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Review

Zebrafish as a model to understand autophagy and its role in neurological disease ☆

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ABSTRACT

In the past decade, the zebrafish (*Danio rerio*) has become a popular model system for the study of vertebrate development, since the embryos and larvae of this species are small, transparent and undergo rapid development *ex utero*, allowing *in vivo* analysis of embryogenesis and organogenesis. These characteristics can also be exploited by researchers interested in signaling pathways and disease processes and, accordingly, there is a growing literature on the use of zebrafish to model human disease. This model holds great potential for exploring how autophagy, an evolutionarily conserved mechanism for protein degradation, influences the pathogeneses of a range of different human diseases and for the evaluation of this pathway as a potential therapeutic strategy. Here we summarize what is known about the regulation of autophagy in eukaryotic cells and its role in neurodegenerative disease and highlight how research using zebrafish has helped further our understanding of these processes.

1. Introduction

1.1. Protein degradation pathways

Efficient degradation of proteins is essential to maintain normal cell homeostasis. In eukaryotes, there are two main degradative pathways; the ubiquitin-proteasome pathway and the autophagy-lysosome pathway. The proteasome is a barrel-shaped multi-subunit protein complex, the core of which contains the components necessary for proteolysis. Proteins are generally targeted to the proteasome after they are tagged by a chain of four or more covalently bonded ubiquitin molecules. In addition to the specificity imposed by the requirement of an ubiquitination signal, the narrow core of the proteasome barrel limits the size of proteins that can be degraded via this pathway. Typically, short-lived and long-lived cytosolic and nuclear proteins are degraded by the proteasome. In contrast, macroautophagy (from hereon referred to as autophagy) can mediate non-specific, bulk degradation of long-lived cytosolic proteins and organelles. Autophagic degradation requires the formation of a double-membraned vesicle, the autophagosome, around a portion of the cytoplasm. Ultimately, autophagosomes fuse with lysosomes to form autolysosomes, acidic compartments in which lysosomal hydrolases degrade any proteins contained within the vesicle (see Fig. 1). Autophagy occurs at a basal level in mammalian cells, but is upregulated in response to various physiological stress conditions e.g. starvation. While it is primarily a mechanism to ensure cell survival, there is increasing evidence for the importance of autophagy as a mechanism for cell death, particularly in insect metamorphosis [1].

1.2. Zebrafish models of neurodegeneration

The optical clarity, speed of development, and fecundity of zebrafish have made them a popular vertebrate model for the study of developmental biology, and, more recently, as an animal model to study disease processes [2,3]. The creation of transgenic zebrafish is relatively straightforward [4–7], and has been used to successfully generate models of a range of human neurodegenerative disorders. Diseases caused by dominant mutations can be modeled by expressing the mutated human gene under the control of a zebrafish promoter. Such an approach has been used to model polyglutamine expansion diseases, like Huntington's disease [8], tauopathy [9–11] and amyotrophic lateral sclerosis (ALS) [12]. Furthermore, since zebrafish larvae are transparent, fluorescent transgene constructs can be used in a variety of ways to examine disease pathogenesis, *in vivo*. For example, reporter lines where particular neurons are fluorescently labeled have been used to investigate the sensitivity of monoaminergic neurons to the neurotoxin MPTP [13]. Bi-directional transgenic constructs have been used to create lines in which a fluorescent protein signal is expressed with the same spatial and temporal control as the disease-causing protein [11]. Similarly, direct fusion of a fluorescent protein to the disease-causing transgene has been employed as a read-out of transgene expression, but can also be used as a marker for protein

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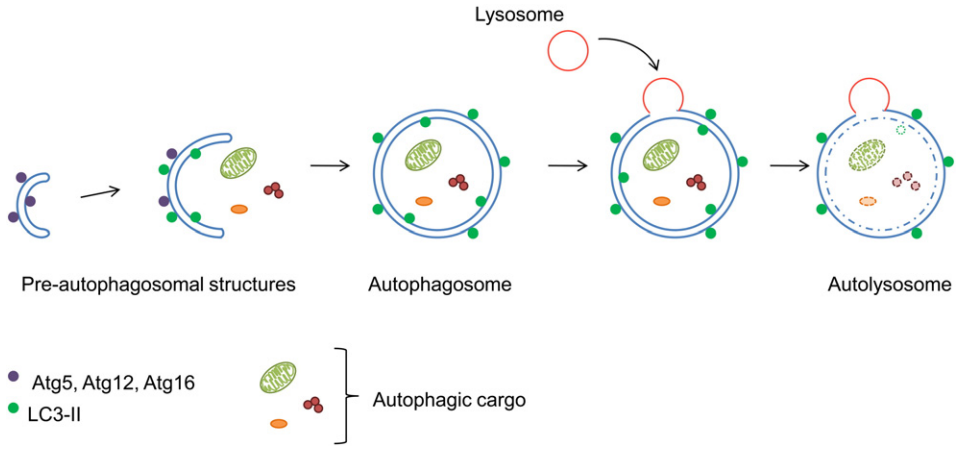

Fig. 1. Schematic model of autophagy. Pre-autophagosomal structures form within the cytoplasm. Atg5, Atg12 and Atg16l proteins are recruited to the structure and facilitate elongation. The elongated membranes enwrap a region of the cytoplasm and its contents in a double-membraned autophagosome. Lysosomes ultimately fuse with autophagosomes releasing lysosomal hydrolases into the vesicle resulting in the degradation of its contents.

aggregation. Such an approach has been used to examine huntingtin aggregate clearance *in vivo*, as a method for validating novel therapeutic strategies [8]. In addition to the creation of stable transgenic lines to model dominant genetic mutations, transient over-expression techniques have been used to this end. In such studies, injection of DNA or mRNA into fertilized eggs results in transient expression of the disease-causing protein during embryogenesis and in early larval stages [14–18]. Although there is an inherent level of variability in gene expression, this method has proved powerful for the study of disease modifiers in models of polyglutamine disease [14] and motor neuron disease [16–19] and in the evaluation of therapeutic strategies [15].

Loss-of-function models of neurodegeneration have also been widely explored in zebrafish (see [20] for review). The most widely used technique for the study of loss-of-function is that of transient knockdown using antisense technologies. Morpholino oligonucleotides are the most commonly used, validated and accepted antisense technique in zebrafish [21], although other antisense technologies, such as peptide nucleic acid mimics (gripNAs) [22], are now gaining popularity. Recently, spatial and temporal control of morpholino knockdown has been described, by combining a neutralizing strand with the morpholino oligonucleotide that is photocleavable by irradiation with UV light [23]. Transient knockdown techniques have been used to develop zebrafish models of Parkinson's disease [24–29], ALS [30] and spinal muscular atrophy (SMA) [31–33]. In addition to their use for developing disease models for loss-of function disorders, antisense knockdown technologies have been used to investigate normal gene function and elucidate novel signaling pathways in a range of neurodegenerative disorders including Huntington's disease [34–37], Alzheimer's disease [38–41] and SMA [32,42–44] and to investigate the role of progranulin and TDP-43 in the pathogenesis of ALS and frontotemporal lobe dementia [17–19,45].

The limitation of such antisense techniques is that knockdown is transient, usually only lasting to 5–7 d.p.f. Therefore efforts have focused on the development of methods for targeted gene knockdown in zebrafish [46–48], with the recent reports of zinc finger nuclease (ZFN) technology holding promise for the widescale and specific generation of heritable loss-of-function alleles [49–51]. In addition, random mutagenesis screens [52,53] have yielded numerous mutant zebrafish lines in which the mutated gene is implicated in a human disease, e.g. muscular dystrophy [54–57], and it is hoped that the continuing screening of mutagenized libraries (e.g. TILLING—Targeting Induced Local Lesions in Genomes) will yield mutations in specific genes of relevance to neurodegenerative disorders.

In addition to the ease of genetic manipulation, zebrafish are also highly amenable to pharmacological manipulation [58]. This allows up and down-regulation of cell signaling pathways by chemical agonists and antagonists, in addition to the use of zebrafish disease models to test therapeutic strategies [8,11] and perform compound screens [15]. The zebrafish offers advantages over equivalent rodent models, since the manifestation of disease phenotypes is typically more rapid than in their rodent equivalent, larvae can be arrayed in multi-well plates, and compound requirement is small. Since zebrafish are highly amenable to both genetic modification [59,60] and direct compound screening in a tractable fashion [61], they are potentially a powerful tool for the investigation of the autophagy pathway and its role in neurodegeneration. The remainder of this article is dedicated to the application of such zebrafish models to the study of autophagy.

1.3. The molecular control of autophagy in eukaryotes

Under normal conditions, autophagy occurs at basal levels, but can be induced rapidly in response to stress conditions and extracellular signals. Target of rapamycin (TOR), a serine/threonine protein kinase, is a central component controlling autophagy, integrating signals from multiple upstream pathways and inhibiting autophagy (see Fig. 2). The regulatory pathways controlling autophagy are well described elsewhere [62], hence this review focuses on aspects of the pathway where the zebrafish model has or could be employed to further our understanding of this process.

mTOR forms two distinct complexes (mTORC1 and mTORC2), which vary both in their subunit components and their function. The mTORC1 complex consists of 3 subunits: mTOR, G protein β-subunit-like (mSLT8) and the regulator-associated protein mTOR (Raptor). Under normal conditions, the mTORC1 complex blocks autophagy by phosphorylating Ulk1 [63–65], but this inhibitory activity is repressed by rapamycin treatment (a specific TOR inhibitor) or starvation conditions, leading to an upregulation of autophagy. mTORC1 is itself inhibited by the action of the tuberous sclerosis complex 1 and 2 proteins (TSC1 and TSC2), which together form a complex (TSC1/2) (Fig. 2). The mTORC2 complex consists of 4 subunits: mTOR, mSLT8, Rictor (rapamycin-insensitive companion of mTOR) and mSin1 (mitogen-activated-protein-kinase-associated protein 1). The mTORC2 complex regulates actin cytoskeleton dynamics and is not involved directly in the regulation of autophagy [66].

The conservation of TOR signaling pathway has been explored in zebrafish using both pharmacological manipulation and morpholino gene knockdown [67]. Zebrafish have a single homologue of mTOR which, although expressed ubiquitously during early embryogenesis,