Emerging role of mitophagy in cardiovascular physiology and pathology

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ABSTRACT

Healthy mitochondrial function is imperative for most tissues, but especially those with a high energy demand. Robust evidence linking mitochondrial dysfunction with cardiovascular disease has demonstrated that mitochondrial activity is highly relevant to cardiac muscle performance. Mitochondrial homeostasis is maintained through coordination among the processes that comprise the so-called mitochondrial dynamics machinery. The most-studied elements of cardiac mitochondrial dynamics are mitochondrial fission and fusion, biogenesis and degradation. Selective autophagic removal of mitochondria (mitophagy) is essential for clearing away defective mitochondria but can lead to cell damage and death if not tightly controlled. In cardiovascular cells such as cardiomyocytes and cardiac fibroblasts, mitophagy is involved in metabolic activity, cell differentiation, apoptosis and other physiological processes related to major phenotypic changes. Modulation of mitophagy has detrimental and/or beneficial outcomes in various cardiovascular diseases, suggesting that a deeper understanding of the mechanisms underlying mitochondrial degradation in the heart could provide valuable clinical insights. Here, we discuss current evidence supporting the role of mitophagy in cardiac pathophysiology, with an emphasis on different research models and their interpretations; basic concepts related to this selective autophagy; and the most commonly used experimental approaches for studying this mechanism. Finally, we provide a comprehensive literature analysis on the role of mitophagy in heart failure, ischemia/reperfusion, diabetic cardiomyopathy and other cardiovascular diseases, as well as its potential biomedical applications.

1. Introduction

The heart is highly dependent on mitochondrial metabolism to meet its tremendous energy requirements. Cardiac mitochondria —which occupy 30% of the cardiomyocyte volume— synthesize 6–7 kg of ATP per day by oxidative phosphorylation, using fatty acids as their primary substrate (Taegtmeyer, 1994). Matching cardiac ATP supply to demand on a beat-to-beat basis is critical to fulfilling the demands of cardiac excitation-contraction coupling. Beyond their critical role as an energy source, cardiac mitochondria serve as calcium reservoirs, participate in apoptosis and necrosis pathways and function as a metabolic hub for the Krebs cycle and fatty acid β-oxidation (Spinelli and Haigis, 2018). Maintaining a healthy mitochondrial population is of paramount importance for cardiac homeostasis, since damaged mitochondria produce less ATP and generate dangerous amounts of reactive oxygen species (ROS). Accumulated ROS may damage mitochondrial DNA, membrane lipids and respiratory complex proteins, leading to a catastrophic feedforward cycle of oxidative damage and ultimately cell death (Whelan et al., 2010). This general mechanism is particularly relevant in terminally-differentiated tissues such as the brain and heart, as demonstrated in neurodegenerative and cardiovascular diseases (López-Otín et al., 2013).

Several homeostatic mechanisms promote proper mitochondrial network functioning. For instance, a repertoire of mitochondrial chaperones and quality-control proteases ensures correct folding of mitochondrial proteins in a challenging oxidative environment. If the capacity of these intrinsic mechanisms is surpassed, cells mount a dedicated mitochondrial unfolded protein response that allows for nuclear expression of mitochondrial chaperones and proteases to re-establish protein homeostasis (Pellegrino et al., 2013). Other mechanisms involve changes in mitochondrial morphology. Mitochondrial fusion, for example, contributes to maintaining a robust network, enabling the

exchange of matrix enzymes and mitochondrial DNA between individual mitochondria, and mitochondrial biogenesis allows for continuous incorporation of new mitochondrial units into the network (Eisner et al., 2018). This complex interplay provides a critical flexibility that facilitates cellular responses to various metabolic conditions. However, if all of these mechanisms to rescue a damaged mitochondrion fail, a removal mechanism known as mitophagy allows autophagosomes to selectively eliminate dysfunctional organelles, maintaining mitochondrial network fitness.

Degradation of mitochondria is only one piece of the mitochondrial physiology puzzle. Mitophagy is not an isolated phenomenon, as it is connected to processes such as modulation of dynamics, subcellular location and mitochondrial biogenesis (Nunnari and Suomalainen, 2012). Furthermore, mitochondrial morphology is intimately linked with mitochondrial degradation. The shape of a mitochondrion varies greatly depending on its metabolic status (Eisner et al., 2018), and these morphological alterations rely on specific machinery. Mitochondrial elongation, for instance, is achieved by fusion of adjacent mitochondria. Mitofusins (MFN) 1 and 2 are responsible for outer mitochondrial membrane (OMM) fusion (Santel and Fuller, 2001; Chen et al., 2003; Eura et al., 2003), while optic atrophy protein 1 (OPA1) mediates fusion of the inner mitochondrial membrane (IMM) (Olichon et al., 2003; Meeusen et al., 2006). Mitochondrial fragmentation, on the other hand, depends on the activity of a cytosolic molecule, dynamin-related protein 1 (DRP1), which is encoded by *DNM1L* in humans and *Dnm1l* in mice (Pitts et al., 1999; Smirnova et al., 2001). DRP1 translocates into mitochondria to form spiral-like structures that strangle the organelle until it divides (Smirnova et al., 2001; Ingerman et al., 2005). Strategic translocation of DRP1 to mitochondrial constriction sites is mediated by several OMM proteins, including FIS1, MFF, MiD49 and MiD51 (James et al., 2003; Otera et al., 2010; Losón et al., 2013).

In mammalian cells, mitophagy occurs in coordination with mitochondrial fission. Inhibition of mitochondrial fission by a dominant-negative form of DRP1 prevents mitophagy in pancreatic cells (Twig and Shirihai, 2011). Moreover, mouse embryonic fibroblasts with impaired fusion due to *Opa1* or *Mfn2* knockout exhibit increased mitophagy in response to starvation (Rambold et al., 2011). Although it is tempting to assume that the only role of fission in mitophagy is to isolate and subsequently remove damaged mitochondria, the relationship between mitochondrial fission and mitophagy appears to go well beyond mitochondrial morphology, extending to modulation of mitochondrial potential and bioenergetics. Mitochondrial fission is an asymmetric process that leaves one daughter mitochondrion with high membrane potential, which soon re-fuses into the network, and another daughter mitochondrion with low membrane potential, which typically undergoes mitophagic clearance (Twig and Shirihai, 2011), providing more evidence of the complex relationship among mitochondrial homeostatic mechanisms.

In tissues such as the myocardium that rely mainly on mitochondrial metabolism, it is easy to understand how impaired mitophagy might produce cumulative oxidative damage to the mitochondrial network. Conversely, high metabolic demands on these tissues may exhaust the mitochondria, leading to elevated rates of mitochondrial degradation rate that surpass the capacity of mitochondrial biogenesis to replenish the network. In either scenario, unbalanced mitochondrial turnover results in bioenergetic failure and cell death (Bravo-San Pedro et al., 2017).

All eukaryotic cells depend on mitophagy to respond to changes in the cellular environment, particularly when facing starvation, as rapid recycling of energetic substrates is critical to ensure cell survival under these conditions. Furthermore, mitophagy is required for the lifespan extension effect of caloric restriction. Cardiovascular cell types are no exception to this dependence on mitophagy, raising the exciting possibility that the mechanism may be a target for regulating the impact of aging in the heart (Shirakabe et al., 2016a). In this review, we critically discuss the role of mitophagy in cardiovascular physiology and

pathology, focusing on the therapeutic potential of mitophagy modulation to respond to unmet challenges posed by the cardiovascular disease epidemic in the Western world.

2. The biology of mitophagy

2.1. General overview and core autophagy machinery

All living organisms need to eliminate waste material. Whether to prevent accumulation of toxic molecules, make room for incorporation of new elements or reuse the building blocks of old structures, both simple and complex organisms have developed sophisticated systems to eliminate and recycle components that are no longer needed. Some of these processes take place at the cellular level, where many degradation mechanisms have been described (Dikic, 2017). Autophagy is one such process, in which cellular components ranging from macromolecules to entire organelles are transformed into smaller constituents (Klionsky, 2007; Klionsky et al., 2011).

From a morphological point of view, eukaryote autophagy can be categorized as macroautophagy, microautophagy and chaperone-mediated autophagy (Parzych and Klionsky, 2014). In all three cases, the structure to be degraded, or *cargo*, will ultimately be delivered to lysosomes, where several types of proteases, lipases and glycosidases, known as lysosomal hydrolases, will perform the catabolic reactions (Xu and Ren, 2015). The simpler end-products of the lytic action of these hydrolases will translocate into the cytosol with the help of permeases, where they will be used as metabolites for energy production or anabolic substrates (Xu and Ren, 2015). As described in the following paragraphs, the most distinctive and visual feature of macroautophagy is the formation of a double-membrane vesicle called an autophagosome, which surrounds the cargo and moves it towards the lysosomes (Eskelinen et al., 2011). For the purposes of this review, only macroautophagy will be discussed and, henceforward, will be simply referred to as autophagy.

Autophagy can also be classified according to the specificity of the cargo to be degraded. *Non-selective* autophagy is usually activated in response to reduced nutrient availability or certain types of cellular stress and consists of engulfment and further degradation of bulk cytosolic material (Klionsky et al., 2011; Feng et al., 2014). On the other hand, *selective* autophagy refers to the *labeling* of specific substrates, ranging from unfolded or damaged protein complexes to whole subcellular structures, which are later destined for lysosomal compartments (Gatica et al., 2018). This type of autophagy involves the ubiquitin (Ub) and Ub-like conjugation and activation systems, which provide the molecular tags and adaptors involved in cargo recognition (Shaid et al., 2013). It is worth noting, however, that these types of autophagy may overlap, establishing an intricate network of regulation. Irrespective of the way the cargo is selected for degradation, both selective and non-selective autophagy consist of the same stages: initiation, elongation, autophagosome-lysosome fusion, degradation and termination (Kim and Lee, 2014; Parzych and Klionsky, 2014).

The ATG (autophagy-related) family of proteins is involved in all of the aforementioned stages. The Ser/Thr-kinase complex ULK, comprised of ULK1/ATG1, ULK2, ATG13, FIP200 and ATG101, incorporates upstream signals by undergoing activating or inhibiting phosphorylation from the metabolic sensors AMPK and mTOR, respectively (Jung et al., 2009; Kim et al., 2011; Wong et al., 2013). Once activated, ULK1 is recruited to the endoplasmic reticulum (ER) and phosphorylates the class III PI3K complex I lipid kinase (Russell et al., 2013). This complex includes VPS34/PIK3C3 as the catalytic subunit, which, in association with BECN1/ATG6 (commonly known as Beclin1, encoded by *Becn1* in mice), ATG14 and other proteins, catalyzes the conversion of phosphatidylinositol phosphate (PIP) into PI3P (Russell et al., 2013; Hurley and Young, 2017). Accumulation of PI3P in specific ER sites is considered a key event in the initiation of autophagy, as it drives formation of the *phagophore*, the structural precursor of the

autophagosome (Axe et al., 2008). This change in lipid composition favors recruitment of ubiquitin-like ATG8 proteins into the expanding phagophore. ATG8 proteins, which include LC3s (microtubule-associated protein 1 light chain 3A and 3B) and GABARAP in mammals, play a pivotal role in autophagosome formation and expansion while also serving as a component of cargo recognition (Geng and Klionsky, 2008). ProLC3 is first cleaved by the protease ATG4B, generating the LC3-I form. Then, through a series of reactions similar to those of the Ub conjugation system (involving ATG7, ATG3 and the ATG5-ATG12-ATG16L complex), LC3 proteins are incorporated into the expanding vesicles through the addition of a phosphatidylethanolamine moiety to its C-terminal glycine, resulting in a variant known as LC3-II (Kabeya et al., 2000; Klionsky and Schulman, 2014).

Crucially, ATG8 and its homologs have the ability to interact with various autophagy proteins, including ULK1, ATG4B and ATG7, and move them into the phagophore (Behrends et al., 2010; Klionsky and Schulman, 2014). As noted, these proteins are involved in autophagosome formation and expansion and therefore can be conceived of as autophagy regulators. However, ATG8/LC3 also participates in substrate recognition by helping to guide cargo to the correct compartment (i.e., autophagosomes) for later delivery to lysosomes. This feature of LC3 confers it with an essential role in selective autophagy, as discussed below.

2.2. Mechanisms of selective autophagy

The delivery of specific cargo to the autophagosome relies on two important processes: placement of a tag-signal on the substrate to be degraded, and recognition of the tagged substrate by the autophagosome. The former is usually the result of ubiquitination, whereas the latter depends on autophagy adaptor proteins, also known as autophagy receptors (Shaid et al., 2013; Rogov et al., 2014).

Ubiquitination is the enzyme-mediated covalent bonding of Ub (a 76 amino acid-long polypeptide) to a misfolded or damaged protein. Various activating (E1), conjugating (E2) and Ub-ligase (E3) enzymes lead to the addition of one or more Ub units, individually or as part of a chain (Kleiger and Mayor, 2014). Ub attaches to its target through specific lysine residues. Depending on which of the seven Ub Lys residues are involved in the labeling, the ubiquitinated protein participates in a signaling event or is sent for degradation by the ubiquitin-proteasome system or autophagy (Dikic et al., 2009). In fact, forced addition of mono-Ub moieties to cytosolic or organelle membrane-bound proteins is enough to target those cargo for delivery the autophagosome and degradation by lysosomes (Kim et al., 2008a).

The proteins involved in recognizing these molecular tags are the counterparts to the proteins that participate in the labeling system. These adaptor proteins usually contain a UBA (ubiquitin-associated) domain that allows them to recognize Ub linked to cargo proteins (Rogov et al., 2014). To target their ubiquitinated cargo to autophagosomes, autophagy receptors also possess a LC3-interacting region (LIR) motif that is highly conserved from yeast to mammals (Noda et al., 2010; Johansen and Lamark, 2011). The sequestosome protein (SQSTM1, commonly referred to as p62) binds directly to poly-ubiquitinated proteins through the UBA domain contained in its C-terminal region (Vadlamudi et al., 1996). On the other side, p62 is able to interact directly with several ATG8 proteins, including LC3A, LC3B and GABARAP, targeting poly-Ub protein aggregates to autophagosomes (Bjorkoy et al., 2005; Pankiv et al., 2007). NBR1 shares some structural features with p62, including the UBA and LIR domains, and therefore also serves as an autophagy receptor (Waters et al., 2009; Noda et al., 2010).

Both the tagging and recognition systems are essential for selective cargo removal through autophagy. As depicted in Fig. 1, mitochondrial elimination is an example of how cargo selectivity can be achieved and how the machinery for specific mitochondrial degradation is intertwined with other aspects of mitochondrial biology.

3. Mitophagy: selective mitochondrial autophagy

As noted above, even whole organelles can be targeted to autophagosomes for lysosomal degradation. Mitochondrial clearance through autophagy is called mitophagy. Mitophagy can be seen as the opposite of mitochondrial generation or biogenesis. Together, mitochondrial biogenesis and mitophagy regulate cellular mitochondrial content and therefore constitute a crucial point of metabolic and signaling regulation.

The molecular mechanisms underlying mitophagy have been established only in recent years. Originally, mitophagy was seen as a mitochondrial degradation mechanism triggered by a reduction in mitochondrial membrane potential (Youle and Narendra, 2011). Under these conditions, the Ser/Thr protein kinase PINK1 (PTEN-induced putative kinase 1) is stabilized at the OMM due to reduced basal degradation (Narendra et al., 2010a; Youle and Narendra, 2011). In turn, PINK1 phosphorylates the E3-ligase Parkin (encoded by *PRKN* in humans and *Prkn,* alias *Park2,* in mice), leading to its activation and mobilization from the cytosol to mitochondria (Narendra et al., 2008; Shiba-Fukushima et al., 2012). Furthermore, PINK1 also phosphorylates ubiquitin, serving as a recruitment signal for Parkin, as well as increasing its enzymatic activity (Koyano et al., 2014). Once at the OMM, Parkin ubiquinates several mitochondrial proteins, such as MFN1, MFN2 (Gegg et al., 2010) and VDAC1 (Geisler et al., 2010; Narendra et al., 2010b), generating a positive feedback loop for recruitment of more Parkin units (Yamano et al., 2016). Another Parkin target is the mitochondrial protein NIX, also known as BNIP3L (BCL2-interacting protein 3 like). This protein belongs to the BH3-only Bcl-2 family, and its role in mitochondrial removal was first discovered in the erythroid cell line (Schweers et al., 2007; Sandoval et al., 2008). In response to depolarization, NIX/BNIP3L is directly ubiquitinated by Parkin, which leads to recruitment of the autophagy receptor NBR1 and LC3-coated vesicles to the mitochondria (Gao et al., 2015). NIX is required for mitophagy when mitochondrial activity increases (often due to a change in substrate availability), to eliminate damaged mitochondria and promote mitochondrial renewal (Melser et al., 2013). Parkin-independent mechanisms have also been described, wherein PINK1 phosphorylates Ub-targeted mitochondrial proteins, triggering the recruitment of the autophagy adaptors NDP52 and optineurin (Lazarou et al., 2015). Therefore, ubiquitination of OMM proteins is a signal that is recognized by autophagy receptors, promoting the delivery of mitochondria to autophagosomal vesicles.

Although the most studied, the PINK1/Parkin mitophagic pathway is not the only known mechanism for mitochondrial degradation. For instance, BNIP3 (BCL2-interacting protein 3), which is highly similar to the NIX protein, also mediates mitophagy (Quinsay et al., 2010; Rikka et al., 2011). BNIP3 is inserted at the OMM through a transmembrane domain shown to be essential for its pro-autophagy function (Hanna et al., 2012). How BNIP3 is activated and drives mitochondrial removal is still an open question, although the mechanism may involve altered bioenergetics as its overexpression leads to reduced ATP levels, as well as a reduction in some electron transport chain subunits (Rikka et al., 2011). As is the case for NIX, at least some of actions of BNIP3 also involve the PINK1/Parkin pathway. BNIP3 is able to interact directly with PINK1, stabilizing its full-length form and therefore promoting its ability to recruit Parkin to the mitochondria (Lee et al., 2011a; Zhang et al., 2016).

The mitochondrial protein FUNDC1 (FUN14 domain containing 1) has also been linked to mitophagy (Liu et al., 2012; Chen et al., 2016). This protein is located at the OMM, and its overexpression leads to enhanced mitochondrial degradation (Liu et al., 2012). Site-specific phosphorylation of FUNDC1 has been described as a mechanism for increasing (Wu et al., 2014a) or decreasing (Chen et al., 2014, 2016; Liu et al., 2012) its mitophagic actions under hypoxia or mitochondrial membrane potential dissipation. The inhibiting phosphorylation at Ser13, for instance, which is generated by casein kinase 2 (CK2) and
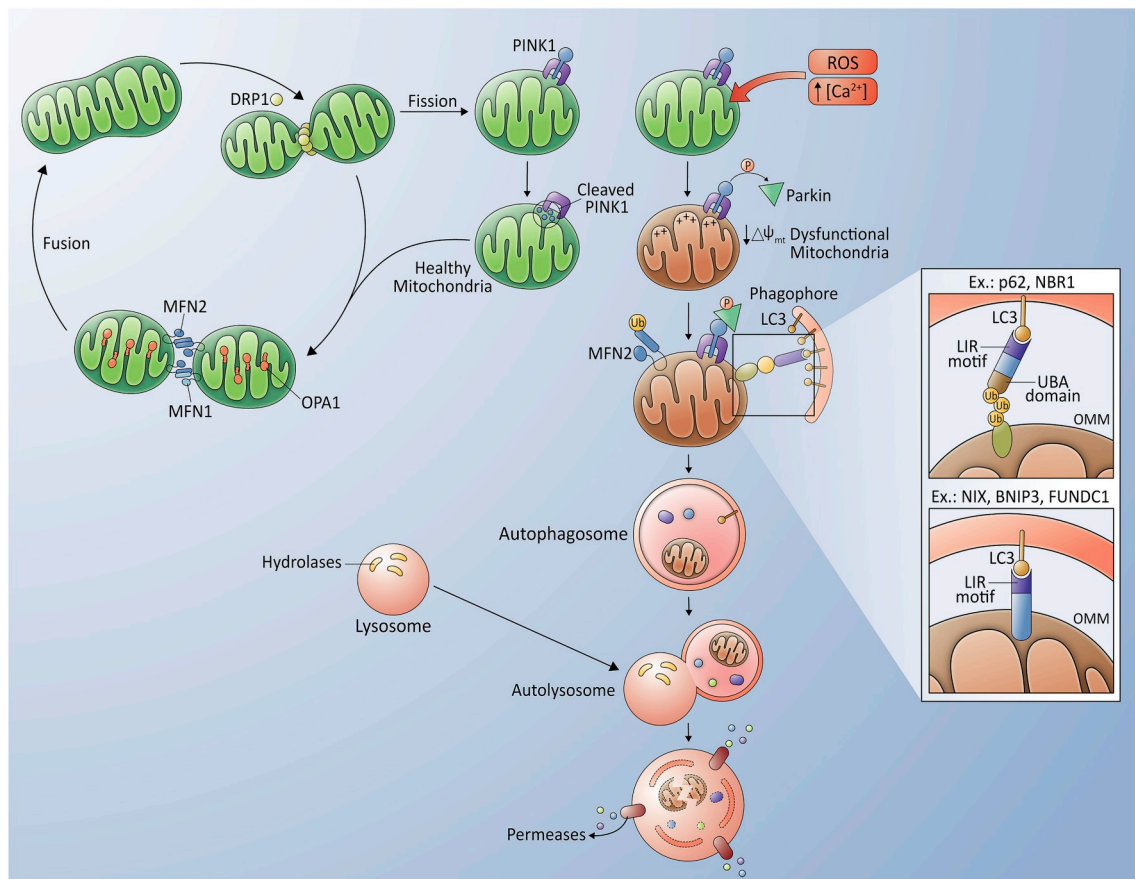
Fig. 1. Selective mitochondrial degradation (mitophagy) and its link to mitochondrial dynamics. Mitochondria are continuously adapting their morphology through fusion and fission. Under favorable conditions, the protein kinase PINK1 is constitutively degraded, and this healthy organelle is able to rejoin the mitochondrial network via MFN1-MFN2-OPA1 fusion proteins. In response to various stressors, mitochondria fragment into smaller entities through DRP1-mediated fission. Reduction of mitochondrial membrane potential results in stabilization of PINK1, which in turn phosphorylates and recruits the E3-ligase Parkin. Polyubiquitinated proteins serve as a "tag" for autophagy receptors that interact with the autophagosome-resident protein LC3. After the mitochondrion is engulfed, the autophagosome fuses with lysosomes, which pour their hydrolytic enzymes into the new compartment. End-products of the catabolic reactions are ultimately released into the cytosol with the assistance of autophagolysosomal permeases. Magnification shows two different types of autophagy receptors/adaptors: top) Polyubiquitinated proteins at the mitochondria are recognized by an autophagy adaptor, which also interacts with LC3 proteins (i.e., p62 or NBR1); bottom) Resident proteins at the OMM directly interact with autophagosome-localized LC3 proteins, without the need for polyubiquitinated proteins (i.e., NIX, BNIP3 or FUNDC1).

removed by the phosphoglycerate mutase family member 5 (PGAM5), promotes FUNDC1-OPA1 interaction while decreasing FUNDC1-DRP1 contact (Chen et al., 2014, 2016). Recruitment of DRP1 to mitochondria is required for mitochondrial network fragmentation, enabling further mitophagy (Chen et al., 2016; Wu et al., 2016). FUNDC1, then, integrates the mitochondrial morphology machinery with the process of mitophagy.

It is worth noting that despite their interrelated roles in mitophagy, there are significant differences between PINK1/Parkin- and BNIP3-, BNIP3L- and FUNDC1-mediated autophagosomal recruitment. As noted, Parkin-catalyzed polyubiquitination of OMM proteins is the signal to engage in mitochondrial removal recognized by autophagy receptors such as p62 or NBR1. However, BNIP3, NIX and FUNDC1 contain a LIR domain within their structures, facilitating direct contact with LC3 without the need for another adaptor protein (Novak et al., 2010; Hanna et al., 2012; Liu et al., 2012; Chen et al., 2016). Since these proteins are themselves located at the OMM, activation leads to recruitment of autophagosomal vesicles at the mitochondrial surface, enabling degradation.

4. Experimental approaches for assessing mitophagy

In the next sections, we discuss associations between mitophagy and various heart conditions. To evaluate the accuracy and validity of the

evidence, it is necessary to understand how the observations were obtained. In general, several approaches are required to definitively demonstrate that autophagy is altered in response to certain stimuli, as discussed in detail by Klionsky (2016). To summarize, electron microscopy visualization is the gold standard for morphological analysis of autophagy-related structures (Eskelinen et al., 2011). Autophagosomal vesicle recruitment to the mitochondria can also be assessed by measuring levels of autophagosome-resident proteins such as LC3 or autophagy receptors in mitochondrial-enriched preparations (Kubli et al., 2013; B. Wang et al., 2018a). Fluorescent-tagged probes or proteins have also been used to identify cells undergoing mitophagy. Table 1 provides a brief description of various methodologies currently used to study mitophagy as well as relevant examples of their use in studies of cardiovascular tissues.

5. Mitophagy in cardiac physiology

5.1. Mitophagy and cardiac mitochondrial dynamics

It has been known for many years that mitochondrial fission and subsequent mitochondrial network fragmentation are prerequisites for mitophagy. However, although the yeast homolog of DRP1, Dnm1, has been identified as an essential mediator of mitophagy in this system (Kanki et al., 2009a), it is unclear whether DRP1 is essential for

Table 1
Experimental approaches for studying mitophagy. The most common techniques used for assessing autophagy/mitophagy in cell biology are listed, with an emphasis on cardiovascular research. EM: electron microscopy; RFP: red fluorescent protein; GFP: green fluorescent protein.

Experimental approach	Methodology	Applications	Relevant examples
Immuno-gold staining/ electron microscopy	Au-labeled antibodies directed against mitochondrial or autophagy proteins, visualized by EM	Identification and quantification of mitochondria inside autophagosomal structures	Liu et al. (2012); Wang et al. (2018b)
Mitochondrial isolation	Extraction of mitochondrial fraction from cell cultures/tissues, followed by autophagy-specific immunoblotting	Measurement of autophagy machinery recruitment to mitochondria; identification of protein complexes	Kubli et al. (2013); Wang et al. (2018a)
Genetic models of mitophagy proteins	Deletion of mitophagy-related gene alleles (hemizygous or homozygous) at the whole-organism or tissue-specific level	Assessment of the involvement of a particular protein in the observed phenomena	Siddall et al. (2013); Tong et al. (2019)
Fluorescent proteins			
GFP-LC3	cDNA transfection or DNA manipulation to overexpress the given protein probe; analysis requires ulterior fluorophore-specific emission quantification	Visualization of LC3 puncta (indicative of autophagosome structures)	Kubli et al. (2013); Lee et al. (2011b); Tong et al. (2019)
mCherry/RFP-GFP-LC3		Visualization of acidic autophagosomal (autophagolysosomal) structures	Ma et al. (2012); Wang et al. (2018b)
Mito-Timer		Evaluation of time-dependent mitochondrial damage; more powerful when colocalized with autophagy markers	Ferree et al. (2013); Laker et al. (2014)
Mito-Keima, based on Keima protein fluorescence		Direct visualization of mitochondria with acidic pH	Kageyama et al. (2014); Tong et al. (2019)
Mito-QC, based on mCherry-GFP fluorescence		Direct visualization of mitochondria with acidic pH	McWilliams et al. (2016), 2018

mediation of mitophagy in mammalian hearts (Tong and Sadoshima, 2016). Multiple studies have shown that DRP1 plays an important role in regulating normal physiological cardiac function at baseline conditions and under stress (Parra et al., 2008; Kageyama et al., 2014; Cahill et al., 2015; Ikeda et al., 2015; Ishihara et al., 2015; Song et al., 2015a, 2015b). Moreover, postnatal cardiac-specific downregulation of DRP1 induces dilated cardiomyopathy and lethality in mice (Kageyama et al., 2014; Ikeda et al., 2015; Ishihara et al., 2015; Song et al., 2015b). More specifically, Ikeda et al. showed that cardiac-specific $Dnm1l^{-/-}$ mice exhibited accumulation of dysfunctional mitochondria due to suppressed mitophagy, developed left ventricular dysfunction and died within 13 weeks. Furthermore, cardiac-specific heterozygous $Dnm1l^{+/-}$ mice had larger infarcts after ischemia/reperfusion (I/R) than wild-type controls (Ikeda et al., 2015).

However, whether DRP1 plays an essential role in mediating heart mitophagy remains controversial. In two different studies, $Prkn$-knockout mice were bred with DRP1-deficient mice to explore the role of Parkin-dependent mitophagy as a mechanism for the cardiomyopathy induced by the cardiac-specific $Dnm1l$ gene deletion. The results of the two studies were significantly different in terms of experimental design and conclusions. In the first study, Kageyama et al. determined that mitophagy was triggered but then interrupted in a small cohort of mice, which developed early cardiomyopathy after both floxed $Dnm1l$ alleles were deleted in cardiomyocytes at birth (using a $Myh6$ promoter-driven Cre transgene) (Kageyama et al., 2014). Crossing these cardiac $Dnm1l$ knockout mice with germline $Prkn$ knockout mice did not reverse the cardiomyopathy, leading the authors to conclude that the mitophagy triggered by $Dnm1l$ deletion was Parkin-independent, whereas Parkin was critical for maintaining mitochondrial respiratory function in the absence of DRP1. In the second study, Gerald Dorn's group analyzed the mitophagy mechanisms involved in the cardiomyopathy provoked by cardiac DRP1 deficiency, using conditional tissue-specific gene manipulation. As in Kageyama, these authors found that perinatal $Dnm1l$ deletion caused early cardiac disease (Song et al., 2015b). However, in this study, the cardiomyopathy was so extensive that it was almost impossible to distinguish between direct and indirect disease-related consequences of $Dnm1l$ deletion. Accordingly, the authors replaced the conventional $Myh6$-Cre system with a tamoxifen-inducible $Myh6$ promoter-modified Cre chimeric protein to delete $Dnm1l$ in adult hearts. This system allowed the mice to develop with normal levels of DRP1 for 8 weeks. After that time, tamoxifen was administered to delete $Dnm1l$ specifically in cardiac myocytes (Song et al., 2015b). Under these conditions, the dilated cardiomyopathy caused by $Dnm1l$ deletion

and interruption of mitochondrial fission progressed for 6 weeks before finally becoming fatal. Mitochondria in the adult DRP1-deficient hearts were enlarged (as expected due to the imbalance in mitochondrial fission/fusion), while respiratory function was normal. However, over time, cardiac mitochondrial mass decreased, whereas markers of mitophagy increased, suggesting that the mitophagy-enhanced reduction in mitochondrial content contributed to cardiomyocyte necrosis and heart failure (HF) (Song et al., 2015b). Cahill et al. also reported that the C452F mutation in DRP1 (causing a resistance of Drp1 oligomers to disassembly) caused dilated cardiomyopathy with abnormal mitochondrial morphology and defective mitophagy (Cahill et al., 2015). Furthermore, a follow-up study in the Dorn laboratory showed that Parkin mediated the accelerated mitophagy observed in DRP1-deficient hearts (Song et al., 2015a). Using a combined conditional cardiac-specific gene deletion approach to simultaneously delete $Dnm1l$ and $Prkn$ in adult mice, the authors reasoned that the tissue-specific Parkin reduction approach would avoid the previously-discussed confounding developmental compensation. As predicted, conditional $Prkn$ deletion prevented $Dnm1l$ deletion-induced Parkin upregulation and delayed and ameliorated the resulting cardiomyopathy (Song et al., 2015a). These findings show that stress-inducible Parkin-mediated mitophagy contributes to mitochondrial depletion and cardiomyopathy in hearts with defective mitochondrial fission.

While the studies by Song et al. in cardiac cells seem quite definitive, the question of whether mitophagy depends on DRP1 in other mammalian cells remains unresolved. In mammalian cells (including cardiac cells), it is not at all clear that mitochondria must be small to allow for mitophagic engulfment, as might be assumed. Electron microscopy has shown multiple nascent autophagosomal membranes engulfing large mitochondrial aggregates (Yoshii et al., 2011), and autophagosomes have the capacity to engulf micrometer-diameter beads (Kobayashi et al., 2010). Moreover, Burman et al. recently reported that although DRP1 does mediate fission upon mitochondrial proteotoxic stress, it is not required for mitophagy in HeLa cells (Burman et al., 2017). The data from this study are consistent with a model in which DRP1 is not required for mitophagy but rather restricts PINK1-Parkin activity to specific mitochondrial subdomains by segregating the PINK1-Parkin positive feedback loop from healthy mitochondria and/or removing misfolded mitochondrial proteins to prevent their spread, which may lead to organelle dysfunction and mitochondrial membrane potential perturbations (Burman et al., 2017). These results are consistent with previous works indicating that individual mitochondrial proteins have different autophagy-mediated turnover rates and that

some mitochondrial protein half-lives, including electron transport chain subunits, are modulated specifically by PINK1-Parkin-mediated mitophagy (Vincow et al., 2013). In summary, further investigations are required to elucidate the role of DRP1 in mammalian mitophagy, both in general and specifically in the heart, as well as the precise molecular mechanism involved.

5.2. Mitophagy and cardiomyocyte death

Mitochondrial triggering of apoptosis is classically associated with cardiomyocyte death and cardiac remodeling (Torrealba et al., 2017). Moreover, the apoptosis rate increases 10 to 100-fold from baseline in cardiomyocytes after myocardial infarction (MI) (Guerra et al., 1999). Mitochondria house several intrinsic apoptosis-inducing factors, such as cytochrome c, the SMAC/Diablo protein and proteases called calpains (Kar et al., 2010). After any intracellular damage signal, such as DNA fragmentation, accumulation of intracellular toxic compounds or unbalanced oxidative stress, the IMM is permeabilized, allowing for the release of pro-apoptotic factors into the cytosol and activation of the intrinsic apoptotic cell death pathway (Marín-García, 2016). In particular, uncontrolled oxidative stress has been shown to cause a rapid destabilization of the IMM (Jacob et al., 2016). Therefore, it seems likely that several ROS-involved cardiac pathological conditions are involved in activating mitochondrial cell death signals.

Historically, mitochondrial fission has been linked to the organelle fragmentation observed during apoptosis (James et al., 2003; Martinou and Youle, 2006; Arnoult, 2007; Parra et al., 2008; Marín-García and Akhmedov, 2016). DRP1 recruitment to FIS1 foci in mitochondria is one of the early events of the process in several cell types, including cardiomyocytes (Arnoult et al., 2005; Gomes and Scorrano, 2008; Parone et al., 2008; Parra et al., 2008; Twig et al., 2008; Lee et al., 2011a). Moreover, additional proteins implicated in mitochondrial dynamics are now known as active players in cell death, forming the complex mitochondrial dynamics-mitophagy-cell death interactome. Apoptotic stimuli can also trigger mitochondrial hyperfusion, which is followed by mitochondrial fragmentation and concomitant OMM permeabilization and cytochrome c release (Martinou and Youle, 2006; Tondera et al., 2009). Consistently, DRP1 is implicated in this process, and its downregulation prevents mitochondrial fission, cytochrome c release and subsequent apoptosis (Frank et al., 2001; Lee et al., 2004; Germain et al., 2005; Cassidy-Stone et al., 2008). Interestingly, DRP1 knockdown does not fully attenuate mitochondrial fragmentation, suggesting that other factors also contribute to this process during apoptosis (Ishihara et al., 2009; Landes and Martinou, 2011). DRP1 collaborates with the proapoptotic Bcl-2 family proteins BAX and BAK by increasing BAX oligomerization during apoptosis (Suen et al., 2008; Montessuit et al., 2010; Martinou and Youle, 2011). BAX activation during apoptosis triggers BAX/BAK-mediated sumoylation of DRP1, leading to its translocation from the cytosol to mitochondria and promoting mitochondrial fission (Wasiak et al., 2007).

In addition to the proteins BAX and BAK, two additional members of the Bcl-2 family are implicated in post-infarction cardiac remodeling and cardiomyocyte death (Dorn and Kitsis, 2015). These BH3-only proteins, BNIP3 and NIX/BNIP3L, have both been described as regulators of mitophagy in the adult heart (Dorn, 2010). BNIP3 is upregulated in the myocardium during hypoxia (Regula et al., 2002), whereas NIX is upregulated during age-related pathological cardiac hypertrophy (Gálvez et al., 2006). As expected, double *Bnip3l/Bnip3*-knockout mice accumulated dysfunctional mitochondria but also developed cardiac dysfunction at about twice the rate of the $Bnip3l^{-/-}$ mice (Dorn, 2010). Additionally, as is the case with MFN2, NIX and BNIP3, these proteins have a dual subcellular localization to the mitochondria and adjacent to the ER/SR. Localization of these proteins to the mitochondria or ER/SR determines whether they mediate cardiomyocyte death predominantly through apoptosis or necrosis, respectively (Diwan et al., 2009, 2008; Chen et al., 2010).

As noted, FUNDC1 is another OMM protein involved in mitophagy. FUNDC1 is dephosphorylated at Ser13 by PGAM5, triggering activation of mitophagy (Chen et al., 2014). PGAM5 has been described as a critical modulator of PINK1-mediated cardiac mitophagy (Lu et al., 2016), but the connection between the two proteins is still murky. Additionally, ULK1 is upregulated and translocated to the mitochondria to interact with and phosphorylate FUNDC1 at Ser17, enhancing the interaction between FUNDC1 and LC3 in MEF cells (Wu et al., 2014a). However, this mechanism has yet to be studied in the heart. Bcl2-like protein 13 (Bcl2-L-13), homologous to Atg32 in yeast (Kanki et al., 2009b; Okamoto et al., 2009), is another mitophagy receptor (Murakawa et al., 2015) that has been shown to bind to LC3, inducing mitochondrial fragmentation independently of DRP1 or Parkin in HEK293A cells (Murakawa et al., 2015).

In sum, homeostatic control of baseline mitophagy tone is critical for maintaining an appropriate number of functional mitochondria, supporting cardiac integrity and contractile function. Mitophagy plays an essential role in cardiac adaptations to stress (Shires and Gustafsson, 2015). However, upon prolonged and/or high stress, mitophagy can be also harmful to the heart. Any imbalance in activation or inhibition of mitophagy can lead to an excessively reduced number of functional mitochondria or an accumulation of damaged organelles, respectively, resulting in cardiac dysfunction and cardiomyocyte death.

5.3. Mitophagy and cardiac metabolism

Metabolic reprogramming is a process by which cells, even in the presence of oxygen, switch their metabolism from oxidative phosphorylation to glycolysis and convert glucose into lactate (aerobic glycolysis). This phenomenon is also known as the Warburg effect in the context of cancer cells, where it was originally described (Esteban-Martínez and Boya, 2018). Under hypoxic conditions, cells must rely on glycolysis to generate ATP as oxidative phosphorylation slows. The transcription factor HIF1α/HIF-1 act as a master regulator, coordinating the cellular response to the lack of oxygen. Given the essential role of mitochondria in cell metabolism, it is not surprising that elimination of dysfunctional mitochondria through mitophagy could contribute to these metabolic adaptations (Esteban-Martínez et al., 2017a).

Programmed mitophagy regulates metabolic reprogramming in at least two different physiological scenarios: embryonic neuronal development and macrophage polarization (Esteban-Martínez and Boya, 2018). Like cardiomyocytes, retinal ganglion cells (RGCs) are terminally differentiated. RGCs are the only neurons that project their axons outside the retina, forming the optic nerve. Several reports indicate that mouse RGC differentiation depends on mitophagy (Deczkowska and Schwartz, 2017; Esteban-Martínez et al., 2017b). This process is triggered by tissue hypoxia, which leads to the stabilization of HIF-1 and increased expression of NIX and glycolytic enzymes, which are known HIF-1 target genes. NIX-dependent mitochondrial elimination results in a metabolic shift towards glycolysis that allows neuronal differentiation to proceed. Accordingly, the retinas of NIX-deficient mice show increased mitochondrial mass, reduced glycolytic enzyme expression and decreased neuronal differentiation (Esteban-Martínez et al., 2017b).

Similarly, NIX-dependent mitophagy also contributes to mitochondrial elimination during polarization of macrophages to the M1 phenotype. This state is characterized by its proinflammatory profile and glycolytic nature, facilitating a faster immune response. In contrast, M2-phenotype macrophages depend on oxidative phosphorylation and do not respond to NIX-dependent regulation (Esteban-Martínez et al., 2017b). In NIX-deficient macrophages, glycolysis decreases expression of inflammatory regulatory genes such as *Tnf*, *Il1b* and *Nos2*, strongly suggesting a causative role for mitophagy in inflammation.

In cardiac cells, metabolic reprogramming and mitochondrial rewiring have been studied only in the context of differentiation. However, some works have evaluated the roles of autophagy and

mitophagy in the metabolic shift induced in cardiac cells by the onset of diabetes mellitus (DM) and diabetic cardiomyopathy. It is widely accepted that the adult heart is a highly energy-demanding tissue and that 60–90% of the energy used by the heart originates from fatty acid oxidation in mitochondria (Tong and Sadoshima, 2016; Vásquez-Trincado et al., 2016). Diabetic hearts shift away from glucose utilization and depend almost completely on fatty acids as their metabolic fuel. This change generally produces oxidative stress during oxidation, eventually provoking mitochondrial dysfunction (Kim et al., 2008b). In addition, due to the imbalance between fatty acid uptake and oxidation, fatty acids are accumulated in the form of lipid droplets, which often develop in the cytosol of cardiomyocytes as well as in the vascular structure, inducing histological and functional disturbances in the heart (Drosatos and Schulze, 2013). Mitochondrial dysfunction also is commonly observed in diabetic cardiomyopathy; however, autophagy and mitophagy may be either downregulated (Xie et al., 2011; Sciarretta et al., 2012; Jaishy et al., 2015; Kanamori et al., 2015; Xu et al., 2013b) or upregulated (Mellor et al., 2011; Russo et al., 2012). Recently, Tong et al. examined two questions: a) Is mitophagy activated during the development of diabetic cardiomyopathy, and if so, what is the underlying molecular mechanism? and b) What is the role of mitophagy during the development of diabetic cardiomyopathy? Using newly-developed mitophagy indicator mice and loss-of-function mouse models of autophagy and mitophagy, these authors showed that activation of cardiac mitophagy is time-dependent in response to a high-fat diet (HFD). Activation of mitophagy is also dependent on LC3, ATG7 and Parkin, suggesting that a conventional autophagic mechanism is activated in the heart during HFD consumption (Tong et al., 2019). However, the authors do not discard that the possibility that other non-conventional mechanisms of mitophagy may be also activated.

In summary, controlled mitophagy promotes metabolic reprogramming (Esteban-Martínez et al., 2017a) and is involved in cardiac metabolic shifts (Tong et al., 2019). These alterations in turn contribute to cell differentiation in various developmental and pathological contexts, suggesting that therapies aimed at mitophagic and metabolic reprogramming have a substantial and interesting therapeutic potential.

5.4. Mitophagy and cardiac myocyte differentiation

Stem cells can proliferate as well as differentiate into other types of cells. Cell proliferation requires energy, nutrients and biosynthetic activity to duplicate the macromolecular components necessary for each step of the cell cycle. Differentiation requires profound changes in cellular components and a shift in metabolic activity, since different types of cells have different metabolic demands. Indeed, metabolic pathways and cell differentiation may be controlled by the same signals (Agathocleous and Harris, 2013). During the development of the heart and formation of the circulatory system, early embryos shift from a largely anaerobic to a partially aerobic metabolism in a hypoxic environment (Simon and Keith, 2008). This change is accompanied by an increase in the number of mitochondria and the first signs of dependence on mitochondrial metabolism (in adults, the heart can only contract for a few seconds without ATP produced by mitochondria). It is also important to note that mitochondria in fetal and adult cardiomyocytes are different. For instance, the preferred substrate for ATP production in the mitochondria shifts from carbohydrates in the fetal heart to fatty acids in the adult heart (Stanley et al., 2005; Lopaschuk and Kelly, 2008; Simon and Keith, 2008). This difference may be attributable to the use of fatty acids to synthesize membrane lipids in fetal hearts, as the main priority during this stage is myocardial growth. After birth, breast milk is rich in fatty acids, which promotes the shift in the substrate for ATP production. Moreover, there are evident morphological distinctions between the organelles at these two stages, as mitochondria are long and thin in fetal cardiomyocytes but ovoid in the adult cells. Subcellular distribution changes during development as

well. The transition from the fetal to adult phenotype occurs shortly after birth, as birth induces massive metabolic changes. Arterial oxygen content increases dramatically as a result of pulmonary circulation and gas exchange (Gustafsson and Dorn, 2019), paralleling the shift in substrate preference from carbohydrates to fatty acids. This adaptation during heart development might reflect the ease with which mitochondria accommodate different metabolic substrates. Alternatively, these changes may reflect the ability of cells to generate new mitochondria in response to particular physiological and pathophysiological contexts and the necessity to "renew" the organelles when environmental circumstances change.

One mechanism that directs the perinatal cardiac metabolic shifts is PINK1/Parkin/MFN2-mediated mitophagy (Gong et al., 2015; Gottlieb and Bernstein, 2015). Removal of Parkin in adult hearts produces no discernible adverse effects. However, total Parkin genetic ablation in cardiomyocytes from the first day of life in mice was found to be lethal by 3 weeks of age, with mitochondrial maturation halted in the few surviving mice (Gong et al., 2015). Parkin-mediated mitophagy in neonatal hearts was also selectively interrupted by expression of a mutant MFN2. This variant lacked the PINK1 phosphorylation sites necessary for Parkin recruitment (mutant MFN2 AA). Similar to complete Parkin ablation, when MFN2 AA was expressed from birth, the postnatal mitochondrial maturation essential for survival did not occur. In fact, MFN2 AA hearts retained the transcriptional expression pattern of fetal mitochondria at five weeks of age, with no increase in genes encoding for fatty acid metabolism or mitochondrial biogenesis. Therefore, mitophagy is a prerequisite for the biogenesis of mature mitochondria (Gong et al., 2015; Gustafsson and Dorn, 2019). However, this mitochondrial maturation seems not immediate. In a recent work by de Carvalho et al., using gene expression analysis and functional assessment in both cardiac tissue and cultured cardiomyocytes, they proved that early postnatal cardiomyocyte proliferative capacity correlates with a high oxidative energy metabolism. Although this increased energy requirement decreases as the proliferation ceases in the following days, both oxidative-dependent metabolism and anaerobic glycolysis start to co-exists and subside in the heart (de Carvalho et al., 2017).

There have also been reports on the roles of mitophagy in adult hearts and in cardiac stem cell differentiation. Mitochondria are essential for the survival and function of progenitor cells. In the process of adult cardiac progenitor cell (CPC) differentiation, at least one mitophagy pathway is activated (Lampert et al., 2019). Mitophagy is rapidly activated in CPCs upon initiation of differentiation —not through the classic PINK1-PRKN/PARKIN pathway, but the pathway mediated by the BNIP3L and FUNDC1 receptors. Abrogating BNIP3L- and FUNDC1-mediated mitophagy during differentiation increases mitochondrial fission, the formation of donut-shaped, damaged mitochondria and susceptibility to cell death through oxidative stress. BNIP3L- and FUNDC1-mitophagy facilitates adequate CPC reorganization and formation of a mature, functional mitochondrial network so that the differentiation process unfolds optimally (Lampert et al., 2019).

5.5. Mitophagy and cardiac fibroblast differentiation

Cardiac fibrosis is defined as the excessive deposition of extracellular matrix (ECM) components inside and around damaged tissue and represents the common and final pathological outcome of several chronic diseases, including HF (Wynn and Ramalingam, 2012; Duffield et al., 2013; Urban et al., 2015; Chang et al., 2014). This process is finely orchestrated by fibroblasts, the abundant connective tissue cells responsible for maintaining the structural integrity of connective tissues (Cannito et al., 2017). Fibroblasts are considered to be a uniform cell type with equivalent function regardless of origin (Urban et al., 2015). Fibroblasts proliferate rapidly and can differentiate into myofibroblasts, which are ultimately responsible for the progression of fibrotic diseases (Chang et al., 2014). These processes are regulated by various cytokines

such as TGF-β1, growth factors, interleukins and chemokines that are released primarily by proinflammatory cells and fibroblasts in an auto/paracrine manner (Wynn and Ramalingam, 2012). The main nutrients used by myofibroblasts are glucose and glutamine, which are essential for various metabolic processes involved in fibrosis (Vallée et al., 2017). From an energy metabolism perspective, the TGF-β1 and WNT/β-catenin signaling pathways are central regulators of glycolytic metabolism in fibrosis. Extracellular milieu acidification, as a result of increased lactate dehydrogenase expression, promotes TGF-β signaling, leading to increased fibroblast-to-myofibroblast differentiation and collagen (COL) deposition (Kottmann et al., 2012; Meng et al., 2016). Activation of WNT/β-catenin reduces the conversion of pyruvate to acetyl-CoA in mitochondria and, therefore precludes its entry into the citric acid cycle. As a result, significant amounts of cytosolic pyruvate are converted into lactate as the main alternative to oxidative phosphorylation (Warburg, 1956). However, counterintuitive evidence has also been reported. Treating fibroblasts with TGF-β1 induces differentiation to myofibroblasts but also increases the number of mitochondria, content of mtDNA and expression of specific mitochondrial proteins, as well as elevating the oxygen consumption rate. Therefore, TGF-β1-dependent differentiation is accompanied by a complex energy remodeling in myofibroblasts (Negmadjanov et al., 2015).

The hemodynamic stress observed in cardiovascular disease onset can promote mitochondrial ROS formation, which may activate mitophagy, in turn preventing the accumulation of damaged mitochondria (Yamaguchi, 2019). This process is activated in HF and aging-associated disorders. Therefore, mitochondrial quality control is important for maintaining both cardiomyocyte and cardiac fibroblast homeostasis. There are few studies on the mitophagic process in cardiac fibroblasts. Liu et al. showed that increased NIX levels stimulate norepinephrine-dependent fibrogenesis in the NIH-3T3 fibroblast cell line (Liu et al., 2014). NIX overexpression promoted COL and fibronectin expression, while NIX suppression reduced this expression (Liu et al., 2014). The same group later reported that BNIP3L promoted cardiac fibrosis in neonatal fibroblast primary cultures stimulated with norepinephrine and *in vivo* HF models (Liu et al., 2017). These authors showed that BNIP3L overexpression promoted cardiac fibroblast proliferation, increased COL and fibronectin levels and activated the TGF-β1/Smad2/3 pathway, suggesting that BNIP3L-induced activation of this mechanism could be a trigger for cardiac fibrogenesis.

The effects of mitophagy on cardiac fibroblast differentiation remain unknown. However, it has been shown that insufficient autophagy is associated with impaired regulation of myofibroblast differentiation in other models of fibrosis (Araya et al., 2013). Additionally, inhibition of mitophagy is involved in PDGFR/PI3K/AKT pathway activation both *in vitro* and *in vivo*, triggering myofibroblast differentiation and proliferation (Kobayashi et al., 2016).

There are several molecules or drugs shown to regulate mitophagy in different fibroblast models. For example, the microRNA miR-1224-5p stimulated silica-induced pulmonary fibrosis, while its repression attenuated the fibrotic progression induced by silica both *in vivo* and *in vitro* (Wu et al., 2017). miR-1224-5p reduced Beclin1 expression, which prevented the translocation of Parkin to mitochondria, promoting accumulation of damaged mitochondria (Wu et al., 2017). Consistent with this finding, the antifibrotic agent pirfenidone, which is used to treat idiopathic pulmonary fibrosis, induced mitophagy by increasing Parkin expression, inhibiting differentiation of the myofibroblasts (Kurita et al., 2017). Moreover, a relationship between mitophagy and pyruvate levels was observed in a study on human dermal fibroblasts, in which pyruvate deprivation stimulated accumulation of damaged mitochondria due to a defective mitophagic process (Kim et al., 2018). It seems, therefore, that inhibition of mitochondrial degradation is required for fibroblast-to-myofibroblast differentiation.

As noted, no studies to date have directly assessed the impact of mitophagy in cardiac fibroblasts on cardiovascular pathologies. However, mitophagy has been studied in various fibroblast models,

which may shed light on mitophagy alterations in other types of diseases, such as cardiac pathologies. For example, fibroblasts from patients with multiple systemic atrophy showed impaired mitophagy and respiratory chain activity (Monzio Compagnoni et al., 2018). These findings point to fibroblasts as a cellular mirror of the intracellular quality control process in other cell types, suggesting that future studies may be able to identify new cellular pathways involved in the onset of various cardiac conditions.

6. Cardiovascular diseases and mitophagy

6.1. Mitophagy in cardiac hypertrophy and heart failure

The role of autophagy, and specifically mitophagy, during cardiac hypertrophy, remodeling and HF has been described as context-dependent (Sciarretta et al., 2018). However, it is known that prolonged and/or high-level cardiac stress can provoke mitochondrial damage and dysfunction in cardiac myocytes (Parra et al., 2008; Kuzmicic et al., 2014; Pennanen et al., 2014; Marín-García and Akhmedov, 2016). Cardiac stress-induced mitophagy helps to remove damaged and dysfunctional mitochondria, thus preventing oxidative damage that could in turn initiate apoptosis and ultimately lead to HF (Hoshino et al., 2013; Kubli et al., 2013; Marín-García and Akhmedov, 2016).

In terms of the mitophagy machinery, impaired PINK1/Parkin-dependent mitophagy leads directly to myocardial dysfunction. Moreover, *Pink1*-knockout mice showed abnormal cardiac mitochondrial function and elevated oxidative stress, along with early left ventricular dysfunction and pathological cardiac hypertrophy (Billia et al., 2011). Supporting the previously-discussed data, PINK1 protein levels are notably reduced in advanced human HF (Billia et al., 2011). On the other hand, Kubli et al. showed that Parkin-deficient mice had abnormal mitochondrial networks with small mitochondria but normal mitochondrial and myocardial function (2013). However, $Prkn^{-/-}$ mice show increased susceptibility to damage from coronary artery ligation-induced MI and cardiac aging (Hoshino et al., 2013; Kubli et al., 2013). Interestingly, PINK1/Parkin-mediated mitophagy activation was reduced in $Mfn2^{-/-}$ mice, associated with a severe myocardial dysfunction related to the onset of HF in mice at around 30 weeks of age (Song et al., 2014; Marín-García and Akhmedov, 2016). These concepts were later supported by a study from the Dorn group (Bhandari et al., 2014) that demonstrated a compensatory upregulation of several Parkin-related E3 ubiquitin ligases from the RING family in the hearts of Parkin-knockout mice (Bhandari et al., 2014). The authors also observed that the presence of enlarged mitochondria was associated with dilated cardiomyopathy in Parkin-knockout tubes from Drosophila mutants. This finding is striking, as, unlike mice, Drosophila flies lack Parkin-redundant genes, reducing the possibility of alternative explanations. Parkin-deficient mitochondria are less polarized and in turn generate more ROS. This altered phenotype was reverted by the suppression of mitochondrial fusion (through silencing of MARF, the Drosophila MFN2 ortholog). Once mitochondrial fusion and function was normalized, the cardiomyopathic phenotype was rescued (Bhandari et al., 2014). Moreover, the same group showed later that Parkin mRNA and protein levels are low in normal mouse hearts but upregulated after a cardiomyocyte-specific *Dnm1l* deletion in adult mice (Song et al., 2015a). Thus, DRP1 deficiency triggers Parkin-dependent activation of mitophagy, leading to a severe myopathic phenotype.

Autophagy and mitophagy have been well characterized in pressure-overload mouse models. Indeed, it has been consistently established that mitophagy is necessary for cardiac cell adaptations to pressure overload, a validated model for cardiac hypertrophy (Sciarretta et al., 2018). However, while pressure overload triggers mitophagy, this activation is transient, occurring during acute pressure overload but later inactivated. After this inactivation, there is a marked decrease in mitochondrial and cardiac function. Consistent with this

finding, injection of the Tat-Beclin1 peptide to induce autophagy improves cardiac function by reactivating mitophagy (Shirakabe et al., 2016b). All of these results point to mitophagy as an adaptive process that preserves mitochondrial function during the development of pathological hypertrophy. However, the "how" and "why" of mitophagy inactivation after its temporary activation during early pressure overload remain under debate. Similarly, mitophagy is implicated in the cardiac remodeling after MI that may eventually lead to HF. Parkin and mitophagy are upregulated in the peri-infarct zone in post-MI hearts, while Parkin-deficient mice show larger infarctions and increased cardiac remodeling (Kubli et al., 2013; Sciarretta et al., 2018).

Interestingly, the onset and time course of mitophagy activation after pressure overload induction do not coincide with the timeline for Parkin translocation to the mitochondria (Shirakabe et al., 2016b). Moreover, recruitment of Parkin to the mitochondria is also independent of PINK1 during MI (Kubli et al., 2015). These results suggest that cardiac mitophagy activation in response to hemodynamic overload may be dependent on a unique signaling pathway that differs from the well-established PINK1-Parkin mitophagic response. Mitochondrial quality-control and degradation mechanisms are key for proper disposal of mtDNA by mitochondrial DNase II. When coordination between the mitophagy process and DNase II activation fails, sterile inflammation is triggered due to the accumulation of damaged and improperly-degraded mitochondrial DNA, thus contributing to the development of HF during pressure overload (Dorn, 2012; Oka et al., 2012; Sciarretta et al., 2018).

6.2. Mitophagy in ischemia/reperfusion and myocardial infarction

The heart is an extremely energy-hungry organ, requiring large amounts of ATP daily to sustain normal ventricular function. Most of the ATP in cardiomyocytes is derived from mitochondrial activity (Lopaschuk et al., 2010), which explains the pronounced vulnerability of cardiac tissue to I/R. In pathological conditions such as MI, drastic changes in nutrient and oxygen availability lead to abnormal mitochondrial ROS production, oxidative damage and ultimately cell death. While the mitochondrial network adapts to metabolic stress in most tissues through the process of fusion, fission and migration that is collectively termed mitochondrial dynamics, adult cardiomyocytes exhibit small, round interfibrillar mitochondria that do not travel within the cell and are markedly hypodynamic (Song and Dorn, 2015). This particularity of the adult cardiomyocyte explains why the mitochondrial quality control exercised by mitophagy is paramount for maintaining the viability and function of cardiac tissue in response to metabolic stress. For instance, postnatal cardiac-specific deletion of *Mfn2* leads to accumulation of abnormal mitochondria, elevated ROS levels and myocardial damage due to the lack of PINK1-mediated MFN2 phosphorylation, a critical signaling hub for initiation of mitophagy (Chen and Dorn, 2013). Conversely, suppressing mitochondrial ROS below physiological levels also deteriorates myocardial function in mitophagic-impaired murine models (Song et al., 2014). These findings should be taken into consideration when assessing the double-edged effect of cardiac mitophagy in response to I/R: excessive activation provokes a catastrophic loss of mitochondria, while an insufficient response leads to accumulation of dysfunctional mitochondria, hindering the ability of cardiac myocytes to adapt to stress.

Reports on general deficiencies in autophagy, such as those seen in *Becn1*$^{+/-}$ mice (Matsui et al., 2007) or in response to miR-188, which targets ATG7 for degradation (Wang et al., 2015), suggest that impaired autophagy may protect against myocardial damage in response to I/R *in vivo*. However, evidence explicitly linking increased mitophagy with myocardial injury in I/R is somewhat scarce: a seminal study using the nonspecific mitophagy inhibitor mdivi-1 showed reduced myocardial infarction size (Ong et al., 2010), but this result may depend on the effect of enhanced mitochondrial fusion rather than the potential decrease in mitophagy (Maneechote et al., 2017). Similarly, amelioration

of I/R-induced myocardial damage in *Bnip3*$^{-/-}$ mice may be linked to a direct effect on apoptosis and not to decreased mitophagy (Bravo-San Pedro et al., 2017). Overtly-activated mitophagy in response to I/R may also impair myocardial function by inducing a steep decrease in total mitochondrial mass (Jin et al., 2018).

Despite the conflicting evidence, most preclinical studies suggest that appropriate mitophagy is a cardioprotective response in the I/R setting. Several models, such as *Pgam5*$^{-/-}$ (Lu et al., 2016) or *Prkn*$^{-/-}$ mice (Kubli et al., 2013), indicate that deficient mitophagy is linked to more extensive myocardial damage in response to coronary artery ligation. Furthermore, transgenic mice overexpressing RHEB, a general autophagy inhibitor that works by activating mTORC1, also exhibit increased susceptibility to myocardial ischemia (Sciarretta et al., 2012). Loss of PINK1 increased infarct size after I/R in one experiment, but the study failed to assess mitophagy (Siddall et al., 2013). Ischemia also induces expression of CK2α, which in turn deactivates FUNDC1, decreasing mitochondrial receptor-mediated mitophagy and increasing fatal mitochondrial fission via NR4A1/MFF activation. Loss of CK2α restores FUNDC1-mediated mitophagy, providing a survival advantage to cardiac myocytes in response to I/R stress (Zhou et al., 2018a, 2018b).

I/R may also activate noncanonical autophagy pathways, although their relative contribution to overall mitophagy in the heart remains is unknown. Nutrient deprivation in cardiac myocytes promotes the assembly of a multiprotein complex consisting of ULK1, RAB9, RIP1 and DRP1; the coordinated phosphorylation of RAB9 by ULK1 and the phosphorylation of DRP1 by RIP1 allows for recruitment of late endosome membranes around damaged mitochondria. In fact, in RAB9 phosphorylation-resistant mice, ischemia-induced mitophagy is attenuated, provoking increased mitochondrial dysfunction and myocardial damage in response to 30 min of coronary artery ligation, suggesting that this uncommon branch of mitophagy is relevant for cardiac survival (Saito et al., 2019).

Well-regulated activation of mitophagy is critical for cardiac ischemic preconditioning, where repeated short episodes of ischemia protect the myocardium against a subsequent ischemic event. Preconditioned cardiac myocytes maintain nearly normal ATP stores, calcium handling and mitochondrial morphology for significantly longer in response to I/R stress. Since all preconditioning models depend on transient mitochondrial membrane depolarization (a signal for mitochondrial removal), it might be expected that mitophagy would induce culling of the more fragile elements within the mitochondrial network, allowing for a more robust adaptive response to ischemia. Several studies support the protective role of macroautophagy in I/R models (Hamacher-Brady et al., 2006; Yan et al., 2009; Huang et al., 2010). The response seems to be largely dependent on PINK1/Parkin/p62-mediated mitophagy since *Prkn*$^{-/-}$ mice are resistant to ischemic preconditioning (Huang et al., 2011). Supporting this evidence, autophagy activators such as caloric restriction, rapamycin (Kanamori et al., 2011; Wu et al., 2014b), resveratrol (Goh et al., 2007) and chloramphenicol (Sala-Mercado et al., 2010) reduce infarct size in MI murine models. Conversely, autophagic inhibitors such as 3-methyladenine and bafilomycin A1 increase myocardial damage (Kanamori et al., 2011; Wu et al., 2014a). Simvastatin, a lipophilic statin known to be effectively transported into cardiomyocyte, induces autophagy via reduction of the small GTPase RAC prenylation, thus decreasing mTORC1 signaling (Wei et al., 2013). In murine models, simvastatin induces ischemic preconditioning, which depends on PINK1/Parkin signaling and is abolished in *Prkn*$^{-/-}$ mice (Andres et al., 2014). The beneficial effect of statins may also rely on increased mitochondrial biogenesis by PGC-1α activation (Bouitbir et al., 2012).

While mitophagy is an appealing therapeutic target for I/R damage, there are significant caveats. Most of the signaling pathways involved in mitophagy, such as mTOR and AMPK, are involved in other cellular processes, raising the possibility of unwanted off-target effects. Moreover, the threshold beyond which excessive mitochondrial

pruning becomes detrimental, as well as the details of the cross-talk between mitophagy and mitochondrial biogenesis, remain unknown. An ideal pharmacological intervention in response to I/R should promote mitophagy of damaged elements while enhancing biogenesis to avoid compromising cell energy availability (Moyzis et al., 2015).

6.3. Mitophagy and hypertension

Vasculature is a complex tissue with a cellular architecture that regulates contraction for optimal tissue perfusion and adaptation to physiological demands. Hypertension affects vascular physiology and is considered an important risk factor for cardiovascular diseases (Messerli et al., 2017). Several studies suggest that mitochondrial dysfunction is associated with hypertension and altered blood pressure phenotypes (Ramachandran et al., 2002; Zimmerman and Zucker, 2009; Puddu et al., 2007). Moreover, mitochondrial-derived excessive ROS production and increased mitochondrial Ca^{2+} accumulation are associated with hypertension risk factors (Lassègue and Griendling, 2004; Dedkova and Blatter, 2008).

Nitric oxide (NO) is the main regulator of vascular tone. NO is generated enzymatically by nitric oxide synthases (NOS). Hypertension is correlated with deficient eNOS function and consequent decreased NO generation (Tabit et al., 2010). Pharmacological inhibition of NOS with L-NAME leads to reduced mitochondrial fusion in aortic tissue, along with increased fission (Miller et al., 2013). Surprisingly, mitophagy is minimally disturbed, as BNIP3 levels decreased, but Beclin1, p62 and LC3 levels remained unchanged (Miller et al., 2013). These results suggest that regulation of eNOS/NO signaling plays a role in vascular physiology through mitochondrial dynamics, although not at the level of mitophagy.

In contrast with this evidence, dysregulation of mitophagy has been attributed to hypertension elicited by angiotensin II. Ang-II infusion stimulates mitophagy in the heart (Zhao et al., 2014). However, ATG5 deficiency significantly reduces mitochondrial degradation and increases production of ROS and inflammation markers, with an effect even greater in magnitude than that observed for angiotensin II alone (Zhao et al., 2014). Therefore, promoting autophagy may be a new therapeutic strategy to regulate the inflammation induced by hypertension and cardiac mitochondrial quality control.

Obesity has been reported as an aggravating factor for mitophagic dysfunction in hypertensive individuals. In a study from Zhang et al. renovascular hypertension was triggered in obese pigs; obesity alone had no apparent effect on mitochondrial dynamics but aggravated reductions in mitochondrial protein and mtDNA in hypertensive hearts as a product of increased mitophagy (Zhang et al., 2015). Furthermore, mitochondrial content and mitophagy activation were correlated with myocardial fibrosis. These findings suggest that obesity increases the cardiac mitochondrial aberrations observed in renovascular hypertension.

Pulmonary hypertension is a progressive disease characterized by pulmonary vascular remodeling and pulmonary vasculopathy, resulting from dysfunction of the right ventricle, among other factors (Suliman and Nozik-Grayck, 2019). In lung endothelial cells, loss of mitochondrial uncoupling protein 2 (UCP2), a protein that mediates calcium influx into the mitochondria, increases PINK1 and Parkin levels and mitophagy (Haslip et al., 2015), leading to the development of spontaneous pulmonary hypertension. This finding underscores the role of endothelial mitophagy in the development of pulmonary vascular remodeling (Haslip et al., 2015). Furthermore, mitochondrial dysfunction and increased mitochondrial fragmentation have been observed in vascular pulmonary artery cells from patients with pulmonary hypertension (Marsboom et al., 2012). Since mitochondrial degradation is associated with fragmentation and isolation of damaged mitochondria (Gomes et al., 2011; Tanaka et al., 2010), the aforementioned results suggest that mitochondrial fission may be associated with increased mitophagy in the context of pulmonary hypertension. Curiously,

Map1lc3b- (gene for LC3B) knockout mice show exacerbated pulmonary hypertension-associated damage in response to hypoxia, suggesting that adequate progression of autophagy is protective against the pulmonary hypertension induced by chronic hypoxia (Lee et al., 2011b).

6.4. Mitophagy and diabetic cardiomyopathy

Cardiovascular complications of DM represent the main cause of morbidity and mortality in diabetic patients (Hölscher et al., 2016). These complications include diabetic cardiomyopathy, a condition characterized by left ventricle remodeling and HF in the absence of coronary disease (Kubli and Gustafsson, 2015; Seferović and Paulus, 2015). As current treatments for cardiomyopathy in diabetic patients are not differentiated from those for non-diabetic patients (Yancy et al., 2017), it is important to discover specific treatments for this pathology. As noted previously, most of the energy used in the heart originates from fatty acid oxidation in mitochondria (Tong and Sadoshima, 2016). In diabetic hearts, glucose utilization decreases, and fatty acids become the main source of energy, producing greater levels of oxidative stress and potentially provoking mitochondrial dysfunction (Kim et al., 2008b). ROS and death-inducing factors released by damaged mitochondria can also increase cardiac myocyte injury (Volpe et al., 2018; Wilson et al., 2018). There is evidence that cardiovascular complications of DM converge in the mitochondria as the epicenter of cardiac myocyte damage (Sung et al., 2015).

In the context of streptozotocin (STZ)-induced DM, levels of several core components of autophagy, such as LC3, ATG5 and ATG12, are reduced in cardiac tissue (Xu et al., 2013a). In parallel, both PINK1 and Parkin protein levels are reduced, but BNIP3 levels remain unchanged, suggesting specific mitophagy signaling alterations in type 1 DM models (Tang et al., 2015; Wang et al. (2018b); Xu et al., 2013a). This finding may also suggest that specific mitophagy signaling is reduced in diabetic cardiomyopathy despite extensive mitochondrial dysfunction, leading to the accumulation of dysfunctional mitochondria at the cardiac level and exacerbating the damage to this tissue.

Contrary observations have been reported in the context of type 2 DM. In mice fed a HFD for various experimental periods, cardiac myocyte autophagic flux initially increased but then decreased after 2 months of treatment (Tong et al., 2019). Notably, the Mito-Keima signal and LC3-II localization to the mitochondria increased, suggesting that mitophagy increased after HFD (Tong et al., 2019). Deletion of ATG7 or Parkin resulted in impaired mitophagy along with increased cardiac diastolic dysfunction in response to HFD, whereas Beclin1 overexpression had the opposite effect (Tong et al., 2019). This evidence points to a protective role for autophagy and mitophagy in obesity-associated diabetic cardiomyopathy. It is important to note that despite these seemingly divergent results, we should hesitate to conclude that mitophagy is inhibited in type 1 DM and activated in type 2 DM models of diabetic cardiomyopathy. In a transgenic model of type 1 DM and in STZ-treated mice, cardiac tissue showed decreased PINK1 and Parkin levels, but expression was increased for the small GTPase RAB9 (Xu et al., 2013a), which has been implicated in an alternative non-canonical autophagy responsible for mitochondrial degradation in the absence of ATG5 (Nishida et al., 2009). Therefore, it is possible that when canonical autophagy is inhibited, an alternative autophagy is activated, potentially triggering activation of mitophagy in the diabetic heart.

7. Future perspectives in cardiovascular autophagy/mitophagy

As described throughout the text, cardiac pathophysiology is inseparably tied to mitochondrial function. In all cardiovascular diseases discussed, autophagy or mitophagy activity is either enhanced or reduced. In most cases, assessing mitophagy machinery is the best way to determine the direction of the modulation. Fig. 2 summarizes the effects of autophagy/mitophagy inhibition on cardiovascular physiology. By grouping the outcomes as beneficial or detrimental, we attempt to
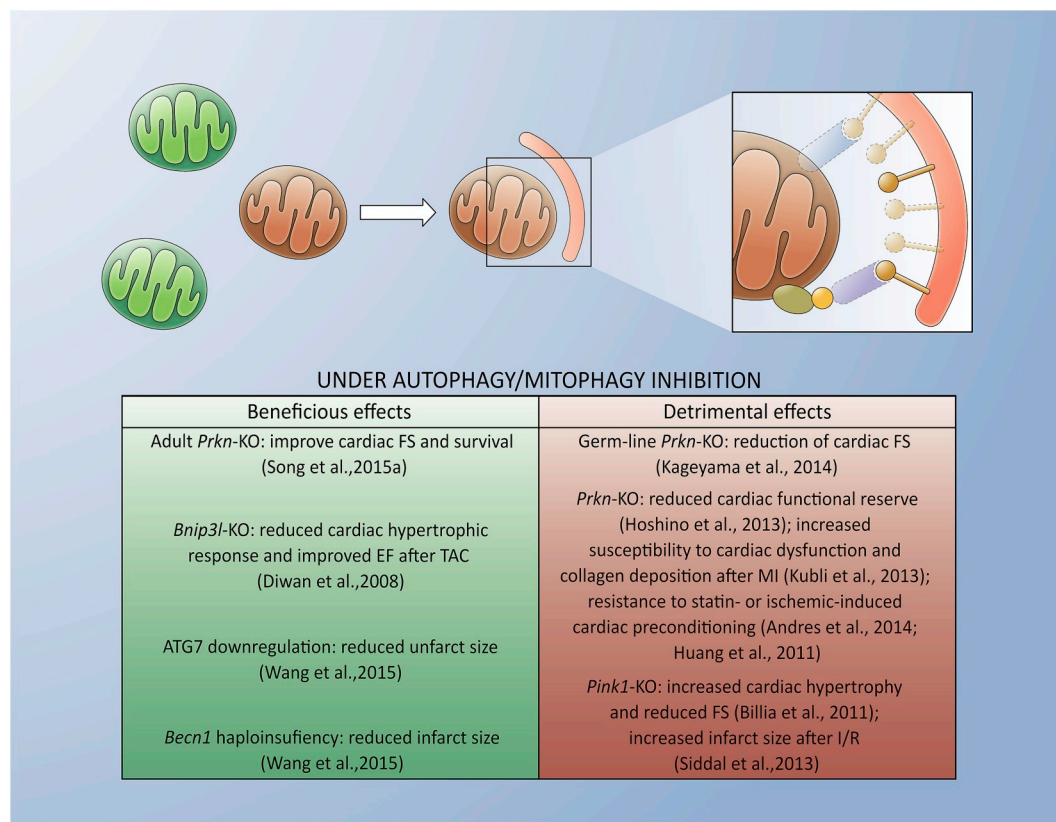
Fig. 2. Cardiac consequences of genetic deletion of autophagy and mitophagy machinery. Elimination of autophagy/mitophagy genes leads to accumulation of dysfunctional mitochondria, producing potentially beneficial (left) or deleterious (right) outcomes for heart physiology, as discussed throughout the text. Abbreviations: EF: ejection fraction; FS: fractional shortening; I/R: ischemia/reperfusion; KO: knockout; MI: myocardial infarction; TAC: transverse aortic constriction.

emphasize the context-dependent effects of modulating mitophagy activity.

The complexity of this regulation on the one hand, and the broad range of consequences of mitochondrial degradation on the other, make it difficult to find a unified treatment target for cardiac pathology. From an optimistic point of view, however, this conundrum invites the discovery of future opportunities. Proteins such as BNIP3 and NIX have been proposed as therapeutic targets at the cardiac level. These proteins heterodimerize with antiapoptotic factors such as BCL2-2 and BCL-XL in the heart, leading to activation of BAX and BAK, formation of pores in the mitochondria and the onset of apoptosis (Dorn, 2010). Despite sharing a common function, these proteins are differentially regulated. For instance, BNIP3 expression is increased by hypoxia in neonatal cardiac myocytes, but not by hypertrophic stimuli such as phenylephrine or angiotensin II (Gálvez et al., 2006). Similar observations have been made in postnatal cardiac myocytes, *ex vivo* hypoxia models and samples extracted from adult rat hearts with chronic HF (Regula et al., 2002). In contrast, NIX transcription increases with phenylephrine but not hypoxia, an observation confirmed by *in vivo* models (Gálvez et al., 2006). Overexpression of both proteins leads to cardiomyopathy and cardiac myocyte death, which is prevented when a truncated variant lacking the mitochondrial-targeting domain is expressed (Regula et al., 2002; Yussman et al., 2002). If BNIP3 or NIX are not bound to the mitochondria, they are presumably no longer able to promote mitochondrial degradation through the autophagy machinery. Therefore, NIX and BNIP3 are upregulated in response to cardiac stressors, and interventions targeting these proteins may contribute to therapies against cardiomyopathy.

AKT, an essential player in insulin and mitogenic signaling in the heart, regulates the autophagy master inhibitor mTOR (Sciarretta et al., 2018). AKT2 deletion improves myocardial contractile abnormalities induced by HFD (Xu et al., 2013a). Specifically, *in vivo* AKT2 elimination prevented the cardiac hypertrophy phenotype and interstitial fibrosis induced by HFD. Knocking out *Akt2* prevented inhibition of autophagy in response to HFD (Xu et al., 2013a). Interestingly, HPD-induced reductions in PGC-1α levels were also blunted in $Akt2^{-/-}$ mice, supporting the idea that coordination between mitochondrial biogenesis and autophagy is needed to sustain mitochondrial health (Xu et al., 2013a).

The transcription factor p53 is another promising candidate for ameliorating the cardiac damage associated with mitophagy. p53 is involved in autophagy activation as part of a DNA-damage stress response, leading to the activation of AMPK and inhibition of mTOR (Feng et al., 2005, 2007). Later observations showed that reducing p53 activity, whether genetically or pharmacologically, increases autophagy in cancer cells (Tasdemir et al., 2008), suggesting that the role of p53 in autophagy could be tissue- and stressor-dependent. As for its function in mitophagy, p53 levels are increased in aged mice hearts, while PINK1/Parkin levels in mitochondria are reduced (Hoshino et al., 2013). Furthermore, p53 interacts with Parkin, precluding its translocation to the mitochondria in response to reduced mitochondrial membrane potential and promoting the accumulation of dysfunctional mitochondria (Hoshino et al., 2013). Accordingly, p53-knockout mice show a greater cardiac reserve capacity and increased resistance to MI-triggered apoptosis and cardiac dysfunction (Hoshino et al., 2012, 2013). Notably, cardiac myocyte-specific overexpression of Parkin was sufficient to restore contractility, preserve mitochondrial respiration and reduce ROS generation (Hoshino et al., 2013).

A few population studies have linked hypertension to the presence of single nucleotide polymorphisms (SNPs) in mitophagy genes. Tayo et al. reported that in a Nigerian population, allelic variants of the *PRKN* gene may be associated with the risk of hypertension (2009). The

presence of two SNPs located in the *PRKN* gene (rs2315314 and rs16892620) were significantly associated with the variability in systolic and diastolic blood pressure in this population (Tayo et al., 2009). Similar results were later described in Korean population cohorts (Jin et al., 2011). Additionally, a hypertension case-control analysis showed that two other *PRKN* SNPs were moderately associated with hypertension (Jin et al., 2011). This evidence provides insight into the genetic basis for hypertension and its potential relationship with mitochondrial quality control in populations of different ethnic backgrounds.

8. Concluding remarks

As a highly ATP-demanding tissue, the crucial role of mitochondrial function in cardiac performance is undeniable. Therefore, removal damaged mitochondria is an essential mechanism for cardiac cell function. The evidence presented here collectively suggests a seemingly ambiguous effect of mitophagy on heart function, as both decreased and increased elimination of mitochondria provoke cardiovascular dysfunction to some extent. However, these contradictory observations may point to a unifying conclusion: maintaining the balance between mitochondrial degradation and accumulation is essential. Accordingly, sustaining a healthy mitochondrial network in cardiac cells may be a better therapeutic goal than promoting the renewal or elimination of damaged mitochondria under various stress conditions, a concept with potential direct implications for cardiovascular disease treatments.

Declarations of competing interest

None.

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