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Abstract

The autophagy pathway is a key regulator of cellular metabolism and homeostasis, and plays a critical role in maintaining normal vascular cell function. It is well recognized that autophagy can regulate endothelial cell homeostasis, vascular smooth muscle cell (VSMC) phenotype transition, and calcium (Ca^{2+}) homeostasis in VSMCs. Emerging evidence has demonstrated that autophagy directly protects against vascular calcification (VC). Crosstalk between endosomes, dysfunctional mitochondria, autophagic vesicles and Ca^{2+} and phosphate (Pi) enriched matrix vesicles (MVs) may underpin the pathogenesis of VC. In this review, we summarize the current experimental evidence in understanding how autophagy maintains normal vascular cell function and its protective role against vascular calcification. We also discuss the underlying molecular and cellular mechanisms through which autophagy inhibits vascular calcification. Pharmacological modulation of autophagy may offer an exciting new strategy for the treatment of vascular calcification.

Key words

Autophagy, Vascular cell function, Phenotype transition, Matrix vesicles, Vascular calcification.

Table of contents

Abbreviations

ALP, alkaline phosphatase; **Ca^{2+}**, Calcium; **CAVD**, calcific aortic valve disease; **CKD**, chronic kidney disease; **EC**s, endothelial cells; **ECM**, extracellular matrix; **LC3**, Light Chain 3; **TOR**, target of rapamycin; **MVs**, matrix vesicles; **PDGF**, platelet derived growth factor; **Pi**, phosphate; **ROS**, reactive oxygen species; **VC**, vascular calcification; **VSMCs**, vascular smooth muscle cells; **VICs**, valve interstitial cells;

1. Introduction

Autophagy is essential for health and longevity (Nakamura & Yoshimori, 2018). This catabolic pathway is initialised by a double membraned phagophore which engulfs misfolded proteins, damaged organelles or unwanted metabolites forming an autophagosome. The autophagosome fuses with single membraned acidic lysosomes, where the engulfed content is degraded into cellular building blocks, thereby, recycling the nutrients and providing a source of energy to the cells (Singh & Cuervo, 2011). Based on the membrane dynamics and the molecular machinery involved, three main types of autophagy have been identified so far, macroautophagy, chaperone-mediated autophagy and microautophagy (Cuervo & Wong, 2014; Feng, He, Yao, & Klionsky, 2014; W. W. Li, Li, & Bao, 2012). In this review we focus on macroautophagy (referred hereafter as autophagy) which could be both selective and non-selective. Non-selective autophagy degrades cytoplasmic contents, however selective autophagy can degrade specific substrates like mitochondria (Lazarou, et al., 2015), ribosomes (Kraft, Deplazes, Sohrmann, & Peter, 2008), bits of ER (Wilkinson, 2019), peroxisomes (Kim, Hailey, Mullen, & Lippincott-Schwartz, 2008), bacteria (Bauckman, Owusu-Boaitey, & Mysorekar, 2015) etc. using specific autophagy receptors. Most of the living cells perform low level of basal autophagy to maintain protein turnover and for recycling of damaged organelles, however autophagy pathway is up-regulated when the cells are challenged with stressors such as nutrient starvation (Shang, et al., 2011), hypoxia (Daskalaki, Gkikas, & Tavernarakis, 2018), reactive oxygen species (ROS) (Filomeni, De Zio, & Cecconi, 2015), protein aggregates (Menzies, Fleming, & Rubinsztein, 2015), infection (Levine & Kroemer, 2008) and increased phosphate levels (Dai, et al., 2013). In the last twenty years, autophagy has been extensively investigated as a common mechanism underpinning the development of important human diseases including cancer (White, 2015), neurodegeneration (Nixon, 2013), cardiomyopathy (Tong & Sadoshima, 2016), diabetes (Gonzalez, et al., 2011), liver disease (Rautou, et al.,

2010), autoimmune diseases (Yang, Goronzy, & Weyand, 2015) and infection (Deretic, Saitoh, & Akira, 2013). Crucially, the role of autophagy in vascular calcification, a significant risk factor for cardiovascular mortality, is just emerging. In this review, we discuss our present understanding of the important role of autophagy in the maintenance of vascular cell function and vascular calcification, and the emerging strategies to target autophagy for the treatment of vascular calcification.

2. The key regulators of autophagy

The autophagic molecular machinery was first elucidated in yeast followed by identification of homologues in humans (Itakura & Mizushima, 2010; Mizushima, Yoshimori, & Levine, 2010; Tsukada & Ohsumi, 1993). To date, there are more than 40 autophagy related (*ATG*) genes known. These genes orchestrate a highly dynamic autophagy pathway which gets activated during stress (Murrow & Debnath, 2013) (Fig 1). When nutrients are limited in the environment, the target of rapamycin TOR complex is inhibited, which results in the activation of ULK1/2 complex. This complex interacts with Atg13, FIP200, Atg101, leading to phagophore assembly (Cheong & Klionsky, 2008; Cheong, Nair, Geng, & Klionsky, 2008; Mao, et al., 2013) (Fig 1a). Phagophore formation is a vital step in the autophagy pathway as it leads to formation of the double membrane sequestering compartment – the autophagosome (Mizushima, Yoshimori, & Ohsumi, 2011; Suzuki, Kubota, Sekito, & Ohsumi, 2007). Several Atg proteins participate in this process including Beclin1, Vps34, Ambra1, Atg6, Atg14 and Atg38 (Z. Xie & Klionsky, 2007) (Fig 1a). After initiation of phagophore formation, the next step is its expansion and maturation. This is carried out by Atg12 and light chain 3 (LC3), the two Ubiquitin-like proteins. Atg12 forms a complex with Atg5 and Atg16L1 (also known as E3 enzyme) with the action of E1 and E2-like enzymes Atg7 and Atg10 (Nakatogawa, 2013) covalently attaches to phosphatidylethanolamine (PE) (Fig 1b) , a lipid, to from a LC3-PE complex on the surface of

the autophagosome, resulting in the formation of a mature autophagosome (Satoo, et al., 2009) (Fig 1b). The conjugation of PE with LC3 requires Atg7, an E1-like enzyme (Tanida, et al., 1999), and Atg3, an E2-like enzyme (Ichimura, et al., 2000). For the efficient expansion of the phagophore and recycling of LC3-PE, Atg4, a cysteine protease (Satoo, et al., 2009) is required. These mature autophagosomes filled with cargo marked for degradation can now fuse with the lysosome, forming a fusion compartment autolysosome (Fig 1c). Cargo engulfed by autophagosome require ubiquitination and is recognised by specific adaptor molecules including p62, NBR1, NDP52, VCP and optineurin (Shaid, Brandts, Serve, & Dikic, 2013). This flagged cargo then binds to LC3/GABARAP/GATE16 family on the internal membrane of the autophagosome (Fimia, Kroemer, & Piacentini, 2013). Inside autolysosomes the cargo is degraded by hydrolases present in the acidic lumen of the lysosomes (Fig 1c). The functional role of the primary autophagy molecules has been extensively reviewed previously (Dikic & Elazar, 2018; Kaur & Debnath, 2015; Mizushima, 2007). Furthermore, autophagy pathway could also be regulated by TOR independent pathways like inositol signalling pathways (Sarkar, et al., 2005), Ca^{2+}/calpain pathway (Williams, et al., 2008), cAMP pathway (Williams, et al., 2008) etc. It is also interesting to note that autophagy pathway, which is mainly a cellular degradation pathway is intimately linked to cell death and is different from other programmed cell death mechanisms like apoptosis, necrosis and necroptosis. Autophagy cell death is often seen associated with increased number of autophagosomes inside the cell (Yonekawa & Thorburn, 2013). However, there is a complex crosstalk between autophagy and apoptosis where the calpain-mediated cleavage of Atg5 can trigger cell death by apoptosis (Yousefi, et al., 2006). In this review we have only focused on autophagy as a cytoprotective pathway.

3. Autophagy in maintenance of vascular smooth muscle cell function

Vascular smooth muscle cells (VSMCs) are the principal cellular components of blood vessels. These cell types both play a key role in maintaining blood flow, vessel tone, and are crucial for restoring vascular homeostasis during mechanical shear stress, vascular injury and blood clots (Cahill & Redmond, 2016). Crucially, autophagy is essential for the maintenance of physiological vascular cell function (Grootaert, et al., 2015; Liao, et al., 2012; Vion, et al., 2017).

VSMCs are the most abundant cells in the medial layer of arteries, where they maintain vessel dilation and constriction, regulating blood pressure and distribution of oxygen and nutrients to surrounding cells (Lacolley, Regnault, Nicoletti, Li, & Michel, 2012). A defining feature of VSMCs is their heterogeneity, i.e. they can switch from a contractile (quiescent) phenotype to a proliferative, synthetic (osteogenic) phenotype. VSMCs with a contractile phenotype express markers including SM-α actin, calponin and SM22α, and have decreased mobility, reduced proliferation, and lower extracellular matrix (ECM) production (Fig 2). However, following phenotypic switching they start to express markers including matrix metalloproteinase, collagenase, alkaline phosphatase (ALP), vimentin, osteopontin and Runx2 (Iyemere, Proudfoot, Weissberg, & Shanahan, 2006) (Fig 2). The cells are now capable of moving to the intima, proliferating and enhancing ECM formation. These osteogenic VSMCs are also a crucial source of calcifying matrix vesicles (Naik, et al., 2012). This phenotypic switching is prompted by various biochemical and physical environmental cues and is seen in several conditions including atherosclerosis, diabetes, hypertension and aging (Durham, Speer, Scatena, Giachelli, & Shanahan, 2018; Lacolley, Regnault, & Avolio, 2018; Reddy, et al., 2016; Touyz, et al., 2018) (Fig 2).

PDGF (platelet derived growth factor) is a principal phenotype switching cytokine elevated during vascular injury and diseases including hypertension, atherosclerosis, and diabetes (Egan, Wainwright, Wadsworth, & Nixon, 2005; Gomez & Owens, 2012; Lacolley, et al., 2018). Studies in VSMCs have shown that PDGF stimulates cell phenotype switching, concomitantly activating autophagy (Salabei, et al., 2013) (Fig 2). The role of autophagy here is likely to be mediated through the degradation of proteins including SM-α actin, calponin and SM22α, required by VSMCs to maintain the contractile phenotype. However, genetic ablation of autophagy in PDGF-treated VSMCs is required to establish whether this phenotypic switching is entirely autophagy dependent. Further studies are also required to elucidate whether autophagy is crucial to VSMC phenotype switching in response to additional stimuli including prostacyclins, statins, amino acids and growth factors.

It has been shown that increased cytosolic concentrations of Ca^{2+} (e.g. thapsigargin treatment) induces autophagosome formation both by TOR dependent and independent pathways (Williams, et al., 2008). However, this increase in autophagosome numbers does not leads to enhanced autophagy, rather causes a decline in autophagic clearance via lysosomes (Ganley, Wong, Gammoh, & Jiang, 2011). The reasons behind autophagy impairment under increased calcium concentrations remains to be elucidated. Interestingly autophagy is shown to regulate Ca^{2+} homeostasis in VSMCs. VSMC specific deletion of *Atg7* (*Atg7$^{fl/fl}$ SM22α-Cre^{+})* in mice causes an imbalance between Ca^{2+} uptake and Ca^{2+} release. The voltage-gated Ca^{2+} channels which promotes Ca^{2+} entry inside the cell from the extracellular space were more sensitive to depolarisation (open for Ca^{2+} entry from extracellular space) in autophagy defective VSMCs. On the contrary there was reduced expression of the plasma membrane Ca^{2+} ATPase required for removal of Ca^{2+} to extracellular space, leading to elevated basal levels of intracellular Ca^{2+} levels inside the cell (Michiels, Fransen, De Munck, De Meyer, & Martinet, 2015). This study clearly shows the crucial role of autophagy in regulating Ca^{2+} flux in VSMCs which further

have consequences on contractile capacity of aorta. It would be interesting to investigate the calcification levels of these cells and also if restoring autophagy pharmaceutically could normalise the Ca^{2+} flux and the aortic contractibility.

4. Autophagy in cardiovascular calcification

Vascular calcification (VC) is a significant risk factor for cardiovascular mortality and morbidity in patients with chronic kidney disease, atherosclerosis and diabetes. Previously considered a passive process due to ageing, recent advances suggest that VC is an actively regulated cell mediated process that shares many similarities with bone formation (Doherty, et al., 2003). According to its location, three principal types of vascular calcification have been reported – intimal (associated with atherosclerosis), medial (also known as Mönckeberg's sclerosis or arteriosclerosis) and calcific aortic valve disease. The cells involved in vascular calcification include ECs, VSMCs, pericytes, calcifying vascular cells and valve interstitial cells, (Meng, et al., 2018; Pillai, et al., 2017; Yao, et al., 2013; D. Zhu, Mackenzie, Farquharson, & Macrae, 2012). These cells form a calcified matrix and undergo a bone-like osteogenic phenotypic transition in the presence of this calcifying environment. This drives apoptosis (Ewence, et al., 2008), matrix vesicle (MV) release (N. X. Chen, O'Neill, & Moe, 2018) and osteogenic differentiation in VSMCs (Liu, Lin, Ju, Chu, & Zhang, 2015) along with hydroxyapatite deposition in tissues and vessels (Lee, Morrisett, & Tung, 2012). Enriched with a concoction of calcifying enzymes, the MVs released from osteogenic cells facilitate hydroxyapatite formation in the ECM (Buchet, Pikula, Magne, & Mebarek, 2013).

Calcifying VSMCs express genes like transcription factor Msx2 (Andrade, Carmo, Farias-Silva, & Liberman, 2017; Zhou, et al., 2013), Runx2 (Speer, Li, Hiremath, & Giachelli, 2010) and phosphate transporter PiT-1 (X. Li & Giachelli, 2007). High Pi increases PiT-1 expression, which leads to elevated levels of intracellular Pi. This further enhances Runx2 expression and

the osteogenic transition of VSMCs (Fig 2). Tissue non-specific alkaline phosphatase (TNAP) expression is also central to the vascular calcification process. TNAP hydrolyses pyrophosphate (PPi), a calcification inhibitor, generating phosphate for hydroxyapatite formation in calcifying VSMCs (Sheen, et al., 2015). Furthermore, the ankylosis protein (ANK) and ecto-nucleotide pyrophosphatase/phosphodiesterases-1 (ENPP1) inhibit vascular calcification through enhancing extracellular PPi levels (Back, et al., 2018; Ho, Johnson, & Kingsley, 2000) and matrix Gla protein (MGP) inhibits vascular calcification possibly by functional inhibition of bone morphogenetic proteins (BMP-2 and BMP-4) in VSMCs (Barrett, O'Keeffe, Kavanagh, Walsh, & O'Connor, 2018; Bjorklund, et al., 2018).

Exciting new evidence suggests that the autophagy pathway may play a pivotal role in regulating the key events underpinning the progression of vascular calcification. Here we discuss current experimental evidence supporting the crucial role of autophagy in regulating vascular calcification.

4.1 Autophagy in CKD medial calcification

Hyperphosphatemia (high serum Pi levels) regulates VC in chronic kidney disease (CKD) patients and predisposes them to progressive VC (Giachelli, 2009). Increased Pi levels induce calcification of VSMCs and the surrounding ECM (Giachelli, 2009) and transforms the cells into an osteogenic phenotype which drives further calcification (Kendrick & Chonchol, 2011). Hyperphosphatemia enhances endothelial dysfunction through the regulation of autophagy. High concentrations of Pi inhibit TOR signalling *in vitro* and thus, enhance the autophagic flux. This flux offers the ECs protection from Pi-induced apoptosis (Hsu, et al., 2015). Further *in vivo* studies demonstrated increased, LC3 expression in the endothelial cells from CKD rats compared to sham-operated controls. Similar *in vitro* protective effects of autophagy has been shown in VSMCs whereby increased autophagy levels counteract VC induced by reactive

oxygen species (ROS) under high Pi levels (Dai et al., 2013). These observations are reinforced by recent *in vivo* experiments on DBA/2 mice which develop uremic media calcification when fed with high Pi diet. These mice show increased expression of the autophagy markers LC3-II, p62, Igfbp3, Atg16l1 in aortic VSMCs compared to control mice (Frauscher, et al., 2018). Treatment with rapamycin significantly reduced aortic calcification and release of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6), with enhanced survival noted in these mice (Frauscher et al., 2018).

These compelling observations suggest a regulatory role of autophagy pathway in the homeostasis of Ca^{2+} and Pi in the population of VSMCs modulated during CKD. Furthermore, these studies establish high Pi as a stress signal for TOR, a key regulator of autophagy.

4.2 Autophagy in diabetic medial calcification

Patients with type-II diabetes mellitus show extensive vascular calcification with disturbed vessel wall homeostasis characterised by endothelial dysfunction and phenotypic switching of VSMCs (Casella, Bielli, Mauriello, & Orlandi, 2015; Dhananjayan, Koundinya, Malati, & Kutala, 2016; Harper, et al., 2016). Recent studies have shown that autophagy induction inhibits both the endothelial dysfunction (Fetterman, et al., 2016; Y. Xie, et al., 2011) in endothelial cells from diabetic patients and phenotypic switching of VSMCs in diabetic vascular lesions (An, Li, Wei, Li, & Xu, 2018; Qiu, et al., 2018). These studies demonstrate a protective role of autophagy in diabetic vascular disorders.

Hyperglycemia, a known inducer of vascular calcification increases posttranslational modification of proteins by O-linked N-acetylglucosamine (O-GlcNAcylation) in diabetic arteries (Heath et al., 2014). Furthermore, they show that O-GlcNAcylation of AKT (protein kinase B) leads to AKT phosphorylation and activation, thereby promoting VC. AKT

activation promotes activation of TOR and thus suppresses autophagy. Inhibition of TOR by rapamycin suppressed this O-GlcNAcylation mediated VC, suggesting a novel mechanistic role of autophagy in abrogating hyperglycemia induced VC (Heath, et al., 2014).

The induction of autophagic flux through the application of tert-butyl hydroquinone (tBHQ) treatment has demonstrated an increase in aortic nuclear factor (erythroid-derived 2)-like 2, using a diabetic mouse model. Treatment with tBHQ provided atheroprotection by reducing both inflammation and the lipid content of atheroma plaques (Lazaro, et al., 2018). Selective uptake of lipid droplets by autophagosomes is an emerging pathway in cellular lipid metabolism (Zechner, Madeo, & Kratky, 2017) which will have crucial implications in metabolic disorders such as diabetes, and requires further investigation.

5. Link between MVs and autophagic machinery

MVs refer to nano (20-200 nm) spherical bodies which are known to bud from the plasma membrane (Fedde, 1992; Thouverey, Strzelecka-Kiliszek, Balcerzak, Buchet, & Pikula, 2009). They are made up of a lipid bilayer enriched with amorphous Ca^{2+} and Pi along with enzymes including TNAP, PHOSPHO 1, Na+/K+ ATPase, ENPP1, and Pit1 (Golub, 2011). MVs are typically found associated with small crystals of calcium phosphate hydroxyapatite mineral (Cui, Houston, Farquharson, & MacRae, 2016; Golub, 2009). In context of atherosclerosis, VSMCs and macrophages are the primary source of these calcified MVs which are released into the collagen rich matrix in the intima. The MVs promote atherosclerotic calcification, directly leading to the formation of calcified plaques (Chistiakov, Myasoedova, Melnichenko, Grechko, & Orekhov, 2017).

It has been shown that MV membranes are enriched in phosphatidylethanolamine (Golub, 2009, 2011), which is also a main constituent of the mature autophagosome membrane (Yin, Pascual,

& Klionsky, 2016). Furthermore, TEM images of MVs reveal that they may have a single, double or a multi-layered membrane structure especially in the context of VC in atherosclerosis (Perrotta & Perri, 2017). Moreover, it is possible that, precursors of calcification such as Ca^{2+} and Pi are formed or processed initially within a subcellular compartment such as endosomes, multi vesicular bodies, autophagosome or autolysosome as part of intracellular control of Ca^{2+} and Pi homeostasis in the cellular energy landscape. Indeed, there is recent evidence to support this view; during Ca^{2+} mediated DNA transfection, calcium phosphate precipitates (CPP), not the DNA, induce autophagosome formation in HEK293 cells (X. Chen, et al., 2014). The CPP-DNA complex enter the cells via endosomes which interact with LC3-positive autophagosomes once inside the cytoplasm (Fig 3a). These endosomes are galectin-3 positive, suggesting that they are damaged. Furthermore, these CPP-induced LC3 positive vesicles co-localise with ubiquitin and p62, a selective autophagy adaptor. Interestingly, these vesicles also colocalise with LAMP1, the lysosomal marker, completing the autophagy cycle. Annexin-V which is a major constituent of the MV is also known to have role in formation of mature autophagosomes and is seen present on the lysosomal membrane where it participates in Ca^{2+} signalling (Ghislat & Knecht, 2012). It is also interesting to note that autophagosomes from calcified mouse primary osteoblasts are packed with calcified hydroxyapatite (Nollet et al., 2014). It is possible that the acidic lysosomes have a role in solubilizing this hydroxyapatite when they fuse with these autophagosomes.

Recent proteomic analysis of MVs released from rat VICs by our laboratory shows expression of autophagic proteins including LAMP1, LAMP2 and LAMTOR1 (Cui, et al., 2016); LAMP-I and LAMP-II are lysosomal membrane proteins required for recycling via the autophagy pathway. LAMTOR1 is part of the regulator complex on the lysosomal membrane (Colaco & Jaattela, 2017). These data suggest that MVs may be entwined with the network of autophagic vesicles either at the stage of their formation or release during the process of vascular

calcification (Fig 3a and 3c). Indeed, the recent emergence of a new field of secretary autophagy (as opposed to degradative autophagy), whereby autophagic machinery participates in conventional and unconventional secretions via plasma membrane (Ponpuak, et al., 2015) may present novel mechanistic avenues to explore (Fig 3).

6. Mitophagy in vascular calcification

Mitochondria have the capacity to accumulate Ca^{2+} in an energy dependent manner and are a crucial regulator of cellular Ca^{2+} homeostasis (Glancy & Balaban, 2012). Excess Ca^{2+} intake by mitochondria triggers the opening of the permeability transition pores and the release of cytochrome C, resulting in cell death by apoptosis or necrosis (Izzo, Bravo-San Pedro, Sica, Kroemer, & Galluzzi, 2016). Using scanning electron microscopy, mitochondria-derived vesicles enriched with Ca^{2+} and Pi have been observed in calcifying skeletal cells including chondrocytes, osteoblasts and osteocytes (Martin & Matthews, 1970; Pei, et al., 2018; Sayegh, Solomon, & Davis, 1974; Sutfin, Holtrop, & Ogilvie, 1971). Indeed it has been proposed during the process of calcification mitochondria release intracellular Ca^{2+} in mitochondrial- derived vesicles (MDV), which are subsequently transported to the ECM where these vesicles deposit hydroxyapatite (Boonrungsiman, et al., 2012). These MDV enriched with Ca^{2+} could be engulfed by autophagosomes or directly taken up by lysosomes, where they could be either degraded or released outside the cell (Fig 3d). Interestingly, Pei *et al* has demonstrated the release of mitochondrial electron dense granules from human dental pulp stem cells after osteogenic induction and their interaction with autolysosomes (Pei, et al., 2018).

In vascular cells, oxidative stress, elevated Pi levels, inflammation, mitochondrial dysfunction and apoptosis are all intimately associated with the calcification process (Byon, et al., 2008; Giachelli, 2009; Madamanchi & Runge, 2007; Proudfoot, et al., 2001; Shanahan, 2007). In a healthy cell, dysfunctional mitochondria are cleared by autophagosomes via mitophagy; it is

not known whether the calcifying vascular cells perform enough mitophagy to remove these dysfunctional mitochondria or do they accumulate and become a source of mitochondria derived vesicles enriched with Ca^{2+}? Recent studies have provided exciting clues in this direction. β-Glycerophosphate (β-GP) a known inducer of VC (Bai, et al., 2015; Shioi, et al., 1995) leads to phenotypic transition of VSMCs. Interestingly metformin, (used to treat type 2 diabetes), has been shown to induce autophagy to restore β-GP-induced impairment of mitochondrial biogenesis and apoptosis in VSMCs, along with blocking the phenotypic transition of VSMCs (Ma, et al., 2019) (Fig 2). Furthermore, lactate, an inducer of calcification, suppresses both autophagic flux and mitophagy in VSMCs (Y. Zhu, et al., 2019). Interestingly, overexpression of BCL2 Interacting Protein 3, BNIP3, which depolarises mitochondria and induces mitophagy (J. Zhang & Ney, 2009) and thus attenuates lactate-induced calcification (Y. Zhu, et al., 2019). These studies suggest that mitophagy is defective during the progression of vascular calcification. Defining the role of autophagy/mitophagy in restoring mitochondrial homeostasis during the phenotypic switching of VSMCs will provide important therapeutic intervention in vascular calcification disorders.

7. Pharmacological modulation of autophagy in vascular calcification

Several studies have investigated whether modulating autophagy by pharmacological agents can prevent vascular calcification (**Table 1**). Treatment with spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques of $ApoE^{-/-}$ mice, this reduction is specific to VSMCs (Michiels, Kurdi, Timmermans, De Meyer, & Martinet, 2016). Spermidine, a polyamine, is a known inducer of longevity, enhancing autophagy and suppressing necrosis in various models of aging (Eisenberg, et al., 2009). Treating mice with spermine and spermidine reverses age-related cardiac deterioration by inhibiting age-related myocardial morphology alterations, myocardial fibrosis, and cell apoptosis (H. Zhang, et al.,

2017). However, the direct effect of these compounds on vascular calcification remains to be elucidated. Furthermore, treatment with estrogen attenuates arterial calcification by blocking the osteoblastic differentiation of VSMCs and this is via induction of the autophagy pathway (Peng, et al., 2017) (Fig 2). This protective effect of estrogen treatment is enhanced by the addition of rapamycin (Peng, et al., 2017) (Fig 2). Valporic acid, an autophagy inducer, has also been shown to inhibit VSMC calcification *in vitro* (Dai, et al., 2013). Additionally, rapamycin has been reported to inhibit vascular calcification in the DBA/2 diabetic mouse model (Frauscher, et al., 2018). Pharmacological modulation of autophagy may therefore have therapeutic efficacy for ameliorating disorders associated with VC and preventing their onset. However, most of these studies are based on pharmacological modulators such as rapamycin and valporic acid, which may induce non-specific effects, for example rapamycin is an immunosuppressant (Dumont & Su, 1996). Tissue/cell-specific ablation of the key regulators of autophagy is therefore required to delineate the specific effects of autophagy. For example, the use of a targeted autophagy inducer such as peptide TAT Beclin (Shoji-Kawata, et al., 2013) or the modulation of master regulator of autophagosomes and lysosomal biogenesis transcription factor EB (TFeb) (Napolitano & Ballabio, 2016) in ECs and VSMCs could shed more light into the intricate involvement of the autophagy process with the progression of VC. High through-put chemical screening for novel autophagy modulators is ongoing for diseases like cancer, neurodegeneration and viral and bacterial diseases (Panda, et al., 2019). It would be interesting to investigate if these novel autophagy modulators have an effect on the process of VC.

8. Concluding remarks

Our knowledge of the role of autophagy in cardiovascular calcification is relatively limited at this time. Nonetheless, there is crucial growing evidence that autophagy may protect

cardiovascular tissue from calcification. It would be interesting to investigate whether membranes from autophagic vesicles contribute to the formation of MVs or if a selective form of autophagy is involved in recycling amorphous Ca^{2+} and Pi or hydroxyapatite (Fig 3a, b, c). Precise understanding of the role of autophagy in VC can be achieved by making novel VC mice models with VSMC specific deletion or overexpression of autophagy genes. It's also crucial to understand the intimate interplay between Ca^{2+} flux, autophagy and the process of VC. *In vitro* and *in vivo* experiments are also required to establish the basal levels of autophagy and mitophagy during the entire length of the VC process. This will require the expression of autophagy/ mitophagy specific markers in VC mice models. This precise temporal information will lead to development of focused treatments in future. Furthermore, the development of specific therapeutic autophagy agents, and novel reliable methods for monitoring and measuring autophagy in patients is required. In conclusion, targeting the autophagic molecular machinery is an attractive therapeutic strategy to inhibit progression or induce regression of vascular calcification.

Disclosure statement

The authors declare that they have no conflict of interest.

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Table 1: Effect of autophagy inducers and inhibitors in VC.

Model	Inducer (+)/ Inhibitor (-) of autophagy	Effect	Reference
ECs, *Apoe*$^{-/-}$; *Atg5*$^{flox/flox}$; *VE-cadherin-cre mice*	Wortmannin (-), *Atg5* shRNA (-)	Failure of EC alignment under high shear stress.	(Vion, et al., 2017)
ECs	*Atg3* siRNA (-)	Impaired endothelial nitric oxide synthase phosphorylation, higher accumulation of ROS and inflammatory cytokines like MCP-1 and IL-8 in response to shear stress	(Bharath, et al., 2014)
VSMCs	PDGF (+)	Induces phenotype switching	(Salabei, et al., 2013)
Cultured rat aortic rings, Bovine aortic smooth muscle cells	Valporic acid (+)	Ameliorate Pi- induced VC	(Dai, et al., 2013)
HMEC-1 cells	High Pi (+)	Blocks Pi induced apoptosis	(Hsu, et al., 2015)
VSMCs *Atg7fl/fl SM22α-Cre*$^{+}$	*ATG7 KO* (-)	Elevated intracellular levels of Ca^{2+}	(Michiels, et al., 2015)
VSMCs, calcified arteries	Estrogen (+)	Inhibits the osteoblastic differentiation of VSMCs	(Peng, et al., 2017)
DBA/2 mice model for uremic media calcification	Rapamycin (+)	Reduced aortic calcification, reduced release of proinflammatory	(Frauscher, et al., 2018)

		cytokines, enhanced survival	
Diabetic arteries	Rapamycin (+)	Suppressed calcification	(Heath, et al., 2014)
Diabetic mouse Aorta	tBHQ (+)	Atheroprotective, reduction in inflammation and lipid content of plaques	(Zechner, et al., 2017)
VSMCs	Metformin (+)	Restores mitochondrial biogenesis.	(W. Q. Ma, et al., 2019)
VSMCs	Overexpression of BNIP3 (+)	Attenuates lactate induced calcification and enhances mitophagy	(Y. Zhu, et al., 2019)
VSMCs *ApoE*$^{-/-}$ mice	Spermidine (+)	Reduced lipid accumulation and necrotic core formation in atherosclerotic plaques	(Michiels, et al., 2016)

Figure legends

Figure 1: Overview of key molecular players in the autophagy pathway. **a)** Phagophore formation is triggered under cellular stress which engulfs cellular debris including damaged mitochondria, bits and pieces of ER, ribosomes etc. **b)** This phagophore matures into a double membrane autophagosome decorated with ATG8/LC3-PE. **c)** Mature autophagosomes fuse with single membrane lysosomes with variety of hydrolases in their lumen which degrade the content of the autophagosome. **d)** This fusion compartment is called autolysosome which returns the recycled building blocks to the cell.

Figure 2: Role of autophagy in the phenotypic transition of VSMCs during the progression of VC. a) Basal levels of autophagy are required by the VSMCs for maintaining both the contractile and the osteogenic phenotype. **b)** Increased concentrations of PDGF and intracellular Pi induces cytoprotective autophagy to counter ROS and apoptosis associated with an osteogenic phenotype**. c)** Inducing autophagy using estrogen, rapamycin, metformin prevents osteogenic phenotypic switching.

Figure 3: A possible crosstalk among endosomes, dysfunctional mitochondria, autophagic vesicles and MVs in VC. a) CPP or hydroxyapatite enters the cells via endocytosis and these endosomes fuse with autophagosomes and are passed on to autolysosomes. **b)** Hydroxyapatite may be degraded inside the autolysosomes. **c)** Or is passed on to autolysosomes packed in nano MVs, these autolysosomes fuse with plasma membrane and release of MVs to the ECM, where they aid in hydroxyapatite formation. **d)** Mitochondria derived-vesicles as a source of calcification could be engulfed by autophagosomes or directly taken up by lysosomes where either they could be degraded or released outside the cell via autolysosomes.

Fig. 1

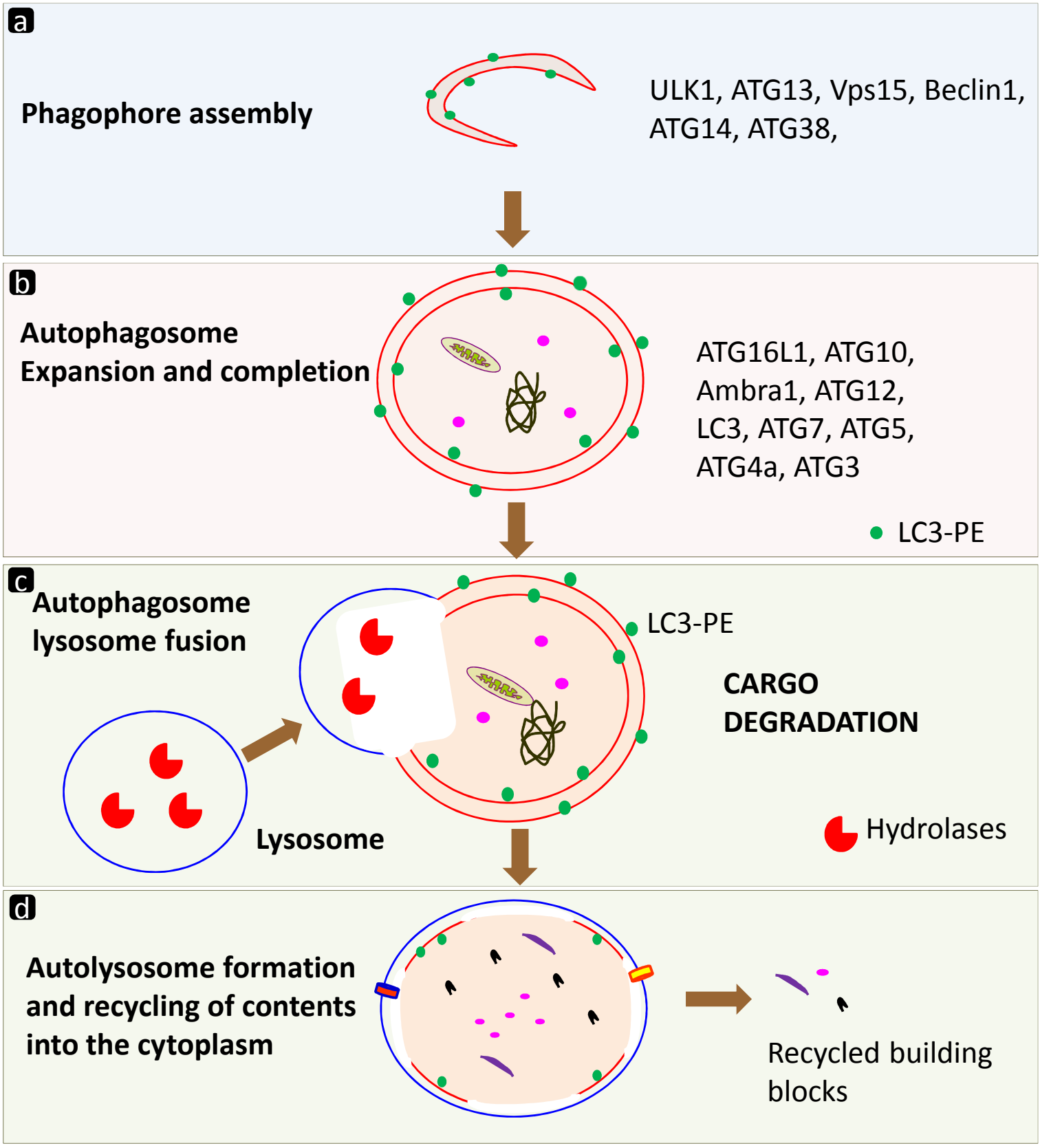

a
Phagophore assembly
ULK1, ATG13, Vps15, Beclin1,
ATG14, ATG38,
b
Autophagosome
Expansion and completion
ATG16L1, ATG10,
Ambra1, ATG12,
LC3, ATG7, ATG5,
ATG4a, ATG3
LC3-PE
c
Autophagosome
lysosome fusion
LC3-PE
CARGO
DEGRADATION
Lysosome
Hydrolases
d
Autolysosome formation
and recycling of contents
into the cytoplasm
Recycled building
blocks

Fig. 2

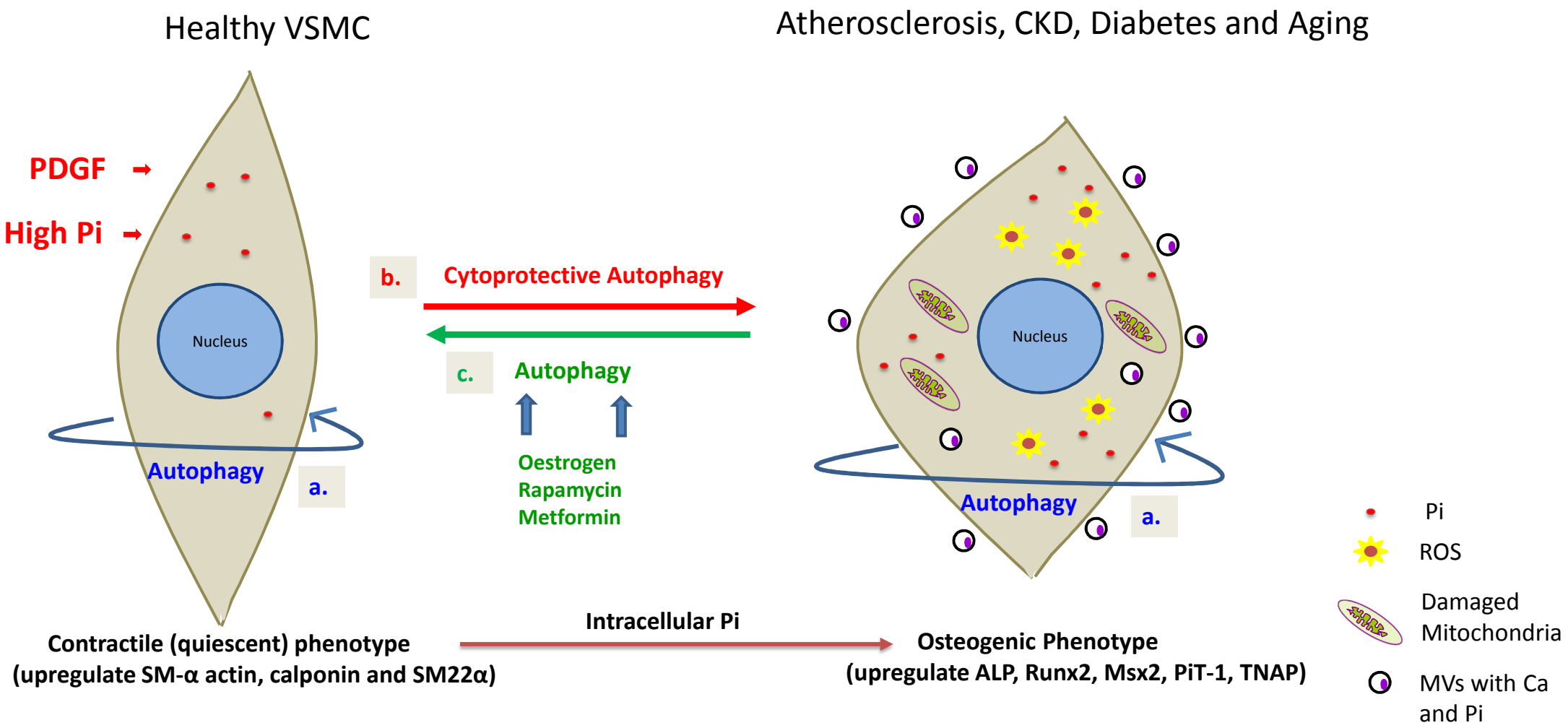
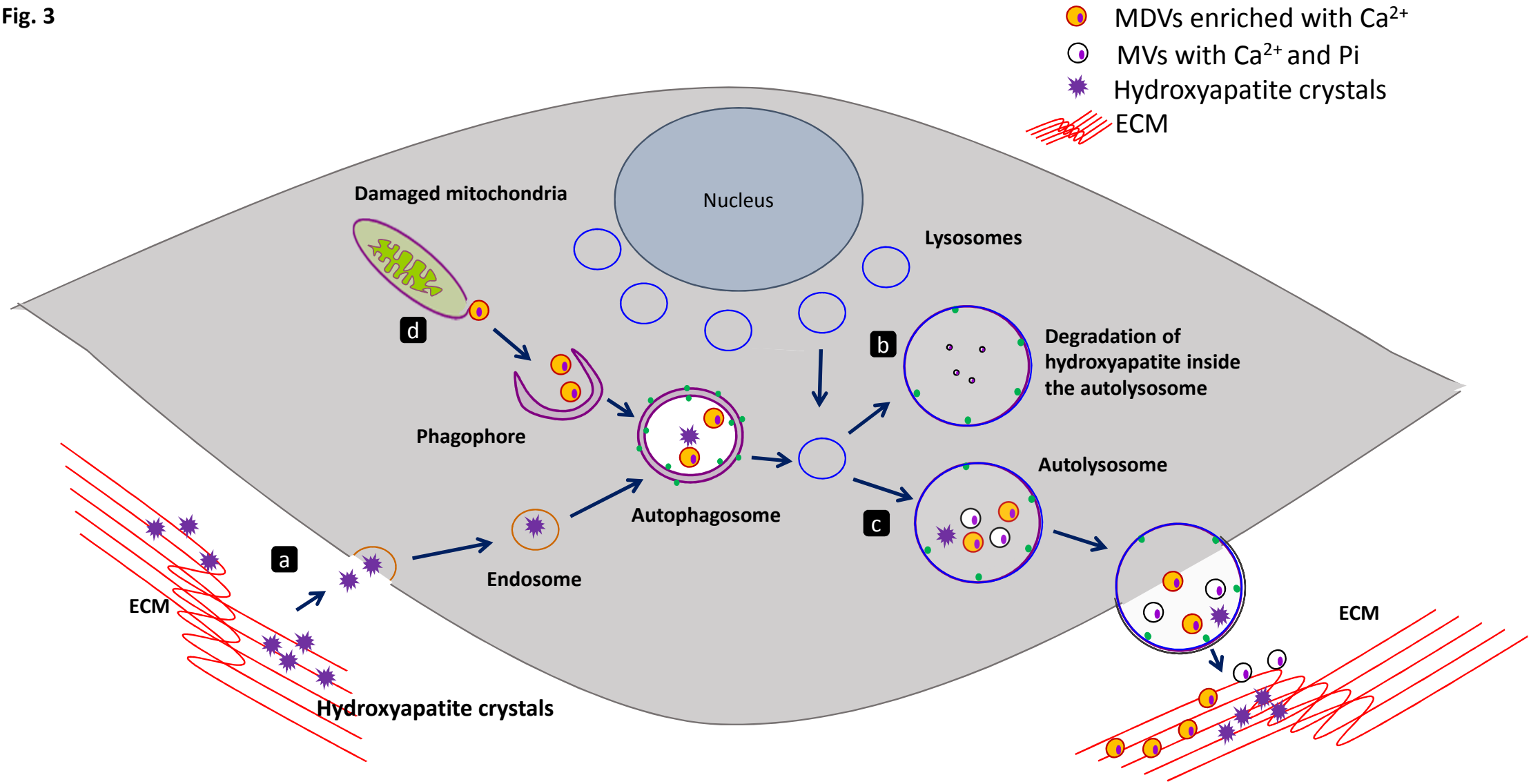

Healthy VSMC
Atherosclerosis, CKD, Diabetes and Aging
PDGF
High Pi
Nucleus
Autophagy
a.
b.
Cytoprotective Autophagy
c.
Autophagy
Oestrogen
Rapamycin
Metformin
Nucleus
Autophagy
a.
Pi
ROS
Damaged Mitochondria
MVs with Ca and Pi
Intracellular Pi
Contractile (quiescent) phenotype
(upregulate SM-α actin, calponin and SM22α)
Osteogenic Phenotype
(upregulate ALP, Runx2, Msx2, PiT-1, TNAP)

Fig. 3


MDVs enriched with Ca^{2+}
MVs with Ca^{2+} and Pi
Hydroxyapatite crystals
ECM
Damaged mitochondria
Nucleus
Lysosomes
d
Phagophore
b
Degradation of hydroxyapatite inside the autolysosome
Autolysosome
Autophagosome
c
a
Endosome
ECM
Hydroxyapatite crystals
ECM