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Characterization of multiple nestin isoforms in the goldfish brain

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ABSTRACT

Nestin is an intermediate filament protein involved in neurogenesis in fish, mice, and humans. In this study we used rapid amplification of cDNA ends PCR to isolate goldfish *nestin* (*nes*). PCR analysis and sequencing revealed three different *nes* transcripts of 4003, 2446, and 2126 nucleotides, which are predicted to generate proteins of 860, 274, and 344 amino acids in length. Sequence analysis suggests that these *nes* transcripts are likely a result of alternative splicing. We next applied a multiple-antigenic peptide strategy to generate a goldfish-specific nestin antibody. Western blotting with this antibody together with mass spectrometry verified the presence of major nestin protein isoforms with differing molecular weights (~70, 40 and 30 kDa). We further examined expression patterns of these nestin protein isoforms in different parts of the goldfish brain and pituitary and found the telencephalon to express all three isoforms at a distinct level and abundance. We report that multiple nestin isoforms are present indicating another level of complexity for the regulation of intermediate filaments in comparison to mammals. Studying the differential roles and regulation of these nestins could lead to a better understanding of cellular remodeling during neurogenesis and the unparalleled regenerative abilities after damage in the teleost CNS.

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1. Introduction

Nestin is a type VI intermediate filament protein that is mostly found in cells undergoing mitosis, for example cells undergoing cellular division in both the central nervous system (CNS) and peripheral nervous system (PNS) (Wiese et al., 2004; Michalczyk and Ziman, 2005; Mahler and Driever, 2007). Nestin is often used as a progenitor cell marker in many species and labels cells such as radial glia that exhibit self-renewal and multipotency capabilities (Michalczyk and Ziman, 2005; Mahler and Driever, 2007; März et al., 2010; Carmona et al., 2011). Teleost fish have abundant progenitor cells and, compared to humans, have a higher capacity to regenerate neurons following injury. Nestin function and the subsequent upregulation of nestin observed following injury could be the key to understanding cellular regeneration. In this study, we characterized the nestin proteins in the goldfish, a species with exceptional capacities for adult neurogenesis and CNS repair (Levine, 1983; Scherer and Easter, 1984; Zupanc and Sîrbulescu, 2011).

Nestin-positive cells have the ability to give rise to neurons and glial cells upon cellular differentiation (Wiese et al., 2004; Mahler and Driever, 2007). The subependymal zone of the lateral ventricle and the subgranular zone of the dentate gyrus in the hippocampus are the only two distinct proliferative areas in the adult mammalian brain that give rise to new neurons (Alvarez-Builla and Garcia-Verdugo, 2002; Taupin and Gage, 2002; Goldman, 2003; Garcia et al., 2004; Merkle et al., 2004; Doetsh and Hen, 2005; Lledo and Saghatelyan, 2005). On the other hand, teleost fish have numerous neurogenic areas especially in the forebrain and these areas are highly dense with progenitor and stem cells. More specifically, the telencephalon has a remarkable neurogenetic capacity especially near the ventral and dorsal telencephalic areas (Mahler and Driever, 2007; März et al., 2010). The proliferative areas are identified as the ventricular zones of the telencephalon, the diencephalon, the midbrain-hindbrain boundary, and the ciliary marginal zone of the retina in teleosts (Michalczyk and Ziman, 2005). This study will describe the expression and distribution of nestin in the goldfish brain including its distribution in highly neurogenetic tissues.

Several studies have explored the various roles of the nestin protein. One study reported that nestin can affect the efficiency of a cell to move particles (e.g., vesicles) intracellularly, thereby providing structural and functional support during cellular proliferation (Michalczyk and Ziman,

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2005; Chen et al., 2010; Carmona et al., 2011). Nestin can also control the localization and partitioning of intermediate filaments within the cytoskeleton, thereby affecting the distribution of cellular components during mitosis (Michalczyk and Ziman, 2005; Chen et al., 2010). This suggests that nestin plays a role in reorganizing cytoskeletal filaments, thus controlling cellular dynamics by regulating the polymerization of various intermediate filaments (Mahler and Driever, 2007). Although several functional properties of nestin protein have been proposed, its mechanism of action at the molecular level still remains unclear. The characterization of 3 novel nestin isoforms in the goldfish could provide insight into neurogenic capabilities in other vertebrates.

Data to date suggest that the nestin protein sequence contains a short amino terminal and a long carboxyl end and is fairly well conserved among species such as chicken, mouse, rat, zebrafish, frog and human (Mahler and Driever, 2007; Chen et al., 2010). Severe developmental malformations such as morphological abnormalities are visible in zebrafish morphants that are injected with a nestin morpholino (Chen et al., 2010). These abnormalities included decreased body and head growth, small eyes, underdeveloped retinal lens, brain defects, and developmental defects of motor neurons, axons, and glial cells in the brain (Chen et al., 2010). These morphants also exhibited decreased proliferative cell niches in the developing nervous system resulting in fewer progenitor cells possibly caused by cellular apoptosis and death (Chen et al., 2010). All of these observations suggest that nestin is involved in the mechanism controlling neurokinetic capacity and regenerative cellular processes.

Characterization of the nestin protein in teleosts could provide a better understanding of the mechanisms underlying brain plasticity. Herein we investigate the nestin protein in the goldfish brain and describe the generation of the first fish-specific nestin antibody, a new tool to study teleost neurogenesis.

2. Materials and methods

2.1. Animal maintenance

Adult female goldfish (*Carassius auratus*) were purchased from a commercial supplier (Mt. Parnell Fisheries Inc., Mercersburg, PA, USA) and maintained at 18 °C under a natural-simulated photoperiod on standard trout food (Martin Mills Profishent Classic Floating Trout Pellets, 3 mm). Fish were kept in 70 L tanks (15–18 fish/tank). All procedures were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee. Goldfish were anesthetized using 3-aminobenzoic acid ethylester (MS-222; 0.05% in water, Sigma Chemicals) for all handling and sampling procedures.

2.2. Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

One female goldfish brain was dissected and frozen on dry ice immediately and stored at −80 °C. Total RNA was isolated using RNeasy mini kit (Qiagen, Toronto, Canada, 74134). The GeneRacer™ Kit (Invitrogen, Burlington, Canada, L150201) was used to obtain the 5′- and 3′-ends of goldfish *nestin* (*nes*) cDNA following the manufacturer's instructions. To obtain the 5′-mRNA end of goldfish *nes*, the mRNA was initially dephosphorylated and then the mRNA cap structure was removed and replaced with GeneRacer™ RNA Oligo using RNA ligase. Reverse transcription was performed on the mRNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Invitrogen, K1642). The cDNA was used to amplify both 3′ and 5′ RACE-PCR using the GeneRacer™ Primer specific to the GeneRacer™ RNA Oligo sequence and gene-specific primers (Table 1). The gene-specific primers were designed using Primer3 Software (http://bioinfo.ut.ee/primer3-0.4.0/) based on the partial goldfish *nes* cDNA sequence (GenBank accession no. **FJ855222.1**). Goldfish *nes* cDNA was cloned using a nested and external PCR approach with a Platinum Taq DNA Polymerase High Fidelity enzyme (Invitrogen). The PCR cycling parameter consisted of 2 min at 94 °C; 5 cycles of 30 s at 94 °C and 1 min at 72 °C; 5 cycles of 30 s at 94 °C and 1 min at 70 °C; 25 cycles of 30 s at 94 °C, 30 s at 65 °C and 1 min at 68 °C and a final extension of 10 min at 68 °C. PCR products were cloned into either pCR4-TOPO vector (Invitrogen) or pGEM-T Easy Vector (Promega, WI, USA) and at least 3 different clones were sequenced by standard methods using the Applied Biosystems 3730 DNA Analyzer at the Ottawa Health Research Institute. Based on the obtained sequence for goldfish *nestin a* (*nesa*) transcript (GenBank accession no. **KT373807**), a second round of RACE-PCR was performed to obtain the complete 3′-end of goldfish *nesa* transcript using new gene-specific primers (Table 2). Procedures were performed as explained above.

Table 1
Primers used for RACE-PCR (Fw, forward; Rv, reverse; gf, goldfish). Total RNA was obtained from female goldfish brain to perform RACE-PCR using the above primer sets.

Primer name	Primer sequence (5′- > 3′)
GeneRacer™ 5′ Primer Fw	GGACTGGAGCACGAGGACACTGA
5′_gfnestin_RACE1 Fw	AAGGACAGCCTGGGGCAGCAGATAGA
GeneRacer™ 5′ Primer nested Fw	GGACACTGACATGGACTGAAGGAGTA
5′_gfnestin_RACE1 nested Fw	CAAGATGTCCCTGGGCCTGGAAGTG
GeneRacer™ 3′ Primer Rv	GCTGTCAACGATACGCTACGTAACG
3′_gfnestin_RACE1 Rv	CTTCCTCCAGGCCTCCTCCAGCATC
GeneRacer™ 3′ Primer nested Rv	CGCTACGTAACGGCATGACAGTG
3′_gfnestin_RACE1 nested Rv	CCTGCTGGTTCCCTGCTGCTCTTG
3′_gfnestin_RACE2 Fw	GGGAAGGATGAGGACACTGA
3′_gfnestin_RACE2 nested Fw	AAATGGCTCGGATTTCACAC

2.3. Production of rabbit anti-goldfish nestin antiserum

A polyclonal antibody against goldfish nestin was generated using a multiple antigenic peptide (MAP) strategy (Basak et al., 1995). The peptide used was composed of 23 amino acids VVSIQRQKAKNAQAEAQRKLMES, located at the N-terminal of goldfish nestin (GenBank accession no. **FJ855222.1**). This peptide sequence was selected based on secondary structure, antigenicity and hydrophilicity analyses using the proteomics tools in the Expasy website (www.expasy.ch). The peptide was synthesized on a 4-branched lysine MAP core to form the immunogenic peptide nestin23-MAP (MW 10,456 g/mol). A total of 6 rabbits were used to generate the anti-nestin antibody. Nestin23-MAP (1 mg) was suspended by vortexing in 0.2 ml of pure glacial acetic acid (ACROS) and then 0.8 ml of sterile distilled water was added to the solution. The solution was further mixed with ~2.2 ml Freund's complete adjuvant for the first injection into rabbits. For all subsequent injections ~2.2 ml of Freund's incomplete adjuvant was used. The injections were administered at the interval of every 4 weeks with 2 intramuscular injections of 0.25 ml and 5 subcutaneous injections of 0.2 ml for a total injection volume of 1.5 ml per rabbit. Blood sample (~1.5 ml) was collected every 14 days following injections until the final bleed at 4 months post the initial injection. Preliminary serum analysis by dot and western blotting indicated that rabbit labeled number 9 had the highest titer and this anti-serum was used in all subsequent experiments.

Table 2
Gene-specific primers used for second round of RACE-PCR to obtain goldfish *nes* transcript A (Fw, forward; gf, goldfish). Total RNA was obtained from female goldfish brain to perform RACE-PCR using the above primers.

Primer name	Primer sequence (5′->3′)
3′_gfnestin_GSP_RACE2 Fw	GGGAAGGATGAGGACACTGA
3′_gfnestin_GSP_RACE2 nested Fw	AAATGGCTCGGATTTCACAC

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