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Embryonic expression patterns of Eukaryotic EndoU ribonuclease family gene *endouC* in zebrafish

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ABSTRACT

Endou proteins belong to the Eukaryotic EndoU ribonuclease family of enzymes that present high sequence homology with the founding member XendoU domain. The enzymatic activity and three-dimensional structure of some Endou proteins have been previously reported. However, their molecular structure and gene expression patterns during embryogenesis remain to be elucidated. Therefore, we took zebrafish (*Danio rerio*) *endouC* as the model to study molecular structure and gene expression dynamics at different developmental stages. Zebrafish *endouC* cDNA contains 930 base pairs encoding 309 amino acid residues, sharing 27%, 27%, 27%, and 25% identity with that of human, mouse, chicken and frog, respectively. A phylogenetic tree showed that zebrafish EndouA was clustered with vertebrate Endou groups, while zebrafish EndouB and EndouC were found to belong to a unique monophyletic group. Furthermore, the *endouC* transcript was detected in one-cell embryos, suggesting that it is a maternal gene. While the *endouC* transcript was only weakly present at early developmental stages, its expression was greatly increased in embryos from 18 to 48 h post-fertilization (hpf) and then decreased after 72 hpf. Finally, *endouC* was ubiquitously expressed throughout the whole embryo during early embryogenesis, but its expression was enriched in brain, eyes and fin buds from 24 to 96 hpf.

1. Introduction

The Eukaryotic EndoU ribonuclease family is a novel protein class which includes several enzymes that share significant sequence homology with the founding member XendoU (Snijder et al., 2003; Renzi et al., 2006). This family includes several proteins from a variety of organisms that range from viruses to humans. For example, *Xenopus* XendoU is a uridylate-specific, divalent cation-dependent enzyme that produces molecules with 2′,3′-cyclic phosphate ends, a unique characteristic of this particular class of RNases involved in generating U16 and U86 small nucleolar RNAs (snoRNAs) through the cleavage of pre-mRNAs encoded within introns (Laneve et al., 2003; Gioia et al., 2005; Renzi et al., 2006). The human Endou, known as PP11, has been recently characterized as a member of the XendoU family. Despite its annotated function as a putative serine protease, human Endou has endoribonuclease activity with placental tissue specificity. The dysregulated expression of PP11 in tumor tissues suggests that it may be associated with carcinogenesis (Inaba et al., 1980). Recent studies also demonstrate that both *Xenopus* XendoU and human Endou play important roles in cellular processes, including the regulation of ER structure, RNA degradation and cell survival (Schwarz and Blower, 2014; Poe et al., 2014). Nsp15 (NendoU), a viral ortholog of XendoU, is unique to the nidovirus family of ssRNA viruses, which includes severe acute respiratory syndrome (SARS) coronavirus (Laneve et al., 2003; Bhardwaj et al., 2004; Gioia et al., 2005). Nsp15 is a component of the replicase–transcriptase complex that plays important roles in virus replication and transcription (Ivanov et al., 2004). Like XendoU, Nsp15 has endoribonuclease activity which can cleave RNA, either upstream or downstream of uridylates, at GUU or GU to produce molecules with 2′,3′-cyclic phosphate ends (Ivanov et al., 2004). NendoU has also been postulated to suppress host immune responses (Ricagno et al., 2006) and facilitate apoptosis in cells expressing the mitochondrial antiviral signaling adapter protein that induces host antiviral responses (Lei et al., 2009).

Recent studies have focused on XendoU enzymatic activity and

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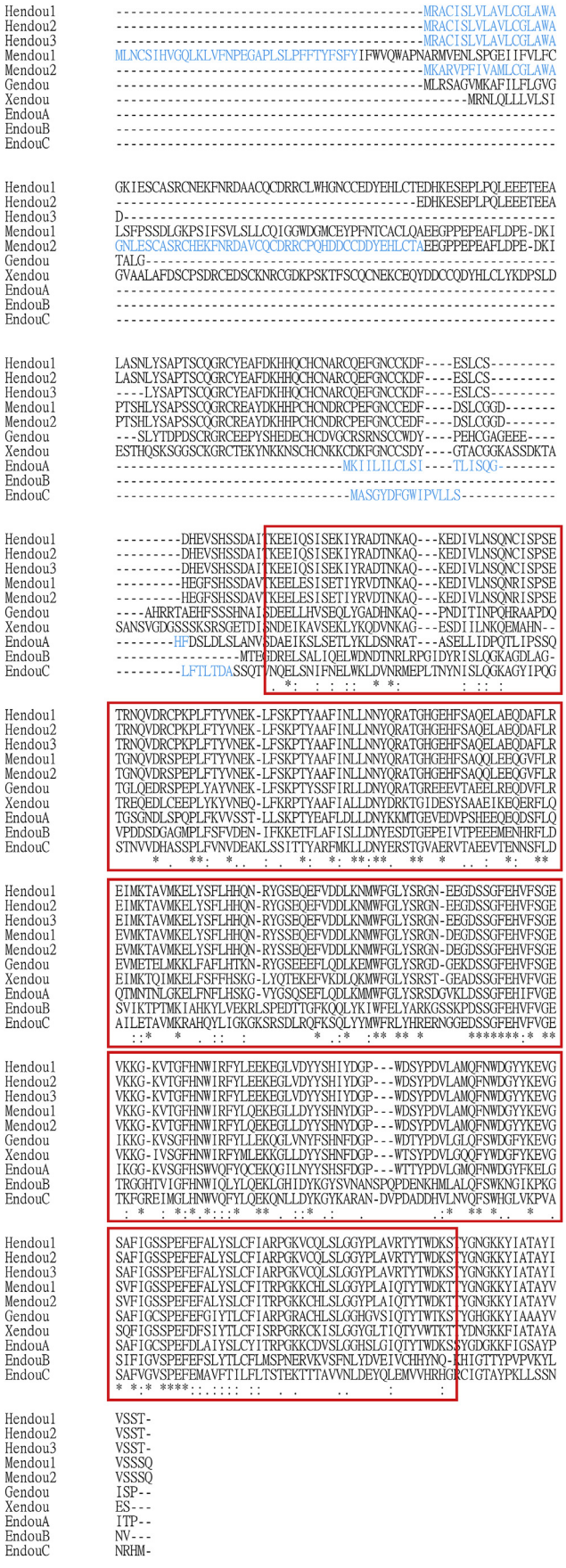

its three-dimensional structure (Laneve et al., 2003, 2008; Gioia et al., 2005; Renzi et al., 2006). Zebrafish (*Danio rerio*) has become a powerful vertebrate model system for the *in vivo* study of gene expression. However, the molecular implications of endonuclease, polyU-specific C (*endouC*) are not well known. Nor has the spatiotemporal pattern of the *endouC* gene been reported. Therefore, in the present work, a phylogenic tree of EndouC among known vertebrate species was elucidated. The spatiotemporal expression pattern of *endouC* in zebrafish embryos at different developmental stages was also analyzed.

2. Material and methods

2.1. Zebrafish husbandry and microscopy

Both zebrafish AB strain and transgenic line *huORFZ* (Lee et al., 2011) were cultured as previously described (Westerfield, 2000). Embryos were grown at 28.5 °C in embryo media (EM) and staged according to standardized morphological criteria (Westerfield, 2000). EM was supplemented with 0.003–0.006% of 1-phenyl 2-thiourea (PTU) (Sigma) to prevent pigment formation in embryos at 24 h post-fertilizationn (hpf). Fluorescence was visualized with a fluorescent stereomicroscope (MZ FLIII, Leica) and a confocal spectral microscope (TCS SP5, Leica). The experiments and treatments of this animal have been reviewed and approved by the Institute of Biomedical Science, Mackay Medical College Institutional Animal Care and Use Committee with ethics approval number A1040009.

2.2. Whole-mount in situ hybridization (WISH)

The full-length coding sequence of zebrafish *endouC* was isolated by RT-PCR, inserted into plasmid pGEMTeasy (Promega), and confirmed by sequencing. After cloning the partial DNA fragments of the desired gene, the probe was labeled by Digoxigenin (DIG). After permeabilization, embryos were hybridized overnight. Then, embryos were incubated with anti-DIG antibody (Roche; 1:8000), stained, and observed under a fluorescent stereomicroscope (MZ FLIII, Leica).

2.3. RNA extraction and RT-PCR

Total RNA isolation, cDNA synthesis and reverse transcriptase polymerase chain reaction (RT-PCR) were performed as previously described (Lee et al., 2011). For RT-PCR and molecular cloning, the primers used were as follows: forward primer: 5′-ATGGCCAGTGGATATGATTTTGGA-3’; reverse primer: 5′- CAGCATGTGTCTGTTGTTGCTGCT -3’.

Fig. 1. The deduced amino acid sequences of zebrafish endouC protein compared with other endou from vertebrates. The alignment of amino acid sequences of endou by CLUSTALW (2.1), including human *H. sapiens* endou1 (Hendou1), *H. sapiens* endou2 (Hendou2), *H. sapiens* endou3 (Hendou3), mouse *Mus musculus* endou1 (Mendou1), *M. musculus* endou2 (Mendou2), chicken *Gallus gallus* endou (Gendou), frog *Xenoupsu tropicalis* endou (Xendou), and zebrafish *Dario rerio* EndouA (EndouA), *D. rerio* EndouB (EndouB) and *D. rerio* EndouC (EndouC). Using Signal-blast software, the predicted signal peptide is labeled with blue color. The conserved regions are boxed with red, Xendou-domain. Asterisks, two dot and one dot were indicated that amino acid residues were 100%, 75%, 50% conserved among all species, respectively.

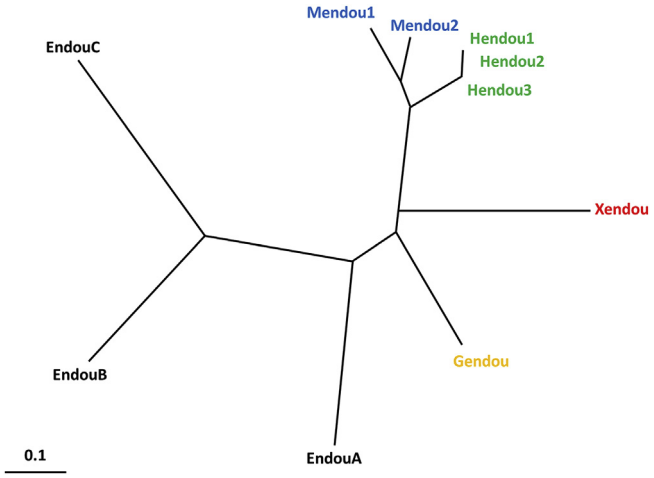

Fig. 2. An unrooted radial gene tree of EndoU among vertebrates. The gene tree was constructed with the neighbor-joining method (Pearson et al., 1999), using 1000 bootstrap values. The marker length of 0.1 corresponds to 10% sequence difference. Human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), frog (*X. tropicalis*) and zebrafish (*D. rerio*) were marked in green, blue, red, brown and black, respectively.

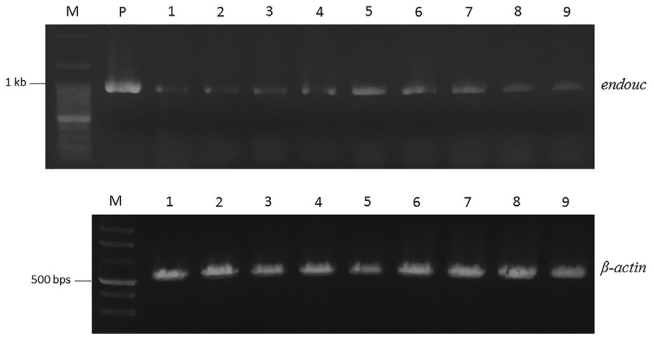

Fig. 3. RT-PCR analysis of zebrafish *endouC* transcript during embryogenesis. Analysis of *endouC* transcript by RT-PCR at different developmental stages of zebrafish embryos as indicated: 1-cell (lane 1), shield (lane 2), 12 hpf (lane 3), 18 hpf (lane 4), 24 hpf (lane 5), 36 hpf (lane 6), 48 hpf (lane 7), 72 hpf (lane 8)and 96 hpf (lane 9). M: marker; P: positive control. β-actin was used as internal control.

3. Results and discussion

3.1. Comparison of deduced amino acids and functional domains of EndouC among known vertebrate species

Zebrafish *endouC* is located on chromosome 18 at 15,490,813–15,498,934, and is composed of eight exons separated by seven introns. The full-length cDNA of zebrafish *endouC* contains 930 nucleotide base pairs encoding 309 amino acid residues.

Three XendoU homologs in zebrafish were reported: EndouA, EndouB and EndouC (Strausberg et al., 2002). Their corresponding deduced amino acid sequences were shown on NCBI Reference Sequence NM_001080698, NM_001020562, and NM_001044974, respectively. When the deduced amino acid residues of EndouA/-B/-C were aligned with the counterpart of other vertebrates, a highly conserved XendoU domain was identified at residues 28–290 (Fig. 1). This result supported the conclusion proposed by Laneve et al. (2003) and Renzi et al. (2006) who demonstrated that Endou family proteins have a highly conserved XendoU domain (residues 131–285) with homologs from eukaryotes. Moreover, this XendoU domain was speculated to have endoribonucleolytic activity (Renzi et al., 2006). Using Singal-blast software, we found that all human three Endou isoforms, mouse two Endou isoforms, and zebrafish EndouA and EndouC contain a signal peptide at N-terminus, while *Xenopus* Xendou, chicken Endou and zebrafish EndouB did not (Fig. 1). Interestingly, three zebrafish Endou proteins lacked the Somatomedin B (SMB) and SMB-like domain otherwise conserved among vertebrate Endou proteins (Fig. 1).

3.2. Comparison of deduced amino acid residues of three zebrafish Endou proteins with those of higher vertebrates

Based on a comparison of the three zebrafish Endou proteins, the deduced amino acid sequence of zebrafish EndouC shared 26% and 42% similarity with EndouA and EndouB, respectively. The deduced amino acid sequence of zebrafish EndouA shared 49–50%, 50%, 54% and 49% identity with human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), and frog (*Xenopus tropicalis*), respectively. Meanwhile, zebrafish EndouB and EndouC respectively shared 28%, 30%, 31% and 30% and 27%, 27%, 27% and 25% identity with human, mouse, chicken and frog. Interestingly, similarity of the XendoU domain among the three zebrafish Endou proteins was low, and zebrafish EndouA was more closely related to vertebrate Endou proteins.

3.3. Phylogenetic analysis of three zebrafish Endou proteins

To examine the evolutionary relationship between Endou of teleost and that of other species, we generated a phylogenetic tree based on the deduced amino acid residues of the three zebrafish Endou proteins in comparison with Endous of other vertebrates (Fig. 2). Results showed that zebrafish EndouA was clustered with vertebrate Endou groups. Interestingly, zebrafish EndouB and EndouC were found to belong to a unique monophyletic group. Further study of this unusual characteristic should provide additional insight into the molecular structure of *endou* genes.

3.4. Expression pattern of endouC in zebrafish embryos

To analyze the spatiotemporal expression pattern of *endouC* during zebrafish embryogenesis, we performed RT-PCR and WISH. We found that the *endouC* was a maternal gene because its transcript was present in one-cell embryos (Fig. 3). Expression of *endouC* was low at early developmental stages (Fig. 3). It gradually increased from 18 to 48 hpf and then decreased after 72 hpf (Fig. 3).

WISH analysis to detect the spatiotemporal expression of zebrafish *endouC* demonstrated that the *endouC* transcript was ubiquitously expressed throughout the whole embryo after 12 hpf (Fig. 4A). At 24 hpf, *endouC* was still strongly expressed at the head and ventral body regions above the yolk sack (Fig. 4B), but it was weakly expressed in somite and spinal cord (Fig. 4C). During 36 to 48 hpf, the expression of *endouC* became more limited, appearing in brain, eyes and fin buds (Fig. 4D–G). In brain, *endouC* was observed at forebrain, midbrain, midbrain hindbrain boundary (MHB), and hindbrain (Fig. 4E and G), while in eyes, *endouC* was expressed in lens and retinae (Fig. 4G). The *endouC* transcript was first detected at the fin bud at 48 hpf (Fig. 4G).

Later at 72 hpf, *endouC* was expressed in the midline of the midbrain and hindbrain, but it was weakly detected in the spinal cord and somite (Fig. 4H). In hindbrain, the *endouC* transcript was visible as a triangular shape emerging from the anterior part of hindbrain midline (Fig. 4I). At 96 hpf, the expression pattern of *endouC* in brain was similar to that of embryos at 72 hpf; however, in head, the signal appeared to be more restricted to pharynx, MHB and hindbrain, and the signal in eye was reduced greatly (Fig. 4J and K).

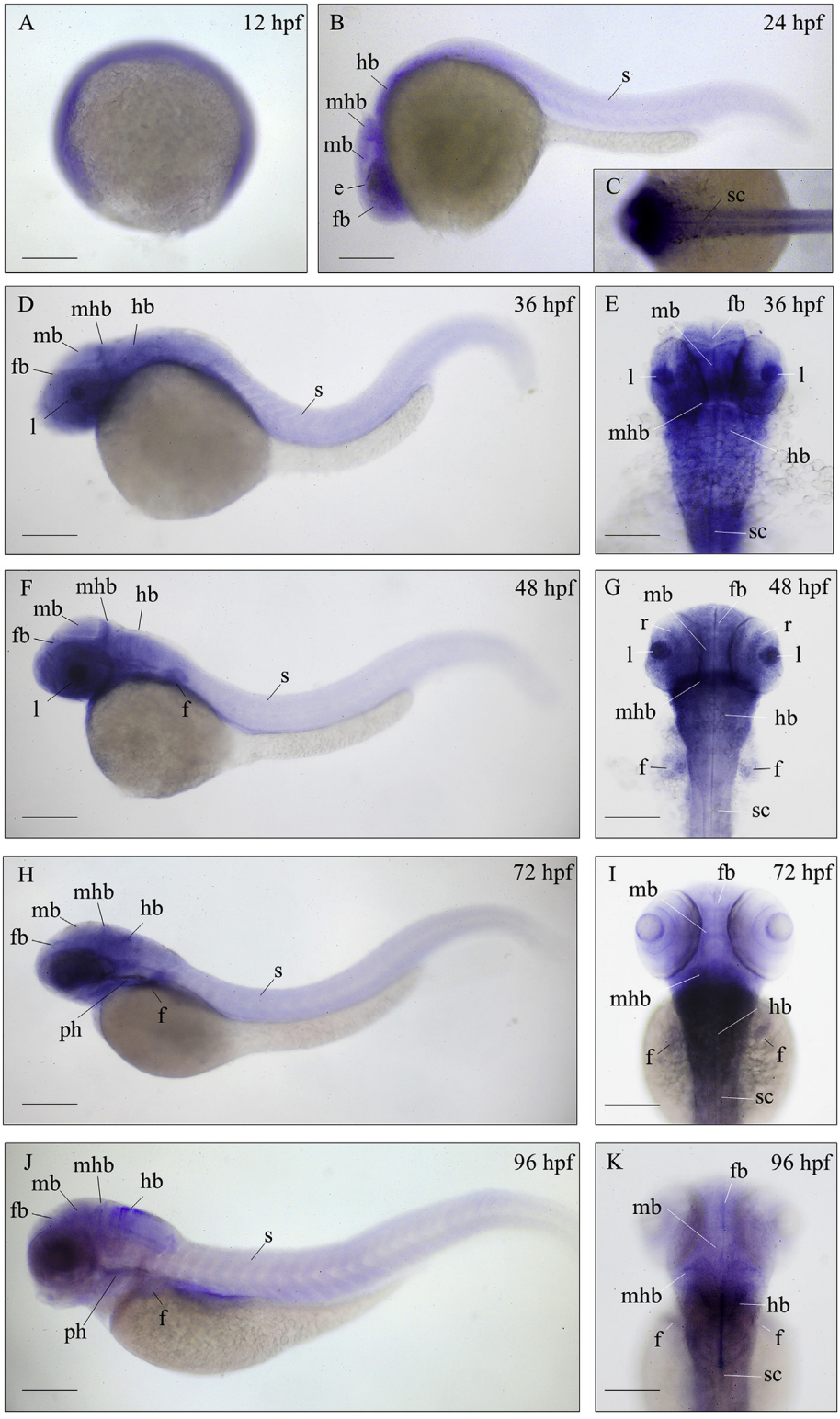

Fig. 4. The expression pattern of *endouC* transcript during the development of zebrafish embryos. Embryos at different stages as indicated were collected and hybridized with *endouC* probe using whole-mount *in situ* hybridization. Panels A, B, D, F, H, and J were lateral views with anterior of embryo on the left; panel C was dorsal view with anterior of embryo on the left; panel E, G, I and K were dorsal views with anterior of embryos on the top. At 12 hpf, *endouC* was expressed in whole embryo. At 24 hpf, *endouC* was highly expressed in head, including forebrain (fb), midbrain (mb), midbrain hibrain boundary (mhb), and hindbrain (hb). The *endouC* transcript was also detected in somite (s) during 24–96 hpf. f, fin bud; sc, spinal cord; r, retina; e: eyes; l, lens; ph, pharynx. Scale bar: 100 μm.

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