Cross talk between autophagy and apoptosis in pulmonary hypertension

Yang Jin and Augustine M. K. Choi

Division of Pulmonary and Critical Care, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

ABSTRACT

Endothelial cell (EC) apoptosis and apoptosis resistant proliferation have been proposed to play crucial roles in the development of featured plexiform lesions in the pathogenesis of pulmonary hypertension (PH). Subsequently, EC injury associated smooth muscle cell (SMC) proliferation facilitates vascular remodeling and eventually leads to narrowed vascular lumen, increased pulmonary vascular resistance, increased pulmonary arterial pressure, and right heart failure. The imbalance between cell death and proliferation occurs in every stage of pulmonary vascular remodeling and pathogenesis of PH, and involves every cell type in the vasculature including, but not limited to ECs, SMCs, and fibroblasts. Despite extensive studies, the detailed cellular and molecular mechanisms on how the transition from initial apoptosis of ECs to apoptosis resistant proliferation on ECs and SMCs remains unclear. Recent knowledge on autophagy, a conservative and powerful regulatory machinery existing in almost all mammalian cells, has shed light on the complex and delicate control on cell fate in the development of vascular remodeling in PH. In this review, we will discuss the recent understandings on how the cross-talk between apoptosis and autophagy regulates cell death or proliferation in PH pathogenesis, particularly in pulmonary vascular remodeling involving ECs and SMCs.

Key Words: apoptosis, autophagy, beclin-1, LC3, pulmonary hypertension

Pulmonary hypertension (PH) is a devastating and often fatal syndrome. At least six groups of PH have been described including an idiopathic form (IPAH), a familial form (FPAH), and a variety of secondary forms (APAH) associated with other medical conditions such as cardiac or pulmonary disorders.[1-5] PH is characterized by elevated pulmonary arterial pressures (PAP) and often progresses to right heart failure.[1-5] All the various forms of PH share some common pathological features involving the intima, media, and adventitia of both large and distal pulmonary vessels.[4-8] These features include but are not limited to the vascular remodeling and excessive arterial muscularization,[4-8] presence of the thrombotic lesions,[4-8] and the plexiform lesions.[9,10] Normal pulmonary vasculature requires maintenance of intimal integrity/EC survival and precise control on media growth/SMC proliferation. While detailed pathogenesis remains not completely understood, the imbalance of cell death and proliferation in ECs and SMCs, as well as the emergence of apoptosis resistant cell, seem to play crucial roles in the development of PH.

Normal cells have complex and delicate regulatory machinery in order to precisely dictate the fate of each cell. Apoptosis (programmed cell death) has been well recognized as a powerful tool to limit the uncontrolled cell proliferation.[11-15] In a variety of disease processes, hyperactive apoptosis leads to massive cell death, tissue injury, and eventually organ failure.[11-15] On the other hand, suppressed apoptosis results in over proliferation and "tumor"-like growth of selected cells, which can be the most obvious feature in malignancy.[11-15] Intriguingly, PH pathogenesis involves both inappropriate apoptosis and over proliferation. Apoptosis in ECs, after initial environmental insults, has been recognized as one of the crucial events that trigger the pulmonary vascular remodeling in PH.[5-10] However, lack of apoptosis in SMCs has been thought as one

Address correspondence to:
Dr. Augustine M. K. Choi
Division of Pulmonary and Critical Care
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
75 Francis Street
Boston, MA 02115, USA
Email: amchoi@rics.bwh.harvard.edu

of the culprits leading to uncontrolled SMC proliferation.[5-10] Therefore, it is not surprising that inhibitors or inducers of apoptosis have not been demonstrated as an effective treatment for PH in humans,[16] despite a few scattered reports on animal models.

In recent years, autophagy ("self-digestion") has caught robust attention in the biological fields. Autophagy is a regulated process for the turnover of cytoplasmic proteins and organelles through a lysosome-dependent degradation pathway.[17-20] At least 27 "Atg" genes and gene products have been identified.[21-25] Of these, the microtubule associated protein light chain-3 (LC3)-family of proteins (Atg8) and becklin-1 are key mediators of autophagosome formation. [26,27] Although its regulatory functions have been implicated in several human diseases, including cancer, neurodegenerative diseases, inflammatory bowel disease, and cardiovascular diseases,[26-30] the role of autophagy in lung disease, particularly in pulmonary vascular remodeling and PH, remains largely unexplored. Autophagy has now been demonstrated as one of the most crucial cellular functions which maintains the cellular homeostasis.[21-25] More interestingly, it possesses differential or somewhat paradoxical functions. It first serves as a cell survival mechanism, via its capability of recycling unused organelles to provide energy resources.[21-25] Furthermore, autophagy is shown to inhibit apoptosis and necrosis. Tanaka et al. demonstrated that autophagic marker protein LC3b physically interacts with Fas apoptotic pathways and subsequently prevents apoptosis.[26] Additionally, autophagy has been shown to interfere with at least two types of necrosis: Necroptosis and poly-(ADP-ribose) polymerase (PARP)-mediated cell death.[27] That said, autophagy has also been known to promote cell death. "Autophagic cell death" (ACD) has been well recognized as a sign of unsalvageable damage to cells.[28,29] Presumably, after obnoxious insults initiate, the cell uses autophagy to generate energy and inhibit death pathways, either apoptosis or necroptosis, as mentioned above. However, when insults continue and when the cell "recognizes" that the injury has been beyond its capability of repair, autophagy becomes a method to "suicide" in order to maintain "healthy" populations. At this moment, autophagy has synergistic effects with apoptosis and necroptosis to augment cell death. Thus, regulation of autophagy, apoptosis, and their cross-talk is essential to maintaining cellular homeostasis. Imbalance of apoptosis and autophagy due to the impaired cross-talks may result in either over proliferation or profound cell death which can be the culprit for many human diseases. Intriguingly, pulmonary vascular remodeling in PH carries the "imbalance" of cell death and survival at multiple stages, yet involving in multiple cell types in the vasculature. Initially, when EC injury occurs, autophagy is supposed to salvage ECs and inhibit apoptosis. Later, when uncontrolled proliferation occurs, apoptosis

and autophagic cell death (ACD) are supposed to limit this pathologic growth. However, the opposite effects exist in PH pathogenesis. We speculate that malfunction of the cross-talk between apoptosis and autophagy in ECs and SMCs leads to pulmonary vascular remodeling in PH, and eventually devastating biological effects.

EVIDENCE ON DEFECTED CROSS-TALK BETWEEN APOPTOSIS AND AUTOPHAGY IN ECS

EC apoptosis in PH

Currently, accumulating interests focus on how the development of PH initiates. Many researchers believe that an initial wave of EC apoptosis triggered by the environmental stress (such as hypoxia, MCT, inflammation) leads to the emergence of apoptosis-resistant and hyperproliferative ECs.[5-10] These abnormal, uncontrolled ECs subsequently result in the characteristic intimal plexiform lesions which have been observed in many forms of PH.[5-10] This theory is supported by a number of studies. Initially, in the early 90s, Arcot et al. and Partovian et al. described a reduced level of vascular endothelial growth factor (VEGF) in both hypoxia- and MCT-induced PH models.[30,31] VEGF has been known to confer a potent protective effect on ECs from apoptosis through the extrinsic pathway.[32] In a variety of experimental PH models, EC apoptosis has been shown to be associated with reduced levels of VEGF.[30,31] Next, Taraseviciene-Stewart et al.[33] demonstrated that inhibition of VEGF receptor 2 (VEGFR2) using sugen 5416 (su5416) robustly potentiates the hypoxia-induced PH and the appearance of intimal plexiform lesions. These proliferative intimal lesions are similar to those often seen in human PAH.[33] More importantly, in their studies, marked EC apoptosis was found coexisting with marked intimal disorganized proliferation (forming of the plexiform lesion.[33] Activated caspase-3 in ECs has been shown in this novel PH animal model induced by VEGF receptor inhibitor su5416 plus hypoxia.[33-35] Interestingly, treatment with Z-Asp-CH2-DCB, a broad-spectrum caspase inhibitor, prevents the development of severe PH.[33] The theory of EC apoptosis as the initial pathological presentation of PH has been further supported by a few studies: (1) MCT active metabolite has been found to cause EC apoptosis;[34] (2) Further, using TUNEL staining, EC apoptosis in MCT-treated rats[35] was detected from smaller arterioles to larger vessels in a time dependent manner in lung sections. This finding supports the hypothesis proposed by Todorovich-Hunter et al. that the distal pulmonary arteriolar endothelium is more vulnerable to apoptosis given the lack of supporting SMCs;[36] and (3) Apoptosis inhibitors significantly blunt the increase in right ventricular systolic pressure (RVSP) and is positively correlated with

improved pulmonary hemodynamic parameters in these MCT treated rats.[37]

Death and survival are probably the most complicated cellular events in mammalian cells. Although novel concepts emerge every decade, the detailed regulation of "survival" or "death" remains largely unclear. Initially, necrosis was described, and apparently underestimated, as the complexity of the tight controlled machinery on cell fate.[38,39] Apoptosis, described as programmed cell death, was demonstrated in the 1990s.[11-15] The role of apoptosis in PH has been widely studied. As noted above, many investigators believe that environmental stimulators which induce EC apoptosis is the initial trigger of the cascade of pulmonary vascular remodeling, followed by marked cell proliferation which ultimately leads to PH and right heart failure.[5-10] However, how this paradoxical switch from apoptosis to proliferation occurs in certain ECs remains unknown. Recently, autophagy, a widely existing yet newly delineated cellular event, provides more understandings on the fine control of cell fate.

Autophagy and the cross-talk between autophagy and apoptosis in ECs

In the past five years, autophagy has caught robust attention from cell biologists. Autophagy includes macroautophagy, microautophagy, and chaperone-mediated autophagy.[17-20] In this article, we will focus on macroautophagy, which is the most relevant to cellular homeostasis involving PH. Macroautophagy is featured by the formation of autophagosomes. The unwanted cellular organelles are enclosed into the double membrane autophagosomes and delivered to lysosomes for degradation and/or recycling.[17-20] Autophagy has been shown to be critical in controlling cell survival and cell death.[17-25] It has been well known as a prosurvival factor under starvation.[17-25] On the other hand, excessive autophagy results in "autophagic cell death" (ACD) which can occur along with apoptosis and necrosis.[28-29] Emerging evidence has demonstrated that the intricate relationship and tight control between apoptosis and autophagy plays a key factor in the development of pulmonary vascular remodeling and PH.

Autophagy is a fine controlled, conserved cellular event. Many environmental factors can upregulate or downregulate EC autophagy.[39,40] One of the most commonly reported offenders is reactive oxygen spices (ROS), a derivative from oxidative stress.[40] Previous studies also demonstrate that oxidative stress and ROS play crucial roles in the pathogenesis of PH.[41] Dysregulation of the ROS redox cycle impairs vascular tone and results in cell proliferation and obliteration of the vasculature.[41] In mammalian cells, mitochondria are the major organelles which generate ROS and regulate cell fate. Mitochondria mediated apoptosis via caspase 9 and cytochrome C release has been reported

in the ROS induced EC death.[42,43] Importantly, autophagy has been shown to be essential in mitochondria associated activities. Autophagy-mediated recycling of cellular substrates can provide "fuel" to mitochondria for energy generation and promote cell survival and proliferation.[44] Further, mitophagy, a specific form of autophagy (selective autophagy) that targets mitochondria, has been speculated to promote cell survival by clearance of damaged mitochondria.[45] Thus, malfunction of autophagy results in cell death due to multiple mechanisms.

The formation of autophagosomes is initiated by Beclin-1 and LC3, also involved in the Atg (Atg4, Atg12, Atg5, Atg16) complex.[20-25] The outer membrane of the autophagosome fuses with lysosomes resulting in degradation of its contents.[20-25] There is emerging evidence suggesting that both Beclin-1 and LC3 are involved in the regulation of pulmonary vascular remodeling in PH.

Beclin-1. As a key component of autophagy, Beclin-1 closely interacts with the members in apoptotic pathways. One example is the Bcl-2 family. Initially, Nguyen et al. found that antiapoptotic protein Bcl-2 forms complexes with Beclin-1.[46] Bcl-2 and Bcl-xL complex with Beclin-1 and lack of Beclin-1 accelerates caspase dependent apoptosis.[46] Deletion of Beclin-1 decreased plasminogen-induced autophagy and accelerated apoptosis, suggesting that interruption of autophagy leads to an antiangiogenic effect in endothelial cells.[47] Additional studies further supported that Beclin-1 interacts with apoptosis in ECs. The same group as above demonstrated that endostatin, an angiogenesis inhibitor, activates autophagy via increasing Beclin-1. Their studies support that inhibition of autophagy promotes a switch to apoptosis.[47] On the other hand, Lee et al. showed that Beclin-1 hemizygous (Becn1 (+/-)) mice develop an increased angiogenesis after hypoxia.[48] Beclin-1 hemizygous ECs carry a feature of increased proliferation, migration, and tube formation comparing with wild-type cells.[48] Pulmonary vascular remodeling in PH is a complex and progressive process involving in multiple cells and different stages. These differential observations suggest that the stages and cell types have to be taken into consideration when evaluating the role of autophagy in PH. The above studies on Beclin-1 and cell death/survival implicate the complexity of autophagy-apoptosis-proliferation network and warrant detailed delineations.

LC3/Atg8. Accumulation of microtubule-associated protein 1 light chain 3-II (LC3-II) has been thought of as a good indicator of autophagy.[20-25] LC3 I/LC3 II conversion is reported to promote EC survival and confers angiogenic and antiapoptotic effects.[49] Kim et al.[50] found that in ECs under hypoxic conditions, LC3-II conversion is detected along with caspase-3 activation. They further concluded that autophagy mediates cell death of ECs in a

dose-dependent manner. Similarly, LPS effectively induces autophagy in cultured ECs.[51] After exposure to LPS, in ECs, there are robust cross-talks between apoptosis and autophagy. For example, BIRC2, an apoptosis inhibitor, promotes LPS-induced autophagy in ECs.[52] Interestingly, Lee et al. have shown elevated lipid-conjugated LC3B and autophagosomes in human PH lungs and in hypoxia or MCT induced PH using murine models.[53] The elevated LC3B is regulated by Egr-1. In this study, the authors further showed that LC3B null mice or Egr-1null mice display exaggerated PH during hypoxia.[53] Mechanistically, deletion of LC3B increases reactive oxygen species (ROS) production and hypoxia-inducible factor-1α (HIF-1α) stabilization, thus resulting in EC and SMC cell proliferation.[53] Therefore, they concluded that autophagic protein LC3B exerts a protective function during the pathogenesis of PH, through the regulation of hypoxic cell proliferation. The same group also found that LC3B interacts with apoptotic pathways by directly binding with apoptosis regulator proteins, such as Fas, via lipid raft marker protein cav-1 in the lipid rafts.[53] As shown in Figure 1, LC3B and Fas competitively bind with cav-1 which functions as the platform and switchboard of cell signaling.[54,55] The Fas-cav-1-LC3B axis thus is crucial in maintaining appropriate cross-talks between autophagy and apoptosis, as well as cellular homeostasis. Interaction between Fas and cav-1 leads to the aggregation of Fas complex and activation of death signaling cascade. Meanwhile, more unbound LC3B is released from cav-1. After interacting with p62, the LC3B/p62 complex induces selective autophagy and recycling of the unwanted death signaling proteins. In this model, Fas mediated apoptosis seems to induce LC3B mediated cytoprotective autophagy and attempts to save the cells by augmented autophagy via LC3B. However, how these interactions are altered in ECs during PH pathogenesis require more investigations.

Atg5, 7, and 12. Besides Beclin-1 and LC3B, more autophagic components and their role in EC death have been shown.
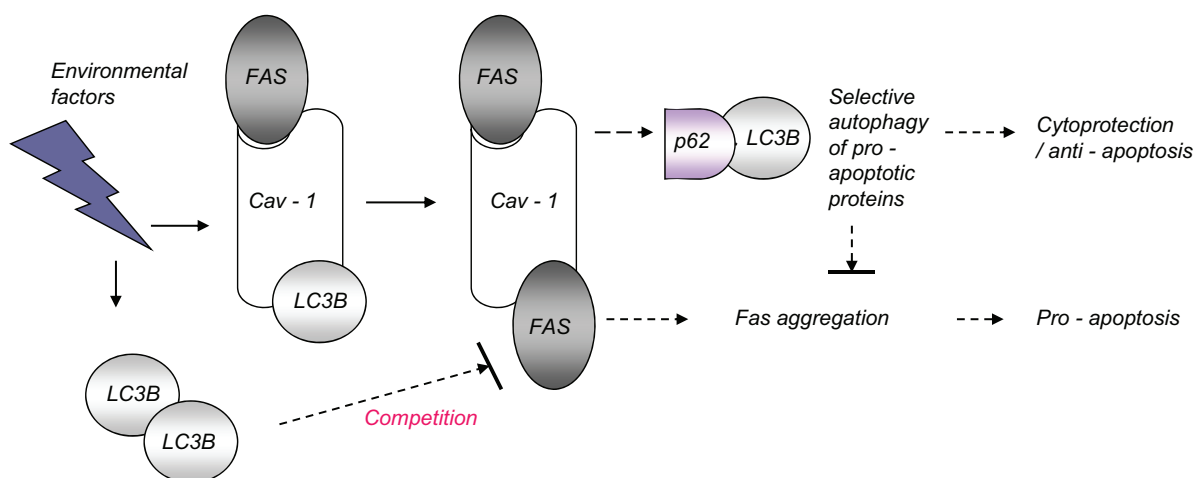
Csordas et al. demonstrated that deletion of autophagy mediator Atg5 significantly delays cell death.[56] Du et al. further concluded that an increase in autophagy is protective under hypoxic and chronic ischemic conditions.[57] Deletion of Atg5 using siRNA has been shown to reduce angiogenesis.[57] In contrast, induction of autophagy by overexpression of ATG5 increased BAECs tube formation and migration.[57] Additionally, inhibition of autophagy impairs VEGF-induced angiogenesis.[58] Thus far, most of the above studies indicate that apoptosis induces autophagy as a "feedback" to confer self-protection. Recently, more direct evidence on the other side of cross-talk between autophagy and apoptosis emerges. Apoptosis, in certain scenarios, induces autophagic cell death (ACD). For example, the well-known apoptosis regulator family Bcl-2 family proteins have been proposed to control ACD.[59] A novel Bcl-2 homology domain 3 (BH3)-only protein, apolipoprotein L1 (apoL1), induces autophagic cell death (ACD) determined by the formation of autophagic vacuoles and the translocation of LC3-II from the cytosol to the autophagic vacuoles.[59] As shown in Figure 2, ApoL1 is found to induce ACD in mouse endothelial cells via Atg5 and/or 7 mediated pathway.[59] Furthermore, ApoL1 is inducible by interferon-gamma and tumor necrosis factor-alpha (TNF-α) in human umbilical vein endothelial cells (HUVEC).[59,60] Thus, in this scenario as illustrated in Figure 2, unlike the Fas mediated pathway above, the TNF-α mediated apoptosis synergizes ACD via apoL1, thus resulting in unavoidable cell death. We now speculate that different environmental factors trigger differential cross-talks between autophagy and apoptosis, thus resulting in differential outcomes on ECs. This hypothesis potentially explains the complexity of pathogenesis of pulmonary vascular remodeling in PH.

Current data support that autophagy actively participates in vascular remodeling involved in ECs in the development of PH. However, it is not entirely clear which pathway is involved in the emergence of apoptosis resistant EC cell proliferation.
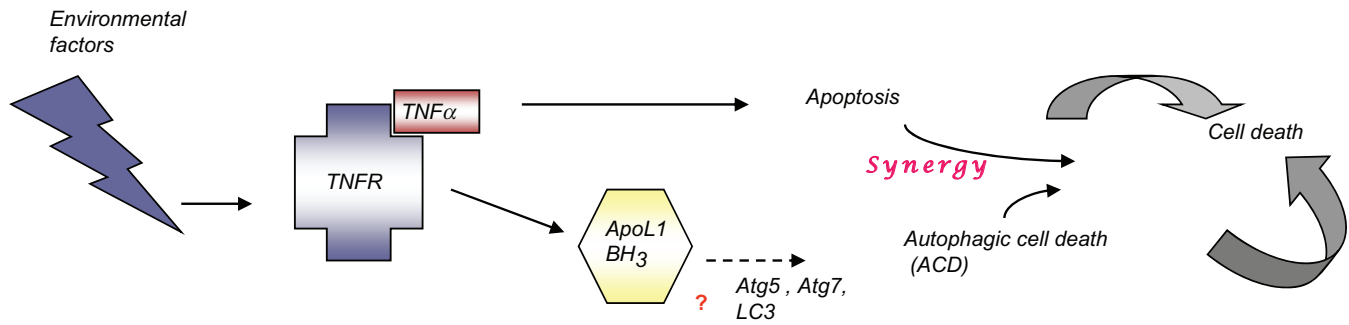

Figure 1: LC3B confers cytoprotection via Fas apoptotic pathways.

Figure 2: Autophagic markers potentially synergize apoptosis.

SMC proliferation in PH

In pulmonary vascular remodeling associated with PH, unlike ECs, SMC proliferation has been a more consistent feature. The thickening of the media occurs at all levels of the pulmonary arterial tree.[5-10] Both hypertrophy and hyperplasia are observed within the SMCs in media, along with the increased deposition of new smooth muscle into the partially muscular and nonmuscular peripheral arteries (muscularization). SMC in idiopathic PH (PAH) has shown characters observed in cancer cells, i.e., decreased apoptosis and increased proliferation.[5-10] Death in the normal turnover of SMCs allows for the clearance of cells with improper growth and proliferation. However, this turnover of SMCs is impaired in pulmonary vascular remodeling in PH. Apoptosis of SMCs occurs in vivo under both physiological and pathological settings. The modulation of apoptosis in pulmonary vasculature is complicated and plays essential effects on the pathologic SMC proliferation in PH. Numerous apoptotic pathways have been studied to delineate the regulation of SMC proliferation. Theoretically, apoptosis inducers should reverse or eliminate the vascular remodeling in PH, if delivering to overgrown SMCs. In fact, in animal experiments, inducing apoptosis of hypertrophied pulmonary arterial SMC in intact pulmonary vessels indeed prevents the progression of the medial hypertrophy.[4-9] However, in human pulmonary arterial SMCs, significant resistance to apoptosis inducers is shown.[8-12] In addition, the balance of cell apoptosis and proliferation in SMCs are crucial in maintaining normal vascular structure and function. Therefore, we speculate that besides apoptosis, other cellular and molecular mechanisms participate in the regulation of SMC death or proliferations, at least in humans. Autophagy, given its dual role in cell death and proliferation, perfectly meets the standard as regulatory machinery in PH pathogenesis, along with apoptosis. It's cross-talk with apoptosis in pulmonary vascular SMCs warrant further studies, and may potentially provide novel insights in the pathogenesis of PH. Nevertheless, the role of autophagy in pulmonary vascular smooth muscle cells remains under-investigated at this moment. Sparse reports have been published so far. Therefore, most of this review on the effects of autophagy, apoptosis, and their cross-talk is from studies using SMCs from a variety of sources, including pulmonary vascular and systemic vascular sources, as well as airway SMCs.

Although SMC proliferation has been well known as a characterized feature in PH, the cellular mechanisms involved in this uncontrolled cell growth remain unclear. Autophagy, as a process which involves both cell death and survival, has been speculated to mediate SMC proliferation. Using aorta smooth muscle cells, an increased amount of LC3 and autophagy are detected during lipid peroxidation.[53] Autophagy in these SMCs apparently confers cytoprotection in aldehyde-induced death via removing the aldehyde-modified proteins.[61] Thus, autophagy promotes survival of arterial SMCs under conditions associated with excessive lipid peroxidation.

PAH has been well known to have a predisposition for women.[1-5] Strikingly, recent studies revealed that the expression of LC3II protein is much higher in male derived vascular SMCs than in female derived SMC.[62] Similar findings are also reported on the levels of Beclin 1.[62] These observations raise the question on whether female derived SMCs use autophagy as an adaptive method to counteract unfavorable environmental stimuli. Upon loss of control on this protective machinery, SMCs proceed to "tumor like" proliferation.

Autophagy and autophagy/apoptosis cross-talk in SMCs

Both autophagy and apoptosis fulfill crucial regulatory roles to maintain cellular homeostasis. These two cellular events are not isolated, but cross-talk constantly. Their regulation understandably shares common upstream regulators. In human airway smooth muscles (HASM), p53, a well known anti-apoptotic gene product, facilitates autophagosome formation via the Atg5-12 complex. Further, integrin/CD44 and p38 MAPK pathways have been shown to stimulate autophagy.[63] On the other hand, chemical inhibition of autophagy increased caspase activation and cell death.[64,65] Deletion of Atg5 using shRNA illustrates proapoptotic effects.[57] A rapidly induced autophagy significantly delayed
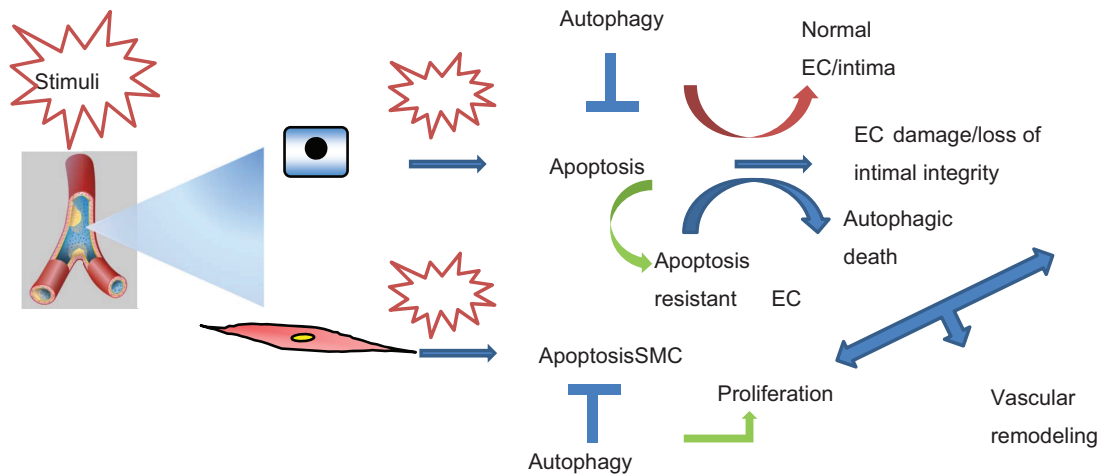
Figure 3: Proposed cross-talk between autophagy and apoptosis in pulmonary vascular remodeling and PH.

apoptosis,[64,65] further confirming the cross-talk between apoptosis and autophagy.

Although recent studies focus on Atg5-12 complex and autophagy in SMCs, LC3B has been reported to play a critical cellular function in SMCs in as early as 1997. Increased fibronectin synthesis is required for the migratory phenotype of SMCs in ductus arteriosus.[66-68] Interestingly, LC3 has been demonstrated to facilitate sorting of fibronectin mRNA onto rough endoplasmic reticulum and translation.[66-68] Further, Xu et al. demonstrated that inhibition of autophagy enhanced both cell apoptosis and necrosis.[64] Additionally, modulation of autophagy affected the cellular organelle stress, suggesting that autophagy in SMCs functions as a cellular defense mechanism via the degradation of dysfunctional organelles such as mitochondria and endoplasmic reticulum.

Although only a small amount of data has been presented on the role of autophagy in SMC proliferation so far, more consistent functions have been revealed comparing the role of autophagy in ECs. Autophagy, via LC3 or ATg5/12 complex, seems to confer protective effect against apoptosis and cell death via a variety of cell signals, including p53, integrin/p38 MAPK, and caspase dependent pathways. Any environmental factors which upregulate these autophagic components presumably lead to activate autophagy in SMCs and subsequently suppress apoptosis, eventually resulting in uncontrolled proliferation of SMC. As shown in Figure 3, augmented autophagy or autophagy flux is supposed to promote autophagic cell death (ACD) to maintain the cellular homeostasis. Why ACD does not occur in SMCs during the development of PH requires further exploration. One of the hypotheses is that "selective" autophagy is the major type of autophagy in SMCs; the selective degradation of damaged organelles in SMCs, such as damaged mitochondria, not only provides more "fuel" as a source of energy but also prevents ROSs generation

induced apoptosis. The combination of these effects results in uncontrolled proliferation of SMCs. Therefore, simple induction of SMC apoptosis may have underestimated the effects of "self-correcting machinery," i.e., the cross-talks between autophagy and apoptosis in SMC. It is not difficult to understand why induction of apoptosis in SMCs is lack of efficacy in treating human PH. Further delineation of the role of autophagy and the cross-talk between autophagy and apoptosis in SMC proliferation may provide novel targets in developing therapeutic agents against PH.

In summary, both apoptosis and autophagy are crucial cellular events occurring in the vascular remodeling of PH. Autophagy is an inducible process upon encountering an unfavorable environmental stimulation in PAECs and SMCs. Emerging evidence suggest that autophagy and apoptosis cross-talk via multiple pathways at the molecular level. The result of an environmental stimulation on pulmonary vascular remodeling may be dictated by the cross-talks between autophagy and apoptosis of ECs and/or SMCs (Fig. 3).

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