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ORIGINAL RESEARCH

Induction of *clusterin* Expression by Neuronal Cell Death in Zebrafish

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ABSTRACT

Clusterin, a protein associated with multiple functions, is expressed in a wide variety of mammalian tissues. Although clusterin is known to be involved in neurodegenerative diseases, ageing, and tumorigenesis, a detailed analysis of the consequences of gain- or loss-of-function approaches has yet to be performed to understand the underlying mechanisms of clusterin functions. Since clusterin levels change in neurological diseases, it is likely that clusterin contributes to cell death and degeneration in general. Zebrafish was investigated as a model system to study human diseases. During development, zebrafish *clusterin* was expressed in the notochord and nervous system. Embryonic overexpression of *clusterin* by mRNA microinjection did not affect axis formation, whereas its knock-down by anti-sense morpholino treatment resulted in neuronal cell death. To analyze the function of *clusterin* in neurodegeneration, a transgenic zebrafish was investigated, in which nitroreductase expression is regulated under the control of a neuron-specific *huC* promoter which is active between the stages of early neuronal precursors and mature neurons. Nitroreductase turns metronidazole into a cytotoxic agent that induces cell death within 12 h. After metronidazole treatment, transgenic zebrafish showed neuron-specific cell death. Interestingly, we also observed a dramatic induction of *clusterin* expression in the brain and spinal cord in these fish, suggesting a direct or indirect role of *clusterin* in neuronal cell death and thus, more generally, in neurodegeneration.

KEYWORDS: Clusterin; Neuronal cell death; Neurodegeneration; Zebrafish

INTRODUCTION

Clusterin was discovered in ram rete testis fluids as a secreted glycoprotein which enhances cell aggregation (Blaschuk et al., 1983). Clusterin is expressed in multiple isoforms, with the secreted isoform of clusterin present as a α/β-heterodimer in human tissue fluids (de Silva et al. 1990). Clusterin is widely distributed in many mammalian tissues, including the central and peripheral nervous system as well as in tissue fluids, such as the cerebrospinal fluid (CSF), vitreous body of the eye and fluid-tissue interfaces, such as intestine, ependyma and endometrium (Aronow et al., 1993; Leskov et al., 2003; Trougakos et al., 2005; Bettuzzi, 2009; Charnay et al., 2012). With its wide distribution among organs and fluids, it is not surprising that clusterin is involved in many functions including, but not limited to lipid metabolism and trafficking, membrane

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recycling, promotion/inhibition of apoptosis, inhibition of complement function, maturation of sperm, and, more generally, carcinogenesis and tissue remodeling (Rizzi et al., 2009). A nuclear isoform of clusterin is a cell death-related protein (Reddy et al., 1996), which is present in prostate and breast cancer cell lines (Rizzi and Bettuzzi, 2010). The analysis of clusterin knock-out mice revealed that clusterin plays a role in pancreas regeneration and autoimmunity (Lee et al., 2011).

Clusterin expression is differentially regulated by cytokines, growth factors, and stress-inducing agents in cell-type specific manners (Thomas-Salgar and Millis, 1994; Sensibar et al., 1995; Humphreys et al., 1997; Michel et al., 1997; Trougakos and Gonos, 2002). Interestingly, clusterin expression is upregulated in neuronal and astroglial subpopulations of the mammalian nervous system in chronic neurological diseases, such as Alzheimer's and Parkinson's disease as well as in acute traumatic injuries, such as spinal cord injury and brain ischemia (Sihlbom et al., 2008; Charnay et al. 2012). In particular, levels of clusterin are increased in the CSF of Alzheimer's disease patients (Sihlbom et al., 2008). Thus, it is plausible to assume that elevated clusterin levels in brain tissue and CSF may be implicated in neurodegeneration. However, the functions of clusterin in the broad spectrum of normal and pathological processes have remained poorly understood.

To investigate the functions of clusterin in neurodegeneration, we used zebrafish as an animal model which is proven to be an excellent vertebrate system for modeling human diseases (Guo, 2004; Xi et al., 2011; Peng et al., 2012; Moro et al., 2013; Sasaki and Kishi, 2013). We isolated zebrafish *clusterin* homologues and performed loss- and gain-of-function analyses in early development. To elucidate the role of *clusterin* in neurodegeneration, we established a neuron-specific cell death system using a zebrafish transgenic line and analyzed the cellular pattern of *clusterin* expression in several neurodegenerative states. To ablate neurons in an inducible manner, we used the nitroreductase/metronidazole (Ntr/Mtz) system in combination with the neuron-specific *huC* transgenic line. Nitroreductase (Ntr) is encoded by the bacterial gene *nfsB* and converts the non-toxic prodrug, metronidazole (Mtz), into cytotoxic agents (Curado et al., 2007; Davison et al., 2007). The *huC* is a pan-neuronal marker expressed in early neuronal precursors and mature neurons of the adult nervous system (Kim et al., 1996). Here we report that *huC* promoter-driven Ntr expression combined with application of Mtz resulted in efficient neuron-specific cell death. In parallel, we observed induction of *clusterin* expression in correlation with neuronal cell death in brains and spinal cords of zebrafish.

RESULTS

Isolation of zebrafish clusterin homologues

To obtain coding sequences of zebrafish *clusterin* and *clusterin-like 1*, RT-PCR was performed using a zebrafish cDNA library. These homologues were subcloned as cDNA into the pCS2 expression vector. From the DNA sequencing analysis and alignment of amino acid sequences, we found that zebrafish *clusterin* is homologous to the secreted isoform of

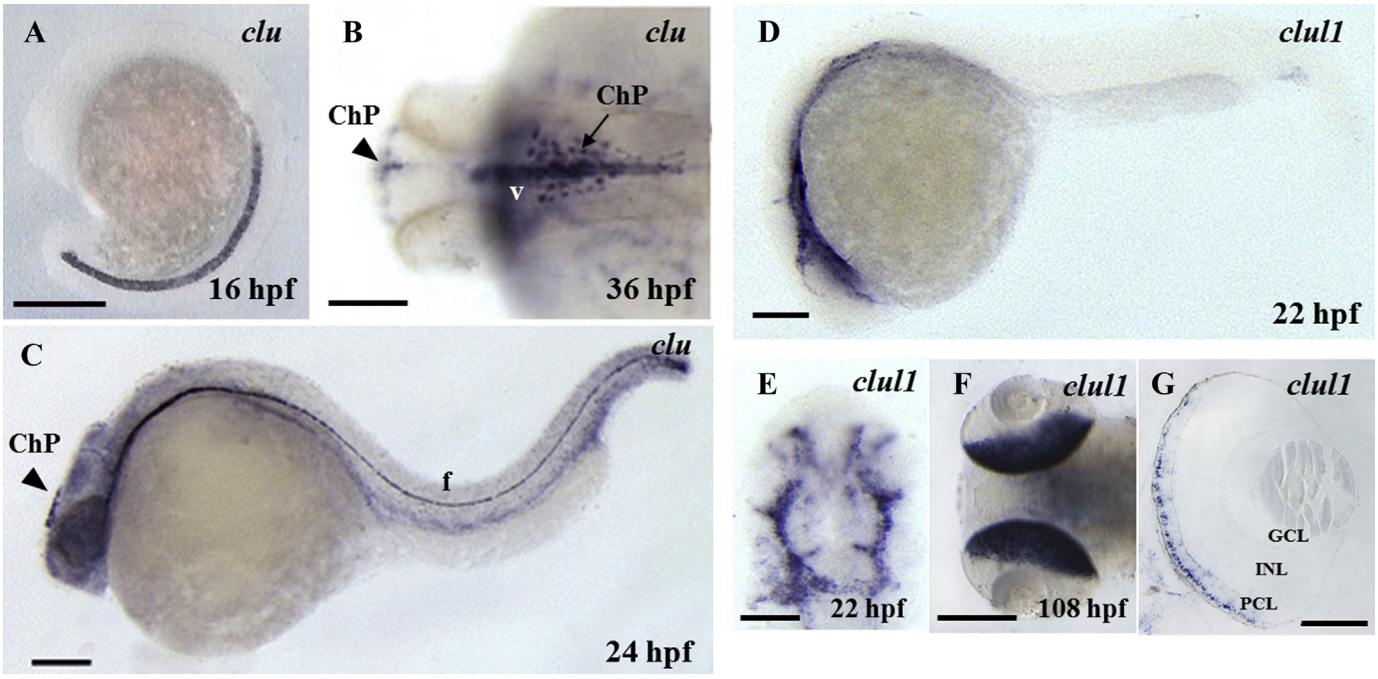

Fig. 1. Expression pattern of *clusterin* and *clusterin-like 1* in zebrafish embryos.

A−C: *clusterin* (*clu*) expression was examined by whole-mount *in situ* hybridization. **A:** *clusterin* transcripts are detected in the notochord at 16 hpf. **B** and **C:** At 24 and 36 hpf, *clusterin* expression is detected in the choroid plexus of the third (diencephalon) and fourth (hindbrain) ventricles. The transcripts are also detected in floor plate cells. **D−G:** *clusterin-like 1* (*clul1*) expression is visible in cranial blood vessels at 22 hpf (dorsal view in **E**) and thereafter in photoreceptor cells at 108 hpf (**F** and **G**). **G:** Transverse section of eye. ChP, choroid plexus; f, floor plate; v, ventricle; GCL, granule cell layer; INL, inner nuclear layer; PCL, photoreceptor cell layer. Scale bars: 200 μm for A−F, and 50 μm for G.