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STATE-OF-THE-ART REVIEW

Autophagy at the interface of endothelial cell homeostasis and vascular disease

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Autophagy is an essential intracellular process for cellular quality control. It enables cell homeostasis through the selective degradation of harmful protein aggregates and damaged organelles. Autophagy is essential for recycling nutrients, generating energy to maintain cell viability in most tissues and during adverse conditions such as hypoxia/ischaemia. The progressive understanding of the mechanisms modulating autophagy in the vasculature has recently led numerous studies to link intact autophagic responses with endothelial cell (EC) homeostasis and function. Preserved autophagic flux within the ECs has an essential role in maintaining their physiological characteristics, whereas defective autophagy can promote endothelial pro-inflammatory and atherogenic phenotype. However, we still lack a good knowledge of the complete molecular repertoire controlling various aspects of endothelial autophagy and how this is associated with vascular diseases. Here, we provide an overview of the current state of the art of autophagy in ECs. We review the discoveries that have so far defined autophagy as an essential mechanism in vascular biology and analyse how autophagy influences ECs behaviour in vascular disease. Finally, we emphasise opportunities for compounds to regulate autophagy in ECs and discuss the challenges of exploiting them to resolve vascular disease.

Introduction

Autophagy is the primary intracellular degradation system of cytoplasmic materials through the lysosomes. However, the primary role of autophagy is not only to eliminate harmful materials, but instead, it serves as a recycling system that produces energy for cellular activation or homeostasis [1]. Three primary forms of autophagy have been defined so far. Macroautophagy, also named basal autophagy, begins with the formation of the phagophore [2]. This specialised membrane engulfs the cytoplasmic material that needs to be eliminated, giving origin to double-membrane sequestering compartments termed the

Abbreviations
AD, aortic dissection; AMPK, AMP-activated protein kinase; ATG, autophagy-related genes; BBB, blood–brain barrier; BECN1, beclin 1; Cav-1, caveolin 1; CCM, cerebral cavernous malformations disease; DCN, decorin; ECs, endothelial cells; EndMT, endothelial-to-mesenchymal transition; eNOS, endothelial nitric oxide synthase; HMGB1, high-mobility group box; I/R, ischaemia/reperfusion; LDL, low-density lipoprotein; $Ldlr^{-/-}$, low-density lipoprotein receptor deficient; miRNA, microRNA; mTORC1, mTOR complex 1; mTORC1, multimeric protein complex mammalian target of rapamycin complex 1; NO, nitric oxide; OGD/R, oxygen-glucose deprivation/reoxygenation; p62, SQSTM1/p62; PCM1, pericentriolar material 1; PIP3P, phosphatidylinositol 3-phosphate; PKA, protein kinase A; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; SIRT1, sirtuin 1; TCHP, trichoplein; TFEB, transcription factor EB; TSC1/TSC2, tuberous sclerosis complex 1-2; ULK1, UNC51-like Ser/Thr kinase; VEGF, vascular endothelial growth factor; VPS, vacuolar protein sorting; VSMCs, vascular smooth muscle cells; VWF, von Willebrand factor.

autophagosomes. The last steps consist in the fusion between autophagosome and lysosome and the lysosomal degradation of the sequestered material [3]. Unlike macroautophagy, microautophagy is mediated by the direct engulfment of cytoplasmic material via invagination of the lysosomal membrane [4]. Microautophagy plays a role in maintaining organelles sizes and cell survival in case of nitrogen starvation and membrane homeostasis. Finally, chaperon-mediated autophagy exploits chaperone proteins to transport the cytosolic components across the lysosomal membrane [5].

Recently, novel research exploring the role of autophagy in the cardiovascular system emerged [6], showing autophagy as a dynamic process to support endothelial cells (ECs) in response to environmental changes and control their function. Under physiological conditions, in ECs, basal autophagy acts as a primary cytoplasmic quality regulation mechanism by recycling macromolecules and degrading potentially toxic reactive oxygen species (ROS)-producing organelles or protein aggregates, thus maintaining EC homeostasis. Nevertheless, in vascular disease, autophagy can be considered a cytoprotective/stress-adaptation mechanism, where an increased autophagic flux may be an attempt to activate ECs to repair a vascular injury or restore ECs homeostasis to prevent disease progression. Under prolonged stress conditions, such as nutrient deprivation, hypoxia/ischaemia or oxidative stress, deregulated autophagy, on the other hand, may be detrimental to EC functions and may contribute to autophagic cell death.

Here, we will first review current knowledge on the role of autophagy in ECs biology. Then, we will explore recent studies revealing a role for deregulated endothelial autophagy in vascular senescence and vascular disease. Finally, we will discuss the therapeutic approaches targeting defective autophagy to restore endothelial function in vascular disease.

Molecular mechanisms of the autophagic process

Macroautophagy consists of multiple steps, including initiation, elongation and maturation of autophagosomes, which are controlled by autophagy-related genes (ATG) [6] and regulated by one of the major anabolic pathway, the multimeric protein mammalian target of rapamycin complex 1 (mTORC1) [7]. mTORC1 complex includes the serine/threonine (Ser/Thr) kinase mTOR, the regulatory protein RAPTOR, the assembly factor mLST8 and two inhibitory subunits: PRAS40 and DEPTOR. It finely integrates growth factors and metabolic availability cues, like glucose and amino acids, to inhibit autophagy and promote cell growth and proliferation [8].

Critically mTORC1 controls the early commitment stage of the autophagosome formation through the phosphorylation of the protein complex formed by UNC51-like Ser/Thr kinase (ULK1), ATG13, FAK family kinase-interacting protein of 200 kDa and ATG101 [9]. When the concentration of growth factor or amino acids fall under a certain threshold, the restraint operated by mTORC1 on ULK1 complex is lifted, and ULK1 subunit undergoes to autophosphorylation at Thr-180 in the kinase activation loop [10]. ULK1 autophosphorylation results in increased kinase activity, enabling the phosphorylation of keys targets like RAPTOR at Ser-855, Ser-859 and Ser-792 and ATG13 at Ser-318, among others, to inhibit mTORC1 signalling and strengthen the activation of ULK1 complex, respectively [11]. ULK1 complex also integrates AMP-activated protein kinase signals (AMPK), a metabolically sensitive kinase activated by increased metabolic demand or decreased energy capacity during stress or glucose or amino acid starvation. AMPK-dependent phosphorylation of tuberous sclerosis complex (TSC1/TSC2) and RAPTOR repress mTORC1 activity [12].

The production of phosphatidylinositol 3-phosphate (PIP3P) by phosphatidylinositol 3-kinase class III in complex with vacuolar protein sorting (VPS) 34, VPS15, Beclin1 (BECN1) and ATG14L is essential for autophagosome formation [13]. The activation and targeting of the complex to the growing autophagosome are driven by ULK1-dependent phosphorylation of ATG14L at Ser-29. BECN1 Ser-15 phosphorylation by ULK1 further enhances VPS34 activity while the phosphorylation of AMBRA1 is thought to expand the pool of available PIK3C3, releasing it from the cytoskeleton [14]. During the autophagosome formation, WD-repeat protein interacting with phosphoinositide (WIPI) functions as autophagy-specific PIP3P-binding effectors at the nascent autophagosome [15].

The phagophore formation relies on two sequential ubiquitin-like conjugation systems. First, the ubiquitin-like protein ATG12 binds to ATG5 by reacting with ATG7 and ATG10, which act as E1 and E3 like enzymes, respectively, resulting in the formation of the ATG12-ATG5-ATG16L1 complex [16]. Then, ATG7, via the E2 enzyme ATG3, catalyses the conjugation of the ATG8 proteins to phosphatidylethanolamine (PE) on the phagophore membranes [17].

While in yeast there is just one ATG8 protein, in mammals there are multiple ATG8 orthologues: LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and

GABARAPL2 [17]. The ATG8 proteins are synthesised as a precursor and the C-terminal glycine residue is cleaved by ATG4 to produce the mature ATG8-I, which will be conjugated to PE, forming the lipidated version ATG8-II on the membranes of autophagosomal structures [18]. The ATG8 acts as a scaffold for the early core autophagy components by binding protein subunits containing the sequence motif called LC3-interacting region (LIR) [19]. The autophagy receptors, like SQSTM1/p62 (p62) and nuclear dot protein 52, which contain a LIR domain, physically link defined cellular material with the autophagy compartment by interacting simultaneously with cargo and ATG8 family proteins on autophagosomes [20]. Finally, mature autophagosomes can fuse with lysosomes, forming autolysosomes. Lysosomes provide the degradative ability of the autophagosome, and this step is also critical to maintain a basal autophagic flux [21].

The two ATG8 subfamilies, LC3 and GABARAP, have a nonredundant function during autophagosome biogenesis; however, the preponderant role of GABARAP is suggested by its preferential binding of essential ATG proteins over LC3 during the initial autophagosome formation [22] and closure [23] as well as fusion with lysosome [24]. However, it is not fully known how ATG8 proteins are translocated to the forming autophagosome. Recent studies have shown that in the centrosome and pericentrosomal region, a pool of GABARAP exists and regulates the development of autophagosomes during amino acid starvation. GABARAP, but not other members of the ATG8 family, binds via a canonical LIR motif directly to pericentriolar material one (PCM1) [25], a large coiled-coil-containing protein that allows pericentrosomal localisation of centrosomal proteins [26]. During autophagosome formation, PCM1 promotes the formation of GABARAP-positive autophagosome, controlling the delivery of GABARAP to the autophagosome [25]. Consistently with this study, in ECs, the keratin-binding protein trichoplein (TCHP) directly binds PCM1 in the pericentrosomal region and regulates its stability [27]. Loss of TCHP accelerates the proteasomal degradation of PCM1 and consequently of its binding partner GABARAP, thereby impairing autophagic flux. The downregulation of TCHP disrupts basal autophagy and autophagosome maturation, leading to the accumulation of unresolved autophagosome in ECs. Notably, in ECs lacking TCHP, the autophagic flux is not entirely blocked, and autophagy and consequently the endothelial function can be pharmacologically restored [27].

Autophagy controls endothelial cells homeostasis and functions

Pioneering studies on endothelial autophagy showed that manipulation of autophagy via regulation of ATG5 in ECs could regulate endothelial functions [28], linking autophagy loss with vascular disease (Fig. 1).

Subsequent studies focused on the role of autophagy in the regulation of nitric oxide (NO) bioavailability. Inhibition of autophagic flux in ECs lacking ATG3 [29] or by pharmacological treatment with bafilomycin [30] impaired the phosphorylation endothelial NO synthase (eNOS) and reduced NO production in response to shear stress. Activation of autophagic flux under steady laminar shear stress showed the opposite effect in ECs, contributing to the upregulation of eNOS expression and improving vascular tone [31].

Shear stress also induces activation of NAD^+-dependent histone deacetylase Sirtuin 1 (SIRT1), which directly deacetylates the promoters of several components of the autophagy machinery, thus promotes autophagy in ECs [32].

Growth of the vascular network is a highly dynamic process, comprising many individual steps promoting the dynamic shift between quiescence and proliferation and sprouting of ECs. Recent research demonstrated that vascular endothelial growth factor (VEGF) activates autophagic flux by an AMPK-dependent mechanism [33]. Notably, the activation of autophagy in response to VEGF is transient, and it is opposed by a delayed activation of mTOR by VEGF itself [33]. Conversely, depletion of cell-autonomous VEGF from the endothelium results in FOXO1-mediated mitochondria fragmentation, leading to cell death by an increased autophagic flux [34].

Protein kinase A (PKA) regulates angiogenesis and the transition of ECs from sprouting to quiescence [35]. During this cellular switch in ECs, PKA regulates autophagy by driving phosphorylation-dependent degradation of ATG16L1 protein [36]. Thus, autophagy stimulates angiogenesis during development [36]. The role of autophagy in vascular development and during endothelial sprouting has also been confirmed in endothelial-specific transcription factor EB (TFEB) knockout mice, showing defective autophagy [37]. The loss of TFEB impairs EC proliferation and retinal vessel sprouting and maturation through the transcriptional control and trafficking of VEGF receptor 2 (VGFR2) [37].

The autophagic state of ECs is also critical for vascular permeability. A recent study demonstrated that ECs require autophagy to regulate tight junction

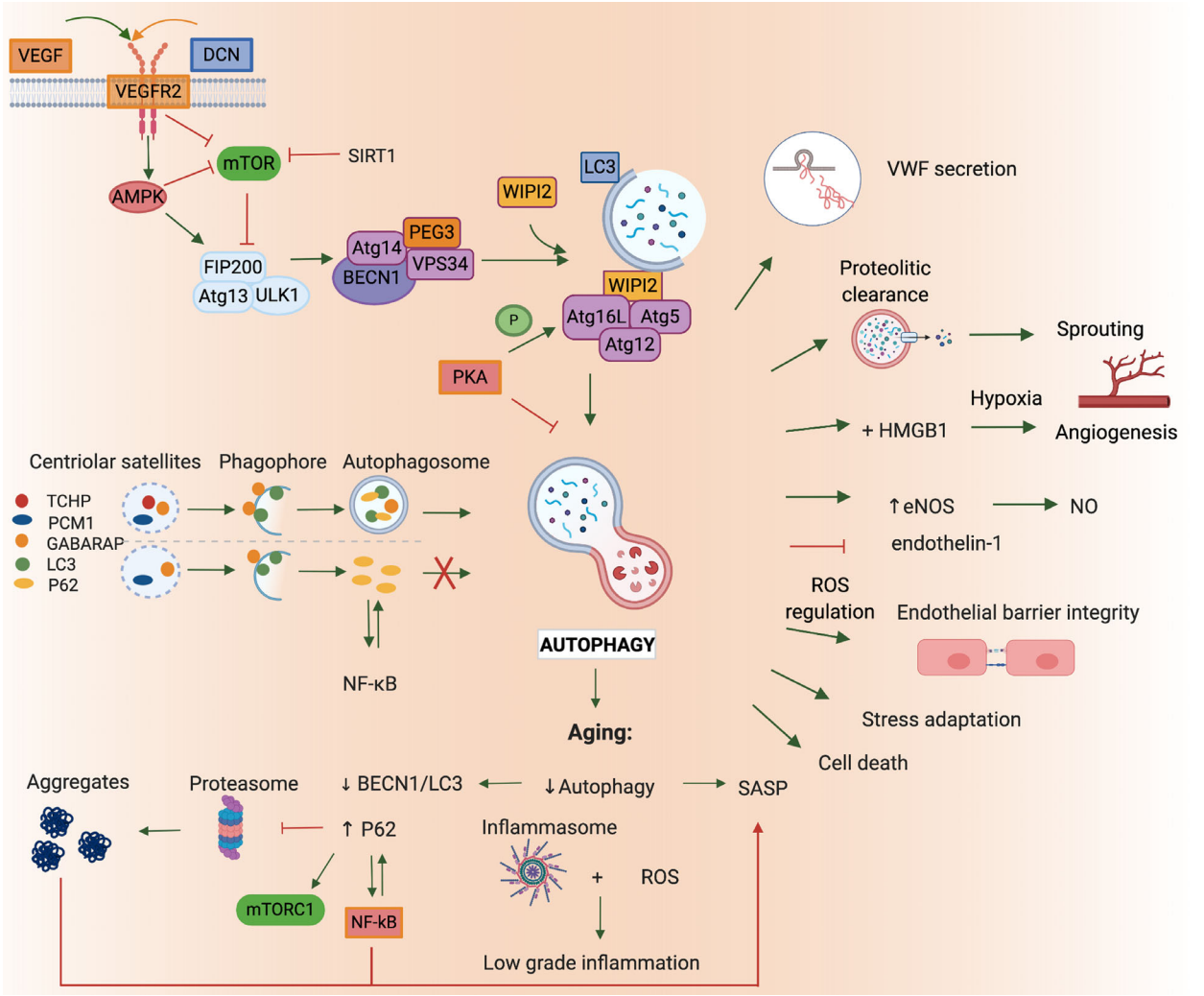

Fig. 1. Interplay of autophagy with mechanisms regulating ECs function, inflammation and senescence. Basal level of autophagy in ECs is necessary to provide primary cytoplasmic quality control and stress-adaptation mechanism, thus ensuring cellular homeostasis. A functional autophagy flux maintains endothelial functions as sprouting in response of angiogenic stimuli, secretion and endothelial barrier integrity. During ageing, the autophagic capacity declines and increased ROS production and aggregated proteins activate inflammasomes which provoke a low-grade inflammation and therefore accelerate EC senescence. Created with BioRender.com.

proteins and maintain endothelial barrier integrity [38]. On the other hand, inhibition of autophagic flux in ECs increases permeability via a ROS-dependent mechanism [38].

The extracellular matrix provides essential signalling to regulate ECs survival, proliferation and migration [39]. The secreted matrix proteoglycan Decorin (DCN), a small leucine-rich proteoglycan, via the paternally expressed gene 3, induces a substantial increase in autophagosome formation in ECs [40]. More experiments have shown that DCN activates autophagy by AMPK activation and simultaneous mTOR inhibition [41].

Finally, autophagy has a fundamental role in ECs secretion. A seminal study showed that von Willebrand factor (VWF) localises in the proximity of autophagosomes in ECs [42]. Based on this observation, the inhibition of autophagy by the knockdown or deletion of either ATG5 or ATG7 in ECs impairs the secretion of VWF from ECs, further suggesting an antithrombotic function of autophagy [42]. As confirmation, the pharmacological inhibition of autophagy

by chloroquine treatment results in a downregulation of VWF secretion [43]. Further support to the importance of secretory autophagy in ECs comes from the analysis of autophagic vacuoles secreted by ECs under starvation, showing that these autophagic vacuoles contain high levels of VWF [44].

Overall, a basal level of autophagy in ECs is required to maintain EC functions. Despite this, prolonged autophagy activation can also induce EC death in some circumstances. In line with this, treatment of human ECs with the endogenous angiogenic inhibitor endostatin induces autophagy and activation of apoptosis [45]. Of note, it has been shown that in ECs during prolonged hypoxia, autophagy shifts from cell survival mechanism to cell death mechanism in a time-dependent manner [46].

Autophagy as a regulator of endothelial cell senescence and inflammation

Several studies have established that autophagy in organs and tissues declines during ageing [47]. Hence, in aged mice, ECs express lower levels of autophagic proteins than younger animals [48]. The causality between ageing and decline of autophagy has been established by pharmacological interventions, showing that compounds increasing autophagy such as rapamycin [49], trehalose [48] or spermidine [50] can reverse aspects of arterial ageing.

A possible connection between autophagy and senescence is that inhibition of autophagy can regulate activation of inflammatory senescence-associated secretory phenotype (SASP) [51]. Based on a recent hypothesis, the build-up of senescent ECs could release SASP inflammatory cytokines and influences nonsenescent neighbouring cells, thus promoting the development and progression of vascular disease [52]. Since it has been demonstrated that activation of autophagy reduced inflammation [53], inhibiting the SASP in senescent ECs through regulation of autophagy, instead of removing senescence cells, should be considered the primary approach in vascular disease.

Although additional mechanistic studies are necessary to determine how defective autophagy promotes ECs senescence, the interplay between p62 with mTORC1 or NF-kB can partially explain SASP transcriptions, defective autophagy and senescent growth arrest [54]. Besides its autophagic function, p62 acts as a signalling hub and activates numerous pathways through different binding domains [55]. It is well known that p62 induces SASP production via TRAF6 polyubiquitination and thereby NF-κB activation [56]. Moreover, increased levels of p62 in autophagy-deficient cells inhibit proteasomal degradation, leading to the accumulation of protein aggregates. Finally, p62 can be part of the mTORC1 complex and is necessary to mediate amino acid sensing for the activation mTOR pathway [57]. Therefore, the accumulation of p62 could lead to hyperactivation of these pathways, promoting cellular senescence. The accumulation of p62 and protein aggregates and a premature senescent phenotype has been associated with defective autophagy in ECs lacking TCHP [27]. The phenotype is due to NF-kB activation, and it has been observed in ECs isolated from patients with coronary artery disease and endothelial dysfunction [27]. A similar phenotype has been observed in human ECs in the cerebral cavernous malformations disease (CCM), a primary cerebrovascular disease, whereby defective autophagy and aberrant p62 accumulation triggers intracellular stress [58], possibly linking CCM with EC senescence [59].

The senescent phenotype of ECs could also be mTOR/autophagy-dependent, as demonstrated in endothelial-specific ATG5 knockout mice [60] or obese mice [61]. Hence, the pharmacological inhibition of mTOR by rapamycin has shown improvement in cardiovascular ageing and age-related disorders with notable results in terms of SASP suppression [62]. Notably, in obese mice, rapamycin also improved the revascularisation response after hindlimb ischaemia [61].

Finally, ageing-induced inhibition of proteostasis with consequent accumulation of protein aggregates in ECs has been associated with defective autophagy and endothelial dysfunction. The accumulation of protein aggregates was resolved by Keap1 depletion, inhibition of protein S-nitrosation or rapamycin treatment [63].

Dysregulation of autophagy in endothelial cells in vascular disease

Autophagy plays a fundamental role in the regulation of several functions in the vascular system. Thus, its impairment is associated with a wide range of vascular pathologies (Table 1).

Atherosclerosis

Endothelial dysfunction has been described as an early event in the development and progression of atherosclerosis [64] and associated with defective autophagy in the endothelium (Fig. 2A).

Robust autophagic flux occurs in the endothelium in vascular regions exposed to high levels of laminar shear stress and is thereby protected from

Table 1. Autophagy in vascular disease.

Disease	Autophagy level	Mechanism	Effect	Ref
Atherosclerosis	↓	Disturbed flow in atheroprone regions	Pro-inflammatory, prosenescent, proapoptotic phenotype in ECs	[65]
	↓	Inhibition of AMPK by high concentration of glucose and fatty acids	Increase of cell apoptosis and inflammation	[66]
	↓	*Atg7*-deficient mice	Accumulation of LDL	[67]
	↑	Cav-1 deficiency	Atheroprotection due to regulation of ATG5-ATG12 complex distribution in lipid rafts	[69]
	↓	Disturbed flow in plaques	Reduction of nuclear import of miR-126-5p and upregulation of caspase 3, apoptosis	[89]
	↑	Upregulation of MALAT1 enhances BECN1 expression by sponging miR-216a-5p	Atheroprotection	[123]
Hindlimb ischaemia	↑	Prolonged hypoxia in mouse model	Proliferation of close skeletal muscle cells	[72]
	↑	Injection of HMGB1	Blood flow recovery, revascularisation	[73]
	↑	AGGF1 regulates the formation of the autophagosome	Postischaemic revascularisation	[74]
Retinopathy	↓	Lack of *Atg5* in mouse	Reduction of pathological neovascularisation	[76]
Cerebral ischaemia	↑	Induction by rapamycin	Decreased ECs apoptosis, restoration of ZO-1 levels, reduction of ROS production	[78]
	↑	Oxygen glucose deprivation	Degradation of claudin-5 and occludin in ECs and capillaries	[79,80]
	↓	Inhibition by 3-methyladenine in diabetic mouse model	Improvement of the recovery after brain ischaemia	[81]
	↓	Depletion of *Atg7* in mouse ECs	Protection against the I/R-induced acute cerebral injury during stroke	[82]

atherosclerosis [60,65]. In contrast, autophagy is deficient in atheroprone regions that experience disturbed flow. Likewise, endothelial-specific deletion of *Atg5* in hypercholesterolemic mice enhances atherosclerosis exclusively in regions of high laminar shear stress [60].

The inhibition of AMPK activity in ECs by metabolic stress due to high glucose and fatty acids impairs basal autophagy, therefore increasing cell apoptosis and inflammation [66]. This mechanism can explain the progression of endothelial dysfunctions to atherosclerosis in patients with metabolic syndrome [66].

Furthermore, it has been shown that endothelial autophagy suppresses lipid retention in the vessel wall [67], showing that increasing the autophagy targeting lipid (lipophagy) [68] may be a crucial mechanism to prevent atherosclerosis progression. Accordingly, exposure to low-density lipoprotein (LDL) induces autophagy in ECs, while defective autophagy in the ECs (*Atg7*-deficient mice) increased the accumulation of LDL in the endothelium with increased atherosclerotic outcomes compared to wild-type mice [67]. The link between lipid trafficking, atherosclerosis and autophagy is explored in the study on the role of caveolin 1 (Cav-1) in regulating autophagy [69]. Using Cav-1 knockout mice crossed with low-density lipoprotein receptor-deficient mice (Ldlr−/−) fed with a high-fat diet, the authors demonstrated Cav-1 deficiency induces endothelial autophagy, and it is athero-protective. The effect of Cav-1 on autophagy is partially due to the regulation of ATG5-ATG12 complex distribution in the lipid rafts [69].

As extensively reviewed recently [70], autophagy plays a fundamental role in atherosclerosis through regulation of vascular smooth muscle cells (VSMCs) homeostasis. Autophagy regulates VSMC phenotype and viability, therefore affecting atherosclerotic plaque stability.

While a moderate autophagy induction could promote the conversion of contractile VSMC to a synthetic phenotype and inhibit cell death, impaired autophagy leads to VSMCs senescence, cell death, contractile phenotype and plaque formation and instability.

Vascular ischaemic disease

After the onset of ischaemia, angiogenesis and atherogenesis act to establish a functional vascular network in ischaemic areas to support tissue regeneration [71].

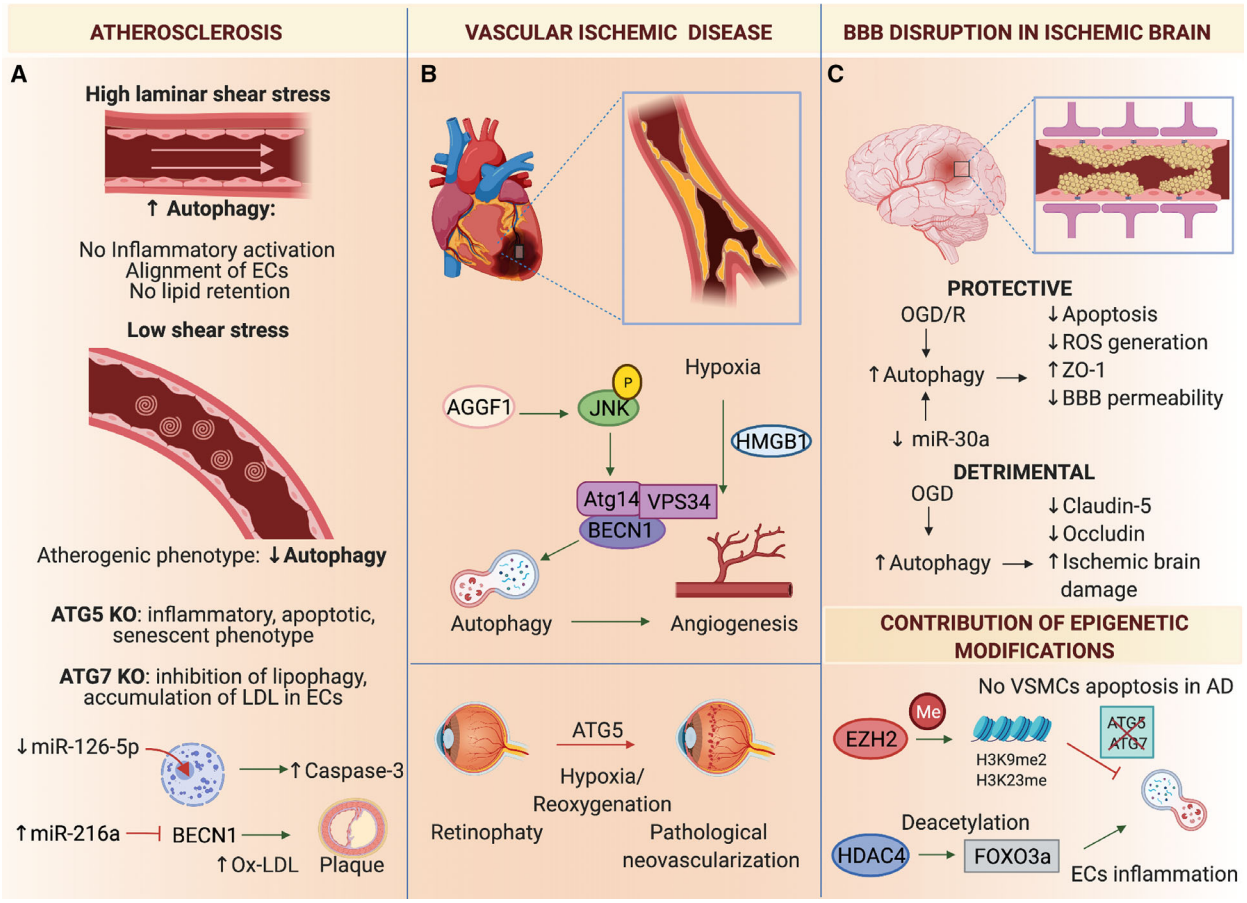

Fig. 2. Role of autophagy in vascular disease. (A) Robust autophagic flux occurs in the endothelium in vascular regions that are exposed to high levels of laminar shear stress and are thereby protected from atherosclerosis. In contrast, autophagy is deficient in atheroprone regions that experience disturbed flow. Reduced levels of autophagy are linked with inflammatory and apoptotic phenotype and with increased incorporation of LDL in ECs. (B) Autophagy in ECs is vital for the repair and regeneration of damaged tissues. Inhibition of autophagy impairs AGGF1-mediated angiogenesis and therapeutic actions postmyocardial infarction, indicating that autophagy acts upstream of and is essential for angiogenesis. Similarly, HGMB1 restore vascularisation after ischaemic injury through an autophagic-dependent mechanism. In contrast, defective autophagy in retinal ECs displayed diminished production of mitochondrial ROS and reduced revascularisation, thus suggesting a specific role of endothelial ATG5 in pathological hypoxia/reoxygenation-related angiogenesis. (C) The role of autophagy in regulating BBB homeostasis is still controversial. EC autophagy has a beneficial effect on BBB integrity increases levels of ZO-1 and protects cells against the generation of ROS. In contrast, studies demonstrated that autophagy promotes the degradation of BBB components such as claudin-5 or occludin in brain ECs and brain capillaries. Several epigenetic regulators, including miRNA or chromatin modification, can regulate autophagy mechanism in vascular disease. Created with BioRender.com.

Despite the documented role of endothelial autophagy in promoting angiogenesis, very few studies explored the relationship between ischaemia-induced autophagy and therapeutic or pathological angiogenesis (Fig. 2B).

A recent study reported that autophagy in ECs is essential to heal and regenerate damaged tissues [72]. Autophagy is activated in the ECs of the adductor muscle in a mouse model of hindlimb ischaemia, thus stimulating neighbouring skeletal muscle cells to proliferate and regenerate. Conversely, the conditioned media from ECs in which autophagy is suppressed, preventing the proliferation and the survival of neighbouring cells [72].

The high-mobility group box 1 (HMGB1), a nonhistone chromosome-associated protein, is released in the cytoplasm of ECs in response to hypoxia and promotes angiogenesis [73]. Injection of HMGB1 into ischaemic limbs increased blood flow recovery and revascularisation partially through an autophagy-depended mechanism [73].

A recent study showed that the angiogenic factor with G patch and FHA domains 1 (AGGF1) could regulate autophagosome formation by activation of Vps34 lipid kinase and the assembly of Becn1-Vps34-Atg14 complex [74]. The association between autophagy and AGGF1-mediated revascularisation has been confirmed in Aggf1 heterozygous mice, showing defective autophagy and inhibition of angiogenesis and larger infarct areas compared to wild -type mice after myocardial infarction. Conversely, inhibition of autophagy impairs AGGF1-mediated postischaemic therapeutic revascularisation [78].

Pathological angiogenesis, such as uncontrolled retinal neovascularisation during proliferative retinopathies, involves endothelial activation by ischaemia/hypoxia or oxidative stress [75]. Lack of *Atg5* in mice endothelium reduced pathological neovascularisation in a mouse model of retinopathy. In contrast, no alterations in physiological retina vascularisation were observed [76]. Defective autophagy in retinal ECs displayed impaired mitochondrial respiratory activity, diminished production of mitochondrial ROS and decreased phosphorylation of the vascular endothelial growth factor receptor 2, thus suggesting a specific role endothelial ATG5 in pathological hypoxia/reoxygenation–related angiogenesis [76].

During cerebral ischaemia due to blood vessel blockage or rupture, the lack of oxygen and nutrients causes pathological processes, including apoptotic neuronal death, neuroinflammation and blood–brain barrier (BBB) disruption [77]. The role of autophagy in regulating BBB homeostasis is still controversial (Fig. 2C). Li *et al*. found that EC autophagy has a beneficial effect on BBB integrity during ischaemia/reperfusion (I/R) injury, showing that autophagy activation attenuates ECs apoptosis, restores decreased levels of ZO-1 and reduces ROS production induced by oxygen-glucose deprivation/reoxygenation (OGD/R). In contrast, suppression of autophagy increases them [78]. In contrast, studies identified a detrimental role for autophagy during ischaemia and oxygen-glucose deprivation in the brain. In these conditions, activation of autophagy promotes the degradation of BBB components such as claudin-5 [79] or occludin in brain ECs and capillaries [80]. In support of this, the autophagic process inhibition in diabetic mice improves the recovery after brain ischaemia [81], and defective autophagy by depletion of *Atg7* in brain ECs protects the neurovascular unit against the I/R-induced acute cerebral injury during stroke [82].

Overall, autophagy activation might be a productive strategy to promote revascularisation after ischaemic injury and reduce the progression of atherosclerosis in the vessels. Defining the role of autophagy in ECs from different tissue (peripheric vs brain vascularisation) or vessels (micro- vs macro- vascularisation) is required to define a role for autophagy in vascular disease.

Epigenetic regulation of autophagy in vascular disease

The fundamental role of epigenetics in the regulation of autophagy has recently come to light. Described for the first time in yeast cells treated with spermidine [83], the activation of autophagic genes by deacetylation of the histone H3 caused by suppression of the histone acetyltransferases was found to play an essential role in oxidative stress response [83]. Interestingly, epigenetic modifications may balance autophagy level upon stimuli, as proved by the presence of bivalent domains in autophagic genes, both activating (H3K4me3) and repressive (H3K9me3) [84]. EZH2 methylates H3K9me2 and H3K23me and consequently inhibits ATG5 and ATG7 expression to finally suppress autophagy and prevent VSMCs apoptosis in aortic dissection (AD) [85]. Yang *et al*. [86] linked the increased expression of HDAC4 and autophagy to vascular inflammation . In response to Ang II, HDAC4, a class IIa family histone deacetylases member, activates autophagy by the deacetylation forkhead box O3a, thus promoting inflammation in ECs [86].

Recent studies have described the mechanistic regulation of autophagy by microRNA (miRNA). miR-216a binds directly Beclin 1 (BECN1) and can regulate ox-LDL induced autophagy in EC. Overexpression of the miR-216a could increase early plaque formation of atherosclerosis by ox-LDL accumulation, as well as elevated monocyte adherence [87]. miR-30a binds BECN1 as well, and miR-30a inhibition can reduce the injury associated with cerebral ischaemia [88].

Finally, a fascinating study showed that autophagy regulates the localisation in the nucleus of a specific miRNA, miR-126-5p. Hence, the inhibition of autophagic flux in ECs reduces the nuclear import of miR-126-5p, thus aggravating endothelial apoptosis and atherosclerosis through upregulation on its target caspase 3 [89].

Overall, novel research highlights the importance of epigenetic modifications in the autophagic process (Fig. 2): they represent potential targets in autophagic-mediated modulation of cardiovascular disease; however, further studies are necessary to define their precise mechanism of action.

Targeting defective autophagy to restore endothelial function in vascular disease: the AMPK/mTOR way

As discussed in the previous paragraph, the mTOR pathway provides tight control during the different autophagy steps. During starvation, when energy production is compromised, activation of AMPK and consequent inhibition of mTOR leads to autophagy stimulation. Increased cellular amino acid levels and growth factors stimulate the mTOR signalling pathway, thus inhibiting autophagy [90]. Several studies have reinforced the idea that targeting AMPK/mTOR signalling provides a direct regulation of autophagic flux [91]. Hence, mTOR inhibitor or AMPK activator has been studied in preclinical models of vascular diseases (Fig. 3). Of note, targeting mTOR activity in ECs will activate autophagy and regulate ECs metabolism, effectively switching ECs from quiescence to proliferative behaviour.

The first evidence of the cardioprotective benefits of rapamycin, an mTOR inhibitor, in humans was found in kidney transplant patients. The treatment induced an improvement in hypertension, lowering central arterial stiffness and reducing blood pressure in brachial and carotid arteries [92]. Everolimus, a rapamycin derivative delivered by drug-eluting stents in patients with coronary artery disease [93], inhibits not only smooth muscle cells proliferation but also endothelial inflammation [94], acting on plaque neovascularisation and stability [95,96].

Polyphenols such as resveratrol, SRT1720 and nicotinamide mononucleotide modulate autophagy through the activation of SIRT1, which consequently inhibits mTOR [97]. At the vascular level, resveratrol can attenuate arterial stiffness and vascular endothelial dysfunction through the enhancement of NO-mediated vasorelaxation by reducing vascular oxidative stress and inflammation and suppressing endothelial apoptosis [98]. SRT1720 was found to reduce EC oxidative

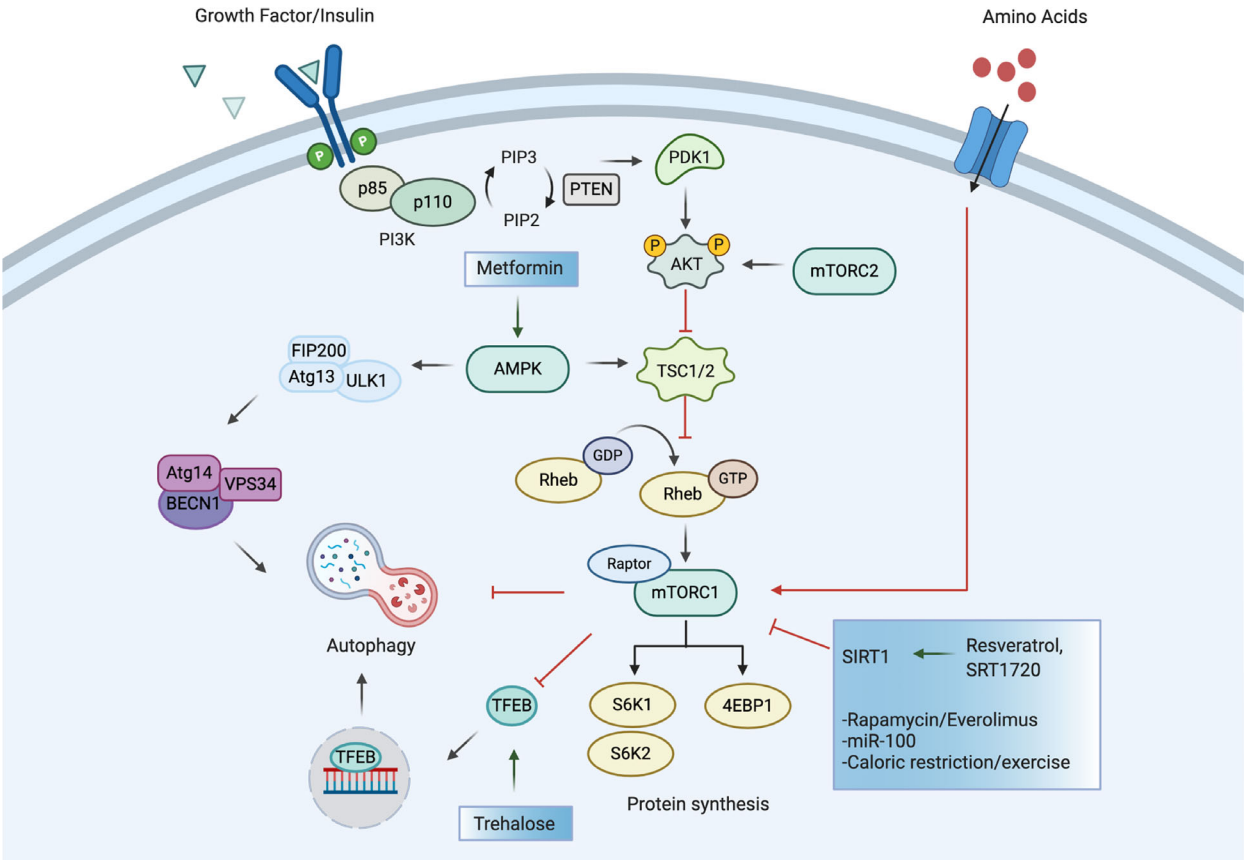

Fig. 3. Targeting AMPK/mTOR pathway to regulate autophagic flux in ECs. Several molecules that modulate the autophagic process mainly through inhibition of mTOR or activation of AMPK have been studied. In line with this, the modulation of autophagic function has been considered a possible therapy for vascular disease. Created with BioRender.com.

stress and inflammation [99], while nicotinamide mononucleotide restores endothelial function and NO-dependent vasodilation, neutralises collagen deposition and limits elastin damage in aged aortae [100].

Metformin is an antidiabetic drug that activates autophagy via activation of AMPK. It mitigates atherosclerosis in diabetic patients and suppresses vascular senescence. Furthermore, it presents an anti-inflammatory function in ECs [101].

Trehalose, a disaccharide, promotes the increase of the autophagic flux through the activation of TFEB, which is typically kept inactivated by mTOR [102]. This leads to restore endothelial function by increasing NO bioavailability in arteries of aged mice [102].

Pankratz *et al.* [103] demonstrate that miR-100 attenuates inflammation and atherosclerosis by stimulating endothelial autophagy. They report that an additional mTORC1 component, RAPTOR, is also a miR-100 target in the endothelium . Overexpression of miR-100 enhanced autophagic flux-attenuated NF-κB activity and repressed adhesion molecule levels in ECs, resulting in reduced leukocyte recruitment. Furthermore, the global knockout of miR-100 exacerbated atherogenesis in Ldlr−/− mice, whereas upregulation of miR-100 through intravenous injection of miR-100 mimics decreased lesion area macrophage content [103].

Finally, caloric restriction and endurance exercise benefit cardiac ageing through autophagy induction due to mTOR suppression [104]. At the vascular level, it attenuates wall thickening and vascular stiffness, restores endothelial function and elastin remodelling and increases eNOS expression and bioavailability [105].

Concluding remarks, perspective and future directions

Fundamental studies published in the last few years have clarified the role of autophagy in the endothelium, demonstrating that endothelial autophagy is fundamentally cytoprotective and regulates its function in response to blood flow and stress. Moreover, autophagy affects several aspects of EC biology, such as sprouting, permeability and secretion.

From novel research, the regulation of autophagy in ECs is likely to be context-dependent, and in ECs from different tissues, autophagy may play diverse roles.

Hence, it remains elusive whether and how EC autophagy influences angiogenesis during ischaemic revascularisation or atherosclerosis progression *in vivo*. The application of advanced *in vivo* imaging approaches like 2-photon microscopy, and transgenic reporters mouse models for autophagy [106] can help investigate changes in autophagic flux at the different stages of the revascularisation process and during the progression of vascular disease. Most of the studies in ECs are limited to endothelial-specific ATG5 and ATG7 knockout mice. Given the complexity of the autophagic pathway, *in vivo* studies using knockout mice for a different subset of autophagic genes in ECs are needed. Regarding this, in EC-specific ATG16L1 knockout mice, ECs are more susceptible to cell death than wild-type mice after treatment with α-toxin [107].

An even less explored area of endothelial autophagy is its role in critical aspects of EC plasticity. In this regard, defective autophagy in ECs leads to an evident loss of EC markers, usually observed during the endothelial-to-mesenchymal transition (EndMT) [108]. The association between autophagy and EndMT is also present during cerebrovascular disease progression [58]. Recently, the analysis of endothelial-specific ATG5 knockout mice fed with a high-fat diet revealed that defective autophagy promotes IL-6-dependent EndMT and heart fibrosis [109]. Finally, a recent study using single-cell RNA sequencing showed that several genes involved in autophagy were highly upregulated in a subpopulation of EC undergoing endothelial-to-haematopoietic transition, again showing the importance of autophagy in regulating EC plasticity [110].

Several studies have also established that autophagy has non-cell-autonomous roles in ECs, controlling secretion (secretory autophagy) and autocrine/paracrine signals [111]. Moreover, the mechanisms regulating secretory autophagy overlap with the mechanisms involved in the biogenesis and release of extracellular vesicles (EVs) and exosomes [112]. Consequently, it has been demonstrated that ATG5, ATG16L1 and ATG8 are essential autophagic genes in stimulating exosome secretion by regulating the de-acidification of multivesicular bodies [113]. Since the specific role of exosomes in regulating ECs function and vascular regeneration [114], the possibility to modulate exosome biogenesis through regulation of autophagy should be explored in the context of vascular disease.

From a therapeutic viewpoint, re-establish physiological levels of autophagy in the vasculature could be an emerging strategy for vascular disease; however, it is uncertain whether activation of basal autophagy in healthy tissues would be harmful. Also, preclinical studies demonstrated that autophagy-mediated therapy fails when the expression of basal autophagic genes is compromised [115]. Based on this, targeting selective autophagy pathways to cause the specific removal of organelles, protein aggregates and regulates

inflammations might be preferable over the inhibition of the core machinery of autophagy which might have significant side effects. Moreover, diseases that may require acute short-term treatments such as ischaemic vascular disease instead of the diseases requiring long-term therapy could be a favourite therapeutic indication for setting up and conducting clinical trials for compounds activating autophagy.

Regarding the therapeutic potential of modulating autophagy for vascular diseases, further consideration should be given to risk factors such as obesity and diabetes. Based on the recent studies, it appears that autophagy modulation could have opposite effects on these factors. Therefore, increasing autophagy in the liver and β cell could reduce hyperlipidaemia [116] and insulin resistance [117], respectively; however, increase of autophagy can enhance adipogenesis in adipose tissue and obesity [118]. In line with this, endothelial-specific delivery approaches for autophagy activators must be developed to target vascular disease. Although endothelium is accessible to circulating compounds, most compounds have no endothelial affinity, and only a minor fraction is uptaken by these cells. Advanced drug delivery systems have recently been tested for drug delivery to normal and pathological endothelium [119]. Successful examples of endothelial-specific carriers include E-selectin [120] or adhesion molecule-targeted liposomes [121] and polymeric nanoparticles with low molecular weight [122].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

EM, AM and AC conceived the manuscript and wrote the text.

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