Autophagy in Cardiac Development

Authors:

Hongxin Zhu*

Lin He

Bio-X Institutes, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders, Ministry of Education, Shanghai Jiao Tong University, Shanghai, China

***Address for correspondence**

Hongxin Zhu Ph.D.

Bio-X Institutes, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders, Ministry of Education, Shanghai Jiao Tong University, Shanghai, China

Tel and fax: 86-21-34207232

E-mail: hxzhu@sjtu.edu.cn

Abstract

The heart is the first functional organ to form during embryogenesis. Cardiac development is a multi-factorial complex process requiring precise control of proliferation, differentiation, migration and survival of diverse cell types by interactive networks of genetic and environmental factors. Autophagy is a conserved catabolic pathway that degrades cytoplasmic contents in lysosomes for reutilization. It is well established that autophagy regulates the development of the nervous system, osseous tissue, adipose tissue and lymphocyte. Recent evidences on the basis of in vitro cell culture systems as well as in vivo animal models suggest essential roles of autophagy in cardiogenesis. In this review, we summarize the major findings regarding emerging roles of autophagy and autophagy-related genes in cardiac development, which implicates autophagy in human congenital heart disease, the leading human birth defect worldwide.

Keywords:

Autophagy; Cardiac development; Congenital heart disease

1. Introduction

Macroautophagy (hereafter refer to as autophagy) is an evolutionarily conserved lysosomal degradation pathway that digests intracellular cargoes for reutilization. Once autophagy is induced, an isolation membrane appears and is elongated to form a double-membrane structure engulfing cytoplasmic contents termed the autophagosome. The autophagosomes then fuse with lysosomes and the contents inside autophagosomes are degraded. In the past 25 years, around 40 autophagy-related genes (Atg genes) have been identified by genetic screening of yeast autophagy-defective mutants. These Atg genes constitute core machinery of autophagy, which allows this sequential, multi-stage, fundamental biological process to proceed. It turns out that the majority of yeast Atg genes and the core machinery formed are well-conserved in higher eukaryotes. Autophagy has long been considered a non-selective bulk degradation pathway. However, accumulating evidences have shown that autophagy is capable of selectively eliminating unwanted cellular cargoes such as protein aggregates (Lamark & Johansen 2012, Kirkin et al. 2009), damaged or redundant mitochondria (Novak & Dikic 2011, Youle & Narendra 2011, Joshi & Kundu. 2013), lipid droplets [(Singh & Cuervob 2012) and cytosolic pathogens (Lerena et al. 2010).

Under normal physiological conditions, autophagy occurs at basal levels in virtually all cell types to maintain cellular homeostasis by removing intracellular materials. In response to stress, autophagy pathway can be activated or suppressed. Although in most settings, autophagy is considered to be a protective mechanism, impaired or excessive autophagic degradation of cellular contents can be harmful, which is linked to many types of human diseases such as cancer, neurodegeneration, myopathy, metabolic syndrome and cardiovascular disease (Kroemer 2015, Choi et al. 2013).

During embryogenesis, the heart is the first functional organ to form. Mammalian heart development is initiated with the formation of cardiac progenitor cells within nascent mesoderm during gastrulation, which gives rise to cardiac crescent. Cardiac crescent then coalesces to form the linear heart tube, which undergoes rightwards looping for the proper development of the future cardiac chambers. Subsequently, maturation and septation of the heart occurs, resulting in the formation of the four-chambered heart. Lastly, the cardiac conduction system is developed and the coronary vessels are formed. The heart itself is originated from the mesoderm. However, cell types from other origins also contribute. During cardiac looping, the endocardial cushion, formed via epithelial-mesenchymal transformation (EMT), participates in the late formation of heart valves and septation of the chamber. Migratory neural crest cells from ectoderm contribute to the division of the outflow tract into the aorta and pulmonary trunk. A subset of epicardial cells invade into the myocardium via EMT and develop into fibroblast and coronary smooth muscle cells (Icardo et al. 1996, Moorman & Christoffels. 2003, Sieber-Blum et al. 2004).

Heart development is a complex process regulated by interactive networks of genetic and environmental factors. Anomalies of cardiac development lead to congenital heart disease (CHD), the most common type of congenital anomalies in live births.

It has been demonstrated that autophagy is essential for organogenesis during embryonic development (Di Bartolomeo et al. 2010, Aburto et al. 2012). Emerging evidences suggest that autophagy modulates cardiogenesis. In this review, we summarize the recent findings on autophagy in cardiac development based on in vitro cell culture systems and in vivo animal models.

2. Core machinery of autophagy

The understanding of molecular mechanisms of autophagy is dramatically deepened due to identification and characterization of Atg genes, most of which are well conserved from yeast to mammals. The autophagy process can be divided into several sequential steps: autophagic induction (initiation), nucleation of phagophore, phagophore elongation and closure to form a complete autophagosome, autophagosome maturation, lysosomal degradation and release of resultant products. Each step is executed by specific core machinery comprised of Atg proteins. In this section, we briefly describe the core machinery of autophagy in mammalian cells.

2.1. ULK complex

In mammalian cells, the ULK complex, which is composed of ULK1 (or ULK2), FIP200, Atg13 and Atg101, functions at the initiation stage (Hosokawa et al. 2009). In this complex, Atg13 binds to ULK and mediates the interaction of ULK and FIP200. ULK-Atg13-FIP200-Atg101 complex is directly targeted by mTOR. Under nutrient-enriched conditions, mTOR phosphorylates ULK and Atg13, which suppresses autophosphorylation of ULK and FIP200 phosphorylation by ULK. Rapamycin or nutrient deprivation separates mTOR from ULK complex and dephosphorylates ULK and Atg13, leading to ULK activation and FIP200 phosphorylation, which allows autophagy process to proceed (Jung et al. 2009, Ganley et al. 2009).

2.2. PI3KC3/VPS34 complex

Once autophagy is initiated, PI3KC3/VPS34 complex (referred to nucleation complex) is recruited by ULK complex. PI3KC3/VPS34 complex converts phosphatidylinositol (PtdIns) into PtdIns3P, which in turn recruits Atg proteins to assemble and elongate phagophore membrane (Zeng et al. 2006). PI3KC3/VPS34 binds to an adaptor protein Beclin 1 (BECN1), which interacts with a number of other proteins to regulate PI3KC3/VPS34 activities. Among them, BECN1- PI3KC3/VPS34-Atg14L complex promotes PtdIns3P generation and subsequent phagophore assembly (Zhong et al. 2009), while the Bcl-2 family of proteins such as Bcl-2 and Bcl-XL binds BECN1 and separates it from PI3KC3/VPS34, leading to suppression of PtdIns3P generation and autophagosome formation (Pattingre et al. 2005, Maiuri et al. 2007). Ambra1 and Rhes

compete with Bcl-2 for binding BECN1, resulting in enhanced autophagosome formation (Di Bartolomeo et al. 2010, Strappazzon et al. 2011). In addition, BECN1-VPS34-UVRAG-Bif complex has also been shown to stimulate autophagosome formation (Liang et al. 2006, Takahashi et al. 2007).

2.3. Ubiquitin-like conjugation systems

Two ubiquitin-like conjugation systems, Atg12-Atg5-Atg16L (Atg16 in yeast) complex and LC3-phosphatidy-lethanolamine (PE) complex are crucial in phagophore elongation (Mizushima et al. 1998, Ohsumi & Mizushima 2004). Atg12, the first identified ubiquitin-like protein essential for autophagy, covalently interacts with Atg5, which is catalyzed by E1-like activating enzyme Atg7 and E2-like conjugation enzyme Atg10 （Mizushima et al. 1998). Then Atg12-Atg5 forms a complex with Atg16L, which serves as E3 ligase to transfer ubiquitin-like protein LC3 to PE to form LC3-PE complex (also called LC3 II) (Romanov et al. 2012). LC3-PE complex is attached to the membrane of phagophore (Kabeya et al. 2000). In addition, Atg9, a multi-spanning transmembrane protein, mediates the continuous delivery of membrane source for phagophore elongation (Zavodszky et al. 2013). The membrane source, together with the ubiquitin-like conjugation systems allows the elongation of phagorphore membrane, ultimately leading to the closure of the phagophore membrane to form a complete autophagosome.

2.4. Core machinery of autophagosome maturation

Following completion of autophagosome formation, autophagosome maturation occurs, in which the autophagosomes fuse with late endosomes and lysosomes to form autolysosomes. Fusion of autophagosomes with lysosomes allows autophagic contents to be degraded by various types of hydrolases and the resultant products are released into the cytoplasm for reutilization. Currently, the core machinery for autophagosome maturation is not completely understood. However, many molecules directly involved in this process have been identified, including soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins (Guo et al. 2014, Takáts et al. 2014), Endosomal sorting required for transport (ESCRT) proteins (Rusten & Stenmark 2009), the Homotypic fusion and protein sorting (HOPS) complex (Moreau et al. 2011, Jiang et al. 2014) and Rab GTPase (Chua et al. 2011, Ao et al. 2014). In addition, some other proteins such as VCP (VCP/p97), a member of the AAA+-ATPase family of chaperone-like proteins, and SNAPIN, an adaptor protein in SNARE complex have also recently been uncovered to have essential roles in autophagosome maturation (Shi et al. 2016, Tresse et al. 2010).

3. The role of autophagy in cardiac development

It is well established that autophagy regulates the development of the nervous system (Hara et al. 2006, Vázquez et al. 2012), osseous tissue (Liu et al. 2013),

adipose tissue (Singh et al. 2009) and lymphocyte (Miller et al. 2008). Recent evidences suggest that autophagy is involved in cardiac development. Here we focus on the major findings on autophagy in cardiac development on the basis of in vitro cell culture systems and in vivo animal models. The experimental models used and the cardiac phenotypes observed are shown in Table I and Table II.

Table I

Genetic mouse models used to study autophagy in cardiac development

Genes	Methods	Autophagy change	Cardiac phenotypes	References
Atg5	Cardiomyocyte-specific KO	Reduced	None	Nakai et al. 2004
Atg5	KO	Abolished	Enlarged right atria, VSD, thickened valves	Lee et al. 2014
FIP200	KO	Abolished	Ventricular dilation and thinning of ventricular wall	Liu et al. 2013
Atg13	KO	Abolished	Thinning of ventricular wall	Kaizuka et al. 2015
Frs2α and Fgfr	Tissue-specific KO in cardiac progenitor cells	Increased	Enhanced myocardial differentiation	Zhang et al. 2012

3.1. P19CL6 cells

P19CL6 cell line, derived from P19 cells, is a well-established cardiomyocyte differentiation system (Fathi et al. 2009). In this in vitro model, autophagy, monitored by protein abundance of LC3 II, Atg5, Atg7 and p62 combining autophagic vacuoles detected by electron microscopy, begins to elevate at the early stage and remains at high levels at the late stage of cardiomyocyte differentiation. Suppression of autophagy by knockdown of Atg7 or Atg5 inhibits cardiomyocyte differentiation of P19CL6 cells (Jia et al. 2014). These data provide evidence showing that autophagy is required for cardiac differentiation. Mechanistically, autophagy is capable of selectively degrading β-catenin and NICD, two critical components in Wnt and Notch signaling pathways respectively (Kwon et al. 2008, Watanabe et al. 2006).

Table II

Other model systems used to study autophagy in cardiac development

Cells or Animals	Treatment	Autophagy change	Cardiac phenotypes	References
P19CL6 cells	Knockdown of Atg7/Atg5	Reduced	Inhibition of cardiomyocyte differentiation of P19CL6	Jia et al. 2014
Zebrafish	Knockdown of Atg5/Atg7/BECN1	Reduced	Enlarged atria, defective cardiac looping, aberrant valve development and ectopic expression of transcription factors	Lee et al. 2014
Chicken embryos	Ethanol	Increased	Cardiac bifida	Li et al. 2015
Chicken embryos	Rapamycin	increased	Cardiac bifida	Li et al. 2015
Chicken embryos	High glucose	increased	Heart tube malformation	Wang et al. 2015
Chicken embryos	Rapamycin	Increased	Heart tube malformation	Wang et al. 2015

3.2. Atg5/Atg7/BECN1 knockdown zebrafish

Autophagy occurs during cardiac development in zebrafish (Lee et al. 2014). Zebrafish mutants with knockdown of Atg5, Atg7 and BECN1 genes by antisense morpholino oligonucleotides exhibit similar cardiac defects including pericardial edema, defect in blood flow through the heart, defective heart looping, enlarged atria, or linearized hearts, although the penetrance is distinct with 80% of BECN1 morphants, 62% of Atg5 morphants and 30% of Atg7 morphants. In addition, knockdown of Atg genes leads to increased number of apoptotic cell death. Gene expression profiling shows that many genes involved in cardiac development and function, especially transcription factors including foxn4, tbx5, and tbx2, are aberrantly expressed (Lee et al. 2014). Thus, autophagy regulates the transcription program and cardiac morphogenesis during cardiac development, though the precise mechanisms remain to be elucidated.

3.3. Atg5 knockout mice

Cardiac-specific Atg5 knockout mice were first used to examine the effects of autophagy on heart development by Nakai et al (Nakai et al. 2007). In this study, Atg5flox/flox mice are crossed to transgenic mice expressing Cre recombinase under control of α-myosin heavy chain or myosin

light chain 2v. Thus, autophagy is specifically inactivated in cardiomyocyte after E7.5 to E8 (Lyons et al. 1990, Chen et al. 1998). These mice appear normal and no structural abnormalities are observed in the heart. The authors propose that pathological alterations are prevented by compensatory mechanisms (Nakai et al. 2007). In contrast to cardiomyocyte-specific Atg5 knockout mice, conventional Atg5 knockout mice have enlarged atria and display cardiac defects including abnormal valve morphology, membranous ventricular septal defects and thickened valves. Transcription factors tbx2 is aberrantly expressed in the heart at E9.5 (Lee et al. 2014). This study suggests that autophagy is also required in cardiac development in mammals.

3.4. FIP200 knockout mice

FIP200 is a component of the ULK1 complex, which is essential in autophagy initiation (Hara et al. 2008). FIP200 knockout mice are embryonic lethal at mid/late gestation, i.e. from E14.5-E16.5. The body size of homozygous embryos is smaller. More importantly, these embryos show severe cardiac abnormalities including ventricular dilation, thinning of ventricular wall with sparsely cellular myocardium, and generalized edema. In embryos most severely-affected, the compact layer of myocardium is barely observed in the heart. Moreover, cardiac apoptotic cells are increased. In addition to cardiac defects, FIP200-defiecient embryos also show liver defects (Gan et al. 2006).

3.5. Atg13 knockout mice

Atg13 is another component of ULK1 complex (Hosokawa et al. 2009). Kaizuka et al investigate the role of Atg13 in cardiac development using Atg13-defiecient mice generated by (CRISPR)/Cas9 system and gene trapping. Mice with Atg13 deficiency are embryonic lethal and die after embryonic day 16.5 and no live birth is obtained. Generally, the mouse embryos show growth retardation and the body size is smaller. Importantly, Atg13-deficient embryos show thinning of ventricular wall after E13.5, which is similar to FIP200-deficient mice. However, unlike FIP200-deficient mice, no structural abnormality other than the heart is observed in Atg13-deficient mice (Kaizuka & Mizushima 2015). This study suggests that Atg13 is essential for cardiac development during embryogenesis.

3.6. FGFR and FRS2α knockout mice

Using mice with tissue-specific depletion of FGFR and FRS2α, Zhang et al have shown that FGF signaling axis suppresses differentiation of cardiac progenitor cells into cardiomyocytes (Zhang et al. 2012). Moreover, FGF signaling inhibits autophagy in outflow tract. In vitro differentiation study using cultured embryoid bodies reveals that FGF signaling promotes mesoderm differentiation while suppresses cardiomyocyte differentiation of the mesoderm cells. Inhibition of FGF signaling promotes myocardial differentiation, which relies on autophagy activation, since inhibition of autophagy suppresses myocardial differentiation and conversely, activation of autophagy enhances myocardial differentiation (Zhang et al. 2012). Thus, this study provides the

first in vivo evidence that autophagy regulates differentiation of cardiac progenitor cells.

3.7. Chicken embryo model

Pre-gestational diabetes mellitus and excessive ethanol consumption during gestation have been recognized as major environmental risk factors for CHD (Jenhins et al. 2007). Two recent studies have been performed to determine the role of hyperglycemia and ethanol exposure in cardiac development during chicken embryogenesis (Wang et al. 2015, Li et al. 2015). Wang et al have shown that high glucose exposure leads to malformation of the heart tube. Meanwhile, high glucose treatment induces autophagy. Moreover, treatment of rapamycin, an autophagy inducer, results in similar heart tube malformation phenotypes. The heart tube malformation is resulted from abnormal migration of gastrulating precardiac mesoderm cells during development of bilateral primary heart field primordial (Wang et al. 2015). In the setting of ethanol exposure, the incidence of cardia bifida is increased, which is partially attributed to induced autophagy as evidenced by increased Atg gene expression and mTOR inhibition. Consistently, Rapamycin treatment induces cardia bifida. Cell differentiation and spontaneous beating of cardiac progenitor cells are attenuated by ethanol and rapamycin treatment. Genes regulating cardiac differentiation such as Bmp2, GATA4, GATA5 and FGF8 are abnormally expressed in chicken embryos exposed to ethanol (Li et al. 2015). Thus, during cardiac development, autophagy is tightly controlled and autophagic activity is maintained below a certain threshold. Overstimulation of cardiac autophagy may be detrimental to heart development through disturbing migration of precardiac mesoderm cells and differentiation of cardiac progenitor cells.

4. Mechanisms by which autophagy regulates cardiac development

Although increasing evidences have shown that autophagy is essential for heart development, however, the precise mechanisms remain unclear. Emerging evidences indicate that autophagy regulates cell differentiation, proliferation, migration and death of a myriad of cell types, which are tightly controlled for proper cardiac development.

4.1. Cell differentiation

Cell differentiation is essential for normal cardiac development during embryogenesis. As mentioned above, autophagy promotes cardiomyocyte differentiation of P19CL6 cells. The molecular mechanisms involve selective degradation of β-catenin and NICD, both of which have been demonstrated to regulate cardiomyocyte differentiation during cardiac development (Jia et al. 2014, Kwon et al. 2008, Watanabe et al. 2006). It would be interesting to examine whether selective degradation of regulatory factors for heart development occurs in vivo, which will provide insights into comprehension of cardiac development.

Mitophagy, a form of selective autophagy specifically degrading

mitochondria, may be important for cardiomyocyte differentiation. Mitophagy is required for differentiation of a number of cell types including myoblast (Sbrana et al. 2016, Phadwal et al. 2013, Sin et al. 2016, Kasahara & Scorrano. 2014). During early myogenic differentiation, autophagy is activated and selectively clears mitochondria. Then mitochondria biogenesis and subsequent reformation of mitochondrial networks occur upon differentiation, which allows metabolic switch from glycolysis to oxidative phosphorylation to meet the demands for increased energy of skeletal muscle contraction. Autophagy suppression impairs mitochondria remodeling and interferes with myogenic differentiation (Sin et al. 2016). Similar to skeletal muscle, cardiac muscle is also a type of striated muscle, which contains numerous mitochondria for robust energy production during contraction. Moreover, mitochondria remodeling is an indispensable event for cardiomyocyte differentiation. Thus, it is reasonable to postulate that developmental mitophagy is required for cardiomyocyte differentiation during cardiogenesis.

4.2. Cell proliferation

During normal development, cell proliferation is inversely related to cell differentiation. However, there is no evidence regarding direct regulation of cell proliferation by autophagy during cardiac development. Suppression of FGF signaling axis promotes cardiomyocyte differentiation of cardiac progenitor cells, which, however, is independent of proliferative capacity of these cells (Zhang et al. 2012). Mice deficient for FIP200 or Atg13 show thinning of ventricular wall (Gan et al. 2006, Kaizuka & Mizushima. 2015). However, increased apoptosis rather than decreased cell proliferation is responsible for this phenotype observed in FIP200-deficient mice (Gan et al. 2006). Neither apoptosis nor cell proliferation is shown in the heart from Atg13-deficient mouse embryos (Kaizuka & Mizushima. 2015). More future work is required to investigate the role of autophagy in the proliferation of distinct cell types during heart development.

4.3. Cell death

In most physiological settings, autophagy functions as a cell survival mechanism by degrading cellular components to generate energy or macromolecular building blocks for the cells (Anding & Baehrecke 2015). Thus, autophagy defect may promote cell death and decrease the cell number in the heart, which disrupts normal cardiac development. Indeed, cell death is increased in FIP200-deficient mouse embryos (Gan et al. 2006). It should be noted that the increased apoptotic cells may be caused by failure in the clearance of cell corpses due to autophagy deficiency since autophagy is required for dying cells to release signals to phagocytic cells during embryonic development. However, in this situation, the increased cell corpses could interfere with normal heart development as demonstrated in embryonic development (Qu et al. 2007).

4.4. Cell migration

During cardiac development, migration of several cell types occurs including neural crest cells, cardiac

progenitor cells, epithelial cells and endothelial cells. Autophagy disturbance affects migration of cardiac progenitor cells during chicken heart tube formation, although the molecular mechanisms remain unclear (Wang et al. 2015). A couple of recent studies have shown that selective autophagy degrades focal adhesion proteins including paxillin, vinculin and zyxin, which destabilizes focal adhesion, ultimately leading to motility of various cell types (Kenific et al. 2016, Sharifi et al. 2016). Future work is needed to be done to determine if this mechanism is involved in the regulation of cell migration during heart development and contributes to the cardiac anomalies observed in the model organisms with deficiency in autophagy.

4.5. Autophagy-independent functions

Cardiac defects observed in autophagy-deficient mice may be resulted from autophagy-independent functions of the perturbed genes. Cardiac defects observed in mice deficient for distinct Atg genes are not identical. For instance, the penetrance of cardiac defects is discrepant in Atg5, Atg7 or BECN1 knockdown zebrafish, with 100% of Atg5 mutants, over 80% of BECN1 mutants and approximately 30% of Atg7 mutants displaying cardiac defects (Lee et al. 2014). Moreover, mice deficient for FIP200 and Atg13 show distinct cardiac defects from Atg5-deficient mice (Gan et al. 2006, Kaizuka & Mizushima 2015, Lee et al. 2014). Considering that mammalian orthologues of almost all Atg genes identified so far have autophagy-independent activities, cardiac malformations observed in autophagy-deficient mice may be ascribed to defects in autophagy-independent functions of these genes. However, it should be pointed out that cardiac development is an extremely complex process precisely controlled by genetic and environmental exposures. For genetic factors, in addition to rare variants, common variants, which may modify the phenotypes, also contribute to cardiac defects. Thus, even the identical CHD pathogenic mutations lead to distinct cardiac defects (Marguerie et al. 2006, Misra et al. 2012, Feng et al. 2002, Schott et al. 1998). The discrepancy in cardiac malformations observed in the model organisms deficient for distinct Atg genes may reflect the influences of animal strains, age, gender, cell types in which autophagy is suppressed, change in the extent of autophagy and animal feeding environment on cardiac development.

5. Conclusions

Despite recent progress in the understanding of autophagy in cardiac development, comprehensive works needs to be done to elucidate the underlying molecular mechanisms. In addition, abnormal cardiac development leads to CHD, the most common forms of birth defect, affecting approximately 1% of newborns. CHD arises from genetic variations and exposure to environmental risk factors, with genetic variations playing a predominant role. However, genetic factors have so far been discovered only account for 20% of CHD. With remarkable advances in contemporary genomic technologies, which allow comprehensive analysis of large

cohorts of CHD patients with various malformations, identification of novel genetic and genomic variations including Mendelian mutations, single nucleotide polymorphisms, copy number variations and translocations involved in CHD is accelerating. Although currently no direct evidences have been developed to link autophagy with CHD, given the essential roles of autophagy and Atg genes in cardiac development, it is possible that defects in autophagy and Atg genes is involved in human CHD. Thus, efforts are required to investigate whether autophagy deregulation contributes to human CHD, and if so, what roles autophagy plays in the cause of CHD. Prospective findings will provide insight into our understanding of the etiology and mechanisms of this disease.

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