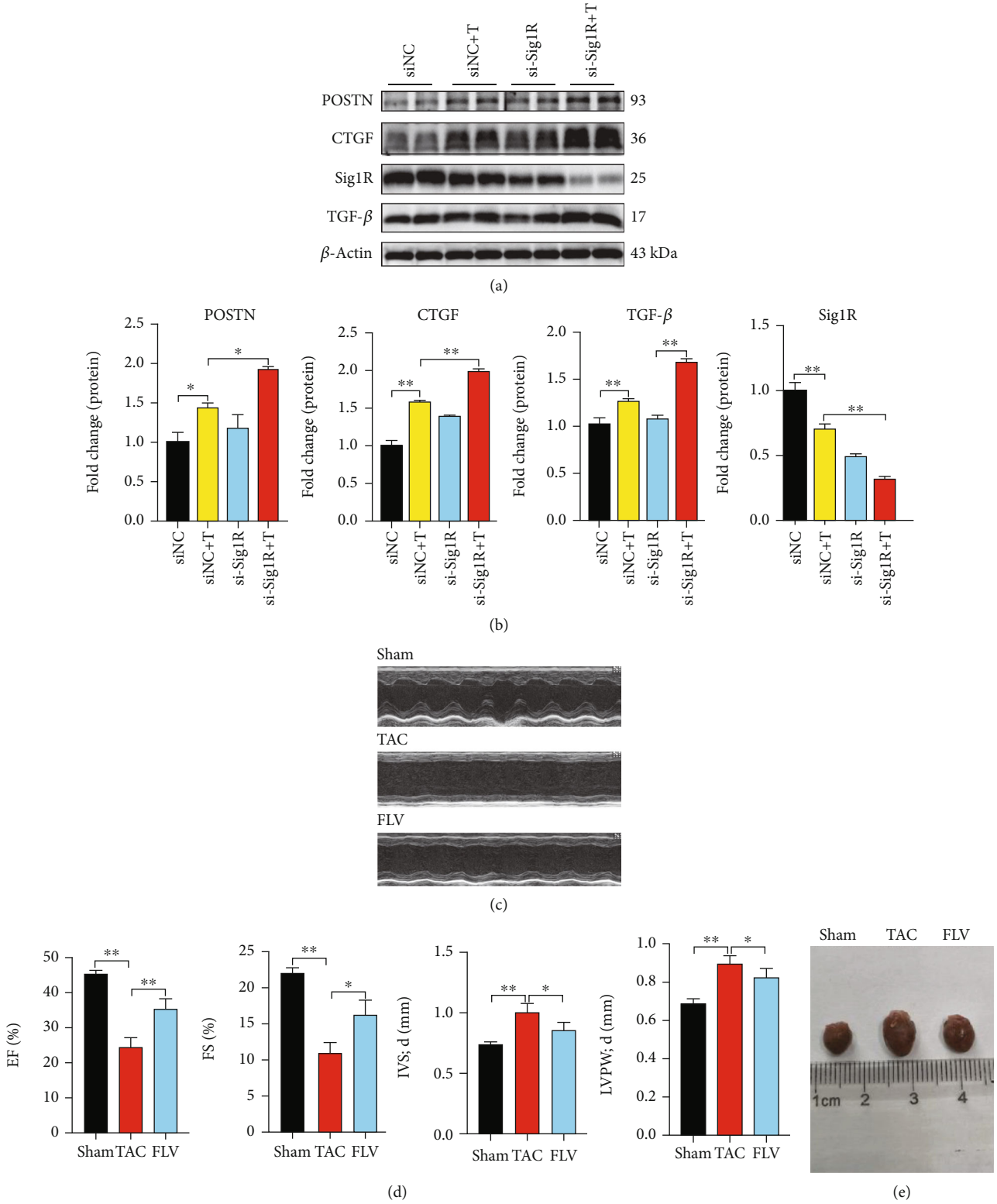


Figure 4: Inhibition of Sig1R further promotes cardiac fibroblast activation. Cardiac fibroblasts were randomly divided into four groups: control, TGF-β, NE-100, and NE-100+TGF-β1. (a, b) The representative western blot results of POSTN, CTGF, and TGF-β in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, NE-100 treatment (NE-100), or NE-100 combined with TGF-β1 treatment (N+TGF-β) groups. $n = 3$; (c) Representative of immunofluorescence staining showed α-SMA (green) in cardiac fibroblasts from different groups. Scale bar = 20 μm, $n = 100$ (d, f) The proliferation rate of cardiac fibroblasts from different groups was assessed by EdU assay. Scale bar = 100 μm, $n = 200$; (e, g) Scratch wound-healing assay in cardiac fibroblasts from different groups; images were taken at 0 and 24 h postscratch. Black lines denote the wound borders. Scale bar = 100 μm. $n = 6$. Shown are representative pictures, p was determined by one-way ANOVA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.



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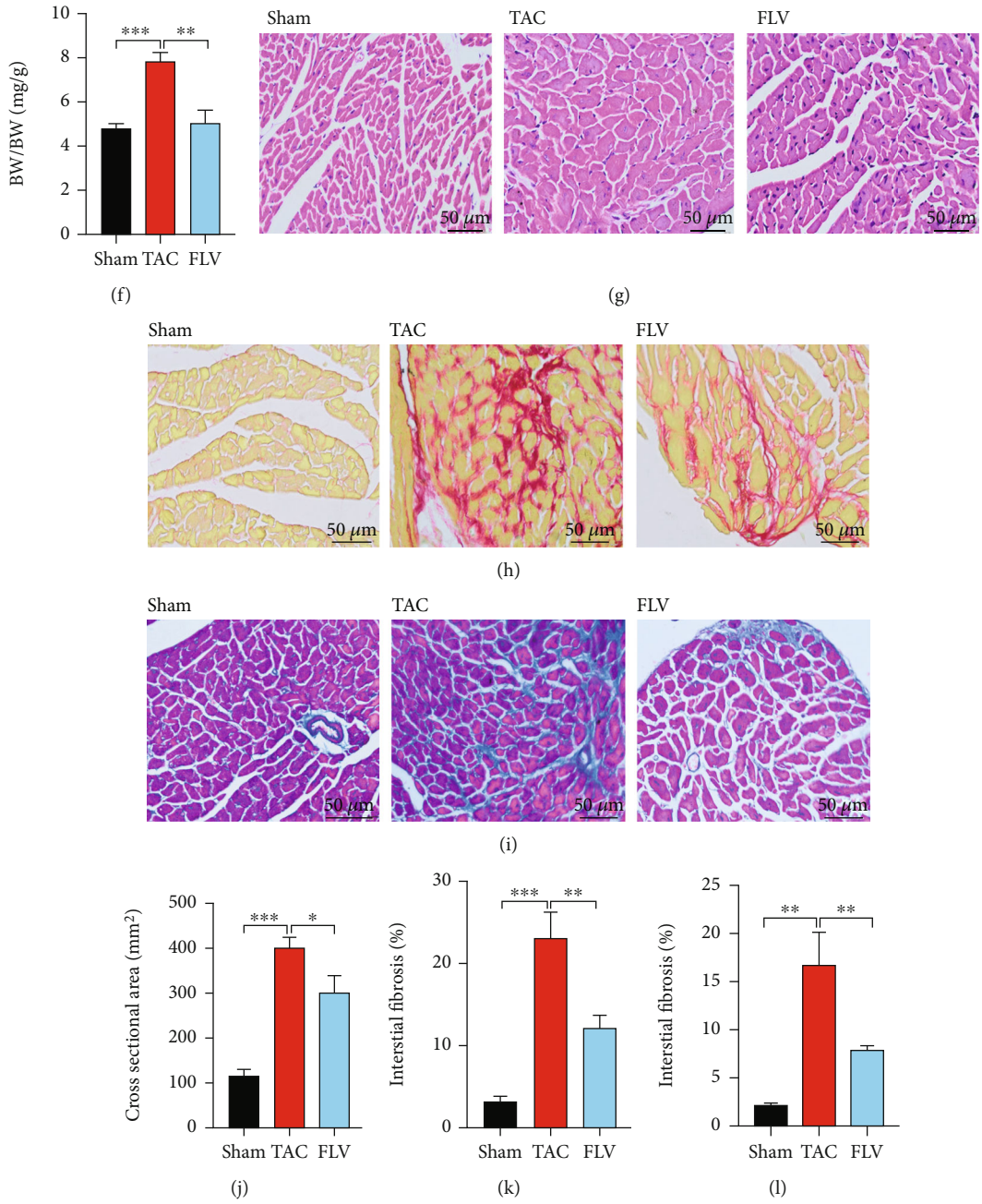

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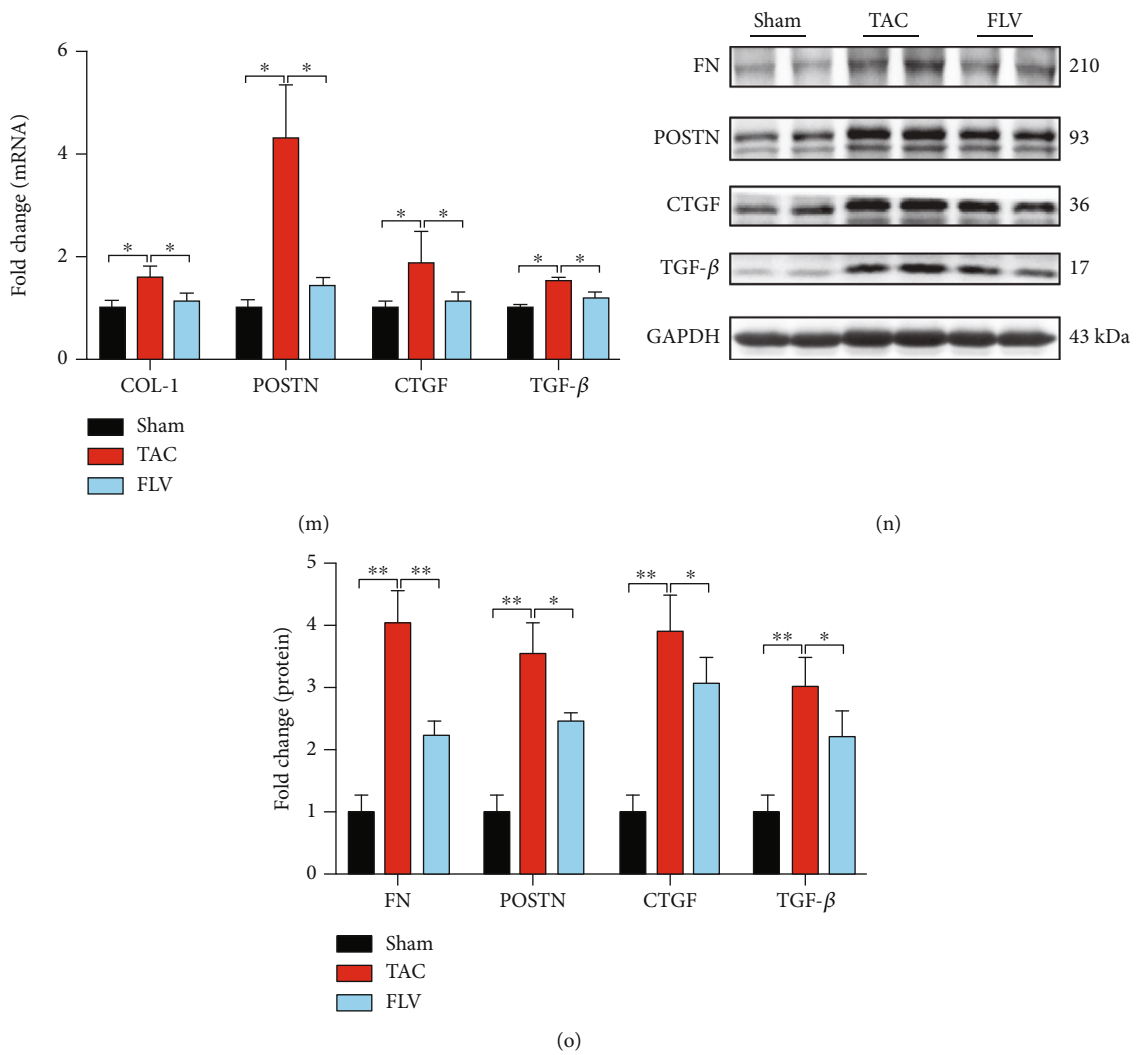
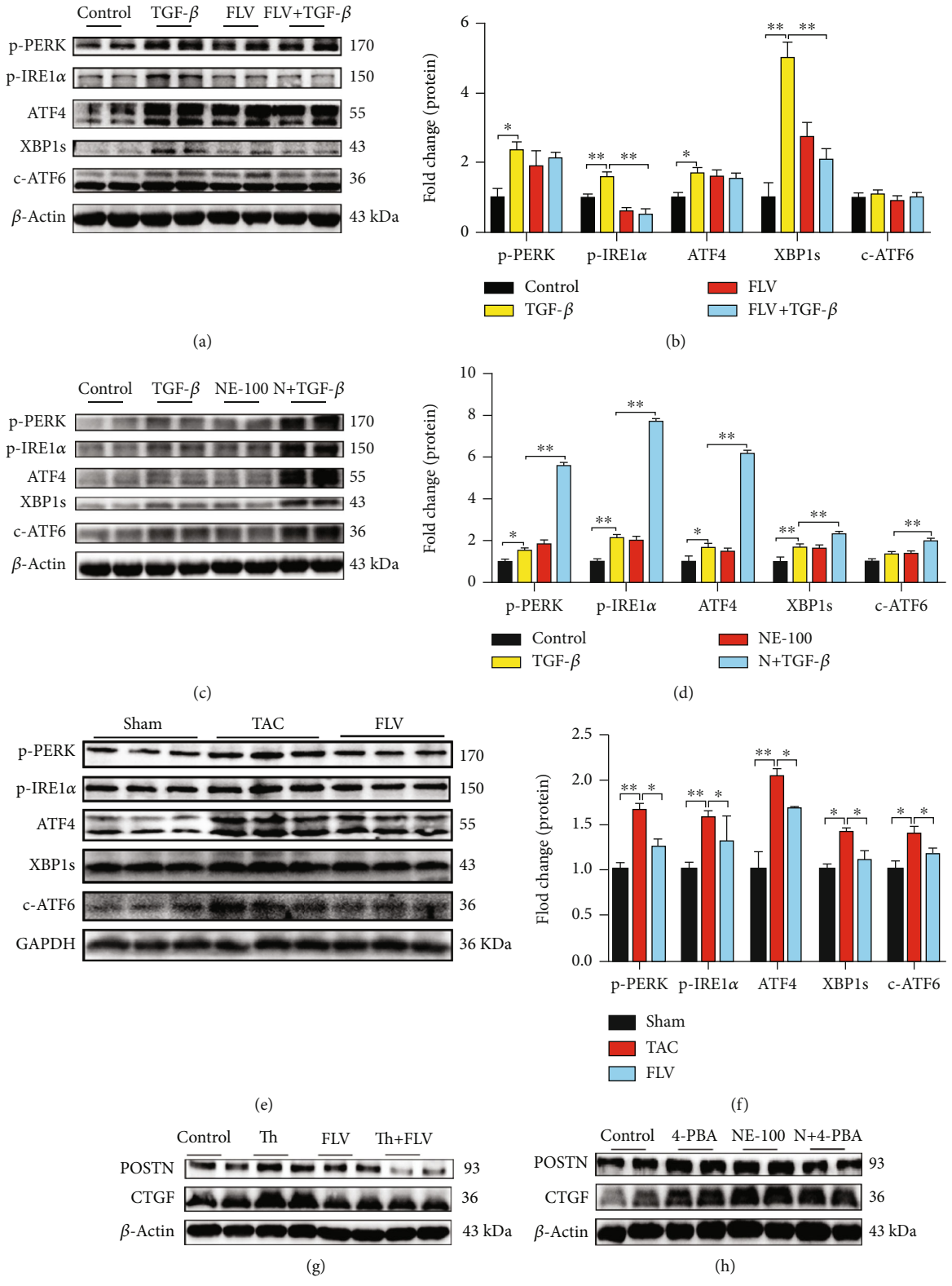


Figure 5: The siRNA of Sig1R further promotes cardiac fibroblast activation; treatment with the Sig1R agonist fluvoxamine reduces cardiac fibrosis and preserves cardiac function 4 weeks post-TAC. Mice were randomly divided into three groups: Sham, TAC, and FLV. (a, b) The protein levels of POSTN, CTGF, Sig1R, and TGF-β in activated cardiac fibroblasts transfected with Sig1R siRNA. $n = 3$; (c, d) Cardiac function and hypertrophy evaluated by echocardiography shown by percent EF and FS; diastole IVS and LVPW thickness. $n = 6$; (e, f) Cardiac images and cardiac hypertrophy index, HW/BW, $n = 6$; (g, j) Representative cross-sectional images of hematoxylin/eosin-stained cardiomyocytes. Scale bar = 50 μm. $n = 6$. (h, i, k, and l) Representative images of Sirius red and Masson trichrome staining of heart tissue are shown to visualize fibrosis (red and blue). Scale bar = 50 μm. $n = 6$; (m) The mRNA levels of COL-1, POSTN, CTGF, and TGF-β in mice heart tissue. $n = 6$; (n, o) The protein levels of FN (Fibronectin), POSTN, CTGF, and TGF-β in mice heart tissue. $n = 6$. Shown are representative pictures, p value was determined by one-way ANOVA analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.

FLV+TGF-β group than in the TGF-β group, but P62 was decreased in the FLV+TGF-β group (Figures 7(a) and 7(b)). Conversely, NE-100 aggravated the autophagic influx impairment induced by TGF-β1 treatment (Figures 7(c) and 7(d)). Additionally, *in vivo* study, the stimulation of fluvoxamine also attenuated the autophagic flux impairment (Figures 7(e) and 7(f)). As shown in Figures 7(g) and 7(h), activated cardiac fibroblasts transfected with mRFP-GFP-LC3 adenovirus showed more autophagosomes (yellow dots) and fewer autolysosomes (red dots). Notably, the numbers of autophagosomes were reduced, and autolysosomes were increased in fluvoxamine pretreated GFP-mRFP-LC3-transfected cardiac fibroblasts, indicating that fluvoxamine restored the autophagic flux in activated cardiac fibroblasts. To further investigate the impact of Sig1R on autophagy, we employed TEM to examine the presence of autophagosomes in cardiac fibroblasts. Relative to the TGF-β group, this analysis revealed a significant increase in the number of autophagosomes in the FLV+TGF-β group, and the number of autophagosomes is significantly reduced in the N+TGF-β group (Figure 8).

4. Discussion

The present investigation of the role of Sig1R in the activation of cardiac fibroblasts revealed the following major



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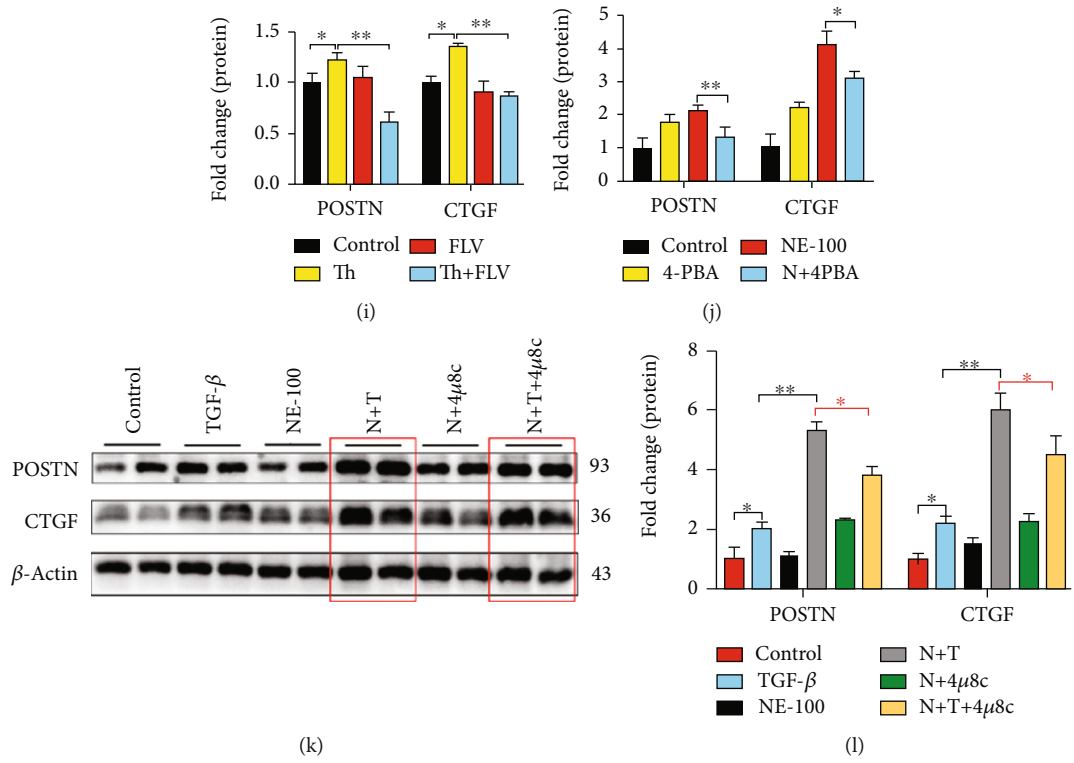
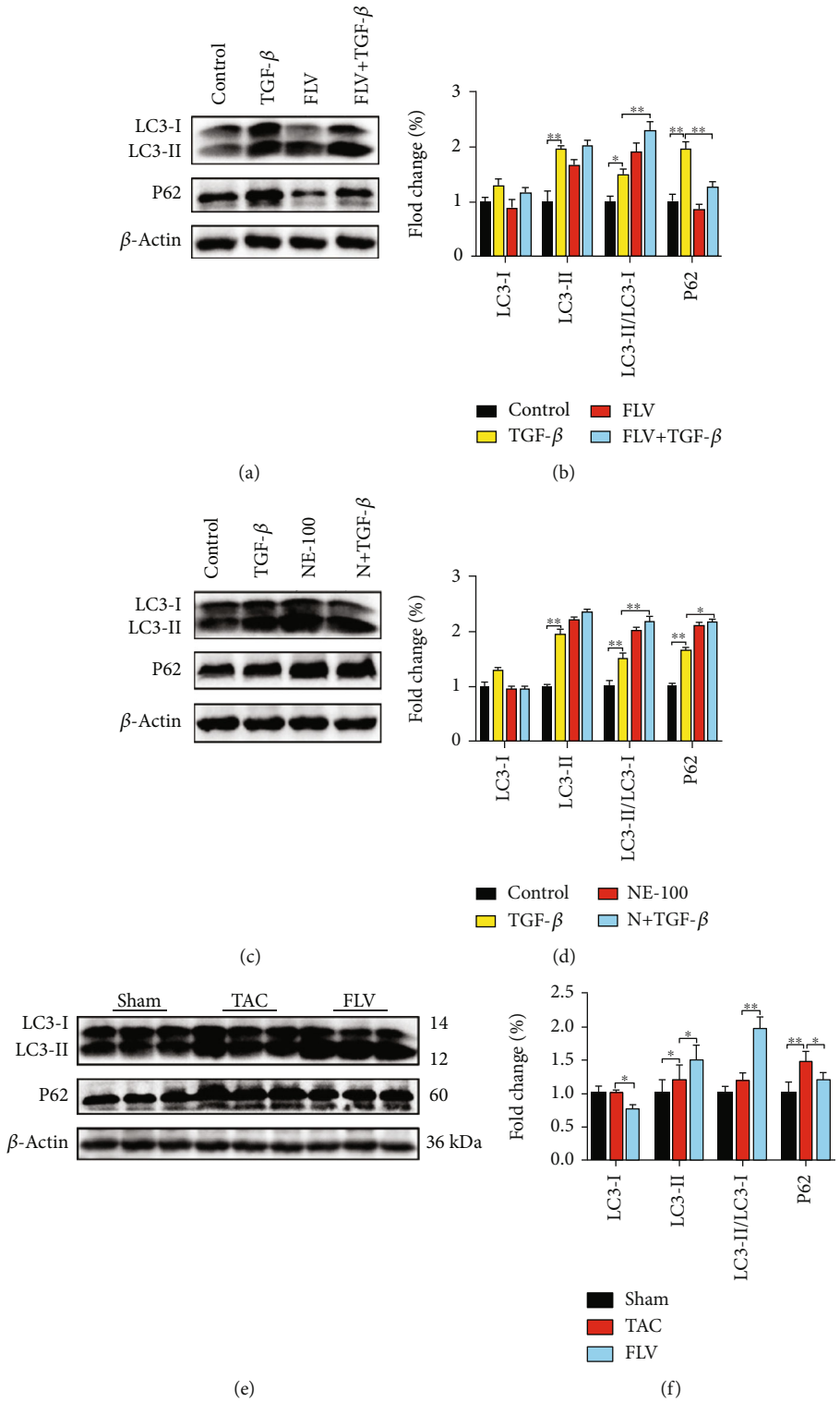


Figure 6: Sig1R protects against cardiac fibrosis by inhibition of ER stress. (a, b) The representative western blot results of p-PERK, p-IRE1α, ATF4, XBP1s, and c-ATF6 in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, fluvoxamine treatment (FLV), or fluvoxamine combined with TGF-β1 treatment (FLV+TGF-β) groups. $n = 3$; (c, d) The representative western blot results of p-PERK, p-IRE1α, ATF4, XBP1s, and c-ATF6 in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, NE-100 treatment (NE-100), or NE-100 combined with TGF-β1 treatment (N+TGF-β) groups. $n = 3$; (e, f) The representative western blot results of p-PERK, p-IRE1α, ATF4, XBP1s, and c-ATF6 in heart tissue from sham-operated (Sham), TAC, and intraperitoneal injection with fluvoxamine after TAC (FLV) groups. $n = 6$; (g, i) The representative western blot results of POSTN and CTGF in cardiac fibroblasts from control, thapsigargin treatment only (Th), fluvoxamine treatment, and thapsigargin combined fluvoxamine treatment (Th+FLV) groups. $n = 3$; (h, j) The representative western blot results of POSTN and CTGF in cardiac fibroblasts from control, 4-PBA treatment only (4-PBA), NE-100 treatment (NE-100), and NE-100 combined 4-PBA treatment (N+4-PBA) groups. (k, l) The protein levels of POSTN and CTGF in cardiac fibroblasts from different groups. $n = 3$. Shown are representative pictures, p value was determined by one-way ANOVA with Tukey post hoc analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.

findings illustrated in Figure 9: (1) The expression of Sig1R is decreased in mice heart tissue following TAC operation and in the activation of cardiac fibroblasts induced by TGF-β1; (2) Stimulation of Sig1R attenuates the activation of cardiac fibroblasts and cardiac fibrosis; (3) The IRE1α pathway mediates the role of Sig1R in the activation of cardiac fibroblasts; (4) Stimulation of Sig1R alleviates the autophagic flux impairment in the activation of cardiac fibroblasts.

Sig1R acts as a pluripotent modulator in many diseases, including Alzheimer's disease and cardiac hypertrophy induced by pressure overload [36, 37], suggesting a pivotal role of Sig1R dysfunction in these diseases. Our previous study showed a decreased expression of Sig1R in hypertrophic rat hearts after TAC [9]. In the present study, a similar reduction in Sig1R expression was observed in mouse hearts showing TAC-induced cardiac fibrosis and in activated cardiac fibroblasts. To determine the role of Sig1R in the activation of cardiac fibroblast, we tried to use fluvoxamine, an agonist of Sig1R *in vivo* and *in vitro*, to observe its effect on the activation of cardiac fibroblast. The results showed that fluvoxamine increased the level of Sig1R in cardiac fibroblasts. Upregulation of Sig1R activity can not only significantly improve the cardiac function decline and cardiac fibrosis *in vivo* but also inhibit the proliferation and migration ability of activated cardiac fibroblasts *in vitro*, indicating a potential protective role of Sig1R stimulation against cardiac fibrosis. To further verify this conclusion, we used Sig1R antagonist NE-100 to intervene Sig1R. The results showed that downregulating the activity of Sig1R greatly increased the expression of TGF-β1-induced cardiac fibroblast activation and exacerbated the proliferation and migration ability of activated cardiac fibroblasts.

Furthermore, in most diseases, including depression and mental disorders, the level of Sig1R is downregulated, while upregulating its expression can slow the progression of many diseases [38]. Studies have shown that depression can increase the risk of heart failure, as well as morbidity and mortality [39] In turn, cardiovascular disease will also cause severe depression [40]. Because Sig1R is a common target, some scholars have already proposed the combined use of serotonin reuptake inhibitors (SSRI) in the treatment of cardiovascular diseases to reduce its morbidity and mortality



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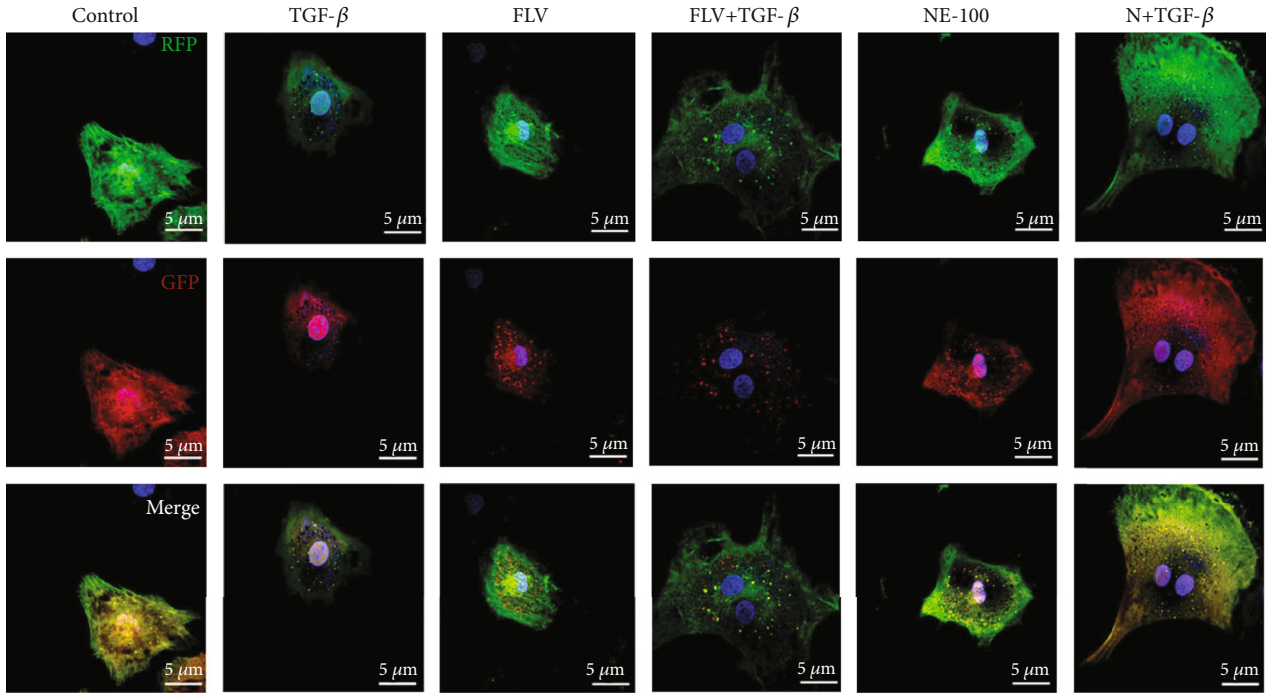

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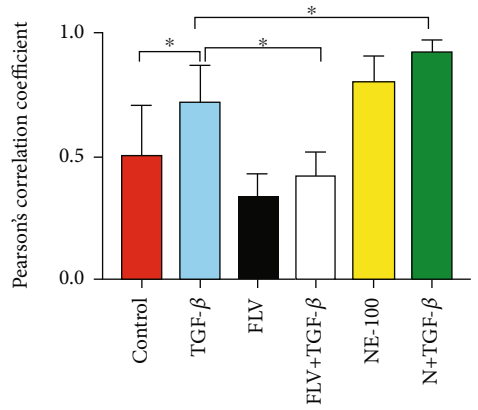

(h)

Figure 7: Sig1R protects against cardiac fibrosis by attenuating autophagic flux impairment. (a, b) The representative western blot results of LC3 and p62 in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, fluvoxamine treatment (FLV), or fluvoxamine combined with TGF-β1 treatment (FLV+TGF-β) groups. $n = 3$; (c, d) The representative western blot results of LC3 and p62 in cardiac fibroblasts from control, TGF-β1 treatment (TGF-β) groups, NE-100 treatment (NE-100), or NE-100 combined with TGF-β1 treatment (N+TGF-β) groups. $n = 3$; (e, f) The representative western blot results of LC3 and p62 in mice heart tissue from sham-operated (Sham), TAC, and intraperitoneal injection with fluvoxamine after TAC (FLV) groups. $n = 6$; (g, h) The mRFP-GFP-LC3 expressing cells were visualized by confocal microscopy. Merged fluorescence from RFP and GFP was assessed with Pearson's correlation coefficient, and 20 cells were used for quantification in each group. Scale bar = 5 μm. Shown are representative pictures, p value was determined by one-way ANOVA with Tukey post hoc analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the mean ± SEM.

[41]. However, there is no report about the role of SSRI in the process of cardiac fibrosis and cardiac fibroblast activation. Fluvoxamine, one of the SSRIs, is a specific agonist of Sig1R and one of the most commonly used drugs in the clinical treatment of depression. In the present study, fluvoxamine attenuates the pressure-overload-induced cardiac fibrosis in mice. Therefore, we recommend using fluvoxamine clinically to treat patients with both cardiovascular disease and depression; of course, this needs more research.

Small molecule drugs are generally considered to have several off-target effects. So, in this study, after blocking the activity of Sig1R with small molecule inhibitors, we further used specific siRNA to silence the Sig1R gene expression. The results showed that silencing Sig1R gene expression aggravated the activation of cardiac fibroblasts, and the results were consistent with the effects of small molecule inhibitors. Although there may be some off-target effects of small molecule drugs, fluvoxamine/NE-100 has been widely

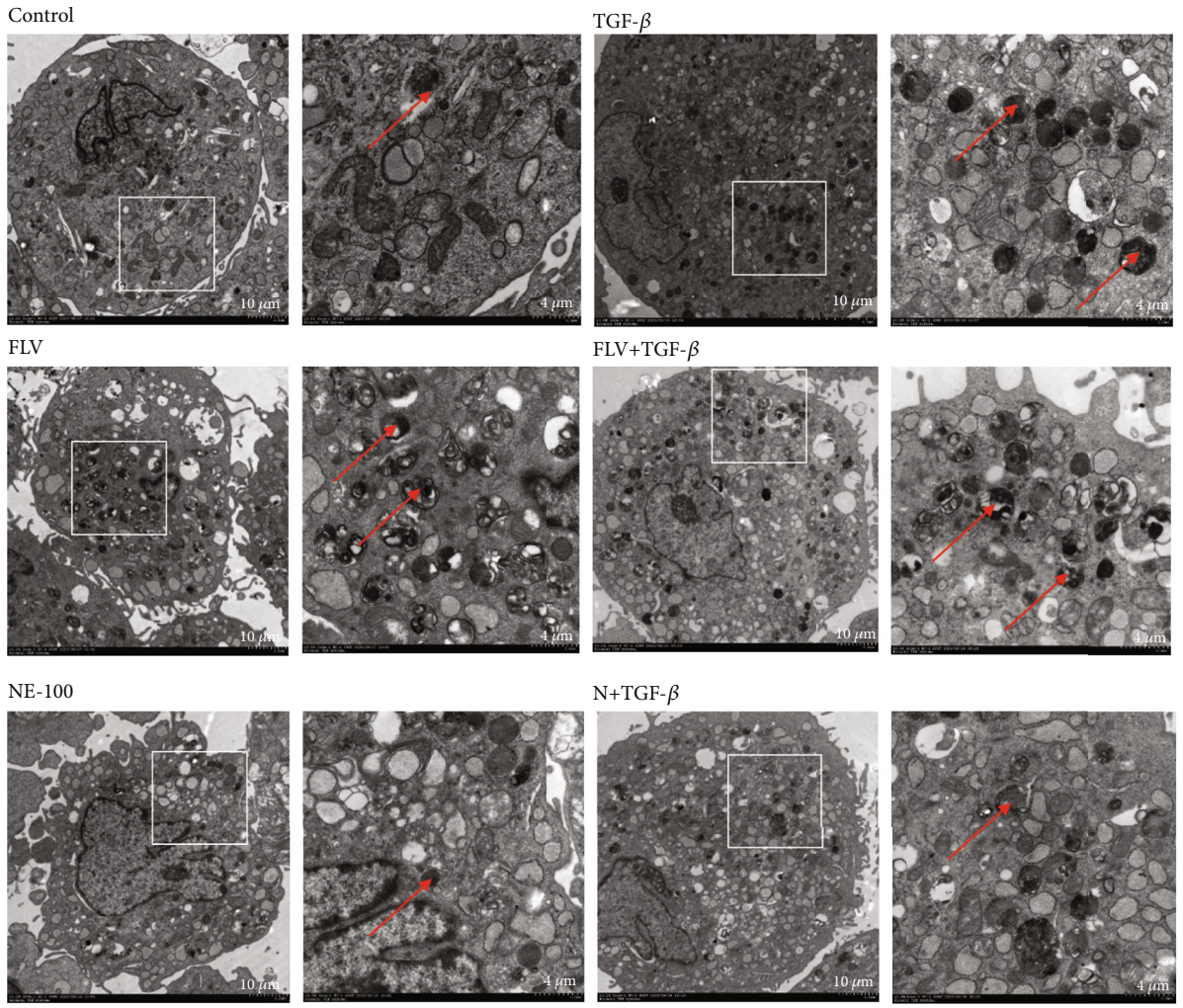

Figure 8: Sig1R protects against cardiac fibrosis by regulating autophagic. A representative image autophagosome was observed by transmission electron microscope in cardiac fibroblasts. Scale bar: 10 μm and 4 μm.

used by other scholars in the research work of Sig1R due to its effectiveness in stimulation or blockage of Sig1R [27, 28]. In addition, compared with overexpressing virus vectors such as adenovirus, fluvoxamine has advantages in clinical translations. Therefore, in the subsequent studies, we continued to use small molecule drugs targeting Sig1R as an intervention.

Sig1R is also expressed in lung fibroblasts and hepatic stellate cells [42, 43], but no role has yet been established in lung or hepatic fibrosis. A recent study reveals that the inhibition of Sig1R promotes atrial electrical remodeling, cardiac autonomic remodeling, and atrial fibrosis, and these changes could be attenuated by fluvoxamine [44]. Therefore, further investigations of the function of Sig1R in other types of tissue fibrosis would be worthwhile.

Another major finding of our present study is that the IRE1α pathway, one of the three arms of the ER stress pathways, contributes to the Sig1R-mediated activation of cardiac fibroblasts. We found that three pathways downstream of ER stress in heart tissue of cardiac fibrosis induced by pressure overload: IRE1α/XBP1, PERK/ATF4, and ATF6 were all activated. While among activated cardiac fibroblasts induced by TGF-β1, only the IRE1α/XBP1 and PERK/ATF4 pathways were activated, with no significant changes observed in the ATF6 pathway. We analyzed and ascribed this phenomenon to different expressions of various types of cells contained in mouse heart tissues, including expressions of cardiomyocytes, cardiac fibroblasts, and vascular endothelial cells. IRE1α can splice the mRNA of transcription factor X-box binding protein 1 (Xbp1), which produces the functionally active spliced form of Xbp1 (Xbp1s). Xbp1s, in turn, translocate into the nucleus to induce the expression of other ER chaperones and antioxidant proteins [44]. IRE1α resides mainly in the MAM [45]. At the MAM, Sig1R binds with and interacts with IRE1 [18]. A recent study has reported that Sig1R restricts the endonuclease activity of IRE1 against inflammation [35], which agrees with our finding that the IRE1 pathway mediates the stimulatory effect of Sig1R on cardiac fibroblast activation.

Most importantly, due to its location in the MAM and ER membranes, Sig1R exhibits a critical role in autophagy [46]. Owing to the highly dynamic process involved in autophagosome synthesis, cargo recognition and transport,

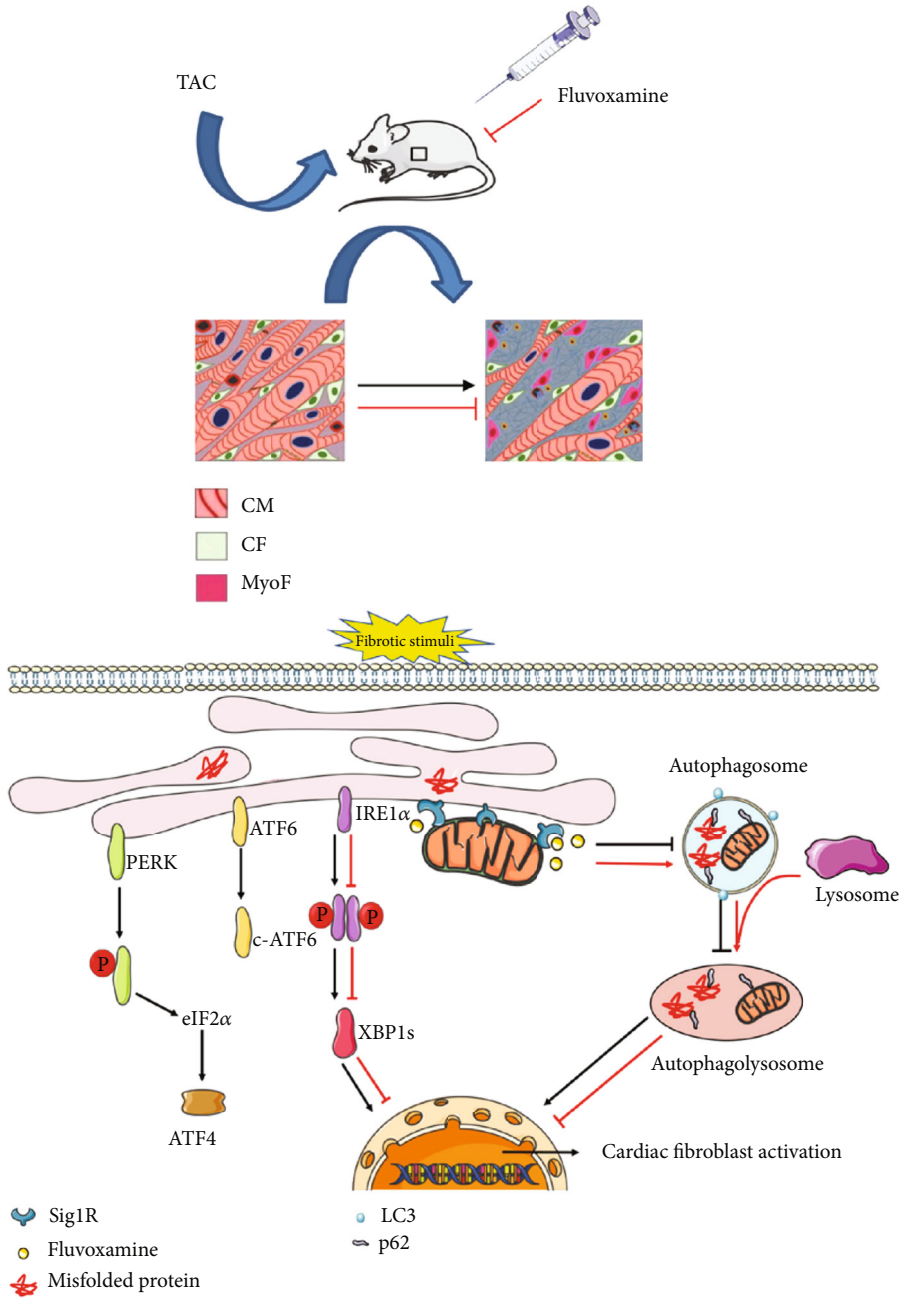

Figure 9: Schematic representation of Sig1R protecting against cardiac fibrosis by regulating IRE1 pathway and autophagic flux. In pressure-overload-induced cardiac fibrosis or TGF-β1-induced cardiac fibroblast activation triggers the ER stress and autophagy impairment. Stimulation of Sig1R with fluvoxamine in TAC mice or activated cardiac fibroblasts primed with TGF-β1 reduces fibrotic extracellular matrix (ECM) gene expression and cardiac fibrosis.

autophagosome-lysosome fusion, and cargo degradation, the quantifying of autophagy becomes a challenge. It is critical to consider not only the number of autophagosomes within the cell but also the autophagic degradative activity, autophagic flux [47, 48]. Therefore, autophagic flux is a commonly used index to monitor the process of autophagy. A previous study reports that Sig1R ablation impairs autophagosome clearance [49]. In the present study, our results have confirmed and extended their results. Sig1R stimulation attenuates the autophagic flux impairment in activated cardiac fibroblasts, whereas Sig1R inhibition aggravates the impairment.

Autophagy plays an important role in cardiac fibrosis, as well as in other fibrotic diseases [50]. Zhang et al. [51] reported that the tribbles pseudokinase 3 (TRIB3) mediates autophagy impairment by not only suppressing autophagic degradation but also promoting the activation of hepatic stellate cells (HSCs). Notably, restoration of the autophagic flux in hepatocytes and HSCs has potent protective effects against hepatic fibrosis [51]. Another study has shown that the activation of Sig1R increases nuclear factor erythroid-2-related factor 2 antioxidative response element (Nrf2-ARE) binding activity in retinal cone photoreceptor cells, and Sig1R

participates in protecting cells from electrophilic or oxidative stress by regulating the expression of antioxidant genes, suggesting an involvement of Sig1R in Nrf2 signaling [52]. Sig1R also reduces the production of reactive oxygen species (ROS) by enhancing the signaling of Nrf2 [53]. Constitutive activation of Nrf2 augments autophagosome formation and promotes autophagic flux in the heart after TAC [54]. Therefore, we speculate that the restoration of autophagic flux in cardiac fibroblasts by Sig1R agonists may also be mediated by the Nrf2 signaling pathway.

Some studies have identified the critical roles of Sig1R in mediating cell survival by a regulation of the interplay between apoptosis and autophagy [55]. The interaction between ER stress (and especially the IRE1 pathway) and autophagy in the activation of cardiac fibroblast clearly needs further study.

Taken together, the findings presented here indicate that the stimulation of Sig1R attenuates the activation of cardiac fibroblasts and cardiac fibrosis induced by pressure overload by alleviating the IRE1 pathway and autophagy impairment. Overall, these results suggest that Sig1R might be a promising therapeutic target for cardiac fibrosis treatments.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Conflicts of Interest

The authors declare that there are no commercial or financial conflicts of interest.

Authors' Contributions

Jing Qu and Miaoling Li performed the experiments and wrote the manuscript. Dongxu Li, Yanguo Xin, Junli Li, and Wenchao Wu contributed to perform the echocardiography and electrocardiogram analysis. Song Lei performed the TEM assay. Xiaojing Liu provided oversight for the project. All authors reviewed the manuscript. Jing Qu and Miaoling Li contributed equally to this work and should be considered co-first authors.

Acknowledgments

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Review Article

Autophagy and Redox Homeostasis in Parkinson's: A Crucial Balancing Act

Natalia Jimenez-Moreno[1] **and Jon D. Lane**[2]

[1]*Edinburgh Cancer Research UK Centre, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XR, UK*
[2]*Cell Biology Laboratories, School of Biochemistry, University of Bristol, Bristol BS8 1TD, UK*

Correspondence should be addressed to Natalia Jimenez-Moreno; natalia.jimenezmoreno@igmm.ed.ac.uk and Jon D. Lane; jon.lane@bristol.ac.uk

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Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated primarily from endogenous biochemical reactions in mitochondria, endoplasmic reticulum (ER), and peroxisomes. Typically, ROS/RNS correlate with oxidative damage and cell death; however, free radicals are also crucial for normal cellular functions, including supporting neuronal homeostasis. ROS/RNS levels influence and are influenced by antioxidant systems, including the catabolic autophagy pathways. Autophagy is an intracellular lysosomal degradation process by which invasive, damaged, or redundant cytoplasmic components, including microorganisms and defunct organelles, are removed to maintain cellular homeostasis. This process is particularly important in neurons that are required to cope with prolonged and sustained operational stress. Consequently, autophagy is a primary line of protection against neurodegenerative diseases. Parkinson's is caused by the loss of midbrain dopaminergic neurons (mDANs), resulting in progressive disruption of the nigrostriatal pathway, leading to motor, behavioural, and cognitive impairments. Mitochondrial dysfunction, with associated increases in oxidative stress, and declining proteostasis control, are key contributors during mDAN demise in Parkinson's. In this review, we analyse the crosstalk between autophagy and redoxtasis, including the molecular mechanisms involved and the detrimental effect of an imbalance in the pathogenesis of Parkinson's.

1. Introduction: Autophagy Forms, Roles, and Regulation

Eukaryotic cells employ a variety of catabolic pathways to degrade altered/damaged proteins and redundant macromolecular components (e.g., organelles). These pathways are critical for cellular homeostasis, and alterations in any have been linked to diverse human diseases [1–4]. Autophagy is one of the major catabolic quality control mechanisms, and is adapted for the degradation of soluble as well as large and/or insoluble cytosolic material, such as aggregated proteins and damaged organelles [1, 2, 5, 6]. It describes several distinct recycling pathways in which cytosolic cargoes are removed through lysosomal degradation, releasing macromolecular precursors such as amino acids, lipids, and nucleosides back to the cytoplasm to be reused. As expected for a process that contributes to removal of toxic cytosolic components, autophagy dysregulation has been linked to numerous diseases, including cancer, bone diseases, cardiomyopathy, infectious diseases, metabolic disorders, and neurodegenerative diseases [7–14]. In this review, we explore the roles of one form of autophagy—macroautophagy—as a prominent pathway for the removal of toxic protein aggregates and damaged organelles, focusing on the interplay between macroautophagy and redox homeostasis, and how imbalances contribute to neuronal decline in Parkinson's.

There are three types of autophagy, each with a distinct mechanism for delivery of substrates to the lysosome. These are microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. In microautophagy, the cargo is directly engulfed into lysosomes through lysosomal invaginations or protrusions [15]. CMA is a highly selective type of

autophagy, where cargoes containing KFERQ-like motifs and/or proteins that have been posttranslationally modified (either by acetylation or phosphorylation) to generate KFERQ-like motifs—becoming de novo CMA substrates [16–18]—are selectively targeted via heat shock cognate 71 kDa protein (HSC70) and cochaperones, and internalized to the lysosome lumen through the lysosome-associated membrane protein 2 receptor (LAMP2A) for their degradation [18]. Thus, CMA plays an important role in the degradation of altered and aggregated proteins, and impairments in this process have been linked to numerous diseases, including neurodegenerative diseases. For example, accumulation of CMA substrates such as α-synuclein (α-syn) and tau are hallmarks, respectively, of Parkinson's and tauopathies [19].

1.1. (Macro)autophagy. Macroautophagy is the best understood of the three autophagy forms. It is commonly referred to simply as "autophagy," and we will adopt the same nomenclature herein. Defects in autophagy are common hallmarks of human diseases, including neurodegenerative diseases [7]. During autophagy, cargoes are sequestered by double-membrane vesicles called autophagosomes, which eventually fuse with lysosomes to generate hybrid degradative compartments (the autolysosomes) (Figure 1) [7, 20–23]. Autophagy is a highly conserved pathway in all eukaryotes, and was first described in detail ~50 years ago by Christian De Duve; however, it was not until the early 1990s that the Nobel Laureate Yoshinori Ohsumi began to unpick the genetic and molecular basis of this process, including identifying the proteins involved and their regulatory interplay using budding yeast [24–26]. Since then, there has been a remarkable progress in this field regarding the molecular control of autophagy and its physiological relevance in multicellular eukaryotes.

Although autophagy occurs in cells under basal conditions, it is dramatically upregulated in response to stresses including starvation, oxidative stress, and pathogen infection [27]. Crucially, autophagy can be nonselective (also known as cargo-independent autophagy), when portions of cytoplasm are randomly encapsulated into autophagosomes based on locality alone, or it can be highly selective. Here, autophagy cargo receptors recognise and bind both cargo and the autophagy machinery, thereby removing specific cargoes such as protein aggregates or damaged organelles [28–31]. Thus, the machineries involved in selective and nonselective autophagy are not identical (e.g., the requirement for specific adaptors and cargo receptors) [32]. In selective autophagy, contributing effector proteins differ depending on specific cargoes, with the process being named according to the organelle affected: mitophagy (mitochondria); pexophagy (peroxisomes); ribophagy (ribosomes); reticulophagy (ER-phagy); lysophagy (lysosomes); xenophagy (bacteria or virus; being distinct from LC3-associated phagocytosis (LAP), where LC3 (see below) is recruited directly to the single-membrane phagosome [33]); nucleophagy (nucleus); proteaphagy (proteasome); lipophagy (lipid droplets); ferritinophagy (ferritin); and glycophagy (glycogen) [28, 34]. Selective autophagy is also implicated in, e.g., noncanonical secretion [35, 36], and LAP for the degradation of bacteria or dead cells [37]. Relevant to a range of human diseases, autophagy also selectively degrades aggregated/misfolded proteins, by a process referred to as aggrephagy [38]. Aggregated proteins that are common hallmarks of neurodegenerative diseases, and known autophagy substrates, include amyloid-β [39, 40], that forms amyloid plaques in Alzheimer's disease; HTT (huntingtin) [41], the causative agent in Huntington's disease; and α-syn [42], a major component of Lewy's bodies associated with Parkinson's and Lewy's body dementia. Befitting such an important process, a dedicated family of protein is required for autophagy (with the majority designated as "AuTophaGy-related" or "ATG" proteins), and their functions are tightly regulated (a summary of the proteins involved, and their functions, can be found in Table 1) [7, 20–23, 43].

1.2. Mechanisms and Regulation of Autophagosome Biogenesis: Initiation and Phagophore Expansion. The process of autophagy consists of several sequential steps: (i) initiation and nucleation; (ii) elongation; (iii) maturation; and (iv) fusion with the endolysosomal compartment. In mammalian cells, autophagy initiation involves the recruitment of several complexes to the autophagy initiation sites and the formation of the phagophore (also known as the isolation membrane). Upon autophagy induction (e.g., nutrient starvation conditions), the Unc51-like kinase 1 (ULK1) complex—formed by the catalytic subunit ULK1, the regulatory subunit ATG13, ATG101, and focal adhesion kinase family interacting protein of 200 kDa (FIP200)—is activated [44]. ULK1 activation depends on its phosphorylation status: (i) it is inactivated by the mammalian target or rapamycin Complex 1 (mTORC1), which also inhibits ATG13 via phosphorylation; and (ii) it is activated by adenosine monophosphate-activated protein kinase (AMPK), which also inhibits mTORC1 directly by phosphorylation, and indirectly via activation of tuberous sclerosis Complex 2 (TSC2) which controls the GTPase activity of the Ras homolog enriched in brain (Rheb) (i.e., Rheb-GDP inhibits mTORC1 activity). The latter process is inhibited in autophagy-inducing conditions [45]. Once activated, ULK1 phosphorylates itself and other ULK1 complex components (i.e., ATG13, FIP200, and ATG101), a step considered important for the catalytic activity of the complex [46–48]. The ULK1 complex is then recruited to the site of autophagosome formation, generally in close-proximity to the endoplasmic reticulum (ER)—or at ER-mitochondria contact sites—triggering nucleation of the phagophore [7, 49–51]. The ULK1 complex activates the downstream machinery including via (i) trafficking of ATG9-positive vesicles from the plasma membrane, recycling endosomes, and trans-Golgi network (TGN) to the autophagy initiation site [52–55]; and (ii) activation by phosphorylation of the autophagic phosphatidylinositol 3-kinase class III Complex 1 (PI3KC3-C1; also known as vacuolar protein sorting 34 Complex 1 (VPS34-I)). This complex comprises (i) the adaptor protein VPS15; (ii) the catalytic subunit VPS34; (iii) ATG14L—required for ER targeting via interaction with syntaxin 17 (STX17) [56]; and (iv) the regulatory subunit coiled-coil myosin-like BCL-2-interacting protein (BECLIN1; itself influenced by AMBRA1 (activating molecule in BECLIN1-

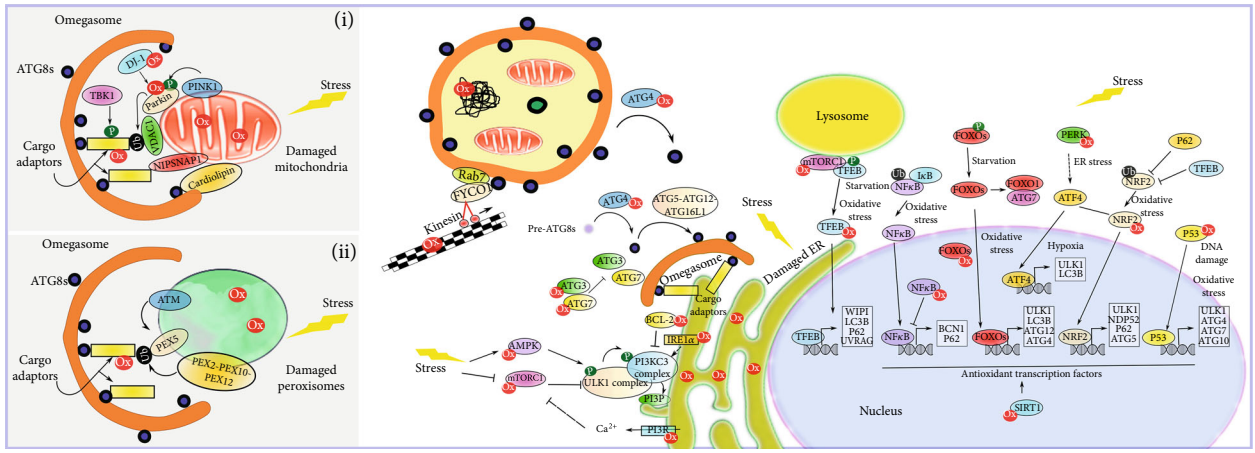

FIGURE 1: Redox regulation of autophagy. Free radicals in the cell are mainly generated in mitochondria, peroxisomes, and ER; thus, a tightly regulated process to ensure proper functionality and turnover is crucial for cell survival (i.e., degradation by selective autophagy, e.g., mitophagy (i) or pexophagy (ii)). Under certain conditions (e.g., oxidative damage), autophagy is induced as an antioxidant pathway, and this leads to the initiation and nucleation of autophagy assembly sites (e.g., at the ER), with subsequent formation of the autophagosome, and eventual fusion with a lysosome to form a degradative autolysosome. ROS/RNS have the potential to regulate autophagy via upstream regulators, including proteins involved in the UPR system and the autophagy inhibitor mTOR, as well as redox modification in the cytoskeleton, affecting autophagosome transport. In addition, direct modifications in proteins involved in the autophagy process have also been identified including those involved in ATG8 cleavage and conjugation (i.e., ATG4 involved in LC3 cleavage; ATG3 and ATG7 involved in ATG8 lipidation), PI3KC3 activation and cargo recognition (e.g., p62/SQSTM1), and in selective autophagy (e.g., ATM in pexophagy and PINK1, Parkin and DJ-1 for mitophagy) (see the text for full description). Finally, autophagy and redoxtasis crosstalk is evident at the transcriptional level, with several transcription factors involved in autophagy regulation subject to redox modification. Some transcription factors regulate both redox levels and the autophagy process (e.g., NRF2, FOXOs, and p53). P (green): highlights phosphorylation events; Ub (black): highlights ubiquitination events; Ox (red): highlights sites for redox regulation of autophagy.

regulated autophagy [57])). When active, it establishes phosphatidylinositol 3-phosphate- (PI3P-) enriched subdomains of the ER, known as omegasomes, from where phagophores emerge [49, 58–60].

At the omegasome, PI3P effector proteins are recruited, including the zinc-finger FYVE domain-containing protein (DFCP1), the autophagy-linked FYVE protein (ALFY), and WD repeat domain phosphoinositide-interacting proteins (WIPIs; here WIPI2 is the exemplar isoform) [7]. DFCP1 resides on ER/Golgi membranes, and is an excellent omegasome marker, but is thought not to be essential for autophagy [58]. ALFY has been reported to be essential for selective degradation of aggregated proteins, and is required for neuronal connectivity [61, 62]. WIPI2 plays an important role in the recruitment and activation of the tandem Ub-like (UBL) conjugation pathways that drive autophagosome assembly, namely, the ATG12 and the ATG8 conjugation systems [43, 63, 64]. In the first UBL conjugation system, the UBL protein ATG12 is conjugated to ATG5 by a process mediated by ATG7 (E1-like activating enzyme) and ATG10 (E2-like conjugating enzyme). ATG12~5 binds to ATG16L1, generating a complex with E3-like activity for the second UBL conjugation pathway [65, 66]. ATG12~5-16L1 complex recruitment is mediated by direct interactions between WIPI2B (WIPI2 splice variant) and ATG16L1 [63]. There, the AT12~5-16L1 complex, together with ATG3 (E2-like conjugating enzyme) and ATG7, coordinates activities in the second UBL conjugation pathway, during which ATG8 family members are covalently attached to lipids (most often to phosphatidyl ethanolamine (PE)) in situ. The ATG8 family comprises the microtubule associated protein 1 light chain 3 (MAPLC3; herein, referred to as LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP) families. These families encompass LC3A (with two variants differing in the N-terminal sequence, v1 and v2 [67]), LC3B (LC3B1 and LC3B2, with only one amino acid difference (C^{113} versus Y^{113} [67]), LC3C, GABARAP, GABARAPL1, GABARAPL2/GATE-16 [68]. Prior to lipidation, ATG8s are first activated (or primed) by members of the ATG4 endopeptidase family (ATG4A-D; with ATG4B being the best characterised family member which displays activity against all ATG8s), which cleave ATG8 proteins at their C-termini to expose a glycine residue (e.g., G120 in LC3B) that is the future site for lipidation (e.g., primed LC3 is referred to as "LC3-I") [7, 22, 69]. The subsequent covalent attachment of ATG8s to lipids at the nascent isolation membrane generates the membrane-bound form (e.g., LC3-II) [70, 71], a step that is followed by coordinating membrane expansion and phagophore closure.

1.3. Mechanisms and Regulation of Autophagosome Biogenesis: Maturation, Trafficking, and Lysosomal Fusion. Autophagosome maturation and fusion with lysosomes involves (i) membrane fission for autophagosome closure; (ii) trafficking of the autophagosome along the cytoskeleton, typically in the retrograde direction (i.e., towards the centre of a typical cell); and finally (iii), fusion with the lysosome to form a degradative autolysosome [43]. Here, cargoes are degraded, and their components transported back into the cytosol. ESCRT proteins (endosomal sorting

Table 1: Key proteins involved in the various stages of autophagy and their general roles.

Protein	Functions	Stages
ATG proteins		
ULK1/2	Serine/threonine kinase that forms complexes with ATG13, FIP200, and ATG101, involved in ATG9 recruitment and the activation of the PI3KC3 complex.	Initiation/nucleation
ATG2A/B	ATG2A interacts with WIPI4, tethering the omegasome to the ER. ATG2-GABARAP interaction is critical for autophagosome closure.	Elongation and maturation (closure)
ATG3	E2-like enzyme coordinating ATG8 conjugation to PE.	Elongation
ATG4A/B/C/D	Cysteine protease that activates (priming) and recycles (delipidation) ATG8s by cleavage of pro-ATG8 and ATG8-PE, respectively for autophagosome formation and possibly maturation).	Elongation
ATG5	Conjugates to ATG12, and acts as an E3-like enzyme for ATG8 conjugation to PE.	Elongation
BECLIN1	Regulatory subunit of the PI3KC3 Complex I.	Initiation/nucleation
ATG7	E1-like enzyme. Coordinates conjugation of ATG12 to ATG5, and ATG8 conjugation to PE.	Elongation
LC3A/B/C	Conjugates to the lipid, PE. Involved in membrane tethering, and phagophore expansion and closure. Coordinates cargo recruitment by binding to autophagy receptors. Binding to FYCO1 promotes microtubule-based transport and autophagosome maturation. Regulates autophagosome-lysosome fusion (binding to PLEKHM1 and HOPS).	Elongation, maturation, and fusion
GABARAPs	Parallel functions with LC3A/BC, although GABARAPs seem to be particularly important for autophagosome maturation.	Elongation, maturation, and fusion
ATG9	Transmembrane protein involved in delivery of membrane to the PAS/autophagosome assembly site.	Initiation/nucleation
ATG10	E2-like enzyme. Coordinates the conjugation of ATG12 to ATG5.	Elongation
ATG12	Conjugates to ATG5, forming the E3-like enzyme for conjugation of ATG8 to PE.	Elongation
ATG13	Regulatory subunit of the ULK1/2 complex.	Initiation/nucleation
ATG14L1	Core component of PI3KC3 Complex I, required for ER localization. Stabilises SNARE complexes for autophagosome-lysosome fusion.	Initiation/nucleation and fusion
ATG16L1	Forms a complex with ATG12-ATG5. Provides E3-like activity for conjugation of ATG8 to PE.	Elongation
ATG17	Scaffolding protein for the recruitment of ATG9 vesicles. ESCRT recruitment.	Initiation/nucleation and maturation (closure)
ATG101	Core component of the ULK1 complex.	Initiation/nucleation
FIP200	Core component of the ULK1 complex.	Initiation/nucleation
WIPI1/2/3/4	PI3P effector protein. WIPI2 recruits the ATG12-ATG5-ATG16L1 complex at the phagophore.	Elongation
Non-ATG proteins		
ALFY	PI3P effector protein involved in the degradation of protein aggregates.	Elongation
AMBRA1	Regulator of the PI3KC3 complex.	Initiation/nucleation
AMPK	Serine/threonine kinase. Autophagy activator via phosphorylation of ULK1 and inhibition of mTOR.	Initiation/nucleation
Basson	Scaffold protein in neuronal active zone. Involved in ATG5 sequestration.	Initiation
DFCP1	PI3P effector protein. Efficient omegasome marker.	Elongation

Table 1: Continued.

Protein	Functions	Stages
Endophilin A	Adaptor protein involved in synaptic vesicle recycling and ATG3 recruitment.	Initiation
ESCRT	Membrane fission.	Maturation (closure)
FBXO7	E3-like enzyme involved in mitochondrial Parkin recruitment.	Initiation/nucleation (mitophagy)
FYCO1	Rab7 effector. Binds to PI3P and LC3. Mediates anterograde kinesin-driven transport.	Maturation (trafficking)
mTORC1	Serine/threonine kinase complex. Autophagy inhibitor via phosphorylation of ULK1.	Initiation/nucleation
Parkin	E3-like enzyme. Ubiquitination of mitochondrial surface proteins.	Initiation/nucleation (mitophagy)
PEX5	Protein family involved in peroxisome biogenesis and pexophagy. PEX5 is ubiquitinated by the PEX2-PEX10-PEX12 E3-like complex and it is recognised by cargo receptors.	Initiation (pexophagy)
Piccolo	Scaffold protein in the neuronal active zone. Involved in ATG5 sequestration.	Initiation
PINK1	Serine/threonine kinase. Drives the phosphorylation of ubiquitin and Parkin, for robust mitochondrial Parkin recruitment.	Initiation/nucleation (mitophagy)
RAB7	Autophagosome trafficking (interaction with FYCO1 or RILP) and autophagosome-lysosome fusion (interaction with PLEKHM1).	Maturation and fusion
Synaptojanin	Enzyme involved in neuronal membrane trafficking. Promotes autophagosome maturation.	Maturation
SNAREs	On the autophagosome, STX17 and SNAP29, and on the lysosome, VAMP7 or VAMP8, mediates membrane fusion supported by HOPS and ATG14L1.	Fusion
TBK1	Serine/threonine kinase. Increases the binding affinity of autophagy receptors	Elongation
UVRAG	Core component of PI3KC3 Complex II.	Maturation and fusion
VPS15	Adaptor protein and core component of PI3KC3 complex.	Initiation/nucleation
VPS34	Catalytic subunit of the PI3KC3 complex.	Initiation/nucleation
Autophagy receptors		
P62, NDP52, OPTN, NRB1, TAX1BP1, NIX, FUNDC1, CCPG1, RTN3, SEC62, ATL3, CALCOCO1, FAM134B, TEX264	Binding to ubiquitinated substrates and ATG8s.	Cargo recruitment to the phagophore

complex required for transport) have been identified as essential for autophagosome membrane fission/closure. These are recruited in a RAS-related protein 5- (RAB5-) dependent manner [72]. Defects in LC3B lipidation have been found to cause the accumulation of unclosed autophagosomes, suggesting that the ATG-conjugation machinery is needed for this process [73]. Despite this, functional autophagosomes do form in the absence of all ATG8 family members [74]. Other interventions that lead to the accumulation of unsealed autophagosomes in the cytoplasm include knockdown of the phospholipid transfer protein ATG2A/B, required for autophagosome expansion [75]. Indeed, it was recently shown that an ATG2-GABARAP interaction is needed for efficient autophagosome closure [76].

Fully formed autophagosomes go through a maturation process during which ATG8 proteins link autophagosomes to motor proteins and the microtubule cytoskeleton. For example, RAB7 is recruited to mature autophagosomes and further recruits the FYVE and coiled-coil domain-containing 1 protein (FYCO1), which in turn binds to LC3 (via LIR- (LC3-interacting region-) type interaction) and PI3P to mediate anterograde kinesin-driven transport [77]. Alternatively, RAB7 binds to the RAB-interacting lysosomal protein (RILP) to mediate retrograde dynactin/dynein-driven transport towards the nucleus [78–82]. Crucially, microtubules are also involved in autophagosome formation (e.g., microtubule transport from the centrosome is necessary for recruitment of GABARAP to the nascent phagophore via the centriolar satellite protein PCM1 [80, 83]).

For membrane fusion and formation of the autolysosome, the SNARE (soluble N-ethylmaleimide-sensitive factor activating protein receptor) fusion machinery is required. On the autophagosome membrane resides STX17 and synaptosomal-associated protein 29 (SNAP29), whereas on the lysosome, vesicle-associated membrane protein 7 or 8 (VAMP7 or VAMP8) mediates membrane fusion supported by the homotypic fusion and protein sorting complex (HOPS) which interacts with STX17 [84, 85]. STX17 also recruits ATG14L (also involved in autophagosome formation as a complex of PI3KC3-C1) to promote membrane tethering and to stabilise the SNARE complex promoting membrane fusion [86–88]. In addition, Wilkinson et al. described that phosphorylation of LC3B by hippo kinases STK3 and STK4 was critical for autophagosome fusion [89]. Meanwhile Wang et al. described that ULK1 mediates autophagosome-lysosome fusion via interactions with STX17, with protein kinase α- (PKCα-) mediated ULK1 phosphorylation reducing this interaction via ULK1 degradation by the CMA pathway [90].

1.4. General Features and Properties of Autophagy in Neurons. Autophagy pathways in general are especially important in neurons, as these are postmitotic cells that cannot dilute cytoplasmic damage through proliferation/division, and thus autophagy is required to maintain long-term neuronal functionality. Although this review focuses on (macro)autophagy, it is important to mention that CMA and microautophagy are also present in neurons [91]. In neurons, autophagy is needed to degrade neurotoxic factors (e.g., α-syn) and damaged organelles that are selected by ubiquitylation and recognised by the autophagy machinery [92, 93]. Misfolded proteins can be refolded by the actions of chaperones, or can be degraded primarily by the ubiquitin-proteasome system (UPS); however, when these processes are impaired, misfolded proteins accumulate to form aggregates (aggresomes) that require removal via aggrephagy [94], otherwise insoluble inclusions are generated [95, 96]. These are defining features of some neurodegenerative diseases, including Parkinson's [2]. In autophagy-deficient dopaminergic neurons (mDANs), derived from *Atg7* knockout mice, α-syn and p62/SQSTM1 both accumulate in inclusions within neurites in an ageing-dependent fashion that is ultimately linked to mDAN loss and motor dysfunction [96].

Autophagy also contributes to axonal regeneration, presynaptic modelling, dendritic spine pruning, and synaptic plasticity [91, 97–100]. Autophagy dysregulation has been linked to the development of neurodegenerative diseases [101–103], and crucially, decreased autophagic activity is a characteristic of ageing [8]. Autophagy supports neuronal survival. For example, neonatal lethality in *Atg5* knockout mice is rescued after restoration of neuronal-specific expression of ATG5 [104]; meanwhile, autophagy activation in the mouse brain protects against mDAN loss mediated by oxidative stress [105], and autophagy induction using a neuronal pharmacophore in amyotrophic lateral sclerosis (ALS) and Huntington's mouse models promotes neuronal survival [106]. Consistent with this, suppression of basal autophagy also causes neurodegeneration. For example, conditional neuronal autophagy deficiency leads to neuronal loss, and mutations in autophagy genes have been linked to several neurodegenerative disorders [103, 107–109].

Previous work in primary rodent neurons points to unique characteristics of autophagosome assembly, maturation, and trafficking in these specialised cells [97, 110]. Importantly, control and substrate targeting appear to differ depending on neuronal cell-type and specific conditions. For example, in neurons of the dorsal root ganglia (DRG), autophagy is triggered almost exclusively at the distal tip, whereas in hippocampal primary neurons, it can be initiated in the cell body, dendrites, and axonal regions proximal to the cell body [111, 112]. However, under stress conditions, mitophagy initiation has been reported to also occur along the axon [113]. In general, autophagosome biogenesis is initiated primarily in the distal axon, and thereafter, autophagosomes undergo dynein-dependent retrograde motility to the lysosome-rich soma following recruitment of neuronal scaffold proteins such as JIP1 [112, 114]. Overall, it is essential that neuronal subtype specification is considered when attempting to generalise about the roles and regulation of autophagy in the brain.

Autophagosome biogenesis is a constitutive process that can be triggered in the soma or distal axon where elements of the core autophagy machinery are actively recruited (e.g., ATG9A-containing vesicles are transported from the soma to the distal axons via the kinesin family member, KIF1A [115]). Supplementing these, several neuron-specific proteins have been reported to be involved in autophagosome biogenesis and maturation (e.g., synaptojanin, endophilin A, Basson, and Piccolo) (Table 1). The presynaptic proteins, endophilin A and synaptojanin (mutations in *SYNJ1* are associated with Parkinson's [116]), are primarily involved in the recycling of synaptic vesicles [117], but have also been shown to mediate ATG3 recruitment to the nascent phagophore [118] and to promote autophagosome maturation [119], respectively. Basson and Piccollo are two proteins involved in active zone assembly for the release of neurotransmitters, and they have each been found to act as autophagy inhibitors by sequestering ATG5 [120]. Autophagosome biogenesis can also occur in dendrites (or alternatively, autophagosomes can also migrate from the soma to the dendrites), and here autophagy activity increases as a function of synaptic activity [111, 121]. Furthermore, recent studies have suggested the existence of an unconventional degradation pathway in which glial cells modulate neuronal autophagy by intercellular regulation and/or direct transfer of cellular garbage from neurons, an idea that builds on previous data supporting autophagosome secretion in nonneuronal cells [97, 122].

Neuronal autophagy properties also appear to vary as a function of ageing, with accumulation of neuromelanin and lipofuscin progressively observed in autophagosomes in aging brain tissues [123]. Overall, autophagosome biogenesis efficiency is seen to decline in aged neurons [124].

2. Selective Autophagy and Its Relevance to Neurodegenerative Diseases

As a key component of cellular and tissue homeostasis, with protective roles in human neurodegenerative diseases, a full

appreciation of mitophagy regulatory control in neurons is desirable. In particular, mitophagy dysfunction is a hallmark of Parkinson's, implicated in a number of early onset genetic forms [125], and is observed in genetic and toxin-induced Parkinson's models [126]. Distinct mechanisms and diverse proteins are involved in the selective degradation of mitochondria, and these have been reviewed in detail elsewhere [32, 103]. Mitochondria can be damaged by numerous factors, including hypoxia, mtDNA damage, chemical uncouplers that dissipate membrane potential (e.g., carbonyl cyanide m-chlorophenylhydrazone (CCCP)), electron transport complex (ETC) inhibitors (e.g., rotenone (Complex I inhibitor) or antimycin (Complex III inhibitor)), or the presence of reactive oxygen species (ROS; mitochondrial superoxide production), as will be described in detail later [127–129]. Thereafter, differing fates are observed, with damaged mitochondria either being rescued by fusion/fission [130] or being degraded via mitophagy.

2.1. The PINK1/Parkin Mitophagy Pathway. The best characterised route for mitochondrial degradation is via the PINK1/Parkin (PRKN) pathway, although several Parkin-independent pathways have been described [131–135]. Crucially, *PINK1* and *PRKN* mutations are linked to familial Parkinson's [9]. When mitochondrial membrane potential is intact, PINK1 is imported into mitochondria via the TOM/-TIM23 system (translocase of the outer membrane and inner membrane, respectively), to be cleaved consecutively by the matrix-localized protease (MPP) and presenilin-associated rhomboid-like protease (PARL) [128, 136]. However, when membrane potential is lost (i.e., as a feature of damaged mitochondria), PINK1 accumulates on the mitochondrial outer membrane where it phosphorylates and activates Parkin (an E3 ligase), driving protein ubiquitylation on the outer mitochondrial membrane. Subsequently, PINK1 phosphorylates target-bound ubiquitin which in turn recruits further Parkin in a positive feedback pathway [137]. Parkin targets mitochondrial surface proteins, such as the voltage-dependent anion channel 1 (VDAC1) [138]. For Parkin recruitment and substrate ubiquitination, an interaction with the Parkinson's-linked protein F-box protein 7 (FBXO7) is involved, although the precise molecular mechanism remains elusive [139]. These ubiquitylated proteins are recognised by cargo receptor proteins, and thereafter ubiquitylated mitochondria are targeted to the nascent phagophore. Zachari et al. suggested that ubiquitylated mitochondria are enveloped by ER strands to facilitate targeting and autophagy [140].

2.2. Cargo Receptors and Their Roles in Mitophagy, Pexophagy, and ER-Phagy. There are several cargo receptor proteins involved in mitophagy, including p62/SQSTM1, NIX (or BNIPL3), Neurabin-1 (NRB1), FUNDC1 (FUN14-domain-containing 1), NDP52, Optineurin (OPTN), and Tax1 binding protein 1 (TAX1BP1) [127]. Recruitment of these receptor proteins occurs subject to specific regulation. For example, receptor binding affinity (particularly OPTN and p62/SQSTM1) is increased via phosphorylation by tank-binding kinase 1 (TBK1) [141, 142]. These receptors bind to ATG8s (via LIR-type interactions) [55], and some of them also recruit the ULK1 complex in a feed-forward pathway to reinforce the autophagosome assembly machinery [143, 144]. Subsequently, mitochondria are degraded by the (macro)autophagy pathway. Ubiquitin is not the only targeting signal for mitophagy, as it has been recently described that the mitochondrial matrix proteins 4-nitrophenylphosphatase domain and nonneuronal SNAP25-like protein homolog (NIPSNAP1) and NIPSNAP2 accumulate on the mitochondrial surface to act as "eat-me" signals through binding to mitophagy cargo receptors [135]. In addition, it has been observed in neurons that cardiolipin externalization on the mitochondrial surface triggers mitophagy via interactions with LC3, thereby targeting mitochondria for degradation [134, 145, 146].

Some cargo receptors involved in mitophagy—including NDP52, OPTN, NRB1, and p62/SQSTM1—also facilitate the degradation of protein aggregates (aggrephagy), or other organelles such as peroxisomes (pexophagy) [147, 148]. Peroxisomes are small single-membrane organelles involved in lipid synthesis and redox homeostasis. Thus, pexophagy is crucial for peroxisome quality control and turnover [149]. In this process, peroxisome membrane proteins, including the peroxisomal biogenesis factor (PEX) 5 and 70 kDa peroxisomal membrane protein (PMP70), are ubiquitylated by the E3-like ubiquitin ligase complex PEX2-PEX10-PEX12, facilitating recognition by cargo receptors and degradation via autophagy [149]. Alternatively, to prevent pexophagy, the deubiquitinase USP30 and the AAA-type ATPase (PEX1-PEX6-PEX26) remove ubiquitylated membrane proteins. Conversely, peroxisomal dysfunction are linked to peroxisome biogenesis disorders. However, the effect of altered pexophagy in neurodegenerative diseases is poorly understood [150].

Other specific proteins implicated in the selective autophagy of different organelles include the following LIR-motif-containing proteins involved in ER-phagy: CCPG1 (cell cycle progression protein 1), FAM134B (family with sequence similarity 134 member B); ATL3 (atlastin 3), SEC62 (secretory 62 homolog), CALCOCO1 (calcium-binding and coiled-coil domain 1), RTN3 (reticulon 3), and TEX264 (testis-expressed protein 264); they are found in different regions of the ER, and they might have different roles and be tissue-specific [151–160]. The ER is a complex organelle that mediates protein folding, processing and transport in the secretory pathway, calcium storage, lipid synthesis, and intracellular signalling via interactions with other organelles. In common with other organelles, the ER is also subject to turnover and remodelling to ensure proper and optimal functional plasticity [161, 162]. The best characterised network for ER remodelling is the unfolded protein response (UPR), triggered by the presence of lumenal misfolded proteins, with the consequent cytosolic signalling cascades originated by ER-sensing proteins: inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These cascades trigger the translational and transcriptional regulation of redox enzymes, chaperones, foldases, lipid synthesis proteins, autophagy-related proteins (e.g., CCPG1 [152]), and ERAD (ER-associated degradation) genes involved in proteasomal degradation [163].

Under nutrient starvation or ER stress (i.e., lumenal misfolded proteins), ER-phagy is induced via different pathways after UPR activation. Calcium released via the inositol trisphosphate receptor IP3R and other calcium channels activates calcium-dependent proteins, namely, calmodulin-dependent protein kinase (CAMKK), which inhibits mTORC1 [164, 165]; death-associated kinase (DAPK) and DAPK2 which regulate BECLIN1 activation and mTOR inhibition, respectively, [166–168]; and CAMK2B, which phosphorylates FAM134B, promoting its oligomerization [169]. On the other hand, IRE1α indirectly activates BECLIN1, thus promoting autophagy initiation, while PERK and ATF6, respectively, activate two autophagy transcription factors, ATF4 (activating transcription factor 4) and CHOP (C/EBP homologous protein) [170, 171] (for a detailed overview, see [172]). ER-phagy can be classified as macro-ER-phagy (commonly referred as "ER-phagy"), where fragments of ER are sequestered into an autophagosome which later fuses with the lysosome, and micro-ER-phagy, when a fragment of the ER is directly engulfed and targeted to the lysosome (for recent reviews, see [173, 174]). Recently, numerous human ER-phagy regulators have been identified in a genome-wide screening after starvation [159], and recent data highlight the importance of ER-phagy in cell survival, with defects in this process being related to infectious diseases and cancer development and progression (for a review, see [175]).

In neurons, the ER extends from the cell body and along the axon to the axonal distal tip. It is crucial for neuronal function (particularly the regulation of the neuronal calcium homeostasis), and the ER tubular network is disrupted in several neurodegenerative diseases [176–178]. Consistent with this, UPR has been recently implicated in memory, synaptic plasticity, dendritic outgrowth and branching, and axonal regeneration [179–182]. In addition, previous studies have highlighted the importance of ER-phagy in neurons. For example, (i) FAM134B deficiency in primary neurons leads to progressive ER stress and affects the survival of sensory neurons [156]; (ii) *RTN3* is linked to AD [183]; and most recently, (iii) Park et al. described that induction of ER stress and consequent ER-phagy is involved in early stages of hypothalamic development and metabolic regulation [184]. However, the role of ER-phagy in neuronal homeostasis and neurodegenerative diseases remain to be fully explored.

3. Redox Homeostasis

Reactive oxygen species (ROS) (e.g., hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$)) and reactive nitrogen species (RNS) (e.g., nitric oxide ($^{\bullet}NO$)) are highly reactive molecules generated under both basal and pathological or stress conditions (for a detailed description of free radicals see [185]). They are involved in numerous pathologies, including Parkinson's, Alzheimer's, ALS, diabetes, cancer, and autoimmune disorders [186]. These radicals are important for cellular homeostasis, regulating several cellular functions including cell signalling, proliferation, and survival in response to stress or injury. Reduction and oxidation reactions, where there is a transfer of electrons between chemical species—also known as redox reactions—are focused at the mitochondria, peroxisomes, and ER, although there are additional contributions from alternative organelles depending on the cell type [187, 188]. In addition, cells have different inherent antioxidant mechanisms to control ROS/RNS levels and avoid/alleviate toxicity. Oxidative stress occurs when antioxidant mechanisms are not sufficient, and ROS/RNS levels accumulate, ultimately impacting on normal biological processes and limiting cell survival. Due to the high oxygen demands and lipid contents in the brain, neurons are particularly sensitive to oxidative stress, with some areas being more susceptible than others (e.g., the hippocampus) [189]. For this reason, high levels of oxidative stress are one of the main hallmarks of neurodegenerative diseases, including Parkinson's, aggravating the disorder by affecting protein aggregation, DNA damage, and ultimately, causing neuronal cell death.

3.1. Sources and Causes of Redox Imbalance. Mitochondria are the major source of cellular ATP, generated via the electron transport chain (ETC), comprising (i) Complex I (NADH dehydrogenase), which uses NADPH generated in the citric acid cycle for proton translocation from the mitochondrial matrix to the intermembrane space, with electrons being transferred to ubiquinone; (ii) Complex II (succinate dehydrogenase), which uses flavin adenine dinucleotide ($FADH_2$) generated from succinate in the citric acid cycle and consequently delivers electrons to the ETC (ubiquinone); (iii) Complex III (cytochrome *c* oxidoreductase), where electrons (from ubiquinone) are transferred to cytochrome *c*; (iv) Complex IV (cytochrome *c* oxidase), where electrons are removed from cytochrome *c* to generate H_2O with energy released used to translocate protons to the intermembrane space; and (v) Complex V (ATP synthase), for the generation of ATP via proton flow to the matrix (at a ratio of 4H+ : 1ATP) [190]. Complexes I, II, and III are among the major ROS production enzymes in the cell, generating $O_2^{\bullet-}$ due to electron leakage [191]. In addition, glycerol-3-phosphate dehydrogenase, which catalyses the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate and the generation of $FADH_2$ while transferring electrons to ubiquinone in the ETC, generates additional $O_2^{\bullet-}$. Similarly, also in the inner mitochondrial membrane, the electron transfer to flavoprotein, ubiquinone oxidoreductase, and dihydroorotate dehydrogenase, that, respectively, links fatty acid β-oxidation and pyrimidine biosynthesis to electron transfer to the ETC, also generates $O_2^{\bullet-}$ [192]. The other major source of ROS in mitochondria is the Krebs cycle (or citric acid cycle). This metabolic pathway is performed by aerobic organisms in the mitochondrial matrix, and consists of a series of chemical reactions for the production of ATP, alongside reduced forms of NADH and $FADH_2$ to be used in the ETC. Particularly, dihydrolipoamide dehydrogenase (DLD), an E3 component of pyruvate dehydrogenase (for the production of acetyl-CoA from pyruvate), and α-ketoglutarate dehydrogenase (catalyses the conversion of α-ketoglutarate to succinyl-CoA, producing NADH), generates unwanted $O_2^{\bullet-}$ via the flavin cofactor of this enzyme. Finally, superoxide dismutase 2 (SOD2) in the mitochondrial matrix, and SOD1 in the intermembrane space, convert $O_2^{\bullet-}$ into

H_2O_2, which can potentially be turned into $^{\bullet}OH$ radicals via the Fenton reaction. Mitochondria are also sources of RNS, including $^{\bullet}NO$ that is produced by nitric oxide synthases (NOS) in the oxidation of L-arginine [188, 193]. Peroxisomes are oxidative organelles involved in lipid metabolism of long-chain and branched fatty acids via fatty acid β-oxidation, lipid synthesis, purine catabolism, and amino acid and glyoxylate metabolism. Importantly, most enzymes involved in these processes produce ROS. For example, acyl-coA oxidase(s), which catalyse the first step in peroxisomal fatty acid β-oxidation, generates H_2O_2 [194]. Similarly, xanthine oxidase, cleaved from xanthine dehydrogenase in response to an increase in calcium extracellular levels (e.g., hypoxia) and involved in the purine metabolism to uric acid, generates $O_2^{\bullet-}$ and H_2O_2 [195, 196]. During peroxisomal amino acid metabolism, D-amino acid oxidase (catalyses oxidation of D-amino acids to imino acids) and the L-pipecolic acid oxidase (involved in lysine degradation) generate H_2O_2. Other peroxisomal enzymes producing ROS/RNS include L-α-hydroxyacid oxidase (involved in oxidation of glycolic acid), polyamine oxidase (involved in polyamine degradation), sarcosine oxidase (metabolises sarcosine, L-pipecolic acid, and L-proline), D-aspartate oxidase (catalyses oxidation of D-aspartate), SOD1, and NOS2 [197, 198].

The ER is involved in diverse functions including protein folding, processing and vesicular transport, calcium storage, lipid synthesis, cell signalling, and xenobiotic toxicity. Particularly, during protein folding, ER oxidoreductin (ERO1) catalyses oxidation of protein disulfide isomerase, involved in disulfide bond formation, generating H_2O_2. Similarly, quiescin sulfhydryl oxidase, also present in the Golgi, generates H_2O_2 for the introduction of disulfide bonds into unfolded reduced proteins and can compensate for the loss of ERO1 [199, 200]. The other main source of H_2O_2 in the ER is the NADPH oxidase 4 (NOX4). Proteins that belong to the NOX family are the only cellular enzymes exclusively involved in the production in ROS by using NAD(P)H for oxygen reduction to produce a superoxide anion [197]. Finally, the microsomal monooxygenase (MMO) system, composed of cytochrome P450 (P450), NADPH-P450 reductase (NPR), and phospholipids, is involved in the oxygenation of several exogenous (xenobiotics) and endogenous substrates (e.g., heme oxygenase and fatty acid desaturase), and is one of the major sources of ROS in the ER via electron leakage from P450 [199].

Other sources of cellular ROS include the plasma membrane and lysosomes, as well as cytosolic reactions [188]. In addition, ROS production can also be induced in response to hypoxia (by acting on the mitochondrial ETC and increasing intracellular calcium levels [196, 201, 202]) and starvation, and more generally following environmental stress (e.g., paraquat), infections, physical exercise, and mental stress; and increased ROS/RNS levels have been observed during aging [203–205].

3.2. Dual Roles of ROS and RNS

3.2.1. Beneficial Activities of Free Radicals: Oxidative Eustress. Crucially, ROS and RNS are not only detrimental to cells, but they are also important for cellular homeostasis, regulating numerous important cellular activities, also known as physiological oxidative stress or oxidative eustress. ROS and RNS act as second messengers in signal transduction pathways involved in cell survival, cell to cell communication, and cell growth and proliferation [206–208]. They influence diverse signalling pathways via oxidation of cysteine sulfhydryl groups in protein kinases, including protein kinase A (PKA), PKC, receptor tyrosine kinase (RTK), and Ca^{2+}/-calmodulin independent protein kinase II (CaMKII). Other pathways that display crosstalk with ROS/RNS include the NF-κB pathway, the MAPK pathway, the PI3K/AKT pathway, ATM signalling, the insulin pathway (e.g., oxidation of protein tyrosine phosphatase 1B (PTP1B)), iron metabolism (e.g., Fenton reaction), calcium signalling (e.g., oxidation of Ca^{2+} channels, pumps, and exchangers), the ubiquitin system (the E1, E2, and E3 enzymes have a group of cysteine residues in their catalytic domains that can be modified by ROS), the UPS (irreversible oxidation of UPS subunits (e.g., 20S)), and the autophagy pathway, as will be described later in detail.

In neurons in particular, physiological levels of ROS are important for (i) axonal growth via cytoskeletal regulation [209]; (ii) progenitor cell growth via PI3K/AKT signalling [210]; (iii) neuronal differentiation (a specific redox state is critical for neuronal development) [211]; (iv) synaptic plasticity, via the control of intracellular calcium release and synaptic vesicle release [212]; and (v) a potential role of NOX and NOS proteins regulating long-term potentiation (LTP), pruning, and dendritic growth [213]. In addition, in the brain, ROS generated by glial cells are also involved in the modulation of synaptic activity and other metabolic compartmentalization/crosstalk with neurons (e.g., astrocytes supply essential GSH precursors for neurons [214] and, in hippocampal pyramidal neurons, Atkins et al. described that ROS are involved in a nonsynaptic glial-neuron crosstalk by modifying the myelin basic protein in oligodendrocytes [215]). Most importantly, the presence of these highly reactive species triggers several antioxidant pathways to counter the accumulation of oxidative stress, and maintain cellular homeostasis, as will be described later.

3.2.2. Negative Effects of Free Radicals: Oxidative Stress. ROS/RNS generated inside organelles can be readily released into the cytoplasm. They diffuse across membranes through aquaporins (e.g., aquaporin 8 for H_2O_2 release in mitochondria) and other specific unidentified channels [216]. In the cytosol, these highly reactive molecules modify all classes of macromolecules (i.e., carbohydrates, lipids, proteins, and nucleic acids), influence organellar homeostasis, and ultimately induce cell death [217–219]. Consistent with this, it has been recently described that ROS-induced autophagy contributes to ferroptosis, a form of programmed cell death based on iron accumulation [220]. In particular, protein oxidation can cause loss of activity and/or protein unfolding, with the tendency to induce intracellular and extracellular protein oligomers and aggregates that compromise cell viability. Indeed, this is a primary characteristic of neurodegenerative diseases (e.g., α-syn in Parkinson's, tau in Alzheimer's,

and HTT in Huntington's) [221]. Lipid peroxidation, triggering degradation of cell membrane components, is also induced in response to oxidative stress, as lipids are susceptible to redox modifications; indeed, such changes have been reported in mDANs in Parkinson's brains [222].

The nucleus is highly susceptible to oxidative stress. Diffusion of ROS/RNS into the nucleus influences diverse pathways/components, including chromatin organisation, DNA methylation, histone function (e.g., nitrated or glutathionylated histones), nucleobases, interactions between DNA and DNA-binding proteins, mutagenesis, transcription via targeting of purines and pyrimidines, single- and double-strand breaks, and abasic site formation [223]. Indeed, oxidative stress can be oncogenic by affecting the expression of oncogenes [224], and the formation of DNA adducts can trigger autoimmune disorders [225].

Yoboue et al. proposed a "redox triangle" formed by ER-mitochondria-peroxisome structures, generating a multiorganellar protein complex called the "redoxosome," where ROS and RNS accumulate to impact organelle function (e.g., ER-mitochondria calcium exchange, oxidative phosphorylation, and protein folding), an idea that awaits mechanistic validation [197, 226]. Indeed, mitochondria-associated membranes (MAM) or mitochondria-ER contacts (MERCs) are modulators of ROS production; calcium cross-talk and autophagosome formation and aberrant MAM structure and function are linked to defective autophagy during neurodegeneration [227–229].

Ultimately, high levels of oxidative stress can induce cell death via apoptosis, necroptosis, and autophagy-associated programmed cell death. Indeed, ROS/RNS activate the extrinsic death receptor pathways (e.g., tumour necrosis factor receptor family), leading to the activation of caspases, as well as the internal mitochondrial and ER cell death pathways. In mitochondria, ROS can induce apoptosis through diverse pathways, including activation of p53 and JNK, which in turn activate proapoptotic Bcl-2 proteins; oxidation of cardiolipin, leading to cytochrome *c* release into the cytosol; ATP depletion; and the induction of mitochondrial membrane depolarization. Low levels of oxidative stress in the ER activate the unfolded protein response (UPR) to inhibit protein translation, and to induce chaperone expression and protein degradation, as will be described later; whereas high levels of ROS trigger the activation of ER stress-mediated apoptosis via different pathways (e.g., prolonged activation of IRE1α triggers proapoptotic cascades, upregulation of the proapoptotic transcription factor CHOP, and activation of proapoptotic Bcl-2 proteins in the ER membrane), some of which are interconnected with mitochondrial pathways, leading to caspase activation and apoptosis [207, 230].

Crucially, the brain is particularly susceptible to oxidative stress damage. Cobley et al. defined 13 reasons why the brain is predisposed to oxidative stress and consequent neurodegeneration: (i) redox signalling (high levels of ROS/RNS can induce proapoptotic pathways via redox modifications); (ii) calcium homeostasis (oxidative stress can lead to calcium overload and affect mitochondrial function, leading to programmed cell death); (iii) excessive glutamate uptake (affecting several cellular pathways and producing excitotoxicity); (iv) glucose metabolism necessary to support neuronal activity (oxidative stress can affect this pathway via the formation of advanced end glycation products (AGE)); (v) mitochondria (there is a high ATP demand in neurons, and elevated ROS/RNS levels affect mitochondrial function and ATP formation); (vi) neurotransmitter metabolism (e.g., generation of H_2O_2 by monoamine oxidase (MOA), whose activity is disrupted in Parkinson's); (vii) neurotransmitter oxidation (formation of toxic intermediates); (viii) lower antioxidant response in comparison to other tissues; (ix) microglia activation and astrogliosis (as a big source for ROS/RNS); (x) presence of redox active transition metals (e.g., iron and Fenton reaction); (xi) lipid peroxidation (high levels of fatty acids in the brain); (xii) NOS and NOX for neuronal signalling; and (xiii) RNA oxidation [231]. In addition, oxidative stress also impacts the blood-brain barrier permeability, leading to increased trafficking of immune cells and neuroinflammation, another characteristic of neurodegenerative disorders [232].

3.3. Antioxidant Pathways for Controlling Redoxtasis. To counterbalance oxidative stress, the cell has developed several antioxidant pathways, including (i) endogenous antioxidant mechanisms (by the presence of molecules and proteins for the removal of free radicals), (ii) a metabolic switch to the pentose phosphate pathway [233], (iii) transcriptional changes by the activation of specific transcription factors, (iv) posttranscriptional regulation via redox-sensitive microRNAs, (v) activation of chaperones and specific degradation systems to avoid protein aggregation, and (vi) the degradation of damaged organelles [234, 235].

Mitochondria are protected from ROS by the presence of antioxidant enzymes that contain cysteine catalytic residues for the reduction of H_2O_2 into H_2O, and by a defence system for the conversion of $O_2^{\bullet-}$ into the less harmful radical, H_2O_2, comprising superoxide dismutase 2 (SOD2) in the mitochondrial matrix, and SOD1 in the intermembrane space. Glutathione peroxidases (GPX1 and GPX4) in the outer mitochondrial membrane, reduce H_2O_2 into H_2O, using reduced glutathione (GSH) as cofactor. Other mitochondrial antioxidant enzymes include the peroxiredoxins (PRX3 and PRX5), which also catalyse the reduction of H_2O_2 into H_2O [197, 236]. Peroxisomes are the other major centre for antioxidant enzyme function. The main ROS defence system here is catalase, which catalyses the reduction of H_2O_2 into H_2O, and indeed deficiencies in this system are linked to cancer and diabetes. In addition, in this organelle, SOD1 and PRX5 are also involved in the formation and reduction of H_2O_2, respectively [197, 237]. The ER also houses antioxidant mechanisms, by the presence of GPX7, GPX8, and PRX4 [197]. Other antioxidant molecules in the cell include ascorbic acid, uric acid, melatonin, ubiquinol, and some vitamins, which neutralize free radicals by donating electrons and other regulators of redox signalling, including the electron donor groups thioredoxins (TXN) and glutaredoxins (GRX) [238]. An additional layer of regulated antioxidant response is via cellular metabolic reconfiguration. Here, cellular metabolism is redirected towards the pentose phosphatase pathway, leading to the formation of NADPH which is

used by glutathione reductase for GSH reduction, a crucial step in responsive redoxtasis [233, 239].

Transcriptionally, NRF2 is considered to be a master regulator of redoxtasis, controlling around 1% of human genes that share in common the Antioxidant Response Element (ARE) in their promoters [240–243]. Crucially, redox regulation by NRF2 via increasing reduced TXN is also crucial for the modulation of apoptosis signal-regulating kinase 1 (ASK1) activity, involved in ER-stress neuronal cell death [244, 245]. One of the main mechanisms of regulation that cooperate to maintain NRF2 levels within physiological values is KEAP1 (Kelch-like ECH-associated protein 1), a redox-regulated E3 ubiquitin ligase substrate adaptor that promotes NRF2 degradation under basal conditions. High levels of oxidative stress modify KEAP1 to impair its function, leading to increased NRF2 that translocates to the nucleus [246]. This factor regulates the expression of genes involved in redox homeostasis (like Heme Oxigenase-1 (HO-1)) as well as in metabolic detoxification (like NAD(P)H quinone oxidoreductase (NQO1)), inflammation, and proteostasis [247]. In addition, posttranscriptional antioxidant regulation via microRNAs also target this pathway [234].

Finally, the two major quality control mechanisms in the cell have antioxidant roles in preventing the aggregation of oxidized proteins and/or the persistence of damaged organelles (namely, the ubiquitin-proteasome system (UPS) and autophagy [1, 2, 5–7]). Here, we focus on the interplay between autophagy, redox homeostasis, and transcriptional control.

4. Autophagy and Redoxtasis Crosstalk

Under stress conditions (e.g., starvation, hypoxia, and uncouplers), ROS/RNS are induced, and have the potential to influence autophagy via core autophagy protein oxidation, or by altering the activities of transcription factors [248–250]. In addition, several lines of evidence suggest that indirect activation of autophagy in response to ROS damage, including DNA oxidation and lipid peroxidation, is crucial for cell survival [251–253]. As ROS damages organelles and biomolecules, their repair and/or removal by fusion/fission or autophagic degradation (e.g., mitophagy; pexophagy; ER-phagy; aggrephagy) is a crucial facet of any ROS response. Thus, a delicate balance is needed between elevated oxidative stress promoting organelle quality control, and its negative effects on components of the autophagy machinery [254, 255] (Figure 1).

4.1. Redox Modifications of Autophagy Proteins: Upstream Pathways/Autophagy Induction. The activity of several proteins upstream of the autophagy pathway is affected by ROS/RNS. These proteins are typically also involved in the regulation of several pathways; thus, their redox modifications influence diverse cellular activities.

4.1.1. Receptor Tyrosine Kinases (RTK) for the Activation of PI3K/AKT via Growth Factors (e.g., EGF). Reversible oxidative and nitrosative modifications include sulfenylation, glutathionylation, disulfide bonds, acylation, and nitrosylation. These affect RTK receptors (including EGFR, FGF, RET, and VEGFR), affecting their activation, localisation, or trafficking, depending on the modification and residues involved (a recent review collecting all known modifications and effects can be found in [256]). In addition, PTP1B activity, involved in the inhibition of RTK signalling, is also affected by oxidation reactions, including sulfenylation, nitrosylation, and glutathionylation [256].

4.1.2. Phosphatase and Tensin Homolog (PTEN). PTEN opposes PI3K activity by dephosphorylating PIP3 and inhibiting AKT signalling. Redox modifications affect PTEN activity; for example, (i) H_2O_2 oxidation inactivates PTEN catalytic activity by the formation of disulfide bonds (C124-C71), leading to autophagy activation in a noncanonical pathway induced my mTOR activation; and (ii) peroxynitrite inhibits PTEN activity and induces neuronal survival and can be oxidized by a lipid peroxide which is prevented by PRX3 [257–259].

4.1.3. Phosphoinositide-Dependent Kinase 1 (PDK1). PDK1 is a Ser/Thr kinase that activates AKT. Redox modifications of this protein include nitrosylation in different residues leading to inhibition of its kinase activity [260].

4.1.4. AKT. Several cysteines within the pleckstrin homology domain of AKT have been identified as being reversibly oxidised, forming new disulfide bonds. These modifications affect protein function, including the stabilisation of the PI3P pocket, or its inhibition, depending on the modification. In addition, AKT can be inactivated via glutathionylation, which is reversed by glutaredoxin 1 [261, 262].

4.1.5. TSC2. Nitrosylation of TSC2 impairs its dimerization with TSC1, leading to mTOR activation [263].

4.1.6. mTORC1. Generation of disulfide bonds affects mTOR stability and activity depending on the residues involved. Oxidised mTOR can be rescued by Thioredoxin 1 [264]. Related to this, lysosomes can also sense redox signalling specifically via redox-sensing lysosomal ion channels [265].

4.1.7. AMPK. Disulfide bonds, sulfenylation, and glutathionylations have been described to be present in both α and β AMPK subunits, affecting AMPK activity depending on the modified residues (e.g., disulfide bonds result in AMPK inhibition, and this is reversed by Thioredoxin 1 but other redox modifications result in AMPK activation). In addition, free radicals induce calcium release (e.g., in hypoxia) leading indirectly to the activation of the AMPK via CaMKII activation. Similarly, it has been reported that the induction of autophagy, as a consequence of ROS production in starvation conditions and ATP depletion, is via activation of the AMPK pathway [266–270].

4.1.8. Ataxia Telangiectasia Mutated Protein Kinase (ATM). ATM is a threonine/serine kinase involved in the DNA damage/repair response. Crucially, ATM is also involved in the induction of pexophagy to maintain redox balance. In response to ROS, ATM activates MAPK, and ATM is transported into peroxisomes via the PEX5 import receptor. Here,

it phosphorylates PEX5 triggering ubiquitylation via the E3-like ubiquitin ligase complex, PEX2-PEX10-PEX12, and later recognition by cargo receptors [271, 272]. H_2O_2 treatment induces nuclear ATM redox disulfide bond formation, indirectly promoting the downstream expression of proteins involved in the pentose phosphate pathway, and an activator effect via nytrosilation has also been suggested [273–275].

4.1.9. Sirtuin 1 (SIRT1). A class III histone deacetylase regulating numerous cell activities (e.g., glucose metabolism, chromatin silencing, inflammation, and lipid metabolism) [276], SIRT1 is involved in autophagy via the release of nuclear LC3 during starvation and the deacetylation of ATG5 and ATG7 [277, 278]. In addition, in response to ROS, it is involved in the activation via deacetylation of several autophagy transcription factors, including FOXOs, p53, NRF2, HIF-1α, NF-κB, PPARs, and FXR [276, 279]. Conversely, SIRT1 translocation to the nucleus is induced indirectly by the presence of ROS, but SIRT1 can also be modified via oxidation, inhibiting its activity [280–282].

4.1.10. UPR. Numerous redox modifications have been described for the ER stress-sensing proteins, IRE1α, PERK, and ATF6 (for a recent review, see [283]). For example, cysteine sulfenylation of cytosolic IRE1α blocks UPR activation, but it induces the antioxidant NRF2 pathway [284]. Other modifications include activation of PERK kinase activity via nitrosylation [285], and disulfide bridge formation in ATF6 in unstressed ER [286].

4.1.11. The Cytoskeleton. It is also important to mention that redox modifications can also affect cytoskeletal dynamics [287], and thus, indirectly, autophagy efficiency.

4.2. Redox Modifications of Autophagy Proteins: Autophagy Proteins Involved in the Assembly Pathway and Selective Autophagy. Proteins involved in the autophagosome assembly pathway can also be targeted by ROS/RNS. These redox modifications affect the efficiency and productivity of autophagosome biogenesis (Figure 1).

4.2.1. ATG4. ATG8 processing mediated by ATG4 proteins needs to be spatiotemporally regulated to support both autophagy initiation and the availability of a pool of primed ATG8 [288]. Scherz-Shouval et al. found that ROS, produced during starvation, are essential for autophagy via regulation of ATG4. They described a cysteine residue near the catalytic site that is a target for oxidation, thus increasing autophagy initiation by blocking ATG4-mediated delipidation in the vicinity of the expanding autophagosome (exemplified by ATG4A) [289]. Later, Qiao et al. described that ROS induces the formation of a prooxidant complex called REDD1-TXNIP, which inhibits ATG4B function leading to autophagy activation [290]. In addition, Perez-Perez et al. described an inhibitory redox modification in ATG4 in yeast, via the formation of disulfide bonds outside the catalytic site, which can be reversed by Thioredoxin 1 [291]. Finally, nitrosylated ATG4 has been observed in the hippocampal neurons of diabetic rats, and in vitro, in neuronal cells in hyperglycemia conditions leading to neurotoxicity [292].

4.2.2. ATG3 and ATG7. Frudd et al. described that these proteins can be modified by oxidation, including glutathionylation and disulfide bond formation, affecting their function, and leading to autophagy inhibition. Conversely, while inactive, ATG3 and ATG7 form covalent thioester complexes with LC3, preventing their oxidation. However, after autophagy induction, their interactions become more transient, thus increasing susceptibility to redox modifications [293].

4.2.3. BECLIN1. Redox modifications affect BECLIN1 function indirectly: under normal conditions, BECLIN1 forms a complex with proapoptotic BCL-2, which inhibits BECLIN1 activity; however, under autophagy-inducing conditions, BECLIN1 dissociates to establish the PI3KC3 complex. Kitada et al. described that BCL-2 is a target of redox modification, particularly nitrosylation, stabilising the interaction with BECLIN1, and thus preventing autophagy induction [276].

4.2.4. p62/SQSTM1. Carroll et al. identified two cysteine residues in p62/SQSTM1 that can be redox modified, forming disulfide bonds that promote its oligomerisation to enable autophagy induction [294]. They further highlighted the potential effect of p62/SQSTM1 oxidation in aging [294].

4.2.5. Parkin/PKRN. Numerous redox modifications have been described for Parkin, including nitrosylation, sulfonation, and methionine oxidation. Particularly, Chung et al. described that S-nitrosylation reduces Parkin E3 ligase activity, thus affecting its protective function [295], although some discrepancies were reported by a different group [296]. Similarly, Ozawa et al. reported a new site of nitrosylation in Parkin that leads to mitophagy induction via activation of its ligase activity [297]. In addition, Meng et al. described that Parkin can be sulfonated in an in vitro Parkinson's model, leading to protein aggregation, and possibly contributing to the formation of Lewy's bodies in Parkinson's [298]. However, in a recent article (at preprint stage at the point of writing this review), Tokarew et al. highlighted the importance of Parkin's own oxidation in neuroprotection [299]. Previously, Vandiver et al. showed that Parkin can also undergo sulfhydration, enhancing its catalytic activity and its protective function [300]. In addition, they described that in Parkinson's brains, Parkin is highly nitrosylated, but that sulfhydration is reduced [300]. Lee et al. recently reported that Parkin can also undergo methionine oxidation at M192, a residue mutated in early onset Parkinson's [301], and that this is reversed by methionine sulfoxide reductase B2 (MSRB2) released in response to damaged mitochondria, thereby promoting mitophagy [302]. Finally, El Kodsi et al. have reported that Parkin can be glutathionylated in an antioxidant reaction (at preprint stage at the point of writing this review) [303].

4.2.6. PINK1. Oh et al. described that PINK1 can be nitrosylated, inhibiting its kinase activity, and this posttranslational modification is present in Parkinson's mice models where Parkin recruitment is reduced, restricting mitophagy [304].

4.2.7. Protein Deglycase (DJ-1). DJ-1 overexpression induces mitophagy via the activation of ERK in mDANs, and this

protects against rotenone-induced cell death [305]. Indeed, mutations in DJ-1 are linked to familial Parkinson's and some studies suggest that oxidised DJ-1 could potentially be used as a biomarker for Parkinson's [306]. Canet-Aviles et al. observed that cysteine-sulfinic acid formation in DJ-1 is necessary for mitochondrial targeting and neuroprotection [307], and consistent with this, Zhou et al. demonstrated that the sulfinic DJ-1 isoform prevents α-synuclein fibrillation [308]. Conversely, in the presence of high levels of oxidative stress, DJ-1 is oxidised to the sulfonic form; this isoform is inactive and predisposed to aggregate formation, and indeed, this overoxidised isoform has been detected in brains from Parkinson's patients [309, 310]. In addition, Ozawa et al. highlighted the crucial role of DJ-1 in the nitrosylation of Parkin, and suggested that DJ-1 inactivation reduces mitophagy, leading to mitochondrial dysfunction and Parkinson's pathogenesis [311].

4.3. Redox Modifications of Autophagy Proteins: Autophagy Transcriptional Control. Autophagy gene expression is influenced in different tissues by diverse transcription factors, microRNAs (miRNAs), and by epigenetic modifications [312]. In the last decade, several studies have pointed out that "nuclear" control of autophagy is key for the regulation of the autophagy process, including short-term and long-term outcomes [312]. Currently, numerous transcription factors involved in the regulation of this process have been described. Notable amongst these is transcription factor EB (TFEB), a member of the basic helix-loop-helix leucine-zipper family of transcription factors. TFEB is considered to be a master regulator of autophagy that, under starvation conditions, translocates to the nucleus, where it regulates more than 200 lysosomal-related genes and autophagy genes (including *ATG4*, *ATG9B*, *BECLIN1*, *LC3B*, *GABARAPL1*, *ATG16*, *WIPI*, *UVRAG*, and *p62*/*SQSTM1*) by binding to CLEAR (coordinated lysosomal expression and regulation network) sequences in their promoters. These genes are involved in autophagosome biogenesis, autophagosome-lysosome fusion, and lysosomal biogenesis [313, 314]. TFEB translocation is regulated primarily by phosphorylation [313, 315], via ERK2 (at S142), and via the autophagy inhibitor mTORC1 (at S211 and S142), to retain TFEB in the cytoplasm by binding to 14-3-3 proteins [314, 316, 317]. Phosphorylated TFEB is also targeted to the proteasome via the E3-like enzyme, STIP1 homology and U-Box-containing protein 1 (STUB1), thereby controlling its stability [318]. Calcineurin, activated by lysosomal calcium release via mucolipin 1 (MCOLN1), binds and dephosphorylates TFEB, causing dissociation from 14-3-3 proteins and translocation to the nucleus [319–321]. Cytoplasmic-nuclear shuttling of TFEB is also observed after refeeding, here modulated via mTORC1 phosphorylation at residues close to the nuclear exported signal (NES) (S142 and S138), with translocation mediated by exportin 1 [322]. An increase in the phosphorylated form of TFEB and dysregulation of autophagy has been correlated with the progression of neurodegenerative diseases, including Parkinson's [323, 324]. In addition, overexpression of TFEB has been reported to be beneficial in numerous disease models via clearance of aggregated protein (e.g., tau in Alzheimer's, α-syn in Parkinson's, and HTT in Huntington's [325–327]).

In this review, we focus on transcription factors whose activity is regulated by redox modifications, thus affecting autophagy transcriptional control.

4.3.1. TFEB. In addition to the well-characterised control of TFEB activities via phosphorylation (above), Wang et al. described the regulation of TFEB (and other members of the MiT family) via ROS-mediated cysteine oxidation (C212). This inhibits the interaction of TFEB with Rag GTPases, and induces its nuclear translocation, thus inducing the expression of autophagy/lysosomal genes independently of mTORC1 (although its role in neurodegenerative diseases remains elusive) [328].

4.3.2. The FOXO Family. In particular, FOXO1 and FOXO3 have been identified as autophagy transcription factors, regulating the expression of numerous autophagy-related genes [329–331]. In addition, FOXOs also regulate the expression of antioxidant genes, including *SOD1*, *SOD2*, and *GPX1* [332]. Under basal conditions, FOXOs are phosphorylated by AKT and retained in the cytosol through binding to 14-3-3 [333]. Under stress conditions, they become activated and can either translocate to the nucleus or regulate autophagy in the cytosol—acetylated FOXO1 (under oxidative stress conditions) can bind to ATG7 and activate it [329, 334, 335]. Crucially, it was shown recently that FOXO3a can be degraded by the autophagy pathway, suggesting a negative feedback mechanism for this transcription factor [336]. Mainly, examples of indirect redox regulation of the FOXO family have been described, although direct modifications via formation of disulfide bonds between FOXOs and other proteins have also been reported (e.g., disulfide bridges between FOXO4 and transportin-1 to induce nuclear translocation in response to ROS [337] and disulfide bond heterotrimers between FOXO3, PRX1, and Importin-7/Importin-8, inducing an antioxidant response [338, 339]). Gomez-Puerto et al. reported that FOXO3 is phosphorylated by MAPK and nuclear translocated in response to H_2O_2 treatment in human mesenchymal stem cells, thus leading to autophagy induction that is crucial for osteogenic differentiation; however, a direct redox modification has not yet been described [340]. Other examples of indirect redox regulation of the FOXO family include redox modifications of signalling proteins upstream of FOXO, including SIRT1 and AKT, as previously described [332].

4.3.3. NRF2. The master regulator of oxidative stress, NRF2 contributes to the regulation of autophagy-gene expression under these conditions (e.g., *p62*/*SQSTM1*, *NDP52*, *ULK1*, *ATG2B*, *ATG4*, *ATG5*, and *GABARAPL1*) [341]. Crucially, as mentioned before, the main canonical redox regulator of NRF2 is the NRF2-inhibitor protein KEAP1. Several cysteine residues in KEAP1 can be oxidised leading to conformational changes and thereby preventing NRF2 degradation [342]. In addition, p62/SQSTM1 also binds to KEAP1, marking it for degradation; meanwhile, TFEB represses the NRF2-ubiquitin ligase, DCAF11 (DDB1- and CUL4-associated

factor 11)), ultimately promoting NRF2 translocation to the nucleus [343, 344] to establish a feed-forward loop. Finally, there is some evidence indicating that NRF2 is also subject to redox cysteine modifications, promoting NRF2 nuclear translocation; consistent with this, mutations in these cysteines enhance interactions with KEAP1, thus increasing NRF2 degradation [345]. Conversely, recent evidence suggests that NRF2 also regulates CMA via *LAMP2A* expression [346]. In addition, NRF2 directly regulates *HIF-1α* expression, and interacts with ATF4 [347, 348], master regulators of O_2 homeostasis and contributors to autophagy-gene expression (in mild hypoxia, HIF-1α activates the transcription of mitophagy genes (e.g., *NIX*); whereas in severe hypoxia, ATF4 regulates the expression of autophagy genes (e.g., *ULK1* and *LC3B*) [349, 350]). Both, in turn, are regulated by ROS, and act as antioxidant and antiapoptotic proteins [351–354].

4.3.4. P53. As one of the best characterised transcription factors, p53 has been reported to regulate antioxidant genes (e.g., *GPX1*) [355], autophagy genes after DNA damage (e.g., *ULK1*, *ATG4*, *ATG7*, and *ATG10*), and to induce TFEB nuclear translocation [356]. It also stabilises NRF2 indirectly by regulating the expression of p21 and SESN2, prominent KEAP1 interactors [357]. However, cytoplasmic p53 inhibits autophagy via posttranscriptional downregulation of LC3A [358]. Several redox modifications have been reported in p53, including glutathionylation and nitrosylation at residues near the DNA-binding domain, with the former causing inhibition of p53 DNA binding [359]. Indeed, previous data suggest that glutathionylated p53 may be involved in Alzheimer's neurodegeneration [360]; however, nitrosylation seems to be essential for DNA binding and antioxidant gene expression [361].

4.3.5. NF-κB. Under basal conditions, the proinflammatory transcription factor, NF-κB, is inactivated in the cytosol where it interacts with IκB (inhibitor of κB) preventing its nuclear translocation. Previous data suggest that oxidative stress can induce or inhibit the NF-κB pathway, depending on conditions [362]. As one example of indirect regulation, IκB is phosphorylated under oxidative stress, leading to its polyubiquitination and consequent degradation [363]. The consequent elevation of nuclear NF-κB upregulates the expression of several anti-inflammatory and antioxidant genes (e.g., *HO-1*, *Thioredoxin 1*, *GPX1*, *NOS2*, and *SOD2*) [364], and can induce or inhibit autophagy depending on the context (i.e., NF-κB mainly inhibits autophagy, but it can also activate the expression of the autophagy genes including *BECN1* and *p62/SQSTM1* [365–367]). ROS can also directly regulate NF-κB activity. Disulfide bonds in cysteine in the DNA-binding domain inhibits DNA binding, and this can be rescued by Thioredoxin 1 [368]. Similarly, other redox modifications in NF-κB including glutathionylation and nitrosylation also inhibit DNA binding [369, 370].

4.3.6. Other Transcription Factors. Examples of other transcription factors involved in both the regulation of autophagy-related and antioxidant genes include (i) the peroxisome proliferator-activated receptors (PPARs) that upregulate autophagy and can be directly regulated by redox modifications (e.g., nitrosylation) [365, 371, 372] and (ii) the transcription factor, farnesoid X receptor (FXR), mainly expressed in liver and intestine, a nuclear receptor involved in metabolism [373–375]. Under fed conditions in liver, FXR inhibits autophagy-gene regulation directly (e.g., *ULK1*, *ATG2*, *ATG5*, *ATG7*, *WIPI*, *GABARAP*, and *TFEB*) [376].

5. Autophagy and Oxidative Stress in Parkinson's

Parkinson's is one of the most common neurodegenerative disorders, second only in prominence to Alzheimer's, and it affects 1-3% of the population aged over 60 [377, 378]. Life expectancy can be lower in many Parkinson's patients [379], due to an increased risk of developing other diseases including infections (pneumonia being the most common cause of death in Parkinson's) [380, 381], certain types of cancers (e.g., brain and breast cancer; although, generally, there is an inverse association between cancer and Parkinson's [382]), and cardiovascular disease [383]. In the UK, the number of Parkinson's patients in 2018 according to the Parkinson's UK website [384] was estimated to be 145,500, and this is predicted to reach 250,000 by 2065. This incidence is affected by age, gender, environmental factors [385], and genetics (10-15% of Parkinson's cases are familial [386], with several recognised Parkinson's-associated genes [387]). In patients, the main symptoms are motor problems including bradykinesia (slowness of movement), hypokinesia (paucity of movements), postural instability (balance impairment), tremor at rest, muscle rigidity, and gait problems (walking abnormalities), with mild cognitive impairments, sleep disorders, and impulsive behaviours also common [388–390].

At the cellular level, Parkinson's is characterised by the loss of mDANs, initially in the substantia nigra pars compacta (SNc). As the condition progresses, defects in serotonergic, noradrenergic, cholinergic, GABAergic, and glutamatergic neuronal pathways can also be observed [391]. Although the exact causes of neuronal loss in Parkinson's are not known, the hallmarks that characterise this disease include (i) the accumulation of α-syn-rich Lewy's bodies; (ii) increased oxidative stress accompanied by a reduction in antioxidants; (iii) neuroinflammation; (iv) mitochondrial dysfunction; (v) ER stress; (vi) and disruption in protein quality control, including autophagy dysregulation [392]. Crucially, mDANs appear to be particularly sensitive to autophagy deficits, and are frequently exposed to high levels of oxidative stress [96, 231, 325, 393–396]. In the following sections, we will summarise how these two processes are interconnected, and the links with other Parkinson's hallmarks (Figure 2).

5.1. Midbrain Dopaminergic Neurons and Degeneration of the Nigrostriatal Pathway. mDANs are localized in the mesencephalon, and are characterised by the production of the catecholaminergic neurotransmitter, dopamine [397]. Dopamine belongs to the monoamine neurotransmitter group that

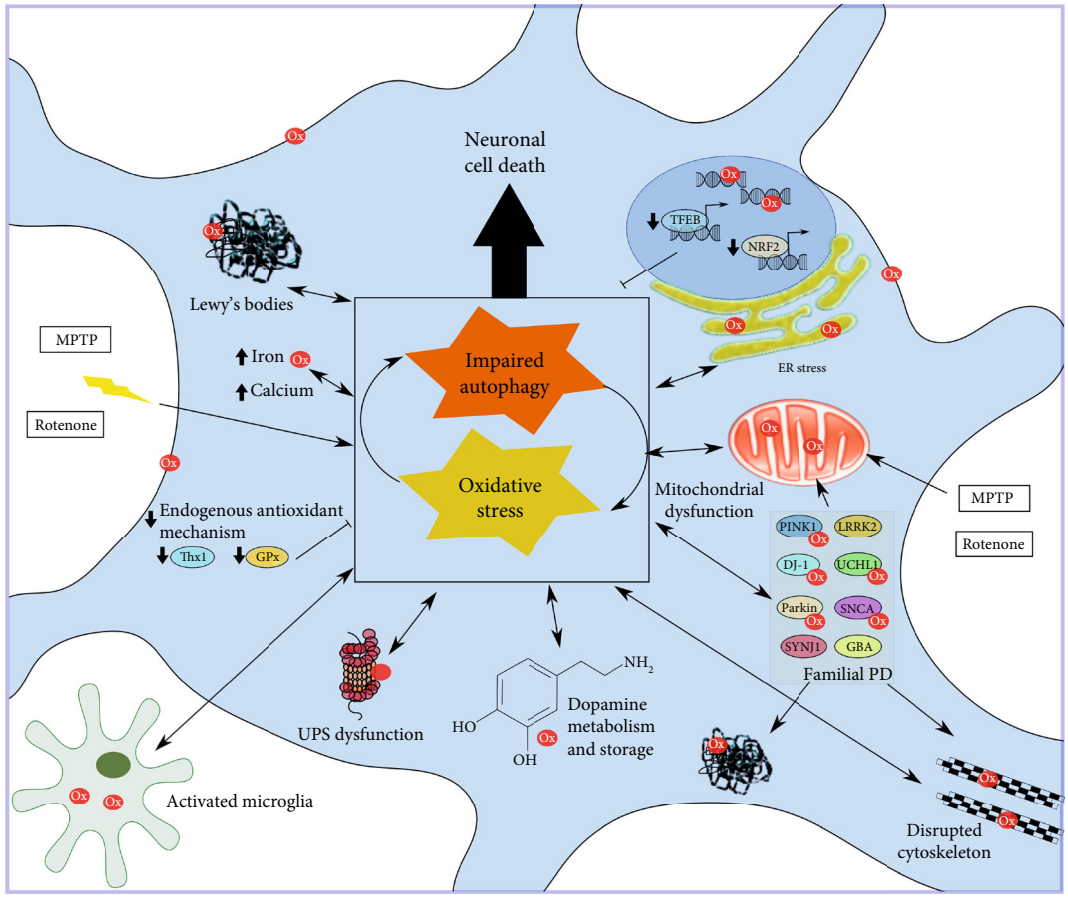

Figure 2: Oxidative stress and autophagy dysregulation in Parkinson's. Oxidative stress and autophagy dysregulation are interconnected in the dopaminergic neurons affected in Parkinson's. In addition, several conditions contribute to this destructive imbalance leading to neuronal death and progressive neurodegeneration, including a reduction in antioxidant pathways (e.g., a reduction in endogenous antioxidant mechanisms and antioxidant transcription factors); ER stress; mitochondrial dysfunction; mutations in key proteins modulating these processes (familial Parkinson's); disruption of the cytoskeleton; UPS dysfunction; neuroinflammation; high levels of calcium and iron, leading to neurotoxicity; neurotoxins (e.g., MPTP and rotenone); and α-syn aggregation in Lewy's bodies.

also contains serotonergic or 5-hydroxytryptamine (5-HT) and noradrenergic neurotransmitters [398]. Dopamine biosynthesis occurs by a two-step process in the mDAN cytosol, and is considered the key element of the oxidative stress theory in Parkinson's: (i) tyrosine is hydroxylated to L-DOPA by tyrosine hydroxylase (TH), an enzyme that can also oxidise L-DOPA leading to ROS production [399]; and (ii) L-DOPA is then decarboxylated to dopamine by the aromatic amino acid decarboxylase (AADC), which can be further oxidised as will be described later. Subsequently, dopamine is incorporated into synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2). Inside these vesicles, dopamine is stabilised by the acidic pH. Ultimately, the neurotransmitter is released into the synapse for signal transduction [400]. Dopamine receptors include D1-like receptors (D1 and D5) and D2-like receptors (D2, D3, and D4). [401], and these differ in their localisations within the brain, their modes of action (D1-like receptors activate adenylate cyclase (AC) whereas D2-like receptors inhibit its activation), and their functional influences (e.g., locomotion, emotion, appetite, learning, attention, reward, and memory) [401]. Dopamine reuptake from the extracellular space into presynaptic neurons is regulated by the dopamine transporter (DAT) [402].

There are three different clusters of mDANs, designated as follows: (i) A8, those originating from the retrorubral field (RRF); (ii) A10, those found in the ventral tegmental area (VTA) forming the mesolimbic (to the nucleus accumbens) and mesocortic (to the frontal cortex) dopaminergic pathways; and (iii) A9, the cells located in the SNc that project to the striatum. Functionally, mDANs involved in emotion-based behavior are found in the A8 and A10 groups, whereas those responsible for voluntary movement control are specified as A9 [403]. As A9 SNc mDANs are the first to be lost in Parkinson's, it is worth focusing on the enhanced vulnerability of these cells. In healthy individuals, voluntary movement is controlled in the basal ganglia via a direct (to increase motor activity) and an indirect (to decrease motor activity) pathway that conveys signals to the motor cortex via the thalamus, and from there, on to the spinal cord [404]. In Parkinson's, loss of mDANs correlates with a reduction in dopamine release, leading to under activation of the direct

pathway and hyperactivation of the indirect pathway, with an overall increase in thalamus inhibition and reduced voluntary movement being the net effect [405, 406].

Perhaps the most compelling explanation for the vulnerability of mDANs is that their atypical morphology and physiology require that these cells operate close to their energy demand/supply threshold [407]. A9 mDANs have very long, unmyelinated axons, with extensive arborisation, and abundant synapses with unique electrophysiological properties [408–411]. They display autonomous low-frequency pace-making activity, controlled by L-type (Cav1) Ca^{2+} channels, to provide tonic dopamine release to the striatum [407]. This burdens them with the additional challenge of coping with excess cytosolic Ca^{2+}. Unfortunately, SNc mDANs have intrinsically low Ca^{2+}-buffering capacity, unlike their counterparts in the neighbouring VTA, thus placing an extra reliance on energy-dependent Ca^{2+} efflux and Ca^{2+} sequestration in mitochondria [412–415]. Although high mitochondrial Ca^{2+} supports enhanced oxidative phosphorylation (OXPHOS) rates [416], this comes at the expense of increased mitochondrial oxidative stress [417]. In addition, increased mitochondrial activity results in high levels of cellular iron content due to the numerous mitochondrial enzymes using it as a cofactor. Numerous publications have reported an increased vulnerability of mDANs to iron-induced oxidative stress (e.g., dopamine oxidation), and iron chelators have a neuroprotective effect [418–421]. Indeed, the generation of ROS may result from a disruption of aerobic metabolism. The resulting steady decline in mitochondrial fitness ultimately leads to apoptosis [412, 414], and for this reason, efficient mitochondrial quality control mechanisms are needed to maintain a healthy mitochondrial population in these cells. In addition, their characteristic neuronal morphology, with extensive arborisation and numerous axonal terminals, creates a high energetic demand to sustain their abundant synapses, indicated by the higher density of axonal mitochondria and high dependency on the cellular trafficking machinery [417, 422].

Perhaps most tellingly, neurotoxins that target mitochondria can induce selective mDAN cell death. A neurotoxin used to generate Parkinson's mouse models, 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibits mitochondrial Complex I in the ETC, causing ROS damage and mitochondrial dysfunction [423]. MPTP can cross the blood-brain barrier, it is metabolised to MPP^+ and transported by DAT into mDANs [424]. Another Complex I inhibitor is the hydrophobic toxin rotenone, and this can also cross the blood-brain barrier to freely diffuse into cells. Several studies suggest a selective vulnerability of dopaminergic neurons to this compound, and it has been widely used as a Parkinson's-inducing model [425–429]. Rotenone acts by inducing an increase in ROS production, elevating mitochondria bioenergetics, dysregulating intracellular calcium homeostasis, dysregulating autophagy, and altering lipid and glutamine metabolism [417, 430–433]. Other examples of neurotoxins affecting mitochondrial function that are commonly used as Parkinson's models include 6-hydroxydopamine (6-OHDA) and paraquat, causing neurodegeneration via mitochondrial dysfunction and increase in free radicals [424, 434]. In the following sections, we discuss how redox imbalance in the Parkinson's brain affects autophagy pathways to exacerbate the disease.

5.2. Autophagy Dysregulation in Parkinson's and Its Interplay with Oxidative Stress. Elevated levels of oxidative stress in Parkinson's correlate with lipid peroxidation, nucleic acid oxidation, elevation of intracellular calcium and increased iron content, and protein oxidation and nitration [395, 435]. In addition, antioxidant deficiencies have been observed in Parkinson's (e.g., low levels of thioredoxin reductase 1 and glutathione peroxidase) [436–438], and might be involved in the aggravation of the disease at early stages (e.g., in early stages of an α-syn Parkinson's model, Nrf2 deficiency increased dopaminergic cell death, neuroinflammation, and protein aggregation [439]),while upregulation of antioxidant pathways appear to be beneficial as a potential therapeutic target (e.g., upregulation of the NRF2 pathway prevents neuronal death in MPTP and α-syn Parkinson's mice models [440, 441] and restores defective locomotor activity in a *Drosophila* Parkinson's model [442]). On the other hand, increased immunoreactivity of ROS-producing enzymes, particularly NOX complexes, has also been observed in Parkinson's [443], and indeed, NOX2 activation in a rotenone-induced Parkinson's model impairs autophagy and induces cell death [444], while inactivation of NOX complexes has a neuroprotective effect [445].

One of the main features of oxidative stress in mDANs is the oxidation of dopamine. In dopaminergic neurons, cytosolic excess dopamine is oxidised to the metabolite aminochrome or other toxic dopamine quinones. These are intermediates in the normal process of dopamine oxidation to neuromelanin, a dark polymer pigment that accumulates with age in the SNc and has neuroprotective roles as a metal chelator (e.g., preventing iron-mediated oxidative damage) [446, 447]. Aminochrome is toxic, and has been proposed to play an important role in the neurodegenerative process through different mechanisms: (i) the formation and stabilisation of neurotoxic protofibrils of α-syn aggregates [448]; (ii) mitochondrial dysfunction by inhibiting Complex I [449]; (iii) cytoskeletal disruption and impairment of axonal transport, with restricted autophagosome-lysosome fusion and lysosomal dysfunction [450, 451]; and (iv) neuroinflammation via the activation of microglia and astrocytes [452]. Consequently, aminochrome contributes to autophagy impairment both cell autonomously [453], and noncell autonomously via neuroinflammation, which itself is linked to glial autophagy dysfunction (e.g., the inflammatory cytokine, TNFα, impairs autophagy flux in microglia via mTORC1 [454]).

Over the previous decade, numerous lines of evidence have highlighted the importance of autophagy in neuronal homeostasis. In the absence of autophagy in dopaminergic neurons (in *Atg7* knockout mice), inclusions containing α-syn and p62/SQSTM1 are observed predominantly in neurites, and these increase with age, preempting neurodegeneration and motor dysfunction [96]. Particularly, the location of autophagic structures and cargo needs to be considered due to the unique characteristics of autophagosome assembly,

maturation, and trafficking in neurons (e.g., fewer autophagosomes in the soma might be an indication of disrupted retrograde transport from the distal axon, including oxidative modifications that can affect the cytoskeleton) [287, 455–457]. This implicates autophagy in mDAN protection, and indeed, genetic autophagy induction (*BECN1*, *TFEB*, and *LAMP2A* overexpression) in *α*-syn (*SNCA*) overexpression mouse models ameliorates synaptic and dendritic pathology [325, 458, 459], while induction via rapamycin (mTORC1 inhibitor) treatment in induced pluripotent stem cell- (iPSC-) derived neurons promotes clearance of *α*-syn aggregates and reduces oxidative stress levels in a paraquat-induced Parkinson's mice model [105, 460]. Conversely, Hunn et al. in 2019 showed that impaired macroautophagy (*Atg7* conditional knockout) in a *SNCA* mouse model led to dopamine release and improved motor movement, while aggravating pathology as reported by increased p62/SQSTM1 inclusions and neuronal death [461]. In addition, it is known that inefficient mitophagy plays an important role in the pathogenesis of Parkinson's, including an accumulation of mitochondrial ROS [462], an observation that correlates with the high vulnerability of SNc mDANs with high energetic demands. Mutations in *PINK1*, *PRKN*/*Parkin*, and *FBXO7*—three proteins involved in the recognition of damaged mitochondria—are linked to familial Parkinson's (identified as *PARK2*, *PARK6*, and *PARK15*, respectively) [463]. In addition, as previously described, redox modifications have been described for PINK1 and Parkin, highlighting the interplay between these two pathways. Indeed, in hiPSC-based Parkinson's disease models, PINK1 nitrosylation—also observed in transgenic Parkinson's mice models—correlates with reduced Parkin recruitment efficiency and mitophagy disruption [304]. Similarly, oxidised forms of Parkin have been described in Parkinson's, including Parkin sulfonation linked to protein aggregation (including Lewy's body formation and associated redox changes) in an in vitro MPTP-induced Parkinson's model (for a description of all oxidised Parkin forms, see Section 4.2) [295, 298, 300]. In addition, in zebrafish, loss of *Nipsnap1*—a mitochondrial matrix protein involved in PINK1/Parkin-independent mitophagy and highly expressed in mDANs—caused Parkinson's hallmarks [9, 135]. In the light of these findings, an autophagy inducer, Nilotinib, is currently in clinical trial for Parkinson's [464, 465].

Apart from the mutations found in mitophagy genes, several genes linked to familial Parkinson's either directly or indirectly modulate autophagy (including *UCHL1*, *DJ-1*, *LRRK2*, *ATP13A2*, *USP24*, *HTRA2*, *VPS35*, *SYNJ1*, *VPS13C*, and *GBA*) [387, 466], and some of them that are also linked to and/or regulated by ROS/RNS are discussed below.

5.2.1. UCHL1 (Ubiquitin C-Terminal Hydrolase L1, PARK5). UCHL1 is a deubiquitylating enzyme [467]. It can impair autophagosome formation, with the *UCHL1* I93M mutant overriding this suppression [468]. In response to oxidative stress, UCHL1 promotes cell survival in cancer cells; however, in rotenone-induced Parkinson's mouse models, Kumar et al. reported that UCHL1 undergoes nitrosylation, disrupting its deubiquitinase activity and causing structural instability and aggregation, thereby promoting *α*-syn aggregation [469, 470].

5.2.2. DJ-1 (Protein Deglycase, PARK7). DJ-1 acts in parallel with the PINK1/Parkin pathway, playing an important antioxidant role to protect mDANs against oxidative damage [471]. However, its roles are widespread in the cell (for a recent review, see [472]). DJ-1 is a cytosolic protein, but under stress conditions (e.g., oxidative stress), it translocates to mitochondria and to the nucleus. It contributes to (i) dopamine production via activation of TH and ADCC; (ii) regulation of mitochondrial activity via interactions with the antiapoptotic protein BCL-xL and Complex I; (iii) the upregulation of CMA, acting as a molecular chaperone interacting with *α*-syn; and (iv) the regulation of transcriptional activity via activation of NF-*κ*B, p53, and NRF2 pathways [308, 472]. Crucially, through NF-*κ*B regulation, DJ-1 controls the expression of mitochondrial uncoupling proteins, UCP4 and UCP5, that decrease mitochondrial membrane potential, thereby suppressing ROS production; meanwhile, DJ-1 binds to Complex I via NDUFA4 to maintain its activity, and is thus crucial for mDAN survival [472]. As previously described, DJ-1 is also involved in the nitrosylation of Parkin1 [311], and DJ-1 itself can be oxidised. This is crucial for neuroprotection, and indeed, levels of oxidised DJ-1 are reduced in Parkinson's patients [307, 473], and low levels of DJ-1 increase vulnerability to oxidative stress [474].

5.2.3. LRRK2 (Leucine-Rich Repeat Kinase 2, PARK8). LRRK2 is degraded by CMA, and its most common mutation (G2019S) increases its kinase activity, restricting its degradation [475–477]. G2019S LRRK2 can also inhibit CMA activity, affecting CMA substrate degradation in general [475, 478]. LRRK2 comprises multiple domains, and thus regulates several, distinct functions, including neurite outgrowth, vesicle trafficking, nuclear organisation, mitochondrial homeostasis, and autophagy, via different pathways [479]: (i) it activates endophilin A, a neuron-specific protein involved in recruitment of ATG3 [118]; (ii) it modulates mitophagy via Rab10 and Parkin interactions [480, 481]; (iii) it regulates autophagy by activating ERK, MAPK, and PI3KC3-C1 [482]; and (iv) it modulates lysosomal pH via interactions with the proton pump [483]. Although yet to be confirmed in mammalian cells, yeast wild-type LRRK2 appears able to protect against oxidative stress, depending on mitochondrial function and endocytosis, and an increase in dopamine oxidation has been reported in mutated LRRK2 neurons [484, 485]. Indeed, LRRK2 function may be regulated by ROS, as arsenite and H_2O_2 treatments downregulate LRRK2 phosphorylation, preventing binding to 14-3-3 in vitro [486, 487].

Mutations in the lysosomal enzyme, glucocerebrosidase (*GBA*)—in which homozygous mutations lead to Gaucher's disease—are one of the most common risk factors for Parkinson's, and GBA deficiency is associated with mitochondrial dysfunction and oxidative stress [488, 489]. In addition, Li et al. reported in iPSC-derived mDANs from Parkinson's patients with mutations in GBA, autophagic and lysosomal defects, with impaired calcium homeostasis and mitochondrial

dysfunction in mouse and neuroblastoma cells and increase in oxidative stress [490].

Finally, as we previously mentioned, glial cells—i.e., astrocytes, microglia, and oligodendrocytes—represent about 90% of all cells in the brain, and are critical for maintaining neuronal homeostasis (e.g., synapse functions, metabolism, neurodevelopment, myelination, neuroinflammation, and axonal regeneration) and alterations of neuron-glia signalling pathways are associated with neurodegenerative diseases, including Parkinson's [491–493]. A potential intercellular regulation of neuronal autophagy by glial cells has been reported in induced pluripotent stem cell- (iPSC-) derived motor neurons using conditioned media from iPSC-derived astrocytes from ALS patients [494]. In addition, overexpression in astrocytes of the oxidative stress regulator and autophagy transcription factor NRF2 promotes α-synuclein degradation in an α-synuclein mutant (A53T) mouse model [341, 495]. Similarly, di Domenico et al. showed that iPSC-derived astrocytes derived from Parkinson's patients presented deficient CMA, impaired macroautophagy, and α-syn aggregates, and this was rescued with a CMA activator [496]. However, the exact mechanism for the regulation of neuronal autophagy by this pathway remains elusive. On the other hand, recent studies support a model of direct transfer of cellular garbage from neurons to glial cells for their degradation, especially relevant for mitochondria, in a process termed "transmitophagy" [497]. In this study, damaged mitochondria in the axons of retinal ganglion cells were engulfed and degraded by neighbouring astrocytes [497]. In addition, in *C. elegans*, neurons release vesicles called exophers in a process that is enhanced during stress or when autophagy is inhibited, and these contain protein aggregates and organelles that are subsequently engulfed by adjacent cells [498].

5.3. Parkinson's Hallmarks Linking Autophagy Disruption with Increased Oxidative Stress. Certain hallmarks of Parkinson's are thought to exacerbate pathology through the disruption of autophagic flux. Here, we will describe how different Parkinson's features are linked to autophagy disruption and oxidative stress damage.

5.3.1. Lewy's Bodies. The best characterised feature of Parkinson's is the presence of Lewy's bodies; however, these are not observed in all Parkinson's cases, and are also found in healthy patients where they are referred as incidental LB disease [499]. α-Syn is a major component of this fibrillar aggregate; however, more than 70 additional molecules have been identified as coconstituents (including DJ-1, PINK1, Parkin (sulfonylated Parkin leads to protein aggregation and contributes to Lewy's body formation [298]), and LRRK2) [500]. Mutations in *SNCA* (e.g., A53T and A30P), posttranslational modifications (e.g., phosphorylation, ubiquitination, and oxidation), and autophagy dysfunction increase the rate of oligomerisation, and thus the formation of inclusions [501, 502]. Importantly, several redox modifications have been described for α-syn. For example, Giasson et al. reported that α-syn can be nitrated at specific tyrosine residues, and these modifications are found in Lewy's bodies [503]. Jiang and Chang demonstrated the presence of disulfide bonds in α-syn that enhance its propensity to aggregate [504]. Ponzini et al. described that α-syn methionine oxidation inhibits secondary structure formation [505]. In addition, mitochondria-associated ER membranes—whose functions are compromised in Parkinson's—are also the residence of a subpopulation of α-syn, and mutations or overexpression of α-syn enhances the extent of contact sites and affect mitochondrial function [506–509].

Overall, accumulation of α-syn in oligomers affects neurotransmitter release, synaptic vesicle recycling and trafficking, and autophagy (both CMA and macroautophagy); meanwhile, it increases ROS/RNS levels (e.g., α-syn oligomers induce Parkin, DJ-1, and UCHL1 nitrosylation), triggers microglial activation, impacts on mitochondrial homeostasis, and induces ER stress and calcium homeostatic imbalance [501, 510–518]. In its aggregated form, α-syn can block CMA, thereby preventing the degradation of itself and other CMA substrates [513]. Aggregates of α-syn also stimulate cell death via oxidative-nitrosative stress, and this [507]further enhances α-syn persistence leading to a compensatory response of increased macroautophagy and an accumulation of aggregates in autophagosomes [2]. Conversely, α-syn aggregates can also impair macroautophagy (e.g., the A30P *SNCA* mutant impairs macroautophagy via inactivation of c-Jun and activation of the transcriptional repressor, ZKSCAN3 [515]) [519, 520]. In addition, several studies have highlighted the presence of different mechanisms for toxic α-syn aggregates to be transferred to other cells, including via exosomes, direct penetration, endocytosis, nanotubes, *trans*-synaptic junctions, or receptor-mediated internalisation, all of which are predicted to spread pathology within the brain [510, 521], and indeed, exocytosis and prion-like intercellular transfer of α-syn increase with oxidative stress and autophagy impairment [522, 523].

5.3.2. Neuroinflammation. Another Parkinson's hallmark that may impact on or be affected by redox imbalance is neuroinflammation [524]. Neuroinflammation is an immune response mainly controlled by microglia and astrocytes in order to respond to an injury, and remove cell debris, and triggered in response to toxic molecules. Microglia and astrocytes act as antioxidant systems to remove excess ROS/RNS; however, in Parkinson's, free radical levels exceed the detoxifying capacity, which can be also compromised due to genetic mutations and autophagy disruption. Oxidative stress exacerbates this chronic response due to the release of oxidised molecules, including neuromelanin, aminochrone, and α-syn [525]. In turn, chronic neuroinflammation is thought to increase oxidative stress by inducing reactive astrogliosis and microgliosis, leading to the production of ROS/RNS, and contributing to mDAN death [526, 527]. In addition, neuroinflammation is also closely related to autophagy dysfunction. Indeed, TNFα impairs autophagy flux in microglia, and autophagy induction promotes microglia polarisation towards a M2 neuroprotective phenotype [454]. Similarly, neuroinflammation in premotor neurons in stress-induced hypertension rats blocks autophagy flux [528], and mitochondrial antiviral signaling (MAVS) in

microglia is involved in microglial activation and this is negatively regulated by autophagy [529]. In addition, autophagy regulates the inflammasome—an innate immune system complex—which in turn inhibits autophagy, and directly regulates IL-1β signalling [530–532]. Indeed, autophagy-deficient microglia lead to an increase in inflammasome activation and causes Parkinson's-like symptoms in mice [533]. Similarly, autophagy inhibition contributes to the exacerbated proinflammatory response in microglia, while autophagy dysfunction in astrocytes might contribute to the progression of the disease; these are reviewed in detail elsewhere [534, 535].

5.3.3. Impairment of the UPS. Disrupted protein quality control in Parkinson's is linked to impairment of the UPS, involved in the selective degradation of the majority of abnormal proteins in the cell [536]. Oxidised and damaged proteins are mainly degraded by the proteasome and the autophagy pathway. α-Syn aggregates, mitochondrial dysfunction, oxidative stress, familial Parkinson's (e.g., Parkin and UCHL1 mutations correlate with reduction in proteasome function), and other conditions impair UPS function, which in turn, leads to an increase in oxidised and damaged protein levels, increased toxic iron, and increasing vulnerability of mDANs [537–539]. Indeed, in an UPS-impaired Parkinson's mouse model, UPS inhibition activated the autophagy-lysosomal system in mDANs [540]. UPS and autophagy are closely related and dynamically interconnected, where p62/SQSTM1 appears to be one of the main modulators [541–543]. In addition, previous evidence indicates that proteasome function is modulated by redox modifications [544].

5.3.4. ER Stress. ER stress/dysfunction and chronic UPR activation are further Parkinson's hallmarks [545]. Disruptions in the protein quality control systems and increase in oxidative stress levels contribute to an increase in misfolded proteins in the ER, leading to an exacerbated ER stress and a chronic UPR activation. When ER stress is too severe, it contributes to the generation of oxidative stress and the UPR initiates programmed cell death [546]. Consistent with this, numerous lines of evidence highlight a specific vulnerability of mDANs to ER stress and protein misfolding [518]. For example, CHOP depletion in mice has a neuroprotective role in mDANs against 6-OHDA, but not in a MPTP Parkinson's model [547]. Similarly, mDANs deficient of the UPR transcription factor X-box binding protein 1 (XBP1) were resistant to 6-OHDA; however, XBP1 downregulation in the SNc caused increased neurodegeneration linked to ER stress, and local SNc XBP1 overexpression had a neuroprotective effect against 6-OHDA or MPTP [548, 549]. Indeed, similar results were found with ATF6 overexpression protecting against MPTP neurodegeneration [550]. MPTP treatment also affects calcium homeostasis in the ER via inhibition of the store-operated calcium entry (SOCE) leading to calcium imbalance [551]. In addition, ER-mitochondria associations—required for calcium homeostasis, mitochondrial function, autophagy, and ER functionality [227]—are altered in Parkinson's, as is the mitochondrial UPR [507, 552]. Overall, the crucial role of ER stress in Parkinson's pathology suggest a key role of ER-phagy; however, the influence of ER-phagy in Parkinson's initiation and progress remains elusive.

5.3.5. Peroxisomal Dysfunction: A New Player? Finally, it is also important to mention that the other major source of ROS/RNS in the cell, peroxisomes, are also affected in Parkinson's. Indeed, peroxisomes are required for neuronal homeostasis and function, and peroxisomal dysfunction has been suggested to contribute to α-syn aggregation [553–555]. In addition, recently, Jo et al. identified the mitochondrial chaperone HSAP9—associated with Parkinson's [556]—acting as a pexophagy regulator in vitro and in vivo; HSAP9 downregulation in neuroblastoma cells increased pexophagy activity, and this could not be rescued by HSAP9 mutated forms found in Parkinson's patients [557]. However, the precise roles of peroxisomes and pexophagy in Parkinson's pathology remain unclear.

Overall, autophagy dysfunction and increased oxidative stress are two closely related hallmarks present in dopaminergic neurons in Parkinson's, aggravated by other Parkinson's features including mitochondrial dysfunction, elevated iron and calcium levels, increase in dopamine oxidation, UPS dysfunction, ER stress, neuroinflammation, and α-syn aggregation.

6. Overview and Conclusions

Autophagy is an intracellular process required for the maintenance of cellular homeostasis, being particularly crucial in neurons as they are postmitotic cells highly vulnerable to stress. Dysfunctional autophagy typically correlates with neurodegenerative diseases, with mitophagy being a particularly important link due to the increased vulnerability of mDANs to autophagy deficits and mitochondrial dysfunction [412, 417]. The regulation of autophagy is closely related to redox homeostasis. To maintain homeostasis, cells have developed antioxidant mechanisms to control the level of free radicals in the cell, including the turnover of ROS-damaged organelles. Upon stress or injury, autophagy is one of the main antioxidant pathways in the cell via the degradation of damaged organelles (e.g., degradation of the major source of free radicals in the cell including mitophagy, ER-phagy, and pexophagy) as well as damaged/misfolded proteins. Basal ROS/RNS levels are also important in the cell as they are involved in cellular signalling, highlighting the beneficial effect of these radicals for cell survival. Indeed, oxidative modifications in redox-sensitive amino acids in proteins involved in the autophagy pathway have been described, including (i) those involved in the upstream pathway; (ii) those directly involved in the process, and iii) those involved with transcriptional regulation of autophagy, highlighting the interplay between these two processes.

However, when the balance of antioxidant mechanisms and ROS/RNS generation is disrupted because the antioxidant defence is reduced (e.g., dysfunctional autophagy), or because ROS generation is increased, oxidative stress is initiated. This can damage the cell, including triggering neuronal cell death programmes, the primary driver in Parkinson's. In

addition, dopaminergic neurons are particularly vulnerable to autophagy deficits and high levels of oxidative stress. Consistent with this, different factors contribute to the disruption of the autophagy pathway linked to an increase in oxidative stress, including (i) dopamine oxidation leading to the formation of the toxic molecules like aminochromone; (ii) familial Parkinson's-associated genes involved in autophagy and oxidative stress regulation; (iii) mitochondrial dysfunction, including an increase in calcium and iron levels; (iv) neurotoxins affecting almost exclusively dopaminergic neurons (e.g., MPTP, rotenone); (v) oxidation of biomolecules (e.g., lipid peroxidation, DNA oxidation, and α-synuclein oxidation); (vi) UPS dysfunction; (vii) disruption in the cytoskeletal transport; (viii) neuroinflammation; (ix) ER stress and chronic activation of the UPR; and possibly (x) peroxisomal dysfunction.

Currently, there are only symptomatic treatments for Parkinson's, and no disease-modifying therapies have been described. The most commonly used approaches to treat motor deficiencies are based on pharmacological stimulation of the dopaminergic pathway—e.g., levodopa (L-DOPA, dopamine precursor), dopamine agonists [558], and nonpharmacological treatments such as deep brain stimulation (DBS) [559]. However, none of these are capable of delaying or stopping the progression of the disease. Other promising therapies, some of them yet not tested in humans, have been developed over recent years, including stem cell-based approaches (stem cell and induced pluripotent stem cells derived from patients' fibroblasts have emerged as a powerful tool to obtain a renewable source of dopaminergic neurons that can integrate in the brain [560, 561]), the use of neurotrophic factors (e.g., BDNF and GDNF (glial-derived neurotrophic factor)), antioxidants as neuroprotective compounds (e.g., NOS inhibitors, iron chelators, and NRF2 activators [438, 441, 562, 563]), gene therapy (e.g., viral-gene expression of TH, AADC, or VMAT2 to induce dopamine release, or NURR1 expression which appears to have a neuroprotective role), and immunotherapy (e.g., for the clearance of α-synuclein aggregates) [564–567]. In addition, another divergent approach is enhancing the autophagy process. The most tested autophagy enhancers are the mTORC1 inhibitor, rapamycin, and the inositol monophosphatase inhibitor, lithium, each of which significantly reduced α-synuclein aggregates and cell death in Parkinson's models [568, 569]. However, they are nonselective for autophagy, affecting other pathways, and thus treatment with these compounds presented numerous side effects [570]. For this reason, recent strategies have focused on specific targeting of autophagy components (e.g., TFEB and Beclin1) [324, 325, 458, 571–573], or the lysosome, including increased acidification and overexpression of LAMP2A (targeting CMA) [459, 571, 574–576].

Overall, there is a strong evidence for the interplay of autophagy and redox homeostasis and how it plays a crucial role in Parkinson's. However, there is still a lot to explore and future research would contribute to a better understanding of this tight relationship and potential target for selective therapies.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Hypoxia Helps Maintain Nucleus Pulposus Homeostasis by Balancing Autophagy and Apoptosis

Han-Jun Kim,[1,2,3] Hye-Rim Lee,[1] Hyosung Kim,[1] and Sun Hee Do[1]

[1]*Department of Veterinary Clinical Pathology, College of Veterinary Medicine, Konkuk University, Gwangjin-gu, Seoul 05029, Republic of Korea*
[2]*Department of Bioengineering, Henry Samueli School of Engineering and Applied Sciences, University of California-Los Angeles, Los Angeles, CA 90095, USA*
[3]*Center for Minimally Invasive Therapeutics (C-MIT), University of California-Los Angeles, Los Angeles, CA 90095, USA*

Correspondence should be addressed to Sun Hee Do; shdo@konkuk.ac.kr

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Intervertebral disc degeneration (IVDD) is a common cause of lower back pain. Programmed cell death (PCD) including apoptosis and autophagy is known to play key mechanistic roles in the development of IVDD. We hypothesized that the nucleus pulposus cells that make up the center of the IVD can be affected by aging and environmental oxygen concentration, thus affecting the development of IVDD. Here, we evaluated the phenotype changes and PCD signaling in nucleus pulposus cells in two different oxygen percentages (5% (hypoxia) and 20% (normoxia)) up to serial passage 20. NP cells were isolated from the lumbar discs of rats, and the chondrogenic, autophagic, and apoptotic gene expressions were analyzed during cell culture up to serial passage 20. Hypoxia significantly increased the number of autophagosomes, as determined by monodansylcadaverine staining and transmission electron microscopy. Furthermore, hypoxia triggered the activation of autophagic flux (beclin-1, LC3-II/LC3-I ratio, and SIRT1) with a concomitant decrease in the expression of apoptotic proteins (Bax and caspase-3). Despite injury and age differences, no significant differences were observed between the *ex vivo* lumbar disc cultures of groups incubated in the hypoxic chamber. Our study provides a better understanding of autophagy- and apoptosis-related senescence in NP cells. These results also provide insight into the effects of aging on NP cells and their PCD levels during aging.

1. Introduction

Lower back pain and disabilities resulting from intervertebral disc (IVD) degeneration are the leading causes of incapacitation in adults [1, 2]. IVD degeneration is characterized by the dehydration of the nucleus pulposus (NP), rupture of the annulus fibrosus (AF), and calcification of the vertebral endplates (EPs). NP cells play an important role in IVD development, maintenance, and degeneration, by promoting the matrix biosynthesis of other IVD cell types [3, 4], indicating that modulating their activity could be a means to treat IVD degeneration. To investigate this, the biological responses of NP cells have been analyzed under various conditions, including microenvironments with altered oxygen and glucose levels [5–8].

The NP, composed of a gel-like, aggrecan-rich extracellular matrix (ECM) and cells, is derived from the notochord and comprises the central avascular structure of the IVD [3, 9, 10]. Its most common ECM component is type II collagen; however, types VI and XI are also present in smaller quantities [11–14]. NP ECM composition is altered by various etiological factors, including aging, infection, abnormal mechanical stress, smoking, diabetes, and trauma [3, 15]. IVD aging begins with changes in the NP, and degenerative NPs are characterized by decreased water content, cytoplasmic loss, and the presence of proteoglycans in the ECM [2, 16, 17].

Autophagy is an intracellular process that delivers cytoplasmic components to autophagosomes and lysosomes to maintain homeostasis. It is a crucial biological mechanism

that is involved in both physiological and pathological conditions [18, 19]. In the articular system, autophagy regulates chondrocyte maturation and promotes the survival of terminally differentiated chondrocytes under stress [20, 21]. Decreased expression of autophagic regulators has been observed in aging joints and osteoarthritis in mice and humans and is accompanied by increased chondrocyte apoptosis [20, 22, 23]. During IVD degeneration, autophagic regulation of the NP helps improve NP cell survival and phenotype maintenance by reducing apoptosis and antioxidant feedback responses [24, 25]. Increased autophagy has been reported in rat NP tissues with aging and degeneration [26]. However, previous studies were conducted in normoxic conditions (20% O_2) or after artificial induction of oxidative stress with H_2O_2, rather than examining NP cells in an environment with lower oxidative stress, as in avascular tissue.

In many cell types, hypoxia induces autophagy as a protection and survival mechanism [27]. However, since the *in vivo* environment of NP cells is hypoxic compared to other tissues, it is important to observe changes in autophagic regulation in these cells under hypoxic conditions. While autophagy has profound effects on NP cell survival and phenotype maintenance, the mechanism of basal autophagy regulation in NP cells and the effects of physiological stimulation on the process are not well understood. In this study, we focused on the effects of autophagy on NP cell survival and phenotype maintenance.

Here, we analyzed the biological responses of NP cells during aging (by serial passaging up to passage 20 (p20)) and environmental stress (normoxia and hypoxia) both *in vitro* and *ex vivo*. We evaluated the transcript and protein expression levels of genes related to the NP cell phenotype, autophagy, and apoptosis in hypoxic (5% O_2) and normoxic (20% O_2) conditions. In addition, vertebrae from juvenile (5 weeks) and young adult (10 weeks) rats were isolated and analyzed for histological and immunohistochemical changes in the NP following injury and hypoxic culture. This work provides an increased understanding of the autophagic pathway during hypoxia and may facilitate the development of novel therapeutic strategies for the treatment of degenerative IVD disease.

2. Materials and Methods

2.1. NP Cell Isolation and Culture. Five 5-week-old male Sprague–Dawley (SD) rats (average weight: 130 g) were obtained from Orient Bio (Seongnam, Korea). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University (Seoul, Korea) under permit numbers KU13116 and KU14075. Under sterile conditions, gel-like NP tissues were separated from the IVDs. NP tissues were pooled, vortexed, and washed twice with phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) and twice in α-minimum essential medium (α-MEM; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) [28–30]. Pooled NP cells were divided into two groups (normoxic culture and hypoxic culture) at p0 and cultured in α-MEM at 37°C in a controlled environment (triplicates for each group, 6 dishes in total). Each cell culture dish was maintained independently throughout the experiment. Control NP cells (C-NPs) were cultured under normoxic conditions (20% O_2, 5% CO_2), while hypoxic NP cells (H-NPs) were cultured under hypoxic conditions (5% O_2, 5% CO_2) in hypoxia chambers (STEMCELL Technology, Vancouver, BC, Canada) [31, 32]. Cells were grown to 70–80% confluence in 100 mm culture dishes. To analyze responses to aging in hypoxia and normoxia, NP cells were cultured until p20 (up to 60 days) [33, 34] and harvested at p5, p15, and p20. In addition, we set 3MA-treated groups (3MA-treated C-NP and 3MA-treated H-NP) to examine the effects of the autophagy pathway in hypoxia. Both C-NP and H-NP cells were treated with 5 mM of 3MA (an inhibitor of autophagy; Sigma-Aldrich, St. Louis, MO, USA) throughout the experimental period [35, 36].

2.2. Cell Viability. The effects of various culture conditions on NP cell viability were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay using a commercial kit (Roche, Basel, Switzerland). Briefly, NP cells (p5) were seeded into 96-well plates (2×10^2 cells/μL) and cultured for 24, 72, and 96 h in hypoxia or normoxia. MTT labeling solution was added to each well, and cells were incubated for an additional 4 h in hypoxia or normoxia. After dissolving the released formazan dye in dimethyl sulfoxide, the absorbance was measured at 595 nm using a Sunrise™ microplate reader (TECAN, Salzburg, Austria).

2.3. Morphometric Analysis. NP cells in a specific passage (p5, p20) were seeded on Lab-Tek chamber slides (1×10^2 cells/μL; Nunc, Rochester, NY, USA), cultured for 72 hours, and stained with Alizarin Red S (Sigma-Aldrich) to visualize mineralization during aging and hypoxia. The slides were fixed in cold methanol (Merck, Darmstadt, Germany) and then stained with 2% Alizarin Red S for 5 min at room temperature. Slides were then dehydrated with a graded series of acetone (Merck) and acetone : xylene (1 : 1; BBC, Mount Vernon, WA, USA) [37]. Stained monolayers were visualized by phase microscopy using an inverted microscope (Leica Microsystems, Wetzlar, Germany). Extracellular calcium deposits were indicated by bright orange-red staining.

2.4. Real-Time Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cells at p5, p15, and p20 using RNAiso Plus (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Isolated total RNA (1 μg) was reverse transcribed into complementary DNA (cDNA) and used in quantitative (qRT-PCR) assays using the SYBR® Green PCR Kit (Qiagen, Valencia, CA, USA) in a Rotor-Gene Real-Time PCR-Cycler® (Qiagen). The reactions (20 μL) comprised 2 μL of diluted cDNA, 2 μL of each primer, 10 μL of 2x SYBR® Green Master Mix, and 6 μL of RNase-free water. The sequences of the oligonucleotide primers used in the qPCR assays are shown in Table 1. Thermocycling conditions were as follows: 50°C for 2 min, 95°C for 15 min, then 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 5 min. All samples were assayed in duplicate, and mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Sequences of rat primers used for real-time PCR.

Target gene	Source	Sequence	Predicted length (bp)
GAPDH	NM017008.4	F: AAC TCC CTC AAG ATT GTC AGC AA R: GGC TAA GCA GTT GGT GGT GC	51
Sox-9	NM080403.1	F: ACG GCT CCA GCA AGA ACA AG R: TTG TGC AGA TGC GGG TAC TG	109
Aggrecan	J03485.1	F: GAC CAG GAG CAA TGT GAG GAG R: CTC GCG GTC GGG AAA GT	72
Type I collagen	NM053304.1	F: TGG CCA AGA AGA CAT CCC TGA AGT R: ACA TCA GGT TTC CAC GTC TCA CCA	81
Type II collagen	NM012929.1	F: GAG TGG AAG AGC GGA GAC TAC TG R: CTC CAT GTT GCA GAA GAC TTT CA	81
Type III collagen	NM032085.1	F: TTC CTG GGA GAA ATG GCG AC R: GGC CAC CAG TTG GAC ATG AT	99
Type VI collagen	XM001079629.4	F: CAA GAA CAC GTG GAC ATG CG R: CAC TGC AGT TTC TTG ACG GC	77
ALP	NM013059.1	F: CAT GTT CCT GGG AGA TGG TA R: GTG TTG TAC GTC TTG GAG AGA	144
Runx2	NM001278483.1	F: GAT GAC ACT GCC ACC TCT GA R: ATG AAA TGC TTG GGA ACT GC	118
BMP-2	NM017178.1	F: CTA TAT GCT CGA CCT GTA CCG R: CAC TCA TTT CTG AAA GTT CCT CG	146
TGF-β	NM_021578.2	F: CGC AAC AAC GCA ATC TAT G R: ACC AAG GTA ACG CCA GGA	204
TIMP-1	NM053819.1	F: TCC CCA GAA ATC ATC GAG AC R: TCA GAT TAT GCC AGG GAA CC	250
TIMP-2	NM021989.2	F: CAG GGC CAA AGC AGT GAG CGA GAA R: TCT TGC CAT CTC CTT CCG CCT TCC	230
MMP-2	NM031054.2	F: GAT CTG CAA GCA AGA CAT TGT CTT R: GCC AAA TAA ACC GAT CCT TGA A	83
MMP-3	NM133523.2	F: TCC CAG GAA AAT AGC TGA GAA CTT R: GAA ACC CAA ATG CTT CAA AGA CA	74
MMP-9	NM031055.1	F: GTA ACC CTG GTC ACC GGA CTT R: ATA CGT TCC CGG CTG ATC AG	68
MMP-13	NM133530.1	F: CTG ACC TGG GAT TTC CAA AA R: ACA CGT GGT TCC CTG AGA AG	96
HIF-1	XM006240199.3	F: AAG TCT AGG GAT GCA GCA C R: CAA GAT CAC CAG CAT CTA G	175
SIRT1	XM017588054.1	F: AGC TGG GGT TTC TGT TTC CTG TGG R: TCG AAC ATG GCT TGA GGA TCT GGG A	111
HMGB-1	NM012963.2	F: CGG ATG CTT CTG TCA ACT TCT R: AGT TTC TTC GCA ACA TCA CCA	292
Beclin-1	NM001034117.1	F: TTC AAG ATC CTG GAC CGA GTG AC R: AGA CAC CAT CCT GGC GAG TTT C	142
Atg7	NM001012097.1	F: GAC CTG GGC TCC TCA CTT TTT G R: CCC TGG GCG GCT CAC TG	135
Atg5	NM001014250.1	F: AGG CTC AGT GGA GGC AAC AG R: CCC TAT CTC CCATGG AAT CTT CT	72
LC3	NM022867.2	F: CAT GCC GTC CGA GAA GAC CT R: GAT GAG CCG GAC ATC TTC CAC T	70
LC3-II	NM022867.2	F: CTT TGT AAG GGC GGT TCT R: GAG GCT TGC TTT AGT TGG	141
p53	NM030989.3	F: CAG CTT TGA GGT TCG TGT TTG T R: ATG CTC TTC TTT TTT GCG GAA A	82
p21	NM080782.3	F: CAG ACC AGC CTA ACA GAT TTC R: TGA CCC ACA GCA GAA GAA G	105

Table 1: Continued.

Target gene	Source	Sequence	Predicted length (bp)
Bax	NM017059.2	F: CCA AGA AGC TGA GCG AGT GTC TC R: AGT TGC CAT CAG CAA ACA TGT CA	147
Bcl-2	NM016993.1	F: TGA ACC GGC ATC TGC ACA C R: CGT CTT CAG AGA CAG CCA GGA G	116
Caspase-3	NM012922.2	F: CTG GAC TGC GGT ATT GAG AC R: CCG GGT GCG GTA GAG TAA GC	104
Caspase-8	NM022277.1	F: TCA GCA ACA TGC GGG ACA G R: TGA AGC AGT CTT TGC CCT TGT G	171
Caspase-9	NM031632.1	F: GGA AGA TCG AGA GAC ATG CAG R: CCG TGA CCA TTT TCT TAG CAG	216

2.5. Immunoblot Analysis. NP cells at p5, p15, and p20 were homogenized in radioimmunoprecipitation buffer containing a protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA). Subsequently, the lysates were centrifuged at 13,572 × g for 10 min at 4°C to obtain soluble protein. Protein concentrations were determined by the Bradford method. Proteins of interest were immunoblotted using 35 μg of protein, following standard protocols. The extracted proteins were resolved by 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 3% bovine serum albumin (Sigma-Aldrich), the membranes were incubated with antibodies against β-actin (sc-130656; 1 : 200), full-length caspase-3 (sc-7272; 1 : 200), polyclonal rabbit anti-beclin-1 (ab55878; 1 : 1000; Abcam, Cambridge, UK), light chain 3 (LC3; ab58610; 1 : 1000), Bcl-2, apoptosis regulator (Bcl-2; sc-492; 1 : 200), Bcl-2 associated X, apoptosis regulator (Bax; sc-526; 1 : 200), and sirtuin 1 (SIRT1; sc-15404; 1 : 200). All antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany) except the beclin-1 antibody (Abcam (Cambridge, UK)). Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (ThermoFisher) and a LAS 4000 chemiluminescent image analyzer (Fujifilm, Tokyo, Japan). Protein band intensities were quantified using the ImageJ software (https://imagej.nih.gov/ij/download.html; National Institutes of Health, Bethesda, MD, USA).

2.6. Monodansylcadaverine (MDC) Staining. MDC staining has been used to monitor autophagy by staining autophagic vacuoles [38]. Specific passage numbers of NP cells (p5, p15, and p20) were seeded on Lab-Tek chamber slides in the same way as the morphometric analysis. Subsequently, autophagic vacuoles were labeled with MDC by incubating the cells with 0.05 mM MDC in α-MEM (Gibco) at 37°C for 60 min. MDC-stained autophagic vacuoles were examined using a fluorescence microscope (BX61; Olympus, Tokyo, Japan) [38, 39]. The MDC-positive cells were calculated by counting cells from at least three random microscopic fields using the ImageJ software (https://imagej.nih.gov/ij/download.html; National Institutes of Health).

2.7. Transmission Electron Microscopy (TEM). NP cells cultured to p5, p15, and p20 were fixed with 4% glutaraldehyde (Sigma-Aldrich) overnight, postfixed in 2% osmium tetroxide, dehydrated with a graded series of ethanol (Merck), and embedded in resin. Images of autophagosomes were captured using a JEM 1010 transmission electron microscope (JEOL, Peabody, MA, USA). Based on the previous studies, a vacuole structure with a double to multimembranous structure in the cytoplasm was defined as an autophagosome [39, 40]. In each group (C-NP and H-NP) with specific passage (p5, p15, and p20), double membranous autophagosomes present in the cytoplasm were quantified in at least three different samples using the ImageJ software.

2.8. Ex Vivo Analysis Using a Disc Microinjection Organ Culture Model. Male SD rats aged 5 weeks (young, Y) and 10 weeks (old, O) were purchased from Orient Bio. Rats were housed at 22 ± 2°C with a 12 h light-dark cycle. Food (PMI Nutrition International, St. Louis, MO, USA) and water were supplied *ad libitum*. Rats were divided into four groups ($n = 3$ /group): 5 weeks old without injury, 5 weeks old with injury, 10 weeks old without injury, and 10 weeks old with injury. In the injured groups, 10 μL of PBS was injected into the vertebral discs with a 26-gauge needle [39, 41]. The vertebrae were dissected and removed from the rats, and the discs between the L1-L2 and L3-L4 lumbar vertebrae were separated from neighboring vertebrae using a scalpel. The isolated discs were maintained for 14 days in α-MEM containing 10% FBS at 37°C in a hypoxic condition (5% O_2) and then subjected to morphometric analysis.

2.9. Histopathology and Immunohistochemistry. Lumbar discs were fixed in 10% neutral-buffered formalin, decalcified in Solution Lite (Sigma-Aldrich), processed using a standard method, and embedded in paraffin. Serial disc sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and Safranin O (Sigma-Aldrich). For immunohistochemistry, sections were subjected to heat-mediated antigen retrieval using 0.01 M sodium citrate buffer (pH 6.0). A monoclonal mouse type II collagen antibody (cp18, 1 : 100, Calbiochem, San Diego, CA, USA) was used as the primary antibody. Antigen-antibody complexes were visualized using the avidin–biotin–peroxidase complex solution from the

VECTASTAIN® Avidin-Biotin Complex Staining Kit (Vector Laboratories, Burlingame, CA, USA) along with 3,3′-diaminobenzidine (Vector Laboratories). Sections were counterstained with Mayer's hematoxylin.

2.10. Protein Extraction from Paraffin-Embedded Tissues. Proteins were extracted from formalin-fixed, paraffin-embedded (FFPE) lumbar disc tissues using the Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany). Protein samples were combined with a polyclonal rabbit LC3 antibody (Abcam) and incubated for 2 h at 4°C. Protein A/G PLUS-Agarose beads (20 μL; Santa Cruz Biotechnology) were added, and the mixtures were incubated at 4°C on a rocker platform for 2 h. The pellets were washed three times, and the buffer was carefully aspirated to avoid disturbing the pellets. Thereafter, the pellets were resuspended in 40 μL of sample buffer. The immunoprecipitated samples were subjected to immunoblot analysis as described above [42].

2.11. Statistical Analysis. Statistical analyses were performed using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA, USA). Multiple comparisons were analyzed using the one-way analysis of variance followed by the Bonferroni post hoc test. All results are expressed as the mean ± standard deviation. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of Hypoxia and Serial Passaging on NP Cell Viability and Mineral Accumulation. To assess the effects of different environmental oxygen conditions on cell viability, NP cells were cultured for 96 h in normoxic and hypoxic conditions. Interestingly, at the earliest timepoint (24 h), the viability of the hypoxic H-NP cells was significantly higher than that of the normoxic C-NP cells ($p < 0.01$). However, after 72 h, both C-NP and H-NP cells displayed slight but insignificant decreases in viability (Figure 1(a)). Next, we cultured C-NP and H-NP cells up to p20 and stained them with Alizarin Red S to examine changes in cell shape and mineralization. As shown in Figure 1(b), mineralization plaques were observed in neither H-NP nor C-NP cells until p20. However, H-NP cells were larger than C-NP cells, with increased cytoplasm, and C-NP cells were more spindle-shaped than H-NP cells at p20. These results indicate that hypoxia does not affect NP cell viability but may affect their phenotype, changing their size and morphology.

3.2. Chondrogenesis-Related Gene Expression in NP Cells under Different Oxygen Concentrations. Given the observed morphological changes with hypoxic culture, we next sought to examine alterations of gene expressions related to chondrogenesis upon aging and hypoxia through the serial passaging of NP cells (Figure 2). This endpoint was chosen based on previous studies regarding the serial passaging of primary isolated cells to senescence [33, 34]. At the earlier passage (p5), compared to H-NP, C-NP showed downregulation of SRY-box transcription factor-9 (Sox-9), type I collagen, and tissue inhibitor of metalloproteinase-2 (TIMP-2) and slight upregulation of matrix metallopeptidase (MMP-3). After aging by serial passaging, C-NP cells exhibited significantly decreased levels of ECM-related genes (e.g., *aggrecan*, *type II collagen*, and *type VI collagen*) as well as ECM-regulating enzymes (e.g., *MMP-3*) at p15. Despite H-NP cells displayed the same tendency as C-NP cells with serial passaging (aging), the levels of ECM-related genes were significantly higher in H-NP cells compared to that in C-NP cells. The results indicate that repeated passaging under normoxia led to the dedifferentiation of NP cells, as they quickly lose aggrecan and type II collagen, while simultaneously transitioning to a fibroblastic phenotype characterized by high type III collagen expression. However, aggrecan mRNA expression at p15 was significantly higher in hypoxia ($p < 0.01$). In addition, hypoxia maintained NP cell homeostasis through increases in catabolic enzymes such as MMP-3 and MMP-13, as well as increased expression of MMP inhibitors, such as TIMP-1 and TIMP-2. These results suggest that hypoxia results in slower ECM protein degradation than normoxia and maintains homeostasis through the coordinated actions of MMPs and TIMPs.

3.3. Gene and Protein Levels of Autophagosome- and Autophagy-Related Genes in NP Cells under Different Oxygen Concentrations. To assess the effects of hypoxia on autophagy in NP cells, the autophagic process was visualized by MDC staining and TEM (Figure 3(a)). First of all, we labeled autophagic vacuoles with MDC, a lysosomotropic agent that is incorporated into the lipids of autophagic vacuoles. Our results showed that the number of MDC-labeled autophagosomes increased with aging in both normoxic and hypoxic conditions and peaked at p15. In particular, under hypoxic conditions, the number of MDC-labeled autophagosomes was significantly higher than in normoxic conditions throughout the experimental periods. Subsequently, double membranous autophagosomes in experimental groups were analyzed using TEM. Similar to MDC staining results, H-NP cells contained more autophagosomes than C-NP cells in all passages examined. Interestingly, the difference increased with the number of passages (5.6- and 14.25-fold higher in H-NP cells vs. C-NP cells at p15 and p20, respectively).

We then investigated whether hypoxia or aging affected the transcript and protein levels of autophagy-related genes in NP cells (Figures 3(b) and 3(c)). Despite repeated passaging, there were no significant changes in the expression of autophagy-related genes in C-NP cells, except for high mobility group box 1 (HMGB1) at p20. Compared to the C-NP, H-NP showed significantly increased gene expression levels of beclin-1, autophagy-related 7 (ATG7), LC3-I, and LC3-II in the early passages (p5). At p15, the LC3-II/LC3-I ratio was significantly increased in H-NP cells compared to C-NP cells ($p < 0.05$). Consistent with this, the beclin-1 protein level ($p < 0.001$ at p5, p10, and p15) and the LC3-II/LC3-I ratio ($p < 0.01$ at p5) were significantly higher in H-NP cells. SIRT1 is a key mediator of hypoxia, which is known to promote autophagy and inhibit apoptosis to protect the cells from hypoxic stress via AMPK activation [43]. In our results, SIRT1 protein expression was significantly upregulated in H-NP cells compared to that in C-NP cells at p15 and p20. Taken together, these results suggest that NP cells

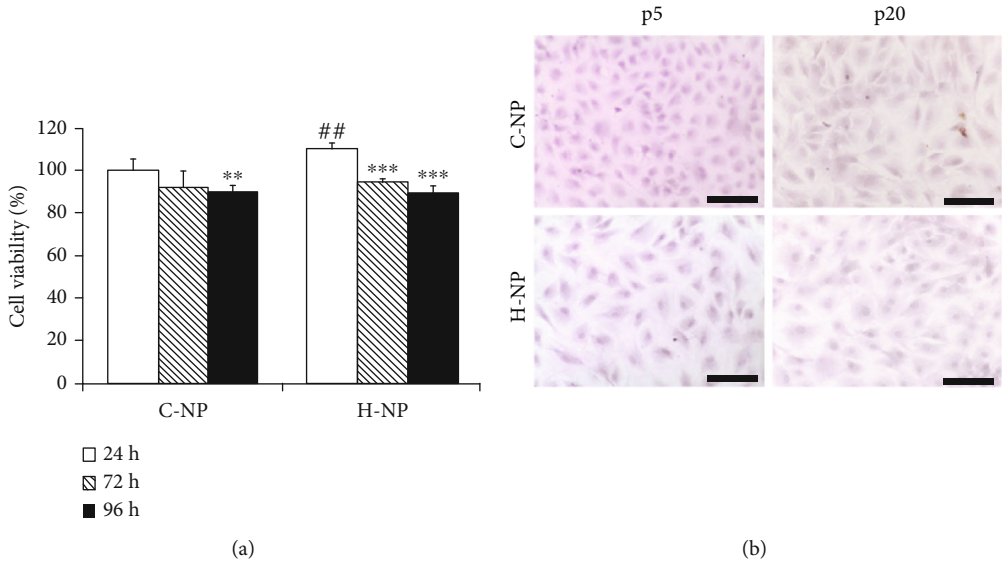


Figure 1: Effect of hypoxia on nucleus pulposus (NP) cell viability. (a) Viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Data are presented as the mean ± standard deviation. (b) Alizarin Red S staining to assess morphological changes in hypoxic and aging conditions. Scale bar = 100 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus 24 h; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus C-NP cells.

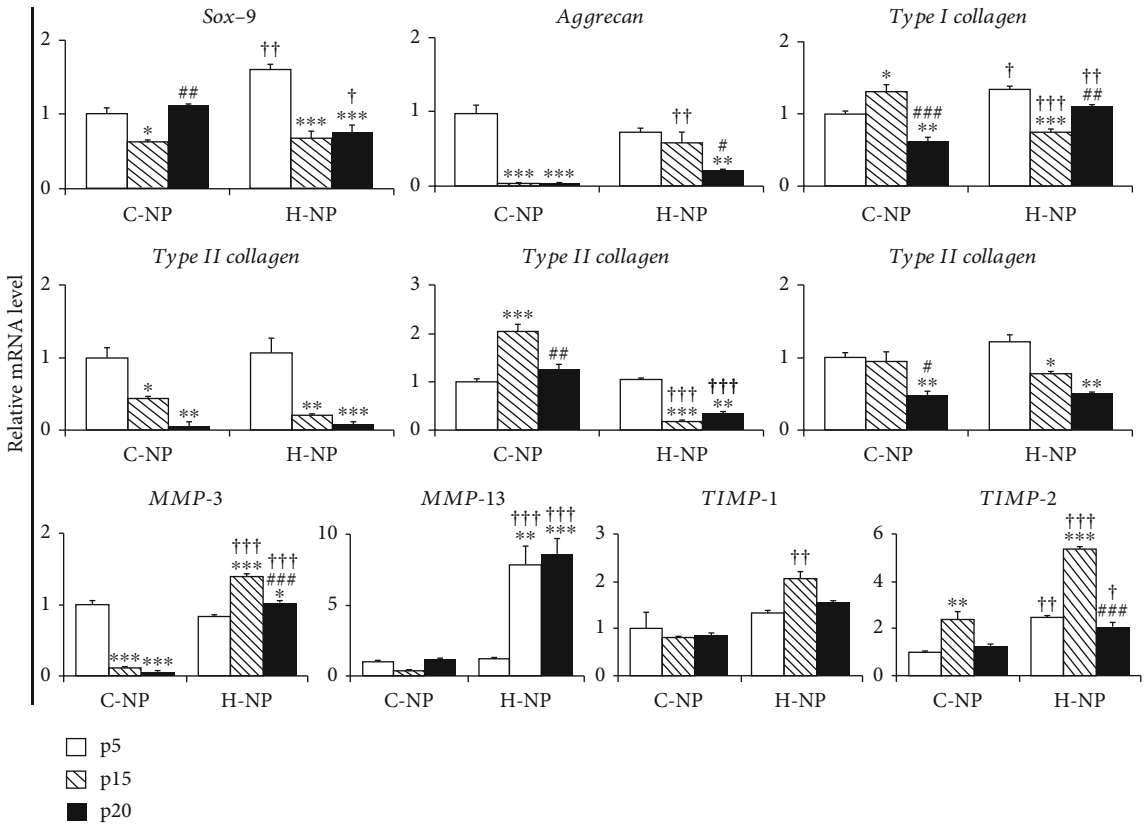
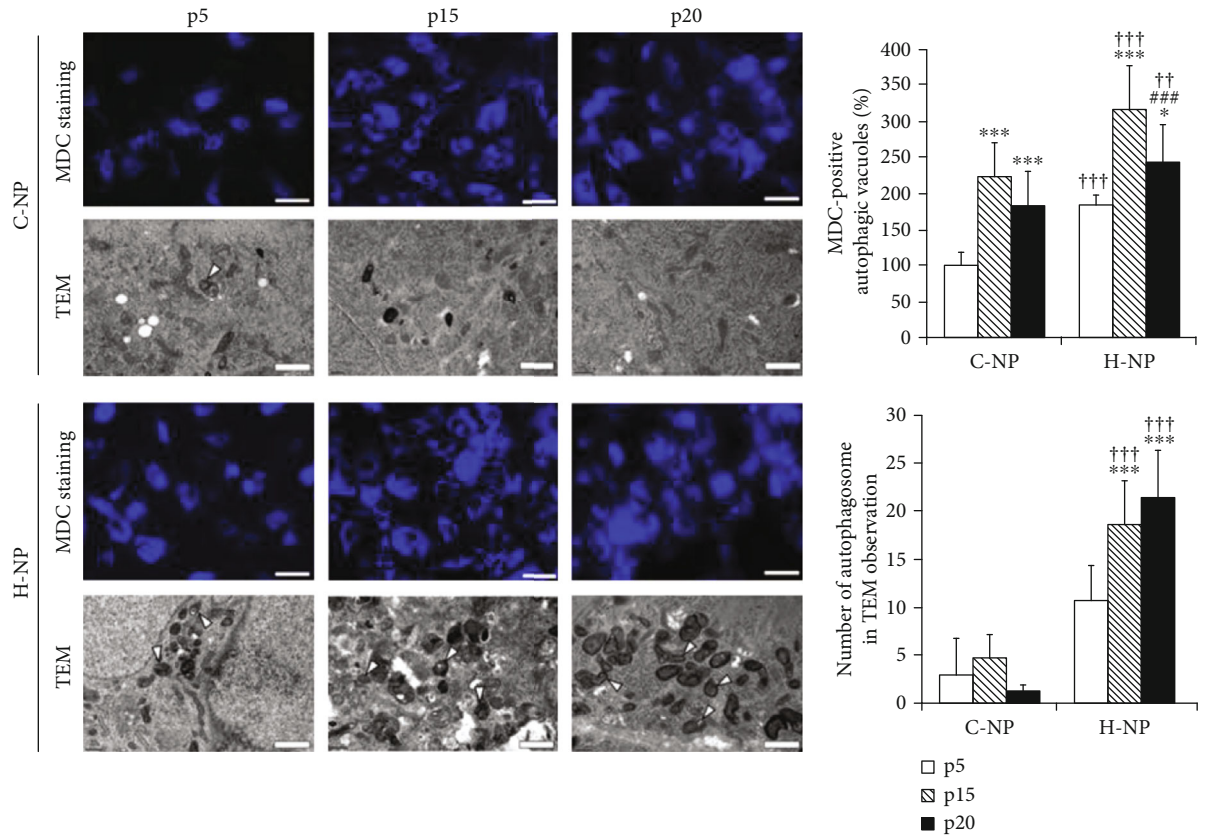
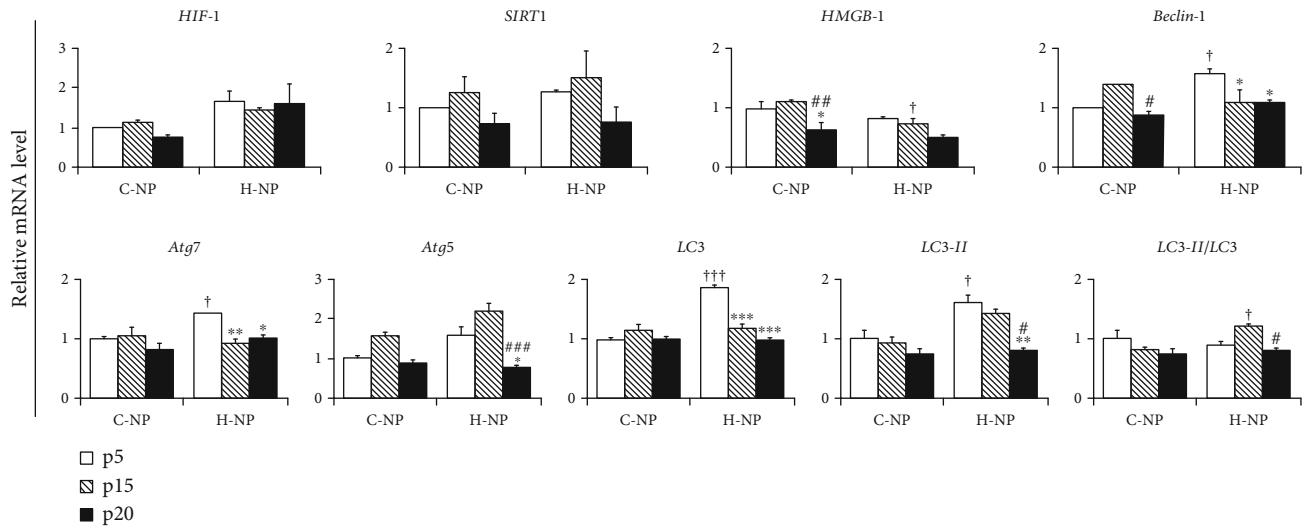


Figure 2: Chondrogenesis-related gene expression analysis in NP cells under different oxygen concentrations. RT-PCR was used to analyze mRNA expression levels in normoxic (C-NP) and hypoxic (H-NP) cells at p5, p15, and p20 ($n = 3$ per group). Primers targeted transcription factors (SRY-box transcription factor 9), ECM proteins (aggrecan and type I, II, III, and VI collagens), and metalloproteinase-related genes (MMP-3, MMP-13, TIMP-1, and TIMP-2). Results are the mean ± standard deviation of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus p5; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus p15; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ versus C-NP cells at the same passage number.



(a)



(b)

Figure 3: Continued.

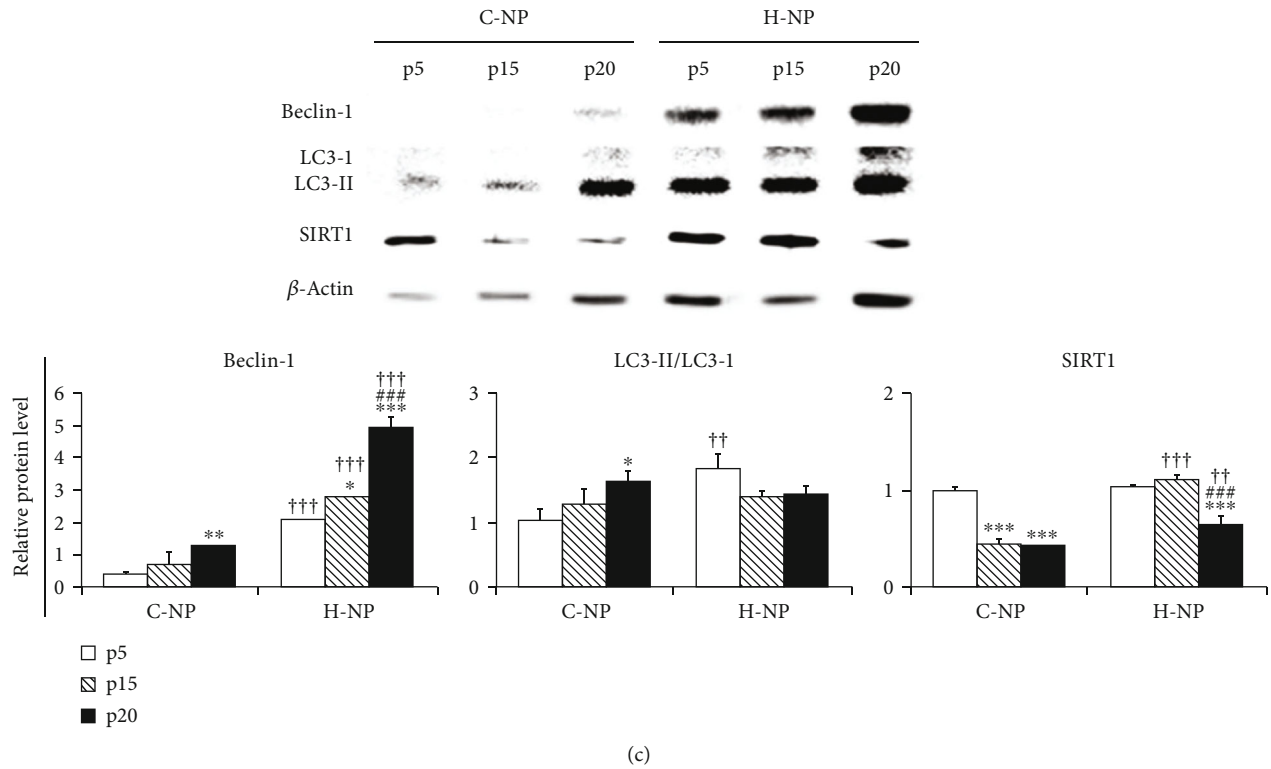


Figure 3: Autophagosome- and autophagy-related genes and protein level analysis in NP cells under different oxygen concentrations. (a) Representative MDC staining and TEM images and statistical analysis of autophagosomes (arrowheads) in p5, p15, and p20 NP cells. Scale bar for MDC staining: 100 μm, TEM: 0.5 μm. (b) RT-PCR was used to analyze mRNA expression levels in normoxic (C-NP) and hypoxic (H-NP) cells at p5, p15, and p20 ($n = 3$ per group). Primers targeted hypoxia- (HIF1, SIRT1) and autophagy- (HMGB1, beclin-1, ATG7, ATG5, LC3-I, and LC3-II) related genes. (c) Representative immunoblots and statistical analysis of protein levels in the experimental groups. Data are presented as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus p5; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus p15; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ versus C-NP cells at the same passage number.

have increased autophagic activation response to hypoxic condition and that this autophagic flux is related to increased beclin-1, LC3-II/LC3-I ratio, and SIRT1 activation.

3.4. Apoptosis and Signaling Pathway in NP Cells under Different Oxygen Concentrations. According to previous reports [24, 25], substances developed to enhance the autophagic flux of NP cells for the treatment of IVD degeneration can reduce the activation of apoptosis-related pathways, in addition to enhancing the autophagy-related pathways. To determine whether this affects our experimental model, we analyzed the expression patterns of apoptosis-related genes and proteins (Figures 4(a) and 4(b)). Consistent with the viability results, C-NP cells did not show significant changes in apoptosis-related gene and protein expression levels with serial passaging. However, compared to C-NP cells, H-NP cells displayed increased gene expression of *Bcl-2* ($p < 0.01$) as well as decreased expression of *Bax* ($p < 0.01$), *caspase-3* ($p < 0.05$), and *caspase-8* ($p < 0.05$) at p15 (Figure 4(a)). In addition, the *Bax*/*Bcl-2* ratio, a measure of apoptotic susceptibility [44], was significantly lower in H-NP cells compared to that in C-NP cells (at p15 and p20, $p < 0.001$). Similar to gene expression analysis, Bax and caspase-3 protein expression levels were also significantly decreased in H-NP cells (Figure 4(b)). However, these antiapoptotic protein expressions (decreased Bax, caspase-3 expression) of NP cells under the hypoxic condition were reversed after 3MA (autophagy inhibitor) treatment. These results could indicate that hypoxic condition not only induces autophagic flux but also could exhibit antiapoptotic signaling activation in NP cells via Bax/Bcl-2 and caspase-3/8 signaling pathways.

3.5. Histological Changes of the Rat Lumbar Disc Ex Vivo Culture Model under the Hypoxic Condition. Finally, we tested an ex vivo IVD culture model under the hypoxic condition as same as in vitro study. To identify changes of NP phenotype upon aging and injury, we set up a control/injury group (with/without injury) and 5-week and 10-week groups (juvenile and young adults). The no injury groups showed relatively well-preserved NP structures than the injured groups in the center of the IVD (Figure 5(a)). Nevertheless, aggregated, serpentine-shaped ECM with clustered NP cells still exist in the injured groups. In the 5 weeks without injury group, the Safranin O-positive area was homogenously distributed with the cells throughout the ECM, whereas in the 10 weeks without injury group, the cells were clustered in localized areas. Even though all 10-week-old and injured groups had ECM inside the NP area, the injured group showed condensed or degenerative features rather than

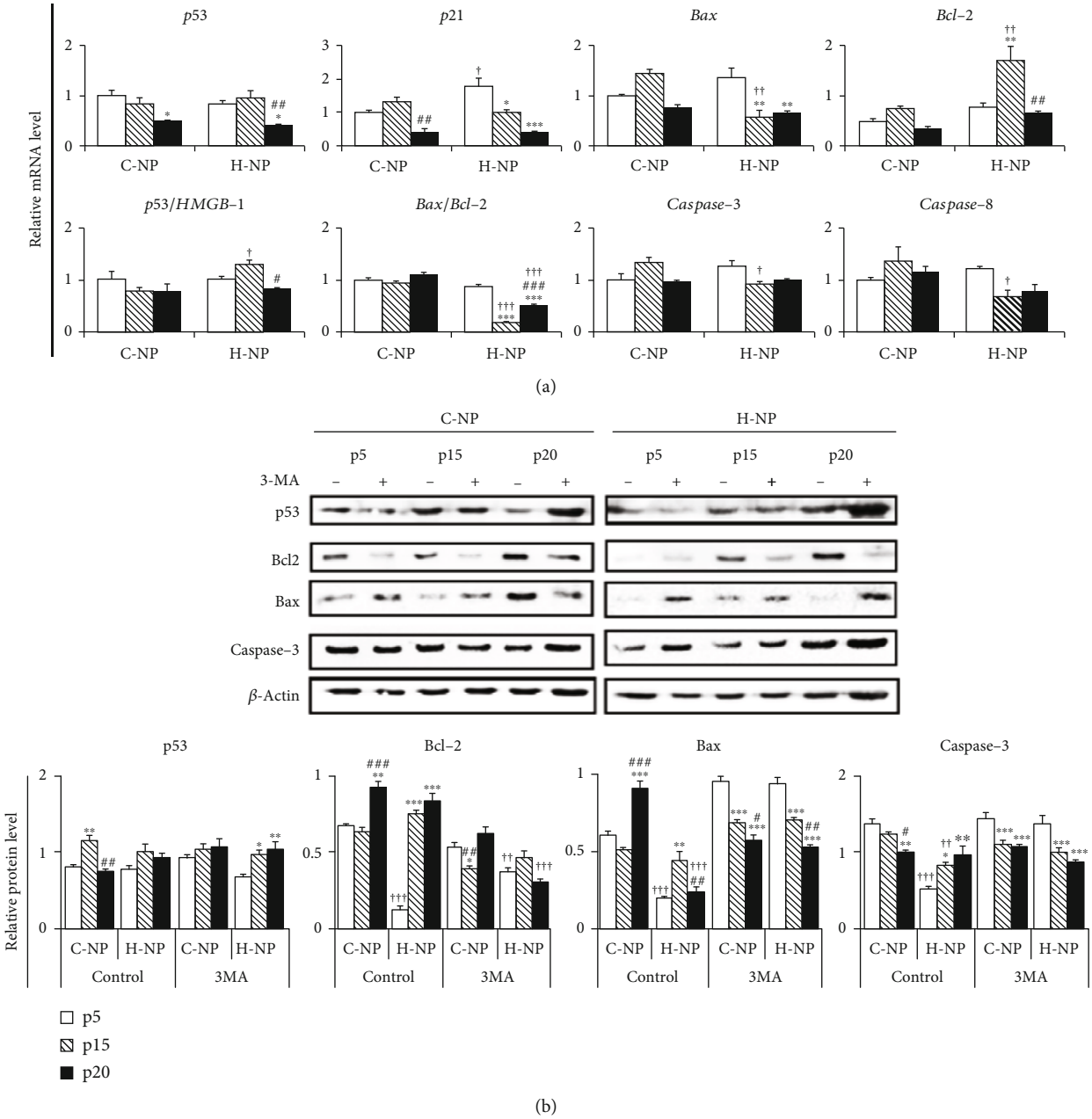


Figure 4: Apoptosis-related gene and protein expression analysis under the different oxygen concentrations. (a) Expression levels of apoptosis-related genes were analyzed using RT-PCR. (b) Representative immunoblots and statistical analysis of protein levels in the experimental groups. Data are presented as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus p5; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus p15; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ versus C-NP cells at the same passage number.

homogenous distribution. These features might indicate that hypoxic conditions could help to maintain the NP cell viability; it could not cure or improve the regenerative capacity of NP cell itself. Similar to the in vitro results, the intensity of type II collagen (a major component of the NP) was higher in the juvenile groups than that in the young adult groups. The LC3-II/LC3-I ratio was higher in the 5 weeks without injury group compared with that in the 10 weeks without injury group (Figure 5(b)). Despite differences in injury and age, discs cultured in hypoxic conditions were found to exhibit a certain level of autophagic activation. These results are consistent with our in vitro TEM and qPCR/protein expression results from early passage cultures (p5).

4. Discussion

IVD degeneration, a major contributor to chronic lower back pain, is an age-related condition characterized by loss of the ECM and the functional cells responsible for its regeneration. The inner NP region of the vertebral disc is composed of type II collagen and proteoglycans. These molecules are responsible for water retention, which maintains the viscoelastic

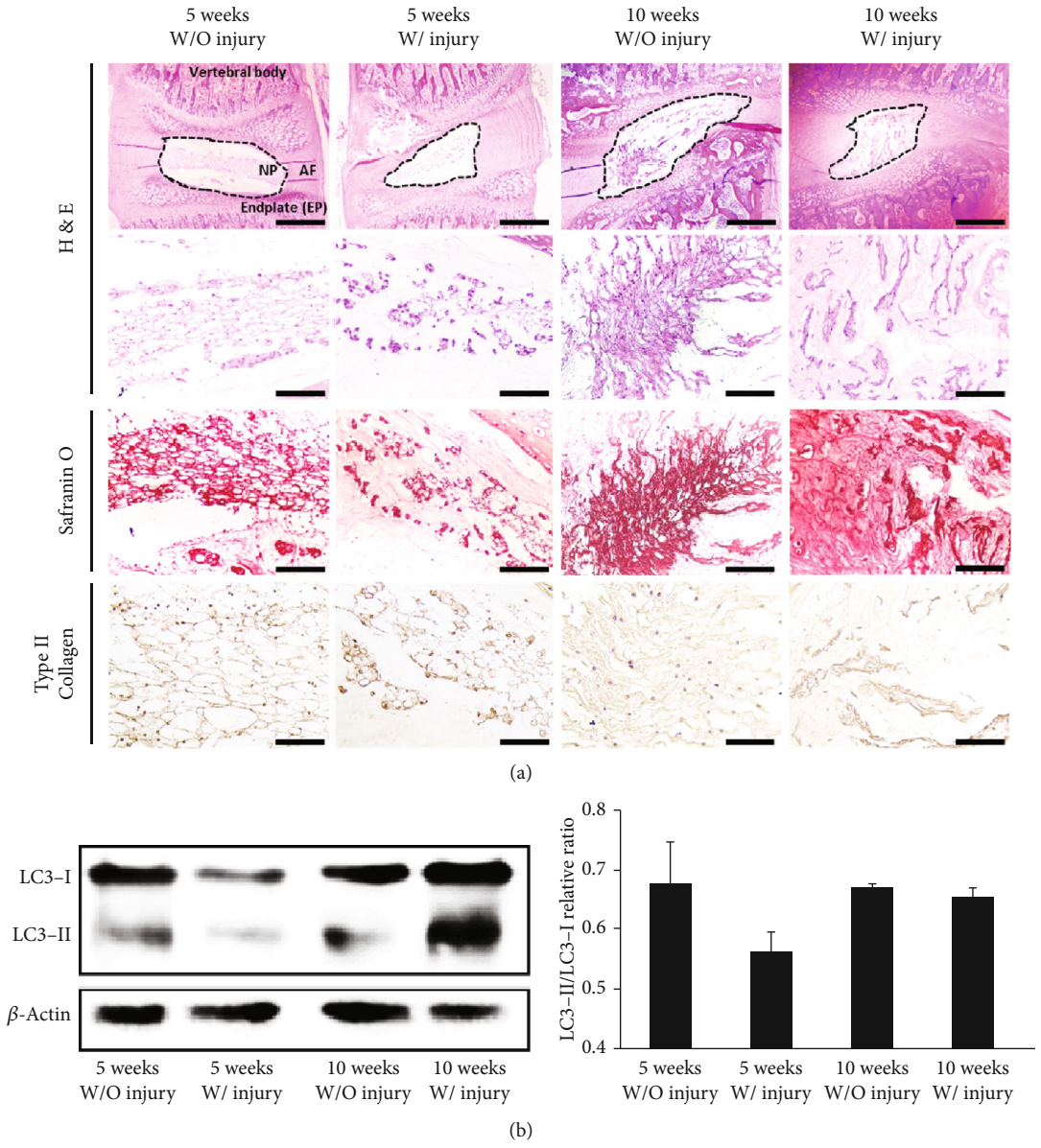


Figure 5: Morphometric and protein expression analyses of an *ex vivo* rat IVD culture model. (a) Representative histological images of *ex vivo* IVD cultures from 5-week-old and 10-week-old rats with or without injury (injection of 10 μL of PBS into the disc). Images shown are at 40x (H&E), 200x (H&E and Safranin O), and 400x (IHC) magnification. The dotted line indicates the NP. (b) Representative immunoblotting images (left) and statistical analysis of protein levels in the experimental groups. Data are presented as the mean ± standard deviation.

properties of the discs [31, 32, 41]. NP tissue is avascular, and the oxygen saturation levels required for its sustenance are relatively low compared to other tissues [35].

The microenvironment of IVD is hypoxic but not completely anaerobic (1% O_2 in central NP), and during IVD degeneration progression, neovascularization of the disc is known to increase oxygen tension in the microenvironment of IVD [5, 6]. High oxygen tension is expected to enhance reactive oxygen species (ROS) generation and subsequently induce oxidative stress in the microenvironment of IVD, which is closely related to the establishment and progression of IVD degeneration [7, 45, 46]. In this study, we evaluated autophagy changes in relation to NP cell phenotype and apoptotic/antiapoptotic signaling during serial passaging in normoxic and hypoxic conditions.

Alizarin Red S staining revealed that although mineralization plaques were not observed until p20 in both H-NP and C-NP cells, the size and shape of the cells differed depending on the culture conditions (Figure 1(b)). RT-qPCR results demonstrated that hypoxia led to increased *aggrecan* and *type II collagen* and decreased *type III collagen* in H-NP cells. These results indicate that C-NP cells exhibit characteristics of fibrocartilage, while H-NP cells exhibited chondrogenic characteristics. In addition, hypoxia resulted in elevated levels of *TIMP-1* and *TIMP-2* (at p15), as well as expression of *MMP-3* and *MMP-13* (at p15 and p20), which

regulate collagen and aggrecan degradation. Collectively, NP cells maintained a partially chondrogenic phenotype without mineralization under hypoxic conditions for 20 serial passages (approximately 60 days). The hypoxic environment plays a crucial role in maintaining the physiological function of the IVD, including cellular metabolism and matrix synthesis [15, 47]. Thus, our results indicate that the use of hypoxic conditions is important to accurately study the IVD microenvironment *in vitro*.

It is well known that apoptosis and autophagy are both closely related to the onset and progression of IVD degeneration [24, 48, 49]. Apoptosis is responsible for decreased NP cell numbers during degeneration [50–52]. Conversely, autophagy is an evolutionarily conserved process that has been implicated in cell growth, development, and stress responses [23, 40]. It is activated by various stresses, such as aberrant mechanical compression, hypoxia, high glucose, and reactive oxygen species [35, 53]. In our study, both MDC staining and TEM results showed that autophagosomes were significantly increased in the late-passage H-NP cells compared to those in the C-NP cells (Figure 3). In addition, beclin-1 expression and the LC3-II/LC3-I ratio increased, whereas Bax and caspase-3 expression decreased in H-NP cells compared with that in C-NP cells. H-NP cells were responsive to hypoxia to protect and promote autophagic influx, as indicated by increased SIRT1 expression. Autophagy is an essential protective mechanism for cell survival after injury, and SIRT1 protects cells by regulating autophagy and metabolism [54]. Furthermore, a Bcl-2/beclin-1 interaction plays a key regulatory role in autophagy, allowing Bcl-2 to inhibit both apoptosis and autophagy [55, 56]. Interestingly, both autophagic flux and antiapoptotic regulation were blocked by the autophagy inhibitor 3MA under the hypoxic condition. These results suggest that homeostasis in hypoxic conditions is promoted through both elevated autophagy and antiapoptotic effects.

There are some limitations to this study. One was the use of NP cells isolated from rat lumbar discs. Species with chondrodystrophoid discs, such as humans, sheep, and dogs, can experience profound, early-onset degenerative disc disease, which often occurs within one year of birth [11, 15]. We used rats in this study, as this model has been used in many previous studies, and rats are one of the few species that maintain an NP cell population similar to that observed in adult humans. Further studies are required to determine the relation between autophagic flux, hypoxia, and aging in humans.

In conclusion, our results provide evidence that NP cells modulate the expression of chondrogenesis-, autophagy-, and apoptosis-related genes under hypoxic conditions. This study provides a better understanding of autophagy- and apoptosis-related senescence in NP cells. These results may also provide insight into the changes that occur in NP cells during aging.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University (Seoul, Korea) with permit numbers KU13116 and KU14075.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

H.-J.K., H.-R.L., H. K., and S.H.D. performed the research. H.-J.K., H.-R.L., H.K., and S.H.D. wrote the manuscript. H.-R.L., H.-J.K., and S.H.D. designed the research. S.H.D. supervised the project. All the authors read and commented on the manuscript. Han-Jun Kim and Hye-Rim Lee contributed equally to this work.

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Research Article

Chaperone-Mediated Autophagy Markers LAMP2A and HSC70 Are Independent Adverse Prognostic Markers in Primary Resected Squamous Cell Carcinomas of the Lung

Tereza Losmanová,[1] Félice A. Janser,[1] Magali Humbert,[1] Igor Tokarchuk,[1,2] Anna M. Schläfli,[1] Christina Neppl,[1] Ralph A. Schmid,[3,4] Mario P. Tschan,[1,2] Rupert Langer,[1,5] and Sabina Berezowska[1,6]

[1]*Institute of Pathology, University of Bern, Bern 3008, Switzerland*
[2]*Graduate School for Cellular and Biomedical Sciences, Bern 3012, Switzerland*
[3]*Division of General Thoracic Surgery, Inselspital University Hospital Bern, Bern 3010, Switzerland*
[4]*Department of Biomedical Research (DBMR), University of Bern, Bern 3008, Switzerland*
[5]*Institute of Pathology and Molecular Pathology, Kepler University Hospital, Johannes Kepler University Linz, 4021 Linz, 4040 Linz, Austria*
[6]*Institut de Pathologie, Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Lausanne 1011, Switzerland*

Correspondence should be addressed to Sabina Berezowska; sabina.berezowska@chuv.ch

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LAMP2A and HSC70 are crucial players in chaperone-mediated autophagy (CMA), a targeted, lysosome-dependent protein degradation pathway. Elevated LAMP2A levels, indicative of increased CMA activity, are observed in several malignancies, and CMA downregulation may be exploited therapeutically. We evaluated the impact of LAMP2A and HSC70 in pulmonary squamous cell carcinomas (pSQCC). Antibodies were validated by knockdown and overexpression experiments using three different cell lines. Expression levels in tissue were analyzed by immunohistochemistry in a cohort of 336 consecutive pSQCC using tissue microarrays. There was no significant correlation between the two markers among each other and no association with pathological parameters (TNM categories, grading). However, both high LAMP2A and HSC70 expression were associated with worse outcome, including overall survival (OS; $p = 0.012$ and $p = 0.001$) and disease free survival (DFS; $p = 0.049$ and $p = 0.036$). In multivariate analysis, both markers and a combination of them were independent adverse prognostic factors for OS (LAMP2Ahigh: HR = 2.059; $p < 0.001$; HSC70high: HR = 1.987; $p < 0.001$; LAMP2Ahigh/HSC70high: HR = 1.529; $p < 0.001$) and DFS (LAMP2Ahigh: HR = 1.709; $p = 0.004$; HSC70high: HR = 1.484; $p = 0.027$; LAMP2Ahigh/HSC70high: HR = 1.342, $p < 0.001$). The negative prognostic impact of high LAMP2A and HSC70 and their variable expression in pSQCC may justify the use of these proteins as potential biomarkers for future CMA-inhibiting therapies.

1. Introduction

Autophagy describes different lysosomal degradation pathways targeting damaged cytosolic proteins and organelles. Deregulation of autophagy pathways is involved in many physiological and pathophysiological mechanisms such as cell aging, neurodegenerative disorders, lysosomal storage diseases, and cancer [1]. However, the role of autophagy in tumorigenesis and its prognostic impact is complex and not fully understood. The term autophagy encompasses three main pathways (macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA)) that differ in how the targeted cytosolic content reaches the lysosome for degradation [2]. In contrast to the highly conserved process of macroautophagy, CMA is only observed in mammalian cells. It is involved in the quality control of proteins by

selectively degrading altered or damaged proteins. The process is induced upon different stresses as for instance hypoxia, and it is maximally activated upon prolonged cell starvation [3, 4]. Briefly, CMA specific client proteins bear a specific pentapeptide stretch, the KFERQ-like motif (Lys-Phe-Glu-Arg-Gln) [5], which is recognized in the cytosol by the heat shock cognate protein of 70 kDa (HSC70, also known as HSPA8) within a cytosolic chaperone complex. Then, the client protein is shuttled to the lysosome where it is unfolded and translocated into the lysosome through a multimeric complex of lysosome-associated membrane protein 2A (LAMP2A) (Figure 1(a)) [6]. The binding of the CMA target protein to LAMP2A monomer initiates a multimerization process involving several LAMP2A proteins. They form a translocation complex through which the unfolded target protein is translocated for degradation into the lysosomal lumen (Figure 1(a)) [7, 8]. Since the expression and degradation of LAMP2A is tightly regulated, this protein is considered the rate-limiting factor of the CMA process.

HSC70 is a heat shock protein (HSPs). HSPs are a large group of chaperones, which are induced upon different stresses. A subclass is formed by the HSP70 family, which includes at least 13 proteins including HSC70. This chaperone is present at the cellular membrane, extracellular exosomes, the nucleus, and the cytosol [9]. Its main function is protein quality control, where it acts as a folding catalyst or targets misfolded proteins for degradation [10, 11].

LAMP2A on the other hand is an alternative splice variant of the protein encoded by LAMP2. LAMP2 is a transmembrane glycoprotein in the lysosomal membrane with three splice variants (LAMP2A, B, and C). The three isoforms share some functions such as antigen presentation, cholesterol trafficking, lysosome biogenesis, and phagocytosis while some are specific to each isoform [12]. For instance, LAMP2A is the unique LAMP2 essential for CMA.

As observed in several human cancer cell lines and in primary tumor samples, CMA seems activated in different cancer types, evidenced by markedly increased LAMP2A levels [13, 14]. In vitro, inhibition of CMA leads to decreased tumor cell survival, and in mouse cancer xenograft models, CMA inhibition results in reduced metastases and tumor shrinkage [13, 15]. However, this rather tumor supportive effect of CMA is not fully understood, and it is important to emphasize that under physiological conditions, CMA is rather tumor suppressive [16]. Data on the expression of CMA-related proteins in human cancers and their potential impact on tumor aggressiveness or response to anticancer treatment are still scarce.

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in all European countries and worldwide [17]. Among NSCLC, pulmonary squamous cell carcinoma (pSQCC) is the second most common histological subtype. It is also a subtype with a strong association to cigarette smoking [18]. The influence of carcinogens in the cigarette smoke results in a high rate of genetic and epigenetic alterations in each tumor [19]. In contrast to adenocarcinomas, pSQCC usually lack any of the main therapeutic targets like mutations in EGFR or ALK fusions [20, 21]. In recent years, new therapeutic options using immunotherapy were developed, but the benefit for most of the patients with pSQCC is still limited, and there is a need to explore alternative approaches [22, 23].

In our study, we aimed at determining the expression patterns and the prognostic relevance of LAMP2A and HSC70, the two key players of CMA, in pulmonary SQCC.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions. The human acute promyelocytic leukemia (APL) cell line, NB4, was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany), and the SKBR3 breast cancer cells were a kind gift of Professor E. Garattini (Mario Negri Institute for Pharmacological Research, Milano, Italy). NB4 cells were maintained in RPMI-1640 with 10% fetal calf serum (FCS), 50 U/mL penicillin, and 50 μg/mL streptomycin, and the SKBR3 cells were cultured in DMEM/F12, 5% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were kept at 5% CO2-95% air humidified atmosphere at 37°C. The human embryonic kidney (HEK) 293 cells expressing SV40-T-antigen (293 T) were a kind gift of Professor B. E. Torbett (Scripps Research, La Jolla, CA). 293 T cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 5% FBS, 1% penicillin/streptomycin, and 1% Hepes (Sigma-Aldrich), and kept in 7.5% CO2-95% air humidified atmosphere at 37°C.

2.2. Cell Lysate Preparation and Western Blotting. Whole cell extracts were prepared using UREA lysis buffer, and 30-60 μg total protein was loaded on a 12% denaturing polyacrylamide self-cast gel (Biorad). Blots were incubated with the primary antibodies in TBS 0.05% Tween-20/5% milk overnight at 4°C (anti-HSC70, Thermofisher MA3-014; anti-LAMP2A, Abcam 125068), incubated with HRP-coupled secondary goat anti-rabbit and goat anti-mouse antibody (cell signaling) at 1 : 5–10,000 for 1 h at room temperature.

2.3. Transient Transfection and Lentiviral Vectors. HEK 293 T cells were transiently transfected with plasmid pLX307 encoding for HSC70 (HSPA8) using the calcium phosphate method [24]. pLKO.1-puro lentiviral vectors expressing shRNAs targeting HSC70 (shHSC70_1: NM_006597.3-976s1c1, shHSC70_2: NM_006597.3-335s21c1, shHSC70_3: NM_006597.3-2040s21c1) were purchased from the Sigma-Aldrich. These vectors contain a puromycin antibiotic resistance gene for selection of transduced mammalian cells. Sequences of shRNAs to target LAMP2A were (1) shRNA: CTGCAACCTGATTGATTA and (2) shRNA: GGCAGG AGTACTTATTCTAGT. These shRNA sequences were cloned into a U6-EF1a-IRES-hygro lentiviral vector backbone. Lentivirus production and transduction were done as described [25, 26]. Transduced NB4 and SKBR3 cell populations were selected with 1.5 μg/mL puromycin for 4 days, and knockdown efficiency was assessed by western blot analysis (Figure 2).

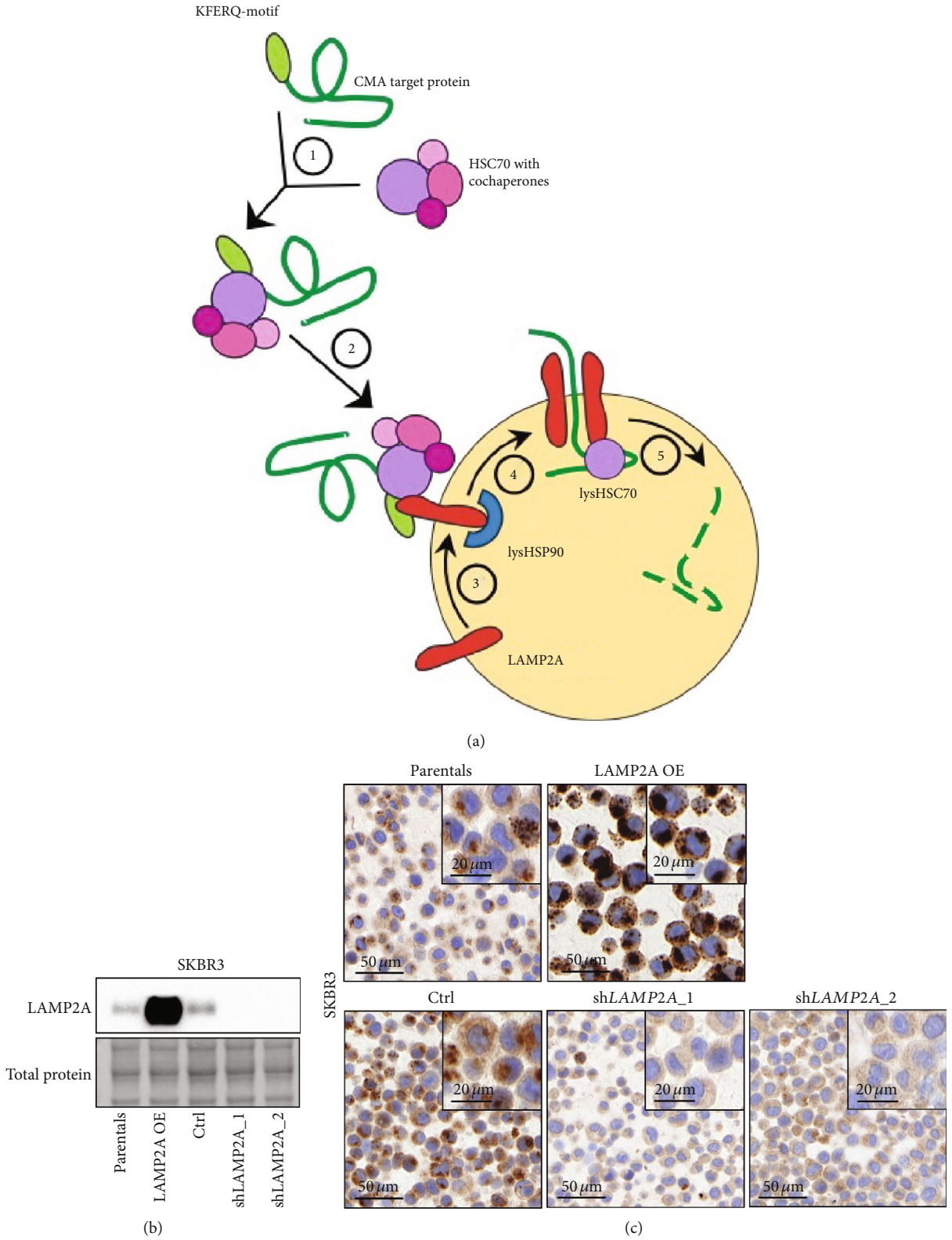

Figure 1: CMA pathway and validation of LAMP2A immunohistochemical staining. (a) Overview of chaperone-mediated autophagy (CMA). ① Recognition and binding of HSC70 to the KFERQ-motif of the target protein. ② Translocation of the complex to the lysosome. ③ Binding of the target protein to LAMP2A at the lysosomal membrane. ④ Formation of a multimeric LAMP2A complex. ⑤ Translocation and degradation of the target protein. (b, c) Specificity of LAMP2A immunohistochemistry. SKBR3 cells were transduced with lentiviral vectors containing a LAMP2A cDNA (OE) construct, an empty vector control, or shRNAs targeting LAMP2A mRNA (shLAMP2A_1-2). After selection, cells were subjected to LAMP2A western blot analysis (b) and immunohistochemistry (c).

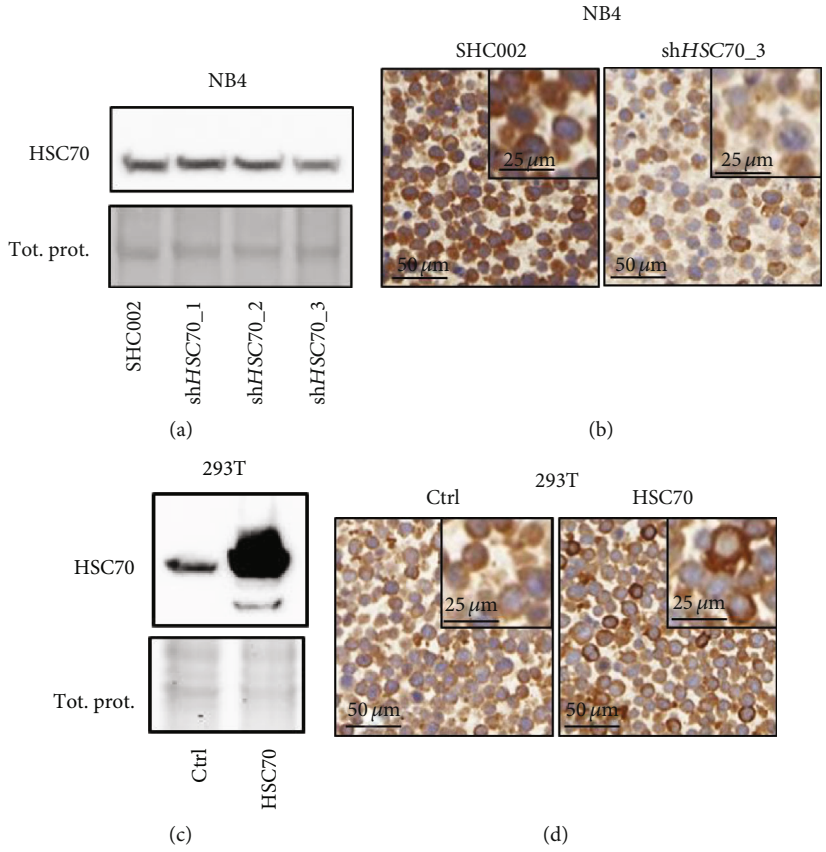

Figure 2: Validation of HSC70 immunohistochemical staining. (a, b) HSC70 knockdown in NB4 APL cells. (a) HSC70 knockdown efficiency of three independent shRNAs (shHSC70_1-3) was determined by western blotting and comparison to scramble shRNA transduced control cells (SHC002). (b) The most efficient HSC70 knockdown (shHSC70_3) was selected and subjected to immunohistochemistry. (c, d) 293 T cells were transiently transfected with an empty vector (ctrl) and HSC70 expression plasmid. (c) HSC70 expression was validated by western blotting. (d) 293 T cells were subjected to HSC70 immunohistochemistry.

2.4. Patient Cohort. In this single center, retrospective study, we investigated a consecutive cohort of patients with primary resected pSQCC, diagnosed at the Institute of Pathology, University of Bern, between 01/2000 and 12/2013. The study was performed according to the REMARK-guidelines and was approved by the Cantonal Ethics Commission of the Canton of Bern (KEK 200/14), which waived the requirement for written informed consent. As previously described, 402 patients met the inclusion criteria of the diagnosis pSQCC according to pathological records [27]. Finally, we included only tumors with confirmed squamous differentiation according to retrospectively performed immunohistochemical staining for p40 and TTF-1, according to current guidelines. Additionally, we excluded patients with previous or concomitant diagnosis of primary SQCC of other organ systems in order to reliably exclude metastatic lung disease and patients whose tumors were resected after neoadjuvant therapy according to reevaluation of clinical files. Tumors were restaged according to the 8th edition of the UICC TNM-classification [28, 29]. Tumor grading was reevaluated in all cases as previously described [30]. In short, grading was performed according to the cancer grading manual that evaluates the microscopic extension of keratinization, similar to the grading of SQCC of other anatomical regions. Grade 1 was assigned to tumors with prominent keratinization and/or prominent intercellular bridges. Grade 2 was assigned to tumors with scattered foci of keratinization, less prominent intercellular bridges, smaller tumor cells, or central comedo-like necrosis. Grade 3 tumors showed only rare or missing intercellular bridges, no keratin pearls formation, sheet-like growth, or single cell infiltration. Grade 1 and 2 corresponded to the WHO classification category of keratinizing carcinomas, and Grade 3 depicted nonkeratinizing carcinomas [31].

Finally, 354 primary resected pSQCC were available for immunohistochemical analysis. Out of these cases, LAMP2A and HSC70 could be evaluated in 336 tumors. For the remaining cases, there was no sufficient tumor material in the TMA cores, or the immunoreactivity of the tissue was insufficient due to technical error. Detailed clinicopathological characteristics are provided in Table 1. Adjuvant chemotherapy or radiotherapy was administered in 116 patients (35%).

2.5. Next-Generation Tissue Microarray. Immunohistochemical staining was applied on a next generation tissue microarray (ngTMA) constructed as previously described, with digital annotation of scanned slides and automatic transfer

Table 1: Description of the case collection.

		n	%
Gender	m	286	85.1
	f	50	14.9
Median age (range)	69 (43-85)		
pT UICC 2017	pT1a	6	1.8
	pT1b	21	6.3
	pT1c	45	13.4
	pT2a	68	20.2
	pT2b	51	15.2
	pT3	78	23.2
	pT4	67	19.9
pN UICC 2017	pN0	192	57.1
	pN1	107	31.9
	pN2	37	11.0
Distant metastases	Absent	328	97.6
	Present	8	2.4
AJCC/UICC TNM stage 2017	IA1	4	1.2
	IA2	17	5.1
	IA3	31	9.2
	IB	48	14.3
	IIA	28	8.3
	IIB	88	26.2
	IIIA	86	25.6
	IIIB	26	7.7
	IIIC	0	0.0
	IVA	6	1.8
	IVB	2	0.6
Grading	Grade 1	7	2.1
	Grade 2	170	50.6
	Grade 3	159	47.3
Resection status	R0	292	86.9
	R1/R2	44	13.1
Total		336	100.0

of the punches [32]. Two separate ngTMAs with a total of four punches per tumor (diameter = 0.6 mm) randomly taken from different tumor regions were used.

2.6. Immunohistochemical Staining and Scoring. Immunohistochemical staining for LAMP2A and HSC70 was done on 4 μm sections using an automated immunostainer Leica Bond RX (Leica Biosystems, Heerbrugg, Switzerland) with the following conditions (dilution, antigen retrieval): LAMP2A (Novus Biologicals, Zug, Switzerland, rabbit polyclonal, #NB600-1384): 1 : 500, tris buffer, 95°C, and 30 min; and HSC70 (LabForce mbl, Nunningen, Switzerland, rabbit polyclonal, #PM0045): 1 : 10,000, citrate buffer, 100°C, and 30 min. For visualization, the Bond Polymer Refine Detection kit (Leica Biosystems, Muttenz, Switzerland, DS9800) was used according to the instructions of the manufacturer.

Scoring of LAMP2A and HSC70 was performed by a pathologist (TL) on a Zeiss Axioscope microscope at 10x objective magnification for each TMA core separately. We assessed the staining intensity in tumor cells ranging from 0 (negative), 1 (weak), 2 (medium) to 3 (strong). The percentage of stained tumor cells was determined using the following increments: 0 ≤ 5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, and 4 = 76-100%. Finally, the immunoreactivity score (IRS) was calculated by multiplication of the scores for intensity with the scores of the percentages of positive tumor cells.

The staining was cytoplasmatic for LAMP2A and HSC70. Some cases showed both cytoplasmatic and nuclear HSC70 staining. The necrotic areas were strongly positive for both markers and discarded from the evaluation. The examples of staining are shown in Figure 3.

The individual IRS was used to assess intratumoral heterogeneity. For the final determination of the marker expression level in the tumor, the sum of the IRS over all cores divided by the number of cores was calculated for each tumor. The IRS sum score was used for the correlation of the marker expression with pathological parameters. For survival analysis, the cohort was first divided into quartiles. The best prognostic differentiation was observed by stratification of the results in low expression (lower three quartiles) and high expression (fourth quartile).

2.7. Statistical Analysis. IBM SPPS Statistics 26 (IBM Corporation, Armonk, USA) was used for statistical analyses. For group comparisons, crosstabs, X2 tests, and Fisher's exact tests were used. Survival analysis (overall survival and disease free survival) was calculated from the day of surgery. For univariate survival analysis, the Kaplan-Meier curves and log-rank tests were used. For multivariate survival analysis, the Cox regression analysis was used. p values of <0.05 were considered as significant for all tests.

3. Results

3.1. Validation of LAMP2A And HSC70 Antibodies for Immunohistochemistry. First, we validated the specificity of the antibodies LAMP2A and HSC70 for immunohistochemical staining. We generated a series of LAMP2A and HSC70 knockdown and overexpression cell lines. For this, we used lentiviral vectors to express LAMP2A cDNA as well as two independent shRNAs targeting LAMP2A in SKBR3 breast cancer cells. We confirmed ectopic expression and knockdown efficiency of LAMP2A by western blot analysis (Figure 1(b)). We detected a marked overexpression of LAMP2A compared to parental SKBR3 cells in cells expressing the exogenous LAMP2A cDNA. In addition, expression of both shRNAs targeting LAMP2A resulted in an efficient depletion of LAMP2A in SKBR3 cells compared to control transduced cells. Next, FFPE cell pellets were subjected to LAMP2A immunohistochemical staining. Consistent with the western blot data, immunohistochemical analysis revealed increased or depleted LAMP2A expression in LAMP2A cDNA and shLAMP2A transduced cells, respectively

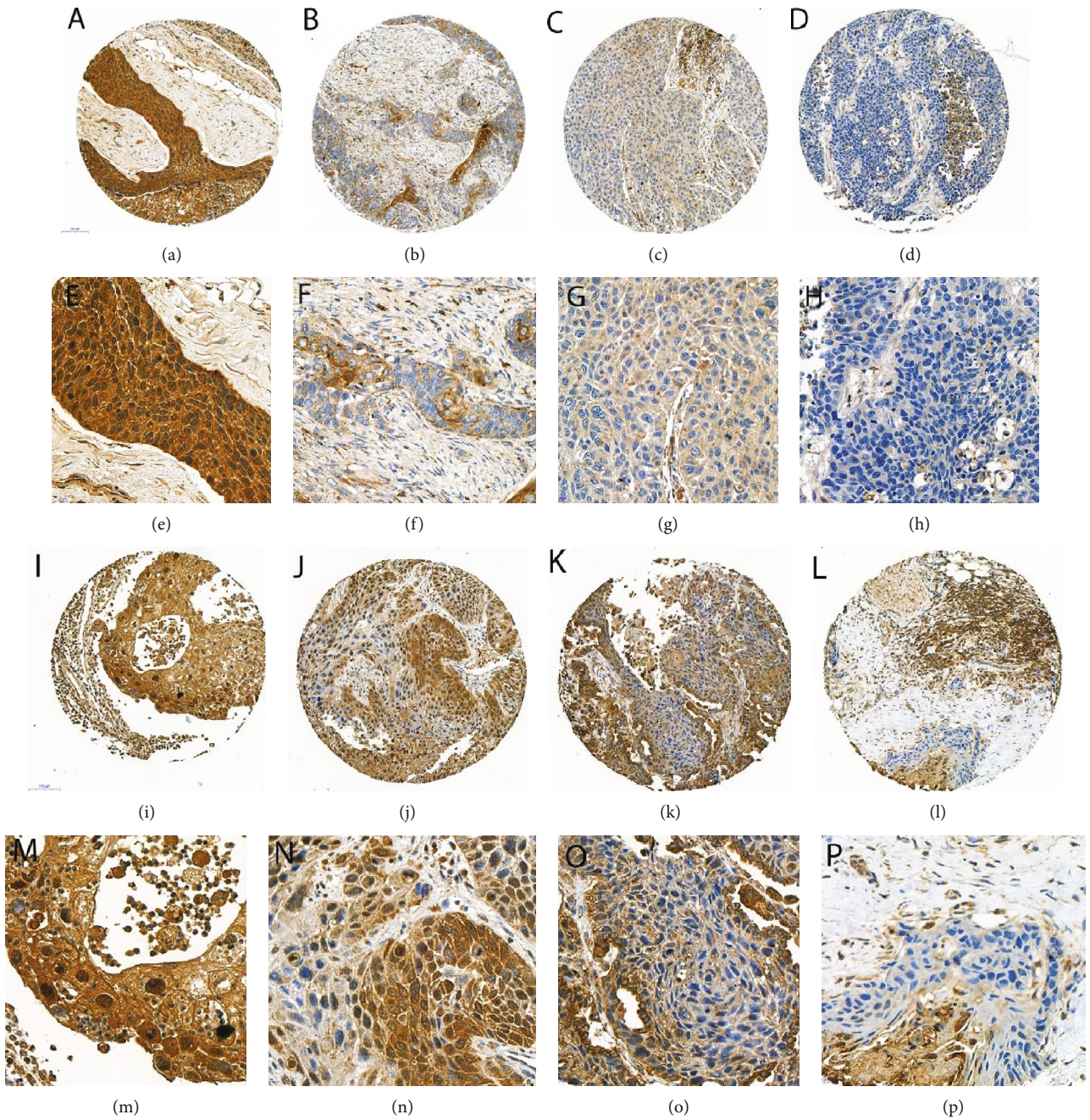

Figure 3: Examples of immunohistochemical staining: (a–h) LAMP2A ((a, e) IRS 3x4 = 12; (b, f) IRS 3x2 = 6; (c, g) IRS 1x4 = 4; (d, h) IRS 0x0 = 0); (i–p) HSC70 ((i, m) IRS 3x4 = 12; (j, n) IRS 3x3 = 9; (k, o) IRS 1x4 = 4; (l, p) IRS 0x0 = 0). Objective magnification: (a–d) 13x, (e–h) 40x, (i–l) 13x, and (m–p) 40x.

(Figure 1(c)). Of note, in agreement with the lysosomal localization of LAMP2A during CMA, a dot-like staining pattern was observed for LAMP2A.

Similarly, we generated HSC70 knockdown and overexpression cells. We transduced NB4 acute promyelocytic leukemia (APL) cells with a control and three independent shRNAs targeting HSC70. Only shHSC70_3 transduced NB4 cells showed a reduction in HSC70 expression compared to the control transduced cells on a western blot (Figure 2(a)). This knockdown was confirmed by immunohistochemical staining of HSC70 (Figure 2(b)). A transient overexpression of an HSC70 expression plasmid in 293 T cells resulted in increased protein expression as assessed by western blotting and immunohistochemistry (Figures 2(c) and 2(d)). Together, our knockdown and overexpression experiments in different cell lines underline the specificity of the anti-LAMP2A and anti-HSC70 antibodies used in immunohistochemical staining.

3.2. LAMP2A and HSC70 Expression and Intratumoral Heterogeneity. For determining LAMP2A and HSC70 expression in 336 pSQCC, a total of 1399 TMA cores stained

with LAMP2A and 1378 TMA cores stained with HSC70 were available for evaluation. LAMP2A expression was absent in 109/1399 (8%) of the TMA cores, weak in 286/1399 (20%), medium in 683/1399 (49%), and strong in 321/1399 (23%) cores. The intensity of HSC70 was weak in 146/1378 (11%), medium in 510/1378 (37%), and strong in 708/1378 (51%) of the TMA cores. Only 14/1378 (1%) of the TMA cores lacked HSC70 expression. IRS multiplying intensity scores with the extent of tumor staining were calculated as described in the Material and Methods. For subsequent analysis, the IRS of the single cores was used for assessing intratumoral heterogeneity and the correlation between the two markers. For the determination of the expression levels with clinicopathologic characteristics, the IRS sum scores were calculated for each tumor. Correlations were performed using either the IRS sum scores or a categorization based on the quartiles of the IRS sum scores.

We identified only 8 cases with additional nuclear expression of HSC70 (Figures 3(i) and 3(m)), and in this small group, there was no statistically significant correlation with other pathological parameters or any valuable prognostic significance.

There was no significant intratumoral staining heterogeneity for LAMP2A and HSC70 when comparing the IRS of the single cores per tumor of the respective markers among each other (p values between 0.155 and 0.82). Rather, there was a highly significant correlation for the IRS within the four TMA cores per tumor for LAMP2A (r = range 0.751-0.895; $p < 0.001$ each) and the IRS of HSC70 (r = range 0.428-0.698; $p < 0.001$ each).

3.3. Correlation between LAMP2A and HSC70. Due to a close cooperation of LAMP2A and HSC70 on the molecular level, the IRS scores of these markers were compared. There was no significant correlation between the expression of LAMP2A and HSC70 in the single cores and overall (p values between 0.388 and 0.875; overall: $p = 0.68$).

3.4. Correlation between LAMP2A and HSC70 Expression Levels and Pathological Parameters. For the assessment of associations between LAMP2A and HSC70 expression and pathological parameters, the IRS scores of each tumor (i.e., the sum of all IRS scores across all TMA cores per tumor) were calculated against the respective factors or were subdivided into quartiles for a categorization into low (lower three quartiles) to high (highest quartile) expression levels. In UICC pT1a tumors, the least advanced subgroups of tumors in the pT category, higher LAMP2A levels and lower HSC70 levels, were observed, but this was overall not statistically significant when analyzing the entire cohort. For all other pT categories, IRS levels were within a comparable range. Similarly, there was no significant association between the expression of LAMP2A and HSC70 with other pathological parameters such as pN categories, presence of distant metastases, UICC/AJCC TNM staging and grading, nor with gender or patients' age. These results were observed using both calculation methods (IRS sum scores and categorization; see supplemental Figures S1- S10).

3.5. Correlation with Survival. Survival data was available for 254 patients. Mean disease free survival (DFS) was 50.1 months, and mean overall survival (OS) was 53.9 months. Survival analysis for DFS and OS was calculated using the expression levels defined by the four quartiles. The best prognostic discrimination was seen for the fourth quartile (then defined as high expression) versus the lower three quartiles (defined as low expression). This threshold was then used for further analysis. IRS cutoffs for differentiating between low and high staining were 28.0 (summarized from all four tumor cores) for LAMP2A and IRS 41.8 for HSC70. Low levels of LAMP2A staining (lower three quartiles) were observed in 255/336 (76%) cases and high levels in 81/336 (24%) cases. Similarly, we found low levels of HSC70 (lower three quartiles) in 252/336 (75%) cases and high levels of HSC70 in 84/336 (25%) cases.

High LAMP2A levels were associated with unfavorable OS ($p = 0.012$) and DFS ($p = 0.049$). High HSC70 levels were also associated with worse outcome, including OS ($p = 0.001$) and DFS ($p = 0.036$) (Figures 4(a)–4(d)).

Applying multivariate analysis, both markers were also independent adverse prognostic factors for OS and superior to UICC/AJCC TNM stage (Table 2). For DFS, both markers, but also UICC/AJCC TNM stage, were independent prognostic factors (Table 3).

The combination of LAMP2A and HSC70 showed an even more significant prognostic impact: patients with LAMP2Ahigh/HSC70high tumors showed the worst prognosis and patients with LAMP2Alow/HSC70low tumors the best prognosis ($p < 0.001$ for OS and $p = 0.012$ for DFS, Figures 4(e) and 4(f)). This combination was also an adverse independent prognostic factor for OS and DFS in multivariate analysis (Tables 4 and 5).

4. Discussion

The role of autophagy and its subtypes, particularly CMA, in tumorigenesis is complex. As described in previous studies, it may play a dichotomous role in cancer by suppressing the initiation of tumor growth but promoting tumor growth and survival in established cancers [33]. The expression patterns of CMA markers are in the majority still unknown but could contribute to a better understanding of these complex and fine-tuned cellular mechanisms.

In our retrospective study, we assessed the immunohistochemical expression patterns of the two CMA key players LAMP2A and HSC70 and their prognostic value in primary resected pSQCC. The strength of our study is the large and histologically homogeneous, well-curated patient cohort with survival data available for 254 cases and the meticulous validation of specificity of the immunohistochemical markers used. In order to guarantee the reliability of our results [34], we generated overexpression and knockdown cell lines for each marker and validated protein expression patterns via western blot and immunohistochemistry on FFPE cell pellets.

We could demonstrate a variable expression of LAMP2A and HSC70 in pSQCC. There was a wide spectrum of staining intensity, even though the best prognostic

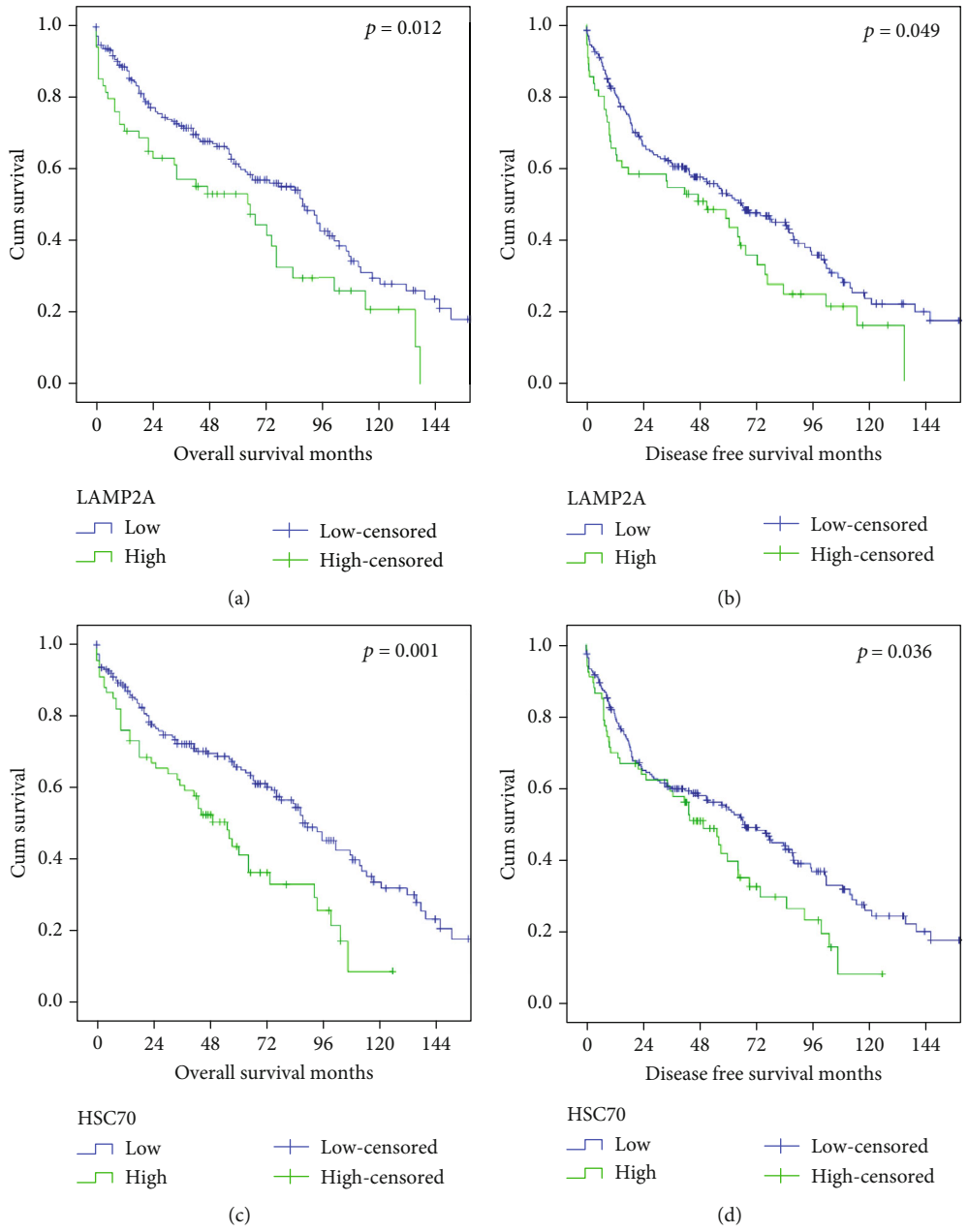

Figure 4: Continued.

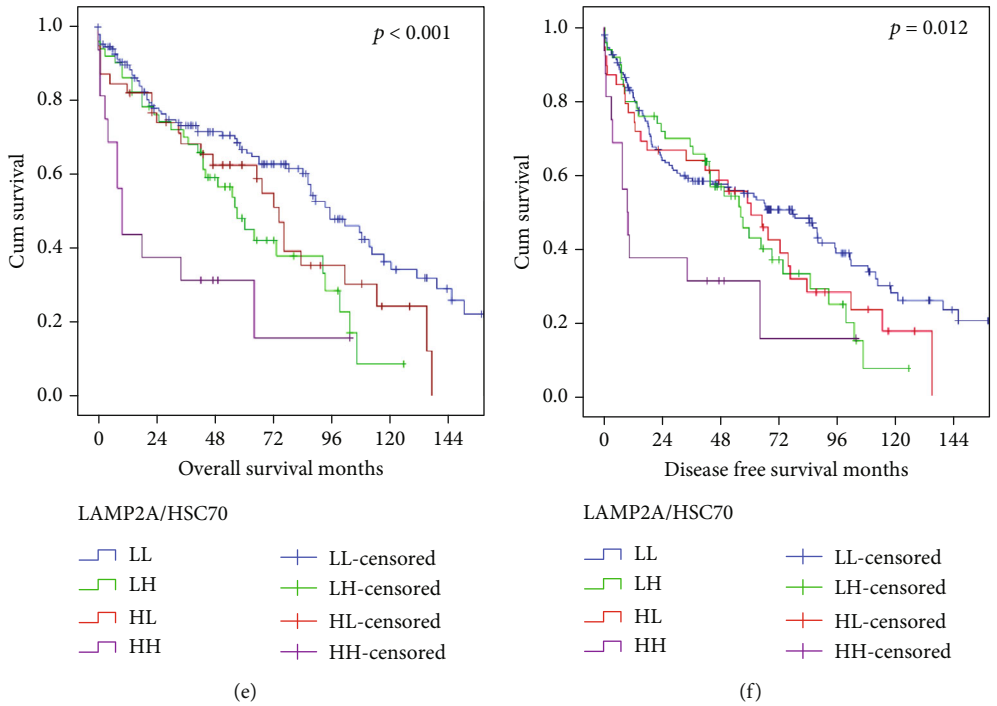

Figure 4: Kaplan-Meier curves (overall survival and disease free survival) for expression of autophagy-related proteins: (a) OS, LAMP2A; (b) DFS, LAMP2A; (c) OS, HSC70; (d) DFS, HSC70; (e) OS, combined; (f) DFS, combined.

Table 2: Results of multivariate analysis for OS.

	HR	95% CI		p value
		Lower	Upper	
Gender	0.578	0.337	0.990	0.046
Age	2.550	1.785	3.642	<0.001
UICC/AJCC stage 2017 (I, II, III, IV)	1.225	0.974	1.539	0.083
R status	1.705	1.062	2.736	0.027
LAMP2A^{high}	2.059	1.396	3.036	<0.001
HSC70^{high}	1.987	1.368	2.885	<0.001

Table 3: Results of multivariate analysis for DFS.

	HR	95% CI		p value
		Lower	Upper	
Gender	0.682	0.423	1.102	0.118
Age	2.059	1.486	2.851	<0.001
UICC/AJCC stage 2017 (I, II, III, IV)	1.233	1.001	1.520	0.049
R status	1.591	1.021	2.480	0.040
LAMP2A^{high}	1.709	1.185	2.467	0.004
HSC70^{high}	1.484	1.046	2.105	0.027

stratification was observed between strong positive tumors versus all other staining patterns. Our key finding is that both markers, LAMP2A and HSC70, are independent adverse prognostic markers in pSQCC including OS and DFS. The combination of both of them (LAMP2Ahigh/HSC70high) showed an even more significant prognostic impact, although this marker profile was observed in only few cases ($n = 21$). This marker profile might correspond to activated CMA in advanced tumors, which could be required to overcome the altered metabolism of the tumor cells [13].

Moreover, we found no significant intratumoral heterogeneity of LAMP2A and HSC70 staining in the examined

Table 4: Results of multivariate analysis for OS and LAMP2A/HSC70 marker combination.

	HR	95% CI		p value
		Lower	Upper	
Gender	0.581	0.338	0.997	0.049
Age	2.629	1.843	3.751	<0.001
UICC/AJCC stage 2017 (I, II, III, IV)	1.221	0.971	1.536	0.088
R status	1.764	1.100	2.828	0.018
$LAMP2A^{high}/HSC70^{high}$	1.529	1.287	1.816	<0.001

Table 5: Results of multivariate analysis for DFS and LAMP2A/HSC70 marker combination.

	HR	95% CI		p value
		Lower	Upper	
Gender	0.688	0.426	1.110	0.125
Age	2.085	1.508	2.882	<0.001
UICC/AJCC stage 2017 (I, II, III, IV)	1.231	0.998	1.517	0.052
R status	1.612	1.035	2.509	0.035
$LAMP2A^{high}/HSC70^{high}$	1.342	1.140	1.579	<0.001

tumors. There was no significant correlation between these two markers and other pathological parameters. Surprisingly, there was also no correlation between these two individual markers, although biologically the two proteins cooperate in CMA, which underlines the need of further functional studies in this field. It is important to mention that high expression levels of HSC70 and LAMP2A are considered indicative of high CMA levels but are not a proof of high CMA activity. As CMA is a dynamic process, it cannot be captured completely using a static method as immunohistochemical staining. High levels of CMA markers could as well occur in a situation of stalled CMA degradation for instance if the lysosomal function is impaired.

Similar results have been reported in other tumor types. LAMP2A is expressed in almost all types of tumors, but the prognostic value of tumoral expression has not been extensively explored yet. In a recent study on esophageal squamous cell carcinomas, high expression of LAMP2A was associated with poor prognosis, similar to our findings [35].

High expression levels of HSC70 were observed in many cancers, e.g., hepatocellular or colon carcinomas [36, 37]. HSC70 was described as a prognostic marker in colorectal cancer (favorable), liver cancer (unfavorable), and renal cancer (favorable) [38]. In our cohort of pSQCC, HSC70 expression was associated with an unfavorable prognosis. This discrepancy of the prognostic value of HSC70 might result from the diversity of HSC70 function in the cell [11].

In lung cancer, the expression of HSP70 was studied before, another HSP70 family member also known as HSPA1A or HSP70-1. However, the results are inconsistent, including a reported better prognosis in patients with HSP70-positive NSCLC as well as an association with a Ki-67 proliferation index and nuclear HSP70 expression [39, 40]. Yet, intense focus has been placed on exploring the potential of HSP70 inhibitors as chemotherapeutic agents [10]. For HSC70, however, pharmacological inhibitors were not available until few years ago. Since this protein is as well involved in the presentation of antigenic peptides by major histocompatibility complex class II (MHCII), it was recently exploited as a target for the treatment of autoimmune disorders [11]. In this context, a phosphopeptide called P140 was shown to directly interact with HSC70 and to inhibit CMA. This new drug showed a significant downregulation of the signaling of autoreactive T cells in vivo in a model of systemic lupus erythematosus, leading to a remarkable improvement of the pathophysiologic condition [41]. Thus, HSC70 may be a possible target to inactivate CMA in future anticancer therapy, warranting the current detailed expression analyses. If those aggressive pSQCC with high LAMP2A and HSC70 expression might be candidates for the new CMA-targeting therapeutics must be further evaluated in functional analyses and subsequent clinical studies.

Our present work has some limitations warranting subsequent validation studies. Importantly, evaluation of the stainings was performed by only one pathologist. Although this ensures the application of homogenously calibrated criteria in scoring of all cases, it precludes any statement on interobserver variability of the scoring method. This will be addressed in the subsequent studies. Additionally, there is a possible bias in the sample collection inherent in the retrospective design of the study, although all consecutive cases were included. Finally, evaluation of the stainings was performed on a TMA, which might not represent the entire tumor when compared to the whole slide. In order to minimalize this limitation, a minimum of 4 cores per tumor were evaluated separately. A comparison of scores between the different cores per tumor failed to show a significant staining heterogeneity, speaking in favor of the robustness of the staining pattern throughout the whole tumor [42, 43].

From a biological point of view, the results of our tissue-based explorative study underline the role of CMA in human tumorigenesis. From a clinical point of view, the two markers

HSC70 and LAMP2A may be exploited as prognostic biomarkers in pSQCC.

5. Conclusions

In our present study, we demonstrated the variable immunohistochemical expression of the key CMA markers LAMP2A and HSC70 in pSQCC. High expression levels of these markers were associated with worse prognosis, including OS and DFS, and could be considered as biomarkers for potential future CMA inhibiting therapies.

Data Availability

The primary data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Parts of this work have been presented as an abstract at the 12th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

The following are available online, Figures S1-S10: IRS values of LAMP2A and HSC70 with pathological parameters (TNM categories, Grading, Stage). *(Supplementary Materials)*

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Research Article

O-GlcNAcylation Is Essential for Autophagy in Cardiomyocytes

Houzhi Yu,[1,2] **Li Wen**,[3] **and Yongxin Mu**[2]

[1]*Department of Cardiology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China*
[2]*Department of Medicine-Cardiology, University of California San Diego, 9500 Gilman Drive, Mail Code 0613-C, La Jolla California 92093-0613, USA*
[3]*Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China*

Correspondence should be addressed to Yongxin Mu; yomu@ucsd.edu

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Since both O-GlcNAcylation and autophagy sense intracellular nutrient level, the alteration of those two pathways plays substantial roles in the progression of heart failure. Hence, determining the relationship between O-GlcNAcylation and autophagy is imperative to understand, prevent, and treat heart failure. However, the mechanism on how O-GlcNAcylation regulates autophagy in the heart is poorly investigated. In this study, we demonstrated that O-GlcNAcylation is required for autophagy in cardiomyocytes by utilizing an O-linked β-N-acetylglucosamine transferase (OGT) cardiomyocyte-specific knockout mouse model for the first time. We also identified that OGT might regulate the initiation of autophagy in cardiomyocytes through promoting the activity of ULK1 by O-GlcNAcylation. In conclusion, our findings provide new insights into the molecular mechanisms underlying heart dysfunction and benefit the development of treatments for heart failure.

1. Introduction

O-GlcNAcylation is an important posttranslation modification of proteins by the addition of O-linked β-N-acetylglucosamine (O-GlcNAc) moieties at serine or threonine residues. Similar to protein phosphorylation, which also occurs on serine or threonine residues, O-GlcNAcylation is dynamic. In contrast to phosphorylation, which is catalyzed and removed by hundreds of kinases and phosphatases with relative substrate specificities, O-GlcNAcylation is catalyzed and removed by a single pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively [1, 2]. Increased O-GlcNAcylation in a hypertrophic or failing heart has been reported from human patients and animal models [3, 4]. Additionally, previous studies using both *in vitro* and *in vivo* models have suggested that increased O-GlcNAcylation plays a cardioprotective role in acute cardiac dysfunctions, yet it may have deleterious effects on cardiac function in chronic conditions [3]. Genetic knockout of OGT at embryonic stage in mice led to congenital heart diseases, resulting in partial postnatal lethality [5] and cardiac hypertrophy among surviving mice [6]. The acute loss of OGT in cardiomyocytes also exacerbated heart failure induced by myocardium ischemia [7].

Autophagy is a conserved mechanism for the degradation of intracellular elements and plays an essential role in protein homeostasis and the quality control of subcellular organelles [8–12]. Although sometimes autophagy can induce cell death with unique morphological changes, in most cases, autophagy plays protective or adaptive roles and prevents cell death [12, 13]. Autophagy is essential in maintaining cardiac structure and function at both baseline by degrading misfolded proteins and damaged organelles and during stress by limiting the cardiac damage in different pathological conditions such as ischemia, starvation, and hemodynamic overload [14, 15]. A decreased level of autophagy was proven to contribute to the progression of heart failure and aging [16, 17]. Hence, autophagy plays an important role in mediating cardiac homeostasis and adaption to aging, stress, and myocardial injury.

Recently, the relationship between O-GlcNAcylation and autophagy is gaining more interests, and studies have shown that O-GlcNAcylation indeed regulates autophagy [18, 19]. Some reports have shown that O-GlcNAcylation negatively

regulates autophagy in the heart. Noteworthy, those studies have used Streptozotocin (STZ) to increase O-GlcNAcylation *in vivo* [20, 21] by damaging pancreatic β cells [22]. However, damaged β cells and STZ's well-known side effects on other organs [22] may compromise the relationship between O-GlcNAcylation and autophagy specifically in the heart or cardiomyocytes.

Here, we used the Cre-Loxp system to specifically knock out OGT and abolish O-GlcNAcylation in cardiomyocytes. We found that the loss of O-GlcNAcylation in cardiomyocytes attenuated autophagy, especially under fasting condition. Also, data from isolated neonatal cardiomyocytes showed that the loss of OGT affected the early stage of autophagy. Moreover, we identified that Unc-51-Like Autophagy Activating Kinase 1 (ULK1), an essential kinase for initiating autophagy flux, was O-GlcNAcylated in cardiomyocytes, and the level of O-GlcNAcylation of ULK1 was diminished in OGT knockout cardiomyocytes. Taken together, our data demonstrated that O-GlcNAcylation is essential for the initiation of autophagy in cardiomyocytes. Our findings provide novel insights on the regulation of autophagy in heart diseases.

2. Materials and Methods

2.1. Animal Models. $Ogt^{flox/flox(f/f)}$ mouse was purchased from Jackson Lab (Stock No: 004860 | OGTF). Floxed female mice were crossed with *α-Mhc-MerCreMer* transgenic mice [23] to create $Ogt^{f/y}$; *α-Mhc-MerCreMer*-inducible KO (icKO) mice. All mice were of a mixed 129/SvJ and C57BL/6J background. Genotypes of the mice were confirmed by polymerase chain reaction (PCR) analysis using tail genomic DNA and *Ogt* primers (forward: 5′-CATCTC TCCAGCCCCACAAACTG-3′, reverse: 5′-GACGAAGCA GGAGGGGAGAGCAC-3′) and *Cre* primers (forward: 5′ -GTTCGCAAGAACCTGATGGACA-3′; reverse: 5′-CTAG AGCCTGTTTTGCACGTTC-3′). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, with an approved protocol# S01049.

2.2. Tamoxifen Induction. 4-Hydroxytamoxifen was dissolved in sesame oil at a concentration of 10 mg/mL. Adult (2-month-old) $Ogt^{f/y}$ and $Ogt^{f/y}$; *α-Mhc-MerCreMer* mice were treated with 4-hydroxytamoxifen by intraperitoneal injection once daily for 5 days with a dosage of 30 mg/kg body weight. Ten days after the last dose of tamoxifen, mice were either given unlimited food or fasted for 12 hours, followed by heart collection for western blot analysis.

2.3. Adenoviral Vectors, Reagents, and Antibodies. Adenoviruses expressing Cre and lacZ (Ad-Cre and Ad-lacZ) were obtained from the UCSD Viral Vector Core. Adenovirus expressing mRFP-GFP-LC3 (Ad-tf-LC3) was provided as a gift from Dr. Junichi Sadoshima. 4-Hydroxytamoxifen (H7904), Thiamet-G (TMG, SML0244), and Bafilomycin A1 (SML1661) were purchased from Sigma-Aldrich. Antibodies used in this study included RL2 (MA1-072, Thermo Fisher Scientific), OGT (61355, Active Motif), ULK1 (4773, Cell Signaling), pATG16L1 (ab195242, Abcam), LC3B (2775, Cell Signaling), SQSTM1 (GP61-C, Progen), ubiquitin (sc8017, Santa Cruz), and GAPDH (sc365062, Santa Cruz).

2.4. Protein Isolation and Western Blot Analysis. Total protein extracts were prepared by suspending ground heart tissue or isolated cardiomyocytes in a urea lysis buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT, 0.03% bromophenol blue, 0.05 M Tris-HCl, pH 6.8). Protein lysates were separated on 4% to 12% SDS-PAGE gels (Thermo Fisher Scientific) and transferred at 4°C overnight onto nitrocellulose membranes (Bio-Rad). After blocking for 1 hour in TBS containing 0.1% Tween-20 (TBST) and 5% dry milk, the membranes were incubated at 4°C overnight with the indicated primary antibodies in a blocking buffer. Blots were washed and incubated with the appropriate HRP-conjugated secondary antibodies (1 : 5000) for 1 hour at room temperature. Immunoreactive protein bands were visualized using an ECL reagent (Thermo Fisher Scientific).

2.5. Immunoprecipitation. After the transduction with Ad-LacZ or Ad-Cre viruses for 48 hours, neonatal cardiomyocytes were lysed in a RIPA lysis buffer (50 mM Tris, 10 mM EDTA, 150 mM NaCl, 0.25% deoxycholic acid, 0.1% SDS, 2% NP-40 substitute, and 0.01% sodium azide). Cell lysates were rotated in the RIPA buffer with 10 μL of RL2 or ULK1 antibody at 4°C overnight. Normal IgG (Santa Cruz) was used as a negative control. Next, 25 μL of PBS-washed protein G beads (Thermo Scientific) were resuspended and incubated in the lysate-antibody complexes for 2 hours at 4°C. After washing 3 times with the RIPA lysis buffer, beads were incubated in 4 × LDS buffer (BioRad) at 70°C for 10 minutes, and the supernatants were collected. The immunoprecipitates and input lysate were gel electrophoresed and immunoblotted with the antibodies against O-GlcNAc (RL2) and ULK1.

2.6. Neonatal Mouse Cardiomyocyte Isolation and Treatments. Neonatal mouse cardiomyocytes were prepared as previously described [24] and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin for 24 hours before adenovirus transduction or other treatments. After being transduced with adenoviruses at MOI of 50, cardiomyocytes were cultured for additional 36 or 48 hours. Wild-type neonatal cardiomyocytes were treated with TMG (25 μM) for 48 hours before other treatments. Right before collection, cardiomyocytes were treated with Bafilomycin A1 (100 nM) for 4 hours or subjected to starvation with serum-free medium for 18 hours.

2.7. Fluorescent Microscopy. 48 hours after the transduction with Ad-tf-LC3 along with Ad-LacZ or Ad-Cre, cardiomyocytes were fixed with 4% paraformaldehyde for 5 min before confocal imaging. For immunostaining of pATG16L1, Ad-LacZ or Ad-Cre, transduced neonatal cardiomyocytes were fixed with 4% paraformaldehyde for 5 min and blocked in the blocking buffer (PBS with 1% BSA, 5% donkey serum,

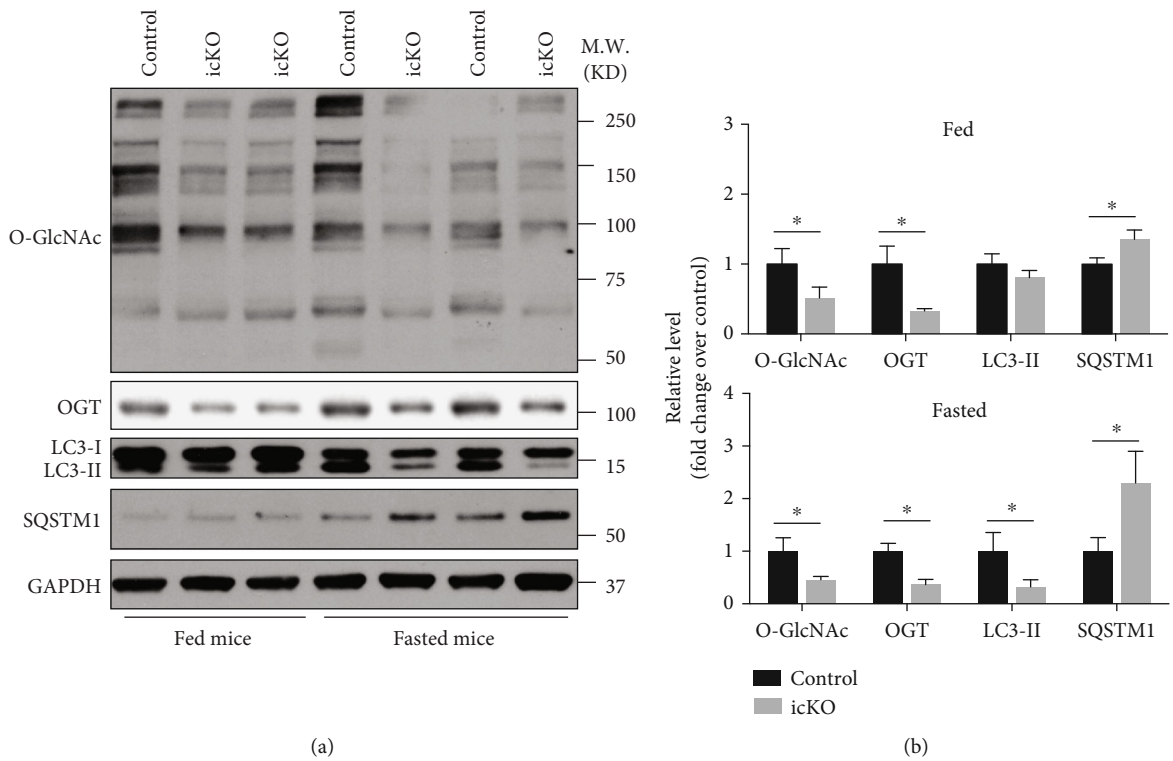

Figure 1: Acute loss of OGT attenuates autophagy in cardiomyocyte. (a) Western blot analysis of O-GlcNAc (RL2), OGT, LC3, and SQSTM1 using whole heart lysates from control and icKO mice with either food (fed) or water only (fasted). (b) Statistical analyses of the western blot results, $n = 4$ for each group. *Significantly different; M.W.: molecular weight.

and 0.2% Triton 100) for 2 hours. They were then incubated with pATG16L1 antibody (1 : 200 in blocking buffer) overnight followed by a secondary antibody (1 : 300 in blocking buffer) incubation for 2 hours and confocal imaging.

2.8. Statistics. Data were presented as the mean ± SEM unless indicated otherwise. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software), with a 2-tailed Student's t test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Deletion of Cardiomyocyte OGT Leads to the Attenuation of Autophagy in the Mouse Heart. To explore the possible effects of O-GlcNAcylation on autophagy in the heart, we crossed $Ogt^{f/f}$ females with inducible *α-MHC-MerCreMer* male mice to generate $Ogt^{f/y}$; *α-MHC-MerCreMer* mice, which were injected with tamoxifen for 5 days to generate OGT cardiac knockouts (hereafter icKO). Meanwhile, age-matched $Ogt^{f/y}$ mice injected with the same doses of tamoxifen were used as controls. Ten days after the last dose of tamoxifen, when the icKO mice did not show heart dysfunction [6], western blot with RL2 and OGT antibodies showed that global O-GlcNAcylation and OGT dramatically decreased in icKO hearts. Under fed condition, the LC3-II level in the heart was not statistically different between control and icKO mice, although LC3-II in icKO mice was decreased (Figure 1). In contrast, the SQSTM1 level in icKO mice increased slightly but significantly (Figure 1). The most potent known physiological inducer of autophagy is starvation, and fasting has been widely used to investigate autophagy in mouse models [25]. Thus, we next investigated whether O-GlcNAcylation affected cardiac autophagy when mice were subjected to fasting. Surprisingly, the LC3-II level was significantly decreased in icKO mice when they were subjected to fasting. The SQSTM1 level in icKO mouse heart was further elevated accordingly (Figure 1). These data suggested that O-GlcNAcylation in cardiomyocytes is indispensable for autophagy under fasting condition, while it has only a mild influence on autophagy at the basal level.

3.2. Deletion of OGT in Isolated Neonatal Cardiomyocytes Attenuates Autophagy. To investigate whether OGT regulated autophagy in cardiomyocytes in a cell autonomous manner, we isolated neonatal cardiomyocytes from newborn pups from $Ogt^{f/f}$ female and $Ogt^{f/y}$ crossings. Those isolated neonatal cardiomyocytes were transduced with adenovirus expressing Cre recombinase (Ad-Cre) to delete OGT. Cells transduced with adenovirus expressing LacZ (Ad-LacZ) were used as control. Western blot showed that the levels of O-GlcNAcylation and OGT were dramatically decreased in Ad-Cre-treated cells (Figure 2(a)). LC3-II was significantly downregulated in knockout cells, regardless whether the cells were cultured in complete medium (nutrient) or subjected to starvation (starved) as described in Materials and Methods. Also consistent with the previous *in vivo* result (Figure 1), SQSTM1 was increased in knockout cells under both nutrient and starved conditions (Figure 2(a)). To further confirm

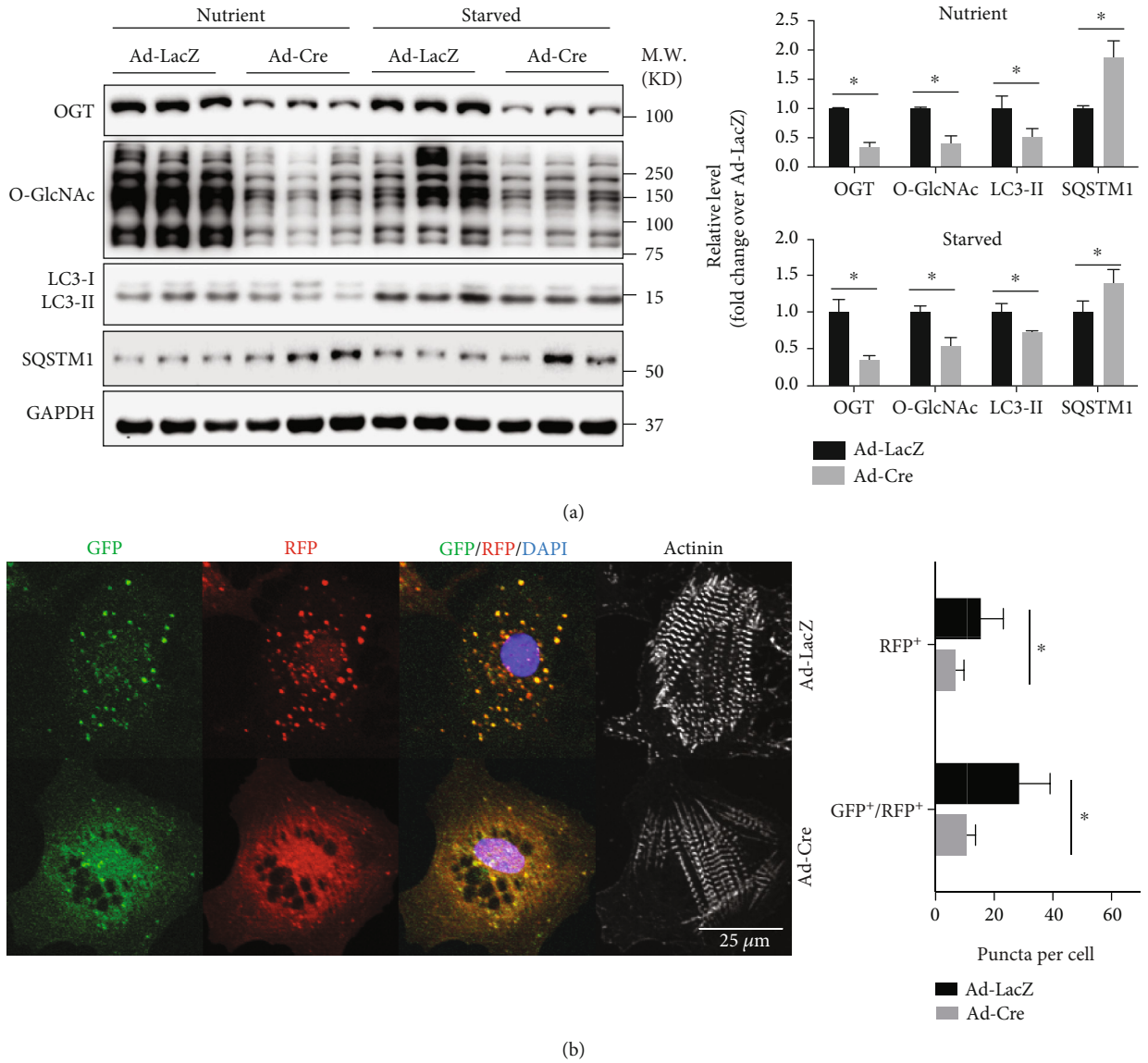

Figure 2: Deletion of OGT in isolated neonatal cardiomyocyte attenuates autophagy. (a) Left panel, western blot analysis for autophagy of Ad-LacZ or Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes with either full medium (nutrient) or starving medium (starvation), using antibodies against OGT, O-GlcNAc, LC3, and SQSTM1. GAPDH was detected as a loading control. Right panel, quantification of western blot results in the left panel, $n = 3$ for each group. *Significantly different; M.W.: molecular weight. (b) Left panel, representative fluorescent images of adenovirus RFP-GFP-LC3-infected control (Ad-LacZ treated) and knockout (Ad-Cre treated) $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes. Quantitative analyses of LC3 puncta per cell were shown in the right panel. 50 cells were analyzed for each group. *Significantly different.

the attenuation of autophagy in OGT knockout cardiomyocytes, the cardiomyocytes were transduced with adenovirus expressing tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3). Ad-tf-LC3 allows the detailed monitoring of autophagy flux, because LC3 puncta labeled with GFP and mRFP represent autophagosomes, whereas those labeled with mRFP alone represent autolysosomes [26]. Indeed, under nutrient condition, the numbers of both mRFP-labeled autolysosomes and yellow autophagosomes were decreased in OGT knockout cardiomyocytes (Figure 2(b)). Collectively, our data suggested that O-GlcNAcylation is required for autophagy in mouse cardiomyocytes.

3.3. OGT Regulates the Early Stages of Autophagy in Cardiomyocytes. To further investigate how OGT regulates autophagy in cardiomyocytes, we treated the control and OGT deleted neonatal cardiomyocytes with or without Bafilomycin A (BafA), a commonly used inhibitor of autophagy through preventing autophagosome-lysosome fusion and acidification of lysosome [27]. Clearly, western blot showed that BafA treatment induced an accumulation of LC3-II in control cardiomyocytes but to a much lower extent in OGT deleted cardiomyocytes (Figure 3(a)). Those data indicated that the loss of OGT in cardiomyocytes attenuated the early stages of autophagy flux. A recent report has shown that

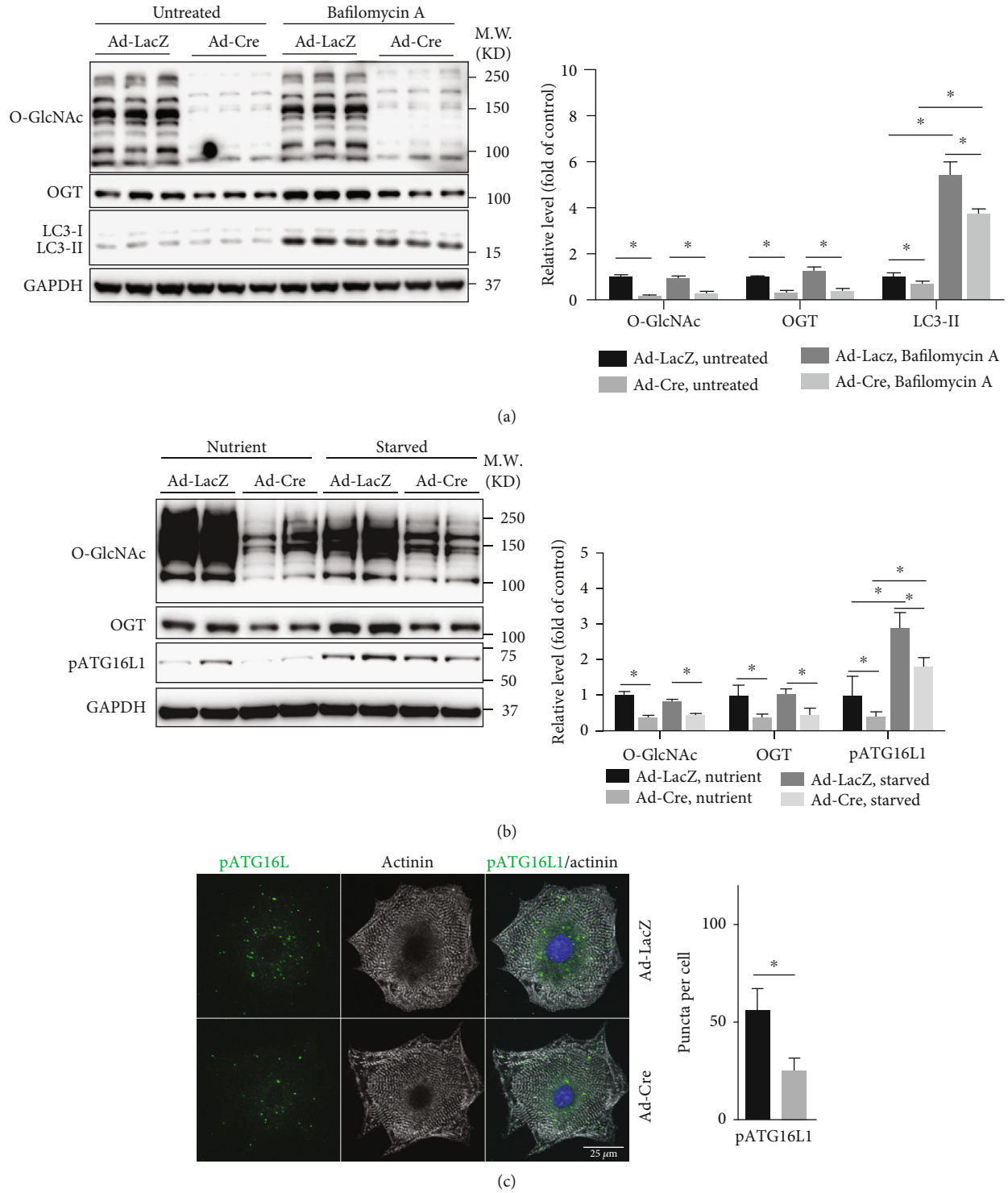

Figure 3: OGT regulates the early stage of autophagy in cardiomyocytes. (a) Left panel, western blot analysis for autophagy of untreated or Bafilomycin A-treated Ad-LacZ or Ad-Cre-infected $Ogt^{f/f}$ neonatal cardiomyocytes, using antibody against O-GlcNAc, OGT, and LC3. GAPDH was detected as loading control. Right panel, quantification of western blot results in left panel, $n = 3$ for each group. *Significantly different; M.W.: molecular weight. (b) Left panel, western blot analysis for autophagy of Ad-LacZ- or Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes with either full medium (nutrient) or starving medium (starved), using antibody against pATG16L. GAPDH was detected as a loading control. Right panel, quantification of western blot results in the left panel, $n = 4$ for each group. *Significantly different; M.W.: molecular weight. (c) Left panel, representative immunomicroscopic images of pATG16L puncta (green) in Ad-LacZ- and Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes, costained with alpha-actinin (gray) and DAPI (blue). Right panel, quantification of pATG16L-positive puncta per cell, $n = 50$ cells each group. *Significantly different.

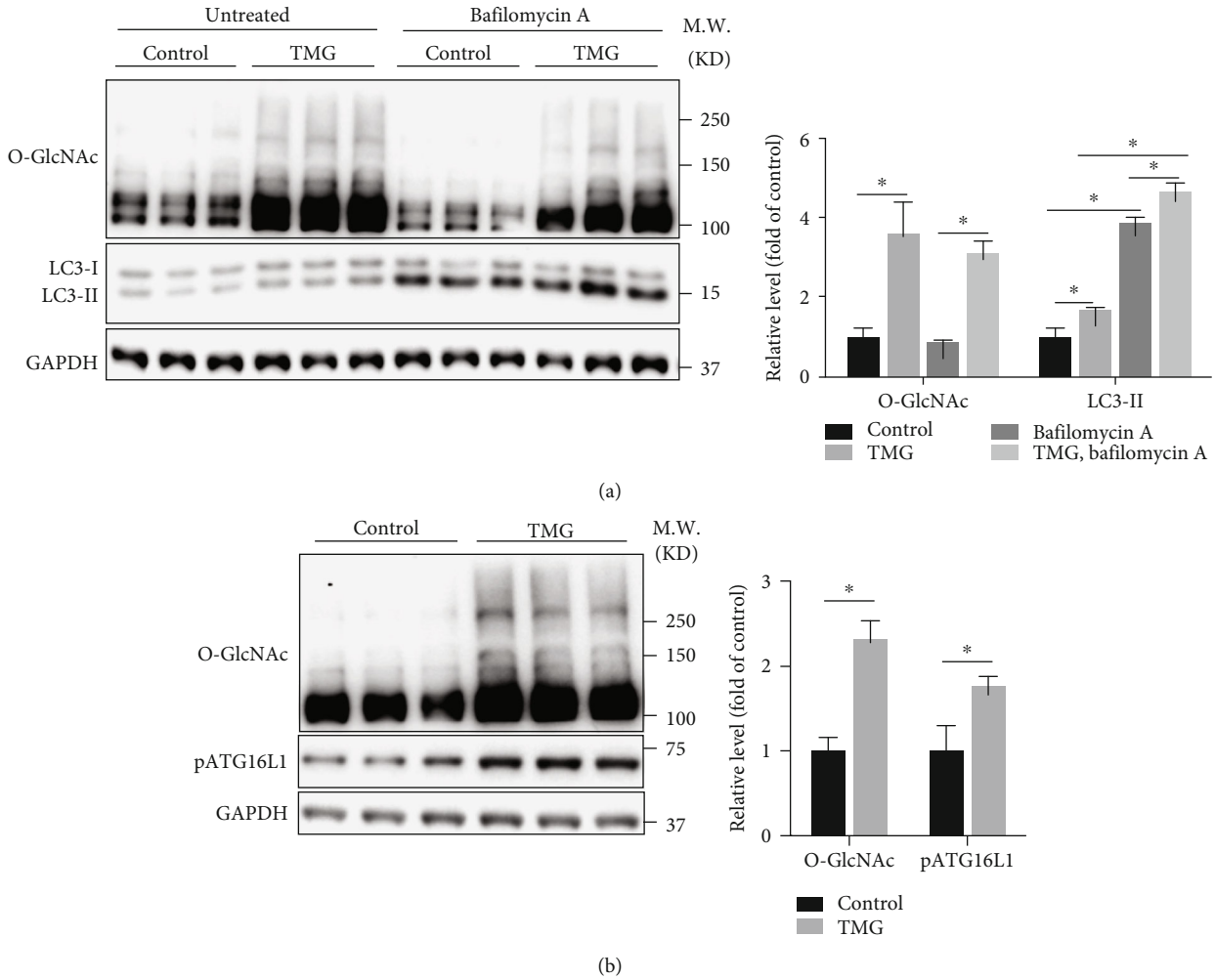

Figure 4: Enhanced O-GlcNAcylation stimulates autophagy in cardiomyocytes. (a) Left panel, western blot analysis for autophagy of wild-type neonatal cardiomyocytes with different treatments as indicated, using antibody against O-GlcNAc and LC3. GAPDH was detected as a loading control. Right panel, quantification of western blot results in the left panel, $n = 3$ for each group. *Significantly different; M.W.: molecular weight. (b) Left panel, western blot analysis of control or TMG-treated wild-type neonatal cardiomyocytes, using antibodies against O-GlcNAc and pATG16L. GAPDH was detected as a loading control. Right panel, quantification of western blot results in the left panel, $n = 3$ for each group. *Significantly different; M.W.: molecular weight.

the level of phosphorylated ATG16L1 (pATG16L1) correlates with the amount of newly formed autophagosome, and it has suggested that the pATG16L1 level could determine the rate of autophagy [28]. Hence, we took advantage of the newly generated antibody that recognizes pATG16L1 to confirm our finding. Consistently, western blot showed that the level of pATG16L1 was decreased in OGT deleted cardiomyocytes, under both nutrient and starved conditions (Figure 3(b)). Immunostaining also showed that the number of pATG16L1-positive puncta in OGT knockout neonatal cardiomyocytes under nutrient condition was decreased (Figure 3(c)). These data suggested that the loss of OGT in cardiomyocytes most likely affects autophagy induction rather than the late stages of autophagy.

3.4. Elevated O-GlcNAcylation in Neonatal Mouse Cardiomyocytes Promotes Autophagy. To check whether elevated O-GlcNAcylation in cardiomyocytes has the opposite effect on autophagy, we treated wild-type neonatal mouse cardiomyocytes with 25 μmol/L TMG, an OGA-specific inhibitor. Western blot showed that TMG treatment indeed significantly increased the level of O-GlcNAcyation in cardiomyocytes (Figure 4(a)). Consequently, the LC3-II level in cardiomyocytes was also increased significantly by TMG treatment, both at the basal level and with BafA (Figure 4(a)). Also, the pATG16L1 level was increased in cardiomyocytes treated with TMG (Figure 4(b)). Collectively, these data indicated that elevated O-GlcNAcylation in cardiomyocytes promotes autophagy.

3.5. ULK1 Is O-GlcNAcylated in Cardiomyocytes. ULK1 is a key factor in controlling the initiation of autophagy [12]. Both *in vivo* and *in vitro* data strongly suggested that ULK1 is required for autophagy in cardiomyocytes [29].

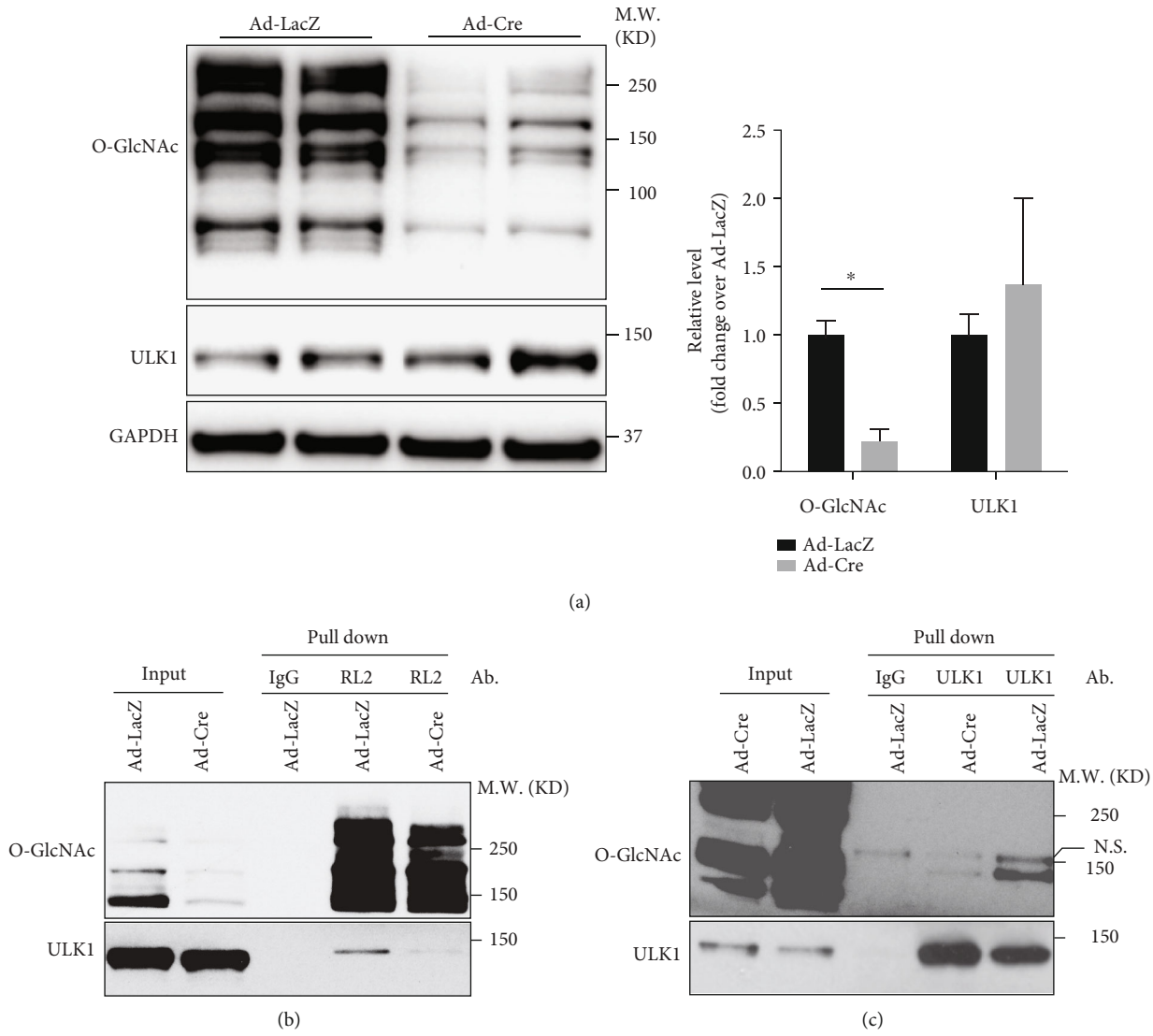

Figure 5: ULK1 is O-GlcNAcylated in cardiomyocytes. (a) Left panel, western blot analysis of Ad-LacZ- and Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes, using antibodies against O-GlcNAc and ULK1. GAPDH was detected as a loading control. Right panel, quantification of western blot results in the left panel, $n = 4$ for each group. *Significantly different; M.W.: molecular weight. (b) Immunoprecipitation of Ad-LacZ- and Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes using antibody against O-GLcNAc (RL2), followed by western blot using antibodies against O-GlcNAc (RL2) and ULK1. Ab.: antibody; M.W.: molecular weight. (c) Immunoprecipitation of Ad-LacZ- and Ad-Cre-infected $Ogt^{f/f}$ and/or $Ogt^{f/y}$ neonatal cardiomyocytes using antibody against ULK1, followed by western blot using antibodies against O-GlcNAc (RL2) and ULK1. Ab.: antibody; M.W.: molecular weight. N.S.: nonspecific band.

In addition, ULK1 is also responsible for the phosphorylation of ATG16L1 [28]. Hence, we checked whether ULK1 was decreased in OGT deleted cardiomyocytes. Surprisingly, ULK1 showed an even higher protein level in OGT knockout cardiomyocytes (Figure 5(a)), excluding the contribution of decreased ULK1 to OGT deletion-induced autophagy attenuation. A recent report has shown that O-GlcNAcylation of ULK1 is required for ULK1-mediated autophagy in liver cells [19], which prompted us to examine whether ULK1 was O-GlcNAcylated in cardiomyocytes. Immunoprecipitation showed that ULK-1 was O-GlcNAcylated in cardiomyocytes. And the level of ULK1 O-GlcNAcylation was dramatically decreased in OGT knockout cardiomyocytes (Figures 5(b) and 5(c)). These data suggested that OGT control the initiation of autophagy in cardiomyocytes through the regulation of ULK1 O-GlcNAcylation.

4. Discussion

Previous studies have showed that cardiomyocyte OGT or O-GlcNAcylation was essential for cardiac function at both the basal level and under stress such as ischemia [6, 7], and the loss of OGT promoted cardiomyocyte's apoptosis [6, 7]. Interestingly, upregulation of autophagy during heart ischemia and reperfusion has also been shown to be cardiac-protective and prevented apoptosis of cardiomyocytes [30–32]. Therefore, we anticipated that the protective effect of O-GlcNAcylation in cardiomyocytes is mediated,

at least partially, via the promoting of autophagy especially under stress. Indeed, our data from both *in vivo* and *in vitro* models clearly demonstrated that the loss of OGT in cardiomyocytes attenuated autophagy.

Interestingly, we found that the loss of O-GlcNAcylation in the heart decreased autophagy only when mice were subjected to starvation, while the loss of O-GlcNAcylation in isolated neonatal cardiomyocytes attenuated autophagy under both nutrient and starved conditions. One possible explanation is that during the process of isolation, the isolated cardiomyocytes might already had gone through stress, which increased the basic requirement of autophagy. Another explanation is that underdeveloped neonatal cardiomyocytes and mature adult cardiomcyotes might behave differently in the extent of autophagy regulations.

Of note, our results were different from the previous studies which showed that elevated O-GlcNAcylation blunted autophagy in the heart and cardiomyocytes [20, 21]. In those studies, STZ was used to elevate O-GlcNAcylation because STZ can induce hyperglycemia by damaging β cells. Consequently, STZ induces altered O-GlcNAcylation globally instead of just in cardiomyocytes, preventing the effects of O-GlcNAcylation in cardiomyocytes on autophagy to be elucidated. Also, STZ treatment in animals has numerous side effects [22], which further compromise the relationship between O-GlcNAcylation and autophagy inside cardiomyocytes. Our results from the OGT cardiomyocyte-specific knockout mouse model demonstrated that O-GlcNAcylation promotes autophagy in cardiomyocyte cell autonomously.

We also showed that O-GlcNAcylation is required for the early stages of autophagy because LC3-II level was decreased even with BafA treatment. Additionally, pATG16L1 level, a novel marker of newly formed autophagosome [28], was also decreased when OGT was knocked out in cardiomyocytes. Consistently, ULK1, a key regulator of autophagy at the early stage, was shown to be O-GlcNAcylated in cardiomyocytes, and O-GlcNAcylation of ULK1 was diminished when OGT was knocked out. In line with our findings, ULK1 cardiomyocyte-specific knockout mice show autophagy defects under stress [29]. Considering that ULK1 is a critical regulator of autophagy initiation and its O-GlcNAcylation is required for the induction of autophagy in other cell types [19, 33], we anticipate that OGT promotes autophagy through regulating ULK1 activity by O-GlcNAcylation in cardiomyocytes. Future studies are needed for confirmation.

In conclusion, using a cardiomyocyte-specific genetic deletion mouse model, for the first time, we demonstrated that O-GlcNAcylation is required for autophagy in cardiomyocytes especially under stress conditions. We also found that O-GlcNAcylation promotes initiation of autophagy probably through the regulation of ULK1 activity in cardiomyocytes. Our findings provide a better understanding of heart dysfunction and should be considered for their potentials in the prevention and treatment of heart failure.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Autophagy in Age-Related Macular Degeneration: A Regulatory Mechanism of Oxidative Stress

Zi-Yuan Zhang, Xiao-Li Bao, Yun-Yi Cong, Bin Fan, and Guang-Yu Li

Department of Ophthalmology, The Second Hospital of Jilin University, Changchun 130000, China

Correspondence should be addressed to Guang-Yu Li; liguangyu@aliyun.com

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Age-related macular degeneration (AMD) is a leading cause of severe visual loss and irreversible blindness in the elderly population worldwide. Retinal pigment epithelial (RPE) cells are the major site of pathological alterations in AMD. They are responsible for the phagocytosis of shed photoreceptor outer segments (POSs) and clearance of cellular waste under physiological conditions. Age-related, cumulative oxidative stimuli contribute to the pathogenesis of AMD. Excessive oxidative stress induces RPE cell degeneration and incomplete digestion of POSs, leading to the continuous accumulation of cellular waste (such as lipofuscin). Autophagy is a major system of degradation of damaged or unnecessary proteins. However, degenerative RPE cells in AMD patients cannot perform autophagy sufficiently to resist oxidative damage. Increasing evidence supports the idea that enhancing the autophagic process can properly alleviate oxidative injury in AMD and protect RPE and photoreceptor cells from degeneration and death, although overactivated autophagy may lead to cell death at early stages of retinal degenerative diseases. The crosstalk among the NFE2L2, PGC-1, p62, AMPK, and PI3K/Akt/mTOR pathways may play a crucial role in improving disturbed autophagy and mitigating the progression of AMD. In this review, we discuss how autophagy prevents oxidative damage in AMD, summarize potential neuroprotective strategies for therapeutic interventions, and provide an overview of these neuroprotective mechanisms.

1. Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness in the elderly population [1] and is becoming a global crisis, with the number of affected people expected to reach 288 million by 2040 worldwide [2]. AMD is classified into two typical forms in the clinic, i.e., dry and wet, both of which can result in visual loss [3]. The wet form, also called exudative or neovascular AMD, is characterized by choroidal neovascularization (CNV) [4] with an abnormally increased expression of vascular endothelial growth factor (VEGF) [5]. The CNVs can leak fluid or blood into the subretinal space (SRS) and lead to sudden vision loss. In contrast, visual loss is usually gradual in the dry form [6]. Yellow subretinal deposits called drusen, or extracellular protein aggregates of retinal pigment epithelial (RPE) cells [7], as well as the accumulation of intracellular lipofuscin [8], can be found under an ophthalmoscope. Larger drusen may become confluent and evolve into drusenoid RPE detachments [9], which often progress to geographic atrophy and less frequently to neovascular AMD. Geographic atrophy is the main pathological feature of dry AMD and can lead to severe visual loss when involving the center of the macula [10].

Many factors determine the risk of developing AMD, including both genetic and environmental factors [11, 12]. Among them, oxidative stress [13–15] and senescence [16] are two major risk factors for AMD, and a growing body of evidence suggests that inflammation also plays an important role in the pathophysiology of AMD [17–19]. Senescence induced by chronic oxidative stress can inhibit cell growth and lead to the release of growth factors, cytokines, chemokines, proteases, and other molecules, inducing inflammation [20]. Additionally, a number of lifestyle factors, including smoking [21], improper dietary intake [22, 23], obesity [24], and lack of exercise [25], are associated with a higher prevalence of AMD. Cigarette smoke can cause accumulation of cadmium (Cd) [26] and further increase the oxidant load in retinal tissues [27]. Dietary zinc deficiency can sensitize

RPE cells to oxidative damage [28]. A high-fat diet with excessive cholesterol may contribute to AMD, as the oxidized form of cholesterol, 7-ketocholesterol, is found at high levels in drusen [29]. The interactions among these factors remain elusive.

RPE cells play a critical role in the pathogenesis of AMD [30]. They are highly specialized pigmented cells located between the neuroretina and the choroid [31]. The physiological functions of RPE cells are essential to maintain the normal health of the retina [32]. These functions include phagocytosis of shed photoreceptor outer segments (POSs) [33], metabolism in the SRS [34], the formation of the outer blood-retinal barrier [35], the exchange of 11-cis retinol and all-trans-retinol during the retinoid cycle [36], and the regulation of ion and metabolite transport [37]. Alterations to retinal metabolism have been reported to be an early feature of AMD [2]. In the pathogenesis of AMD, age-related, cumulative oxidative stress can cause functional abnormalities of RPE cells and induce incomplete digestion of POSs, leading to the continuous accumulation of cellular waste [38]. The major cellular waste is drusen and lipofuscin (a metabolite in lysosomes), containing unfolded and damaged proteins [39] or DNA [40]. Under physiological conditions, these unnecessary proteins are cleared and recycled in RPE cells by two main systems of protein degradation: the ubiquitin-proteasome system (UPS) and autophagy [41]. However, overloaded cellular waste cannot be degraded completely by autophagy or UPS due to the progressive dysfunction of RPE cells in AMD. This will finally lead to cellular degeneration and subsequent death of photoreceptors because RPE cells lose the ability to provide them with oxygen and nutrients and remove waste materials [42].

2. The Role of Oxidative Stress in AMD

Oxidative stress is a major cause of AMD [43]. An imbalance between oxidation and antioxidation is induced when organisms are exposed to biotic and abiotic stress factors such as hypoxia [44]. The main characteristic of oxidative stress is the increased levels of reactive oxygen species (ROS), leading to morphological damage and functional weakness of cellular proteins, lipids, and DNA [45]. ROS include a variety of chemical substances, such as singlet oxygen, superoxide anion radicals, hydroxyl radicals, hydrogen peroxide (H_2O_2), and hydroxyl peroxide radicals [46]. ROS are generated during metabolic processes related to life-sustaining or enzyme-catalyzed reactions [46]. The retina is metabolically very active, maintaining normal physiological function, and thus, it consumes high amounts of oxygen and produces many ROS [47]. These ROS under physiologic conditions are conducive to signal transduction in the retina [48].

RPE cells are responsible for the phagocytosis of POSs as discussed before. POSs contain a lot of unsaturated fatty acids. In the process of POS phagocytosis, nicotinamide adenine dinucleotide phosphate oxidase or peroxidase in the phagocytic bodies will oxidize these fatty acids in POSs and generate large amounts of ROS [49]. Under stress conditions, photoreceptor cells have to metabolize constantly to renew their outer segments, which contribute to a unique source of ROS for RPE cells [50]. Moreover, RPE and photoreceptor cells contain higher levels of mitochondria, which are likely to produce more ROS than other cells [48]. However, unfavorable oxidative stress is triggered when ROS overaccumulate, causing disorders of the cell structure and function, which in turn aggravates ROS production [12]. Photooxidative stress is induced by light and is one of the forms of oxidative stress [51]. Studies have found that photooxidative stress can induce accumulation of deposits in RPE cells and eventually lead to the degeneration of RPE and photoreceptor cells [52]. Additionally, photosensitive molecules (rhodopsin and lipofuscin) interact with light; they are related to oxidative stress induction and the death of photoreceptor cells. Taken together, high oxygen metabolism, continuous light exposure, high concentrations of polyunsaturated fatty acids, and the existence of photosensitizers make the retina prone to be affected by oxidative stress [53].

Aging is related to progressive oxidative stress in the pathology of AMD [54]. With advancing age, the deposition of lipids and proteins in Bruch's membrane and RPE cells has a negative impact on physiological cell functions [55], resulting in reduced cell adhesion, proliferation, and migration and impaired POS phagocytosis. Lipofuscin is a kind of residue from poor lysosomal POS degradation [56]. In AMD, lipofuscin accumulation is induced due to dysfunction of degenerative RPE cells. The lipofuscin promotes oxidative stress by producing free radicals and inhibiting degradation of damaged organelles and proteins [57]. The relationship between lipofuscin and protein degradation systems will be discussed below. The overview of the role of oxidative stress in AMD is presented in Figure 1.

The main methods of inducing oxidative stress in AMD are increasing oxidative stimuli or dysregulating the antioxidant mechanisms [43]. Here, we introduce several common models of establishing AMD mediated by increased oxidative stress. Photooxidative stress models, also called light injury models, are widely used in the investigation of AMD [58]. One of the mechanisms of retinal injury is the interaction between light and photosensitive molecules. The excessive activation of rhodopsin and light conduction can induce photoreceptor cell degeneration [53]. H_2O_2, a component of ROS, is also widely used to stimulate oxidative stress in both in vivo animal models and in vitro RPE cell culture models [59]. Additionally, cigarette smoke, containing powerful chemical oxidants such as hydroquinone (HQ), Cd, and nicotine, can induce oxidative stress and disturb the proteasome pathway in cultured human RPE cells [60]. Furthermore, an autophagy deficiency model is considered to be a potential AMD model. Insufficient autophagy leads to the accumulation of lipofuscin and ROS. Interestingly, ROS and oxidized lipoproteins are also major causes of disturbed autophagy clearance [61]. Liu and colleagues showed that intravitreal injection of wortmannin, an autophagy inhibitor that can irreversibly block phosphatidylinositol 3-kinase (PI3K), transiently suppressed autophagy in C57BL/6J mice within a week, leading to RPE and photoreceptor cell degeneration and death [62].

3. The Role of Autophagy in AMD

Autophagy, which literally means "self-eating," is a lysosome-dependent multistep process that is widely existent

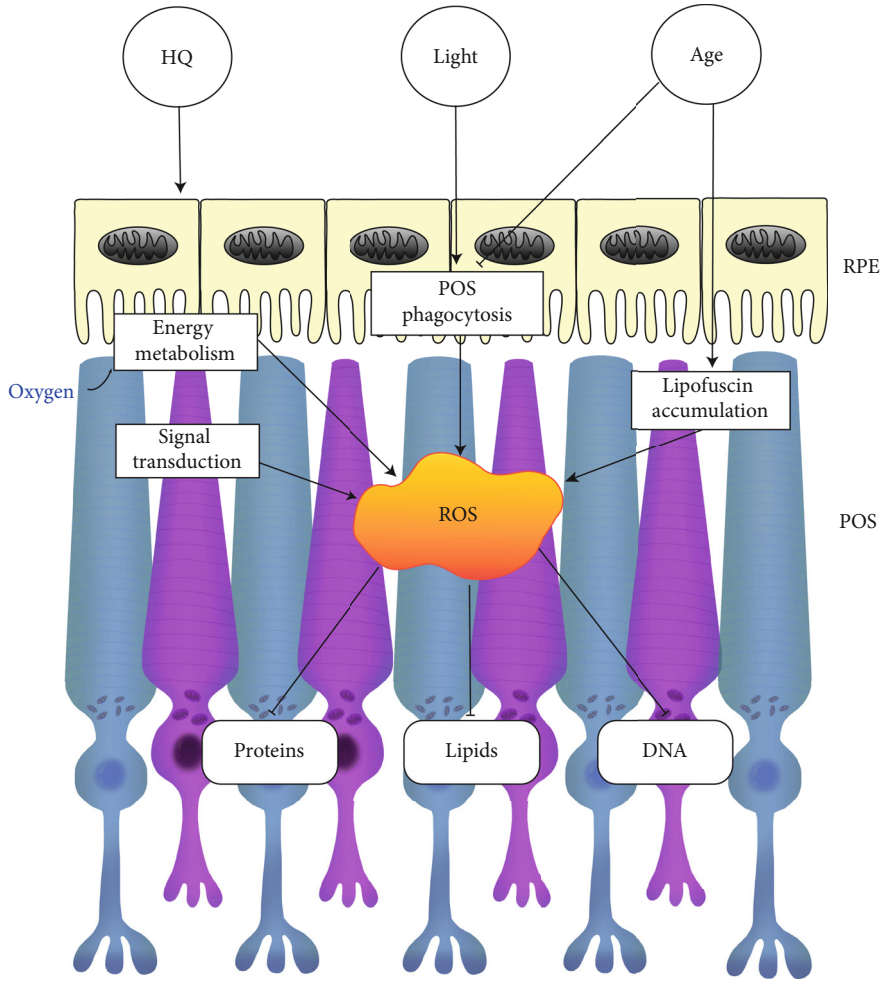

Figure 1: The role of oxidative stress in AMD. Light injury, growing age, and oxidants from cigarette smoke (such as HQ) are the risk factors for AMD. Overactive energy metabolism and excessive signal transduction in RPE and photoreceptor cells produce many ROS. Daily phagocytosis of POSs in RPE cells is also an important source of ROS. RPE cells lose the ability of phagocytizing POSs with increasing age, leading to the accumulation of lipid substances, such as lysosomal deposits (also known as lipofuscin). Taken together, ROS are elevated upon exposure to risk factors, and thus, cellular oxidative stress is triggered, causing injuries to proteins, lipids, and DNA and finally the death of RPE and photoreceptor cells. AMD: age-related macular degeneration; HQ: hydroquinone; RPE: retinal pigment epithelial; POSs: photoreceptor outer segments; ROS: reactive oxygen species.

in eukaryotic cells. It can be divided into macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy, which can be either selective or nonselective, is the most studied and is considered to be the major autophagy pathway [63, 64]. In the process of macroautophagy, damaged organelles and protein aggregates are engulfed in a double-membrane vacuole to form autophagosomes, which are then transported to lysosomes to form autolysosomes for final degradation [65]. Recent studies have found that RPE cells are the major site of pathological alterations in AMD, and autophagy dysfunction in RPE cells plays a key role in the development of AMD [66]. The levels of autophagic flux in RPE cells from AMD donors have been found to be decreased compared with RPE cells from healthy controls [67]. These facts illustrate that autophagy is highly correlated with AMD. In this part, we mainly focus on the role of autophagy in the pathogenesis of AMD.

3.1. Autophagy and Lipofuscin. Lipofuscin, which is composed of covalently crosslinked proteins, lipids, and saccharides, is formed in RPE cells when lipoproteins accumulate due to the disturbed degradation of POSs and extracellular materials [41]. Autophagy is widely considered as a major protein degradation system. With increasing age, lipofuscin accumulates in RPE cells together with its primary spontaneous fluorophore, A2E, and contributes to the pathogenesis of AMD [8, 38]. Once formed, lipofuscin is hard to degrade; it exerts a toxic effect on RPE cells, for example, causing increased DNA damage, inhibiting proteolysis, and reducing cell viability in a time- and concentration-dependent manner [68]. Zhang and colleagues coincubated RPE cells with A2E and found that A2E could induce autophagy in RPE cells at an early stage [69]. It has also been shown that inhibiting autophagy could increase the levels of lipofuscin-like autofluorescence (LLAF), whereas enhancing autophagy by

glucosamine targeting the 5′-adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway could at least partially attenuate LLAF in RPE cells [38, 70]. These findings suggest that elevated levels of autophagy in RPE cells can abate the accumulation of lipofuscin, thereby preventing the adverse effects of A2E in RPE cells and potentially delaying AMD progression.

3.2. The Protective Role of Autophagy in AMD. In addition to age-related oxidative stress, high levels of oxygen consumption, exposure to lipid peroxidation products, and oxidative damage all make RPE cells susceptible to chronic oxidative stress [71]. RPE cells from AMD patients produce more ROS than normal RPE cells and lose the ability to increase superoxide dismutase (SOD) expression when exposed to continuous oxidative stress [72, 73]. Beclin-1 can regulate and induce autophagy [74]. Microtubule-associated protein 1 light chain 3 (LC3) has been considered to be a primary biochemical marker for autophagy activation. The conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II) is indicative of autophagic flux [75]. These are regarded as reliable autophagy markers. Several studies have shown that promoting autophagy through various signaling pathways, such as the PI3K/protein kinase B (Akt)/mTOR pathway [76, 77], the AMPK/mTOR pathway [38], the p62/Kelch-like-ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (NFE2L2) pathway, and the peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) pathway [78, 79], could reduce the occurrence of AMD. The levels of Beclin-1 and the ratio of LC3-II to LC3-I (LC3-II/LC-I) were found to be increased in these studies. Moreover, the NFE2L2 and PGC-1 pathways are also antioxidant pathways, suggesting that autophagy may perform important functions in the regulation of oxidative stress, which will be discussed below.

3.3. The Role of Mitophagy in AMD. Recently, the role of selective autophagy in AMD has been revealed. Double-membrane structures carrying specific cellular components interact with phagophores with the help of selective autophagy receptors and trigger selective autophagy [63]. These components include cytoplasmic aggregates (triggering aggrephagy), lipid droplets (triggering lipophagy), exogenous pathogens (triggering xenophagy), and organelles such as mitochondria (triggering mitophagy) [80]. Mitophagy is essential for maintaining proper cellular functions, since it participates in mitochondrial quality control and clears mitochondria with mutated mitochondrial DNA (mtDNA) [81]. It has been reported that aged RPE cells are more susceptible to oxidative stress, in which the efficacy of mitophagy decreases and mtDNA damage accumulates [82–84]. Hyttinen and colleagues showed that the number of mitochondria in RPE cells from AMD donors was lower than in those from healthy donors, and eight times more mtDNA damage than nuclear DNA damage was observed, indicating that mitophagy has a significant impact on the development of AMD [71]. Interestingly, NAD^+, a critical component that can accelerate the metabolic shift towards glycolysis can also induce mitophagy, restoring homeostasis in RPE cells in AMD patients, and may therefore serve in novel AMD treatment strategies [2].

3.4. The Dual Role of Autophagy in AMD. Improving autophagy can mitigate the degeneration of RPE cells; however, an increasing number of studies have illustrated that excessive autophagy may also lead to retinal cell death [85], particularly overactivated autophagy at early stages of retinal diseases [86]. Zhang and colleagues showed that blocking autophagy directly or inhibiting autophagy by suppressing the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway could protect photoreceptor cells against light-induced damage [87]. Li and colleagues found similar results. They investigated the protective role of epigallocatechin-3-gallate (EGCG), a polyphenolic compound from green tea that protects against ultraviolet light-induced oxidative stress [88]. Surprisingly, although EGCG lowered ultraviolet light damage in an autophagy-dependent manner, it decreased the levels of LC3-II and the formation of autophagosomes instead of increasing them. This notable finding reminds us that autophagy may play a dual role in the protection against retinal degenerative diseases. Appropriate enhancement of autophagy can be beneficial, but excessive autophagy can be harmful and inhibit protective effects.

4. Autophagy Can Regulate Oxidative Stress in AMD

Recently, autophagy has been observed as a crucial regulatory mechanism of oxidative stress in AMD. As mentioned above, autophagy is one of the two major protein degradation systems and is essential to maintain homeostasis in RPE and photoreceptor cells. In AMD, the endocytic/phagosome and autophagy pathways are disturbed in degenerative RPE cells due to impaired cargo handling and processing. Keeling and colleagues proposed that this may contribute to increased proteolytic and oxidative stress, which results in irreversible injury to postmitotic RPE cells [89]. Moreover, autophagy has been found to be enhanced in response to oxidative stress, in order to remove oxidatively damaged proteins and organelles, since RPE cells are exposed to constant oxidative stress during the development of AMD [90].

Mitter and colleagues studied the role of autophagy under oxidative stress by culturing human ARPE-19 cells and exposing them to H_2O_2. They established models of both acute and chronic AMD, exposing cells to H_2O_2 for 6 hours and 14 days, respectively. Interestingly, they found that there was a dynamic alteration of autophagic flux in RPE cells exposed to oxidative stress: acute oxidative stress stimulated autophagic activity, whereas chronic oxidative stress resulted in a reduction of autophagic activity. Inhibition of autophagy by 3-methyladenine or by knockdown of *ATG7* or *BECN1* could increase lipofuscin accumulation and ROS generation. Lipofuscin is believed to inhibit autophagy by blocking the function of lysosomal enzymes and causing excess permeabilization of lysosomal membranes, which can lead to the release of lysosomal content and the subsequent production of more toxic radicals [91]. In contrast, oxidative stress-induced ROS production decreased after treatment with

rapamycin to upregulate autophagy. Their findings demonstrate that autophagy is crucial to the resistance to oxidative stress in RPE cells, and defective autophagy is likely to exacerbate oxidative stress in AMD [92].

Chen and colleagues further confirmed the regulatory role of autophagy in photooxidative stress by using an *Abca4*$^{-/-}$ *Rdh8*$^{-/-}$ mouse model. Photoisomerization of the visual chromophore 11-cis retinol and all-trans-retinol is an essential step of retinal photoelectric conversion to maintain normal vision. However, excessive production of all-trans-retinol may cause retinal cell death. *Abca4*$^{-/-}$ *Rdh8*$^{-/-}$ mice are deficient in ATP binding cassette transporter 4 (ABCA4) and retinol dehydrogenase 8 (RDH8). These are crucial enzymes for all-trans-retinol clearance from photoreceptors. Thus, *Abca4*$^{-/-}$ *Rdh8*$^{-/-}$ mice can develop light-dependent retinal degeneration due to delayed clearance of all-trans-retinol. The team showed that the protein levels of the autophagosome marker LC3-II and the mitophagy regulator Park2 were increased in *Abca4*$^{-/-}$ *Rdh8*$^{-/-}$ mice upon light exposure. They also employed a Beclin-1-deficient mouse model and a rod photoreceptor-specific Atg7-deficient mouse model to inhibit autophagy, and they used *Park2*$^{-/-}$ mice to block mitophagy. These mice all exhibited severe retinal degeneration due to inadequate autophagy or mitophagy. Taken together, both autophagy and mitophagy perform critical functions in regulating photooxidative stress [93].

In summary, accumulating researches support that enhancing autophagic activity can alleviate oxidative stress in AMD and protect RPE and photoreceptor cells from progressive degenerations. However, it is still unknown whether autophagy plays a dual role in regulating oxidative stress. Below, we will illustrate some underlying mechanisms of autophagy regulating oxidative stress.

5. The Mechanisms of Autophagy Regulating Oxidative Stress in AMD

Oxidative stress and autophagy can be therapeutic targets for AMD treatment. Recent studies have investigated the elusive link between autophagy and oxidative stress. Results indicate that autophagy plays an important role in alleviating oxidative stress and reducing retinal cell death. However, the specific mechanisms and signal pathways by which autophagy regulates oxidative stress remain to be studied. We have found that several mechanisms and pathways enhance autophagic activity to protect RPE and photoreceptor cells from oxidative stress. Here, we discuss the interactions among these pathways to explore how autophagy regulates oxidative stress in AMD. The overview of the interactions among these pathways is presented in Figure 2.

5.1. Interactions among NFE2L2, p62/SQSTM1, and mTOR Pathways. NFE2L2 signaling has been found to be a critical pathway that mediates autophagy and oxidative stress [66]. NFE2L2, also known as Nrf2, is a transcription factor that has protective effects against ROS-induced retinal cell death. NFE2L2 can bind to antioxidant response elements (AREs), activate the expression of nuclear and metabolic genes, and regulate DNA replication, transcription, mitochondrial function, and cell growth [94], protecting cells from oxidative damage. Inactive NFE2L2 can bind to the cytoskeletal protein Keap1 and then stays in the cytosol [95]. Upon oxidative stress, cytosolic NFE2L2 is phosphorylated and translocated to the nucleus in response to protein kinase C activation and MAPK pathways. In the nucleus, NFE2L2 activates proteasomal subunits and the expression of autophagy-related genes through AREs [96] by interacting with transcription factors in the bZip family, including CREB, ATF4, and FOS or JUN. Gene activation through NFE2L2 can be blocked by small Maf proteins, such as MafG and MafK, to balance NFE2L2 action and regulate the intracellular oxidation levels [97].

The scaffolding adaptor protein p62, also known as sequestosome 1 (SQSTM1), can be selectively cleared by autophagy [98]. Phosphorylated p62/SQSTM1 can bind to LC3 or ubiquitin, promoting the degradation of unnecessary protein aggregates and malfunctioning mitochondria by autophagy [99]. Thus, the amount of p62/SQSTM1 is inversely proportional to the autophagic flux. In addition to autophagy regulation, p62/SQSTM1 can also stabilize NFE2L2 and activate the expression of ARE genes by binding with Keap1 to block the Keap1 NFE2L2 interaction. Moreover, p62/SQSTM1 may regulate NFE2L2 in a positive feedback manner, as suggested by the fact that p62/SQSTM1 activates *NFE2L* expression by promoting autophagic degradation of Keap1 and NFE2L2 positively regulates *p62/SQSTM1* expression [100]. Taken together, these findings imply that the NFE2L2 pathway may be activated in response to oxidative stress via the autophagy-related p62/SQSTM1 pathway.

More importantly, an interaction between the p62 and mTOR pathways has been reported. In recent years, mTOR signaling has been proven to play a significant role in cell growth and metabolism [101], and it is regarded as a classical pathway to regulate autophagy [102]. mTORC1, one of the two major protein complexes of mTOR, can inhibit autophagy [102]. Raptor, which binds to mTOR in the mTORC1 complex through multiple binding regions [103], is a scaffold that binds and presents substrates to mTORC1 [104]. Deletion or knockdown of *raptor* can abolish mTORC1 activity. Interestingly, upregulation of p62 can activate mTORC1 by directly acting on raptor, a regulatory protein of mTOR, thereby suppressing autophagy [105].

Saito and colleagues showed that the NFE2L2 activator RS9 can accelerate autophagy and protect ARPE-19 cells against $NaIO_3$-induced oxidative damage. ARPE-19 cells exposed to $NaIO_3$ exhibited an increased LC3-II/LC-I. Notably, levels of the autophagy substrate p62/SQSTM1 were transiently elevated in the $NaIO_3$ treatment group at 6 h after treatment, and the levels of LC3-I were upregulated at 24 h after $NaIO_3$ treatment, implying that RS9 accelerated autophagy via transient induction of SQSTM1 expression. They also employed an intense light injury model in zebrafish to mimic in vivo AMD, and the results were in line with the in vitro experiments using ARPE-19 cells [106]. This study supports the idea that the NFE2L2 pathway plays an important role in regulating autophagy and hence preventing oxidative stress in AMD.

5.2. Interaction between the PGC-1 and NFE2L2 Pathways. The PGC-1 pathway, consisting of PGC-1α, PGC-1β, and

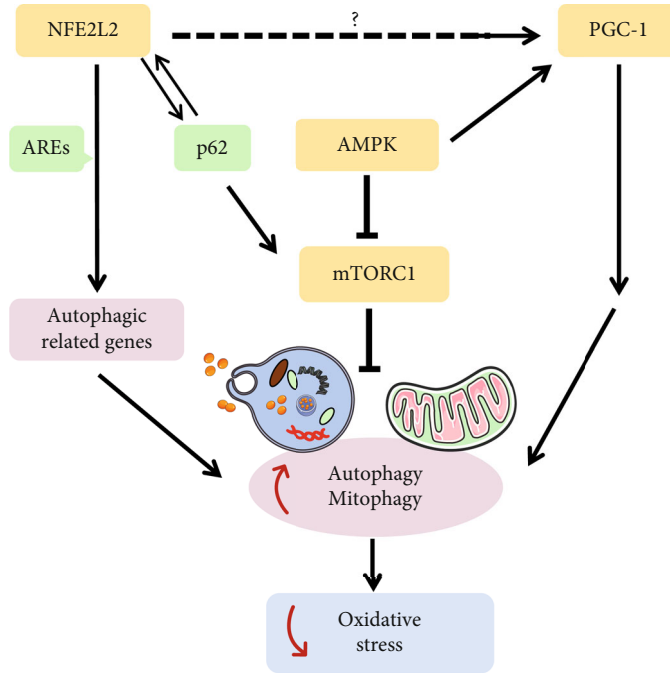

Figure 2: The interactions among the pathways involved in reducing oxidative stress by enhancing autophagy. NFE2L2 seems to be a positive regulator of PGC-1, but the specific functional mechanisms remain to be studied. NFE2L2: nuclear factor erythroid 2-related factor 2; AREs: antioxidant response elements; PGC-1: peroxisome proliferator-activated receptor gamma coactivator 1; AMPK: 5′-adenosine monophosphate-activated protein kinase; mTORC1: mammalian target of rapamycin complex 1.

PGC-1-related coactivator, serves as an antioxidant defense system targeting mitochondrial biogenesis and oxidative metabolism. AMPK and the NAD^+-dependent deacetylase SIRT1 can activate PGC-1α, enhancing autophagy and mitophagy [20]. Increasing evidence supports the notion that suppression of PGC-1α activity contributes to the development of AMD, because loss of PGC-1α induces ROS generation and mitochondrial damage. In contrast, elevated expression of PGC-1α promotes the mitochondrial antioxidant defense by increasing the expression of antioxidant genes, such as *SOD2* and *thioredoxin 1* (*TRX1*) [107]. Thioredoxin-interacting protein can inhibit TRX activity and increase oxidative stress and destructive inflammation [108].

Zhang and colleagues generated a PGC-1$\alpha^{+/-}$ mouse model to study the pathogenesis of AMD. PGC-1$\alpha^{+/-}$ mice expressed lower levels of PGC-1α and were fed a high-fat diet for 4 months. The mice displayed drusen and lipofuscin accumulation, elevated ROS levels, decreased autophagy flux, and increased inflammation, along with obvious RPE and photoreceptor cell degeneration [109]. This research demonstrated that the presence of PGC-1α is necessary for the regulation of autophagy to prevent oxidative damage.

Felszeghy and colleagues further explored the autophagy-regulated function of both NFE2L2 and PGC-1α pathways in the development of dry AMD. They established and characterized a *NFE2L2*/*PGC-1α* double KO (dKO) mouse model to investigate the role of autophagy clearance in regulating the antioxidant response. *NFE2L2*/*PGC-1α* dKO mice developed severe AMD with accumulation of oxidative stress markers and damaged mitochondria. The levels of oxidative stress markers were higher than those in *NFE2L2* KO mice and *PGC-1α* KO mice, implying that *NFE2L2*/*PGC-1α* dKO mice exhibited the highest degree of oxidative stress. The levels of the autophagy marker p62/SQSTM1, as well as the protein aggregate-conjugated marker ubiquitin, were increased, suggesting that the UPS and autophagy clearance were impaired in *NFE2L2*/*PGC-1α* dKO mice. In line with the observed p62/SQSTM1 accumulation, RPE cells from dKO mice exhibited larger autolysosomes and a higher ratio of damaged mitochondria than RPE cells from WT mice, as indicated by transmission electron microscopy [40]. The study not only highlighted the significant role of intracellular degradation systems, including autophagy and the UPS, in reducing oxidative stress, but also revealed a potential cross-talk between the NFE2L2 and PGC-1α pathways. PGC-1α deficiency induces the generation of mitochondrial ROS, while the loss of NFE2L2 leads to impairment of the autophagic degradation system and the accumulation of damaged mitochondria.

5.3. Interaction between Autophagy and Inflammation. Autophagy may also interact with inflammation to regulate oxidative stress [110]. It is widely accepted that inflammation plays a role in the pathogenesis of AMD. Szatmari and colleagues established an in vitro AMD model by exposing human embryonic stem cell-derived RPE (hESC-RPE) cells to H_2O_2. Upon oxidative stress, hESC-RPE cells underwent autophagy-associated cell death. They showed that mature macrophages took up these dying cells and triggered a release of numerous proinflammatory cytokines, including

interleukin- (IL-) 6, IL-8, and tumor necrosis factor-α, resulting in the activation of inflammatory processes. This study demonstrated that autophagy regulation may be a treatment goal to adjust inflammation and protect RPE cells from oxidative damage [14].

In conclusion, the NFE2L2 and PGC-1α pathways play a key role in enhancing autophagy to prevent oxidative injury. NFE2L2 upregulates autophagy by binding to AREs. AMPK activates PGC-1 and thereby promotes autophagy and mitophagy. Additionally, NFE2L2 seems to be a positive regulator of PGC-1, but the specific functional mechanisms remain unclear. Clarifying the interactions among the NFE2L2, PGC-1, AMPK, and mTOR pathways is significant to improve our understanding of the regulatory mechanisms in autophagy that alleviate oxidative stress and mitigate the development of AMD.

6. Potential Neuroprotective Strategies Targeting Autophagy to Alleviate Oxidative Stress in AMD

Wet, or neovascular, AMD is considered to be associated with progressive CNVs and the upregulation of VEGF [111]. Improvements in our understanding of wet AMD pathogenesis could identify and characterize therapeutic targets; for example, anti-VEGF drugs target CNV development. Unfortunately, this is the only effective AMD treatment at present, which means little advances have been made in therapies for dry AMD [17, 112]. As argued above, enhanced autophagy can mitigate oxidative stress in the pathogenesis of AMD, suggesting that stimulating autophagy may be a promising strategy for AMD therapy. Here, we summarize some neuroprotective strategies targeting autophagy to prevent oxidative damage in AMD. The overview of these neuroprotective strategies is presented in Figure 3.

6.1. Inhibitors for mTOR. Compelling evidence has shown that mTOR is a negative regulator of autophagy. AMPK, PI3K, and Akt perform physiological functions upstream of mTOR. The AMPK pathway inhibits mTOR activation [113], while the PI3K/Akt pathway stimulates the activation of mTOR [114]. Rapamycin is a well-known inhibitor of mTOR that has been found to increase the number of autophagic vacuoles and improve RPE and photoreceptor cell survival upon photooxidative stress [115]. Tang and colleagues also found that low doses of proteasome inhibitors, such as clasto-lactacystin-beta-lactone and epoxomicin, could increase the levels of LC3-II/LC-I and decrease the phosphorylation levels of PI3K, Akt, and mTOR in ARPE-19 cells exposed to menadione or 4-hydroxynonenal, suggesting that proteasome inhibitors can activate autophagy through blocking PI3K/Akt/mTOR signaling and prevent oxidative damage [116].

6.2. MicroRNAs. MicroRNAs are small endogenous RNAs that regulate the expression of genes posterior to transcription [117]. MicroRNAs have become novel therapeutic targets for various diseases due to their significant functions in response to outside influences and internal feedback [118]. Cai and Zhang discovered that overexpression of *microRNA-29* (*miR-29*) in RPE cells could rescue degenerative cells by enhancing autophagy through the inhibition of mTORC1 activity. They showed that the levels of p62 declined and LC3-II and autophagy flux increased after transfecting *miR-29* mimics into ARPE-19 cells. Moreover, protein aggregation was also repressed by knockdown of *LAMTOR1*/*p18*, a *miR-29* target located in the lysosome membrane [119]. Zhang and colleagues examined whether *miR-204* plays a role in regulating autophagy in RPE cells and found that knockdown of *miR-204* in both C57BL/6N mice and human RPE cells led to abnormal POS clearance and altered expression of autophagy-related proteins, indicating that high levels of *miR-204* could protect RPE cells from oxidative stress by facilitating autophagy [25]. In summary, microRNAs have proven to be effective in treating AMD, though the therapeutic mechanisms remain to be explored.

6.3. Hormones such as 17β-Estradiol and Melatonin. Wei and colleagues found that 17β-estradiol (βE-2) could enhance autophagy and protect RPE cells from blue light-emitting diode- (LED-) induced oxidative stress. After LED exposure, female ovariectomized rats, which were intravitreally injected with βE-2 in advance, exhibited decreased ROS levels, increased number of autophagosomes, and upregulation of p-Akt, Beclin-1, and LC3-II/LC3-I, implying that the protective mechanism of βE-2 is correlated with autophagy [120]. Melatonin (N-acetyl-5-methoxytryptamine) is a tryptophan-derived neurohormone that plays crucial physiological effects in many systems, for instance, the circadian rhythm, the immune system, the cardiovascular system, and the aging process [121]. Melatonin is a strong antioxidant that scavenges ROS and improves the synthesis of antioxidant enzymes [122]. It has been shown that melatonin also upregulates autophagy, protecting human RPE cells against H_2O_2-induced oxidative damage. Upregulation of LC3-II and Beclin-1 and downregulation of p62 have been observed after treating H_2O_2-exposed RPE cells with melatonin [123]. Moreover, phagocytosis of POS in higher vertebrates is synchronized with the circadian rhythms and usually occurs after dawn, suggesting that melatonin has the potential to modulate POS phagocytosis. As mentioned above, RPE cells have the ability to balance POS phagocytosis and cellular waste clearance, and increasing age can lead to dysfunction of POS phagocytosis in RPE cells. Interestingly, senescence has been reported to be associated with changes in the circadian rhythmicity of melatonin production [124]. Lysosomes, which are among the key organelles involved in autophagy, have also been found to act in a circadian rhythm-controlled manner [125]. These facts illustrate that melatonin can exert antioxidative effects by regulating autophagy.

6.4. Antioxidants in Diet. Several studies have revealed that some food compositions can prevent oxidative injury in AMD through regulating autophagy. Intake of dietary fish and nuts can provide marine n-3 polyunsaturated fatty acids (PUFAs) for humans [126]. Johansson and colleagues showed that physiological doses of n-3 PUFA docosahexaenoic acid (DHA), a type of PUFA, could reduce misfolded proteins

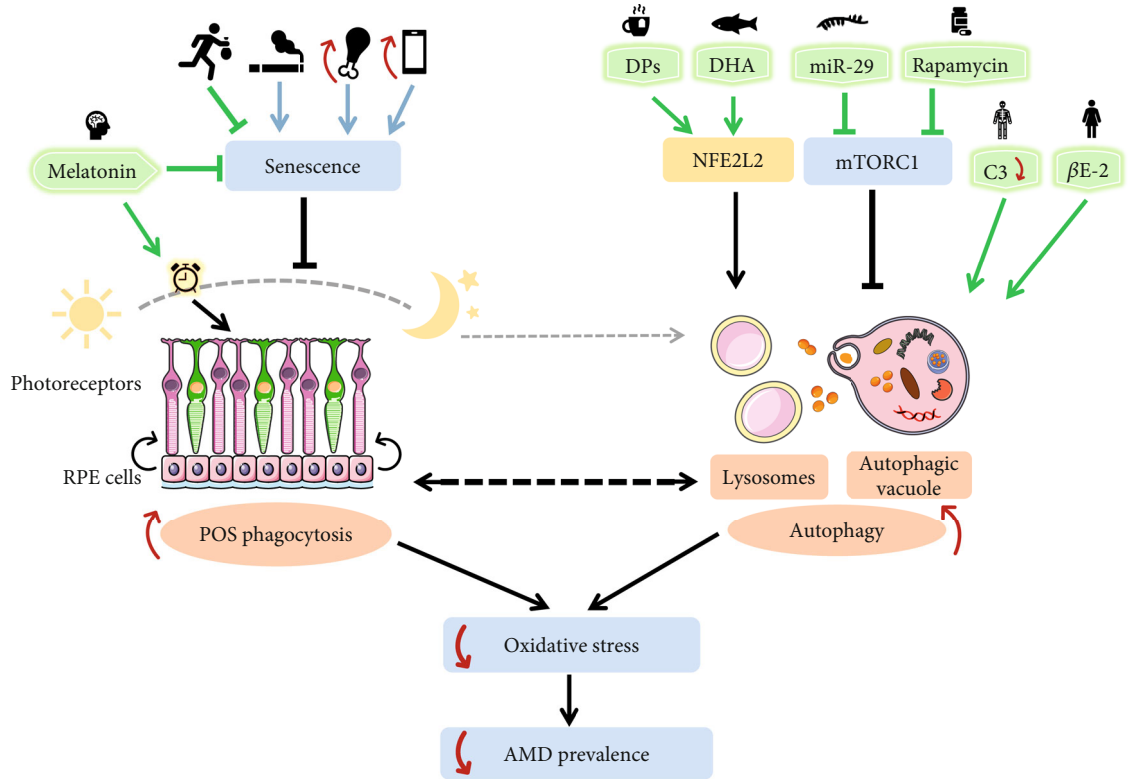

Figure 3: Potential neuroprotective strategies targeting autophagy to prevent oxidative damage in AMD. AMD: age-related macular degeneration; RPE: retinal pigment epithelial; POS: photoreceptor outer segments; NFE2L2: nuclear factor erythroid 2-related factor 2; mTORC1: mammalian target of rapamycin complex 1; DPs: dietary polyphenols; DHA: n-3 PUFA docosahexaenoic acid (PUFA: polyunsaturated fatty acid); miR-29: microRNA-29; C3: the third complement component; βE-2: 17 β-estradiol.

and inhibit oxidative stress by enhancing autophagy through activating the NFE2L2 pathway [8]. Dietary polyphenols (DPs), which are rich in fruits, vegetables, legumes, and plant-derived beverages such as tea [127], were also found to promote autophagy by reducing impairment of the cellular waste clearance and ameliorate oxidative damage through activating the NFE2L2 pathway, thereby preventing the development of AMD [128].

6.5. Complement Depletion. The complement system is widely believed to be responsible for regulating the immune system and inflammation [129]. Complement depletion has been found to improve autophagic activity, reduce cellular oxidative stress, and mitigate age-related retinal degeneration. McHarg and colleagues investigated the role of the third complement component (C3) in AMD and found that *C3* transcription is upregulated in aged retinas [130]. They also evaluated the thickness of retinas in C3-deficient mice through spectral domain optical coherence tomography and showed that the retinas of C3-deficient mice aged 12 months were thinner than those of WT mice aged 3 months, implying that complement activation plays a role in the natural process of retinal aging. Additionally, LC3-II/LC-I in C3-deficient mice was higher than that in WT mice [131]. These findings indicated that C3 is associated with autophagy regulation and may be a promising therapeutic target for AMD.

7. Conclusions

Many risk factors contribute to the development of AMD, including light injury, growing age, and cigarette smoke (as shown in Figure 1). They can aggravate ROS production and thus trigger excessive cellular oxidative stress, causing disorders of the cell structure and function. Enhanced autophagy can alleviate oxidative damage in AMD and protect RPE and photoreceptor cells from degeneration and death. Remarkably, overactivated autophagy may also lead to cell death at the early stages of retinal degenerative diseases. Thus, defining the precise dynamic role of autophagy in the pathogenesis of AMD is essential to choose optimal time points for neuroprotection. As illustrated in Figure 2, the crosstalk among the NFE2L2, PGC-1, p62, AMPK, and PI3K/Akt/mTOR pathways may play a crucial role in enhancing autophagy to prevent oxidative injury. Recently, some novel neuroprotective strategies (as shown in Figure 3) targeting these signaling pathways to activate autophagy and improve RPE and photoreceptor cell survival have been described. However, further studies are still needed to elucidate the precise interaction among these pathways in order to provide more therapeutic interventions,

considering that currently there are no effective treatments for dry AMD.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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