Nestin Is Essential for Zebrafish Brain and Eye Development through Control of Progenitor Cell Apoptosis

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Abstract

Background: Nestin is expressed in neural progenitor cells (NPC) of developing brain. Despite its wide use as an NPC marker, the function of nestin in embryo development is unclear.

Methodology/Principal Findings: As nestin is conserved in zebrafish and its predicted sequence is clustered with the mammalian nestin orthologue, we used zebrafish as a model to investigate its role in embryogenesis. Injection of nestin morpholino (MO) into fertilized eggs induced time- and dose-dependent brain and eye developmental defects. Nestin morphants exhibited characteristic morphological changes including small head, small eyes and hydrocephalus. Histological examinations show reduced hind- and mid-brain size, dilated ventricle, poorly organized retina and underdeveloped lens. Injection of control nestin MO did not induce brain or eye changes. Nestin MO injection reduced expression of ascl1b (achaete-scute complex-like 1b), a marker of NPCs, without affecting its distribution. Nestin MO did not influence Elavl3/4 (Embryonic lethal, abnormal vision, Drosophila-like 3/4) (a neuronal marker), or otx2 (a midbrain neuronal marker), but severely perturbed cranial motor nerve development and axon distribution. To determine whether the developmental defects are due to excessive NPC apoptosis and/or reduced NPC proliferation, we analyzed apoptosis by TUNEL assay and acridine orange staining and proliferation by BrdU incorporation, pcna and mcm5 expressions. Excessive apoptosis was noted in hindbrain and midbrain cells. Apoptotic signals were colocalized with ascl1b. Proliferation markers were not significantly altered by nestin MO.

Conclusion/Significance: These results suggest that nestin is essential for zebrafish brain and eye development probably through control of progenitor cell apoptosis.

Introduction

Nestin is an intermediate filament protein which exhibits structural similarities to vimentin, desmin and neuro-filaments and is classified as a type IV neuro-filament [1]. Nestin is assembled into intermediate filaments by forming heterodimers with vimentin and desmin [2,3] and together with microtubules and microfilaments it forms the cytoskeleton. Nestin was originally cloned from the central nervous system of rat embryos [1]. Its expression was found primarily in neuroepithelial stem cells [1] and proliferating neural progenitor cells (NPC) [4]. The expression of nestin can also be found in the parenchyma [5]. In developing murine embryos, nestin expression coincides with the onset of neuralization and is dramatically down-regulated in mature neural cells [1]. Nestin is conserved across diverse species including mammals, chicken and zebrafish. Phylogenetic analysis reveals that the predicted amino acid sequence of zebrafish nestin is clustered with the mammalian nestin orthologue [6]. Furthermore, similar to mammals, zebrafish nestin is expressed widely in developing nervous system and its expression is restricted to neural proliferation zone in adult fish [6,7].

Despite extensive investigations to ascertain nestin as a marker of neural stem and progenitor cells, the functions of nestin are not clearly understood. Recent reports suggest that nestin may be involved in positioning and functioning of subcellular organelles and may serve as a scaffold for kinases that regulate diverse cellular functions such as proliferation, survival and differentiation [8,9,10,11]. However, it is unclear whether nestin is important in neural development. In this study, we evaluated the function of nestin in zebrafish embryogenesis by injecting nestin morpholino (MO) into fertilized eggs. Nestin MO treatment resulted in brain and eye developmental defects. These developmental defects were accompanied by reduction in NPCs and increase in neural and retinal apoptosis.
Materials and Methods

Zebrafish Husbandry, Experimentation and Care/Welfare

AB strain fish Danio rerio and transgenic Tg(gfp:GFP) fish were purchased from Zebrafish International Resource Center (ZIRC), Oregon. Tg(is1-L-GFP) line was kindly provided by Dr. Hitoshi Okamoto [12]. Heterozygous gfp:GFP was generated by crossing Tg(gfp:GFP) with wild type AB strain. All the fish were maintained at 28°C under continuous flow in our zebrafish facility with automatic control for a 14-hour light and 10-hour dark cycle. To generate embryos for injection, male and female fish were placed the night before injection in a one liter fish tank with the inner mesh and divider. Zebrafish embryos were obtained from natural spawning by removing the divider and light stimulation. The embryos were kept at 28°C before and after microinjection. All the experiments involving zebrafish had been conducted according to the guidelines of Institutional Animal Care and Use Committee (IACUC) of National Health Research Institutes (NHR). The zebrafish experimental protocols were approved by IACUC (approval number: NHRI-IACUC-095050-A, 096037-A, 098017-A and 098087 under Dr. Chiou-Hwa Yuh).

Morpholino (MO) Injections

Zebrafish standard control MO (5'-CCTCTTTACACTGT- TACAATTTATA-3'), nestin MO1 (5'-CGAGAGATATGAGT-GAAATCTCAG-3'), 5-bp mismatch (5-mi) nestin MO1 control (5'-CGACAGATATCAATGAGT-GAAATCTCAG-3'), lower case letters indicate mismatch bases), and nestin MO2 (5'-TGTGCAAGACCC-GAGAACTGCATCT-3') were purchased from Gene Tools, LLC (Philomath, OR), dissolved to 4.12 µg/µl in sterilized ddH2O and stored at −20°C. For microinjection, MOs were prepared in 1x PBS with 0.03% phenol red. They were injected into embryos of one-cell stage with PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL). After injection, embryos were incubated in egg water (60 µg sea salt/ml distilled water) supplemented with 0.003% 1-phenyl-2-thiourea (PTU) at 28°C to prevent pigment formation. Embryo development was evaluated at approximately 24 hpf, 48 hpf, and 72 hpf.

DNA Construction, Microinjection and Western Blot Analysis

To evaluate the knockdown efficiency of nestin MO1, we amplified by PCR a 330 bp fragment of zebrafish nestin cDNA (−58 to +272, +1 indicates translation initiation site) encompassing MO1 targeting sequence (−28 to −4) using primers: nestin-XhoF (5'-AATAGCTAGGTCGAAGATGTCGGCCTTTCTCAG-3'), bold letters are XhoI site) and nestin-KpnR (5'-AATAGGTACTC-TACCCTCAGTCCACAGA-3'), bold letters are KpnI site), and cloned it into pEFGP-C1 plasmid. 200 µg of linearized nestin-GFP plasmid was microinjected into one-cell stage embryos, together with 10 ng of nestin MO1 or 5-mi nestin MO1. Embryos were harvested at 30 hpf and lysed with radio-immunoprecipitation assay buffer plus a complete protease inhibitor cocktail (Roche, Mannheim, Germany), and GFP was analyzed by Western blotting. In brief, 100 µg proteins were loaded onto each lane. Rabbit anti-GFP antibody (1:500, Santa Cruz, Santa Cruz, CA) and mouse anti-β actin antibody (Sigma-Aldrich, St. Louis, MO) were used for GFP protein detection and internal control. Goat anti-rabbit and goat anti-mouse IgG conjugated with horseshad peroxidase (Santa Cruz) were used in protein detection. Protein bands were visualized by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

Bright Light and Fluorescent Microscopy

Embryos were anesthetized with 0.168 mg/ml tricaine (Sigma-Aldrich), mounted in 2% methylcellulose and examined on a Leica fluorescent microscope (DMIRB) equipped with a CoolSNAP TM cooled CCD camera (Roper Scientific, Trenton, NJ).

Histologic Sections and H&E Staining

Embryos were fixed with 4% paraformaldehyde overnight at 4°C and mounted in 1% agarose. The agarose block was gradually dehydrated with 70%, 90%, 100% ethanol and 100% xylene and embedded in paraffin [13]. The brain was cut into 7 µm sections which were stained with hematoxylin and eosin (H&E stain).

Whole Mount In Situ Hybridization (WMISH)

Embryos were fixed by 4% paraformaldehyde overnight at 4°C and dehydrated in methanol at −20°C. WMISH procedure was performed according to the protocol described in detail by Thiese B and Thiese C on ZFIN (http://zfin.org) with some modifications. In brief, embryos were dehydrated gradually with PBST (1x PBS, 0.1% Tween 20). For 25-hpf embryos, they were digested with 10 µg/ml proteinase K (Sigma-Aldrich) in PBST for 12 minutes at room temperature. For 49-hr embryos, they were treated with proteinase K for 30 minutes at 30°C. After postfixed with 4% paraformaldehyde, the embryos were incubated in HYB* (50 µg/ml heparin, 500 µg/ml wheat germ tRNA and HYB− containing 50% formamide, 5x SSC and 0.1% Tween 20) which contained 100 ng DIG-labeled antisense RNA probe at 60°C overnight. Embryos were then washed with 75%, 50% and 25% HYB SSC for 15 minutes, 2x SSC for 15 minutes and two times with 0.2x SSC for 30 minutes at 65°C and transferred gradually to 2x blocking buffer (2% Blocking Reagent (Roche) in 100 mM maleic acid, 150 mM NaCl, and 0.1% Tween 20) at least for 1 hour. Embryos were incubated in 1:5000 anti-DIG-AP (Roche) in 2x blocking buffer overnight at 4°C. After 6 washes with PBST for 15 minutes and 3 washes with alkaline Tris buffer for 5 minutes, bound antibody was detected by SIGMA FASTBCIP/NBT. After staining, labeled embryos were mounted in 90% glycerol and examined on an Olympus stereomicroscope (SZX-ILLD100, Tokyo, Japan). The images were captured using an Olympus DP70 digital microscope camera and processed by Helicon Focus software. The sequences of primers used in synthesizing probes are as follows: asil1b-forward (F): 5'-ACGACGACGACTGACA- CAAGA-3' and reverse (R): 5'-GGAACACAGTTCGTCA- TCA-5'; isl2-F: 5'-CAGACGATAAACACGGGGA-3' and R: 5'-GGAACGAAAACACCTGTCAC-3'; mcm5-F: 5'-GCAAGAA-TGTCGGGATTTGA-3'; mcm5-R: 5'-TGGCTTCTACATTGAGTT-3'. Whole- Mount Immunohistochemistry

For Elavl3/4 staining, embryos were fixed in 4% paraformaldehyde overnight at 4°C and dehydrated in methanol at −20°C. For acetylated tubulin staining, embryos were fixed in Dent’s fixative (80% methanol, 20% DMSO) overnight at 4°C. The embryos were permeabilized with proteinase K followed by post-fixation with 4% paraformaldehyde, and washed with PBSX (PBS +0.3% Triton X-100). After treating with 4% normal goat serum (NGS) in PBSX for 2 hours at room temperature, embryos were incubated with mouse anti-Elavl3/4 antibody (1:500, Molecular Probes, Eugene, OR) or mouse anti-acetylated

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tubulin antibody (1:1000, Sigma-Aldrich) in 4% NGS/PBSTX overnight at 4°C. The embryos were washed five times with PBSTX for 10 minutes each and incubated with goat anti-mouse FITC (1:200, Jackson ImmunoResearch, West Grove, PA) in 2% NGS/PBSTX for 2 hours at room temperature. After washing 5 times for 10 minutes each, embryos were mounted in 1% agarose and examined by confocal microscopy (Olympus Fluoview FV300).

Figure 1. Nestin expression in zebrafish embryos. A–D. Analysis of nestin transcripts by whole-mount in situ hybridization in embryos injected with control MO. A and C lateral view from left; B, dorsal view, head at top and D, dorsal view, head at left. Scale bar: 200 μm. E. Nucleotide sequence around the translation start site of zebrafish nestin cDNA and corresponding sequence of nestin MO1 and MO2. F. Western blot analysis of nestin:GFP fusion proteins in 30 hpf embryos treated with nestin MO (10 ng) or its 5-mis MO control (10 ng). The proteins were immunoblotted (IB) with a GFP (α-GFP) or actin antibody (α-actin).

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Figure 2. Embryo morphological changes induced by nestin MO. A–D. One-cell stage embryos were injected with 10 ng nestin MO and morphology was examined at 24 hpf and 28 hpf. Compared to normal morphology of wild-type (WT) (A: lateral view and C: dorsal view), nestin morphant exhibited head malformation (B: lateral view) with unclear boundaries between brain subdivisions, especially the hindbrain and midbrain boundary (D: dorsal view). E–J. Embryos were examined at 51 hpf after injection of 10 ng nestin MO1 or an equal amount of control MO and 5-mis nestin MO. The head abnormality had become more pronounced in nestin morphants with overt hydrocephalus (G and H). G illustrates mild and H, severe hydrocephalus. The morphology of control MO and 5-mis nestin MO remained normal (E and F). Dorsal view of nestin morphants revealed reduced size of tectum (J) compared with control morphants (I). K–N. Magnified views of E–H to illustrate the gross head and eye defects and hydrocephalus in nestin morphants. Blue circles in I and J refer to tectum. Blue double-head arrows in K–N denote diameter of the eyes. * denotes mild and ** severe hydrocephalus. The scale bar is 200 μm.

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For BrdU incorporation, 24 hpf embryos were treated with 10 mM BrdU in 15% DMSO/egg water for 20 minutes at 4°C and washed three times with egg water for 5 minutes each, followed by 4% paraformaldehyde fixation overnight at 4°C and dehydration in methanol at −20°C. After gradual rehydration, embryos were permeabilized with proteinase K followed by postfixation with 4% paraformaldehyde, washed with PBSTX, blocked with 4% NGS in PBSTX for at least 2 hours at room temperature, and incubated with rat anti-BrdU-FTIC antibody (1:200, Abcam, Cambridge, MA) in 4% NGS/PBSTX overnight at 4°C. After washing five times with PBSTX for 10 minutes each, they were mounted in 1% agarose and examined by confocal microscopy.

**Detection of Cell Death**

To detect apoptotic cells in live embryos, embryos were dechorionated and soaked in egg water containing 2 µg/ml acridine orange at 28°C for 30 minutes. After washing with egg water eight times 5 minutes each, embryos were anesthetized with tricaine, mounted in 2% methylcellulose and examined by stereomicroscopy, or confocal microscopy.

Apoptotic cells were also examined by TUNEL assay. Embryos were fixed with 4% paraformaldehyde overnight at 4°C and dehydrated with methanol at −20°C. After gradual rehydration, the embryos were permeabilized with 25 µg/ml proteinase K for 30 minutes at 30°C followed by 4% paraformaldehyde, and incubated with 90 µl labeling solution plus 10 µl enzyme solution (In Situ Cell Death Detection Kit, Fluorescein, Roche) at 37°C for 2 hours. They were washed three times with PBST for 5 minutes each and the images were examined by confocal microscopy.

To determine whether apoptosis was localized to neural precursor cells, embryos were first in situ hybridized with fluorescence asc1b RNA probe followed by TUNEL assay. The protocol of fluorescence in situ hybridization is as described above with some modifications [14]. The RNA probe was labeled by dinitrophenyl (DNP). After conjugated with anti-DNP-POD antibody (PerkinElmer, Shelton, CT), the signal was detected by TSA-fluorescein system (PerkinElmer). This was followed by TUNEL assay, using TMR red staining (Roche). The embryos were manually de-yolked and the images were captured on an Olympus confocal microscope.

**Results**

**Brain and Eye Developmental Defects Induced by Nestin MO**

*Nestin* expression was detected in embryos at 13 hpf (Fig. 1A and 1B) and was confined to nervous system at 24 hpf (Fig. 1C and 1D). To determine the role of nestin in embryo development, we tested several nestin MO sequences and identified nestin MO1 and MO2 to be active. Their sequences relative to nestin cDNA are shown in Fig. 1E. To test the knockdown efficiency, nestin MO1 or its 5-mis MO control was co-injected with nestin-GFP plasmids into fertilized eggs. Nestin-GFP fusion proteins analyzed in 30 hpf embryos became undetectable after nestin MO treatment (Fig. 1F). The 5-mis control did not suppress nestin-GFP proteins (Fig. 1F).

The causal significance of *nestin* is established by specific transperturbation using morpholin-substituted antisense oligonucleotides. Nestin MO1 or MO2 (10 ng each) was microinjected into the yolk of one-cell stage zebrafish embryos and the phenotypes were examined at various time points. After knockdown of Nestin by either nestin MO, the embryos exhibited brain developmental defects at 24–28 hpf (Fig. 2A, C vs. 2B, D). By 51 hpf, the developmental defects had become pronounced (Fig. 2G, 2H and 2J), while neither control MO (Fig. 2E) nor 5-mis nestin MO (Fig. 2F) caused any morphological changes when compared to untreated wild-type embryos (data not shown). A magnified view of nestin MO-treated embryos at 51 hpf showed characteristic morphological abnormalities including small head, reduced brain size and hydrocephalus as well as small and poorly organized eyes (Fig. 2M and 2N). The hydrocephalus varied in size from mild (Fig. 2M) to severe (Fig. 2N). A dorsal view of nestin morphants revealed smaller tectum (Fig. 2J) than control (Fig. 2I). The morphological changes induced by nestin-MO persisted at 72 hpf (data not shown). Using hydrocephalus as an index of brain abnormalities, we found the effect of nestin MO on brain development was dose-dependent (Fig. 3A). Reduction of eye size was also dose-dependent (Fig. 3B). As treatment with 10 ng nestin MO1 induced hydrocephalus and eye changes in a majority of embryos (Fig. 3), we performed subsequent experiments by microinjection of 10 ng MO1.

Morphological changes of the brain and eyes in fish treated with 10 ng nestin MO were further evaluated by staining of the histological sections. H&E stained sections prepared from nestin MO1-treated embryos show dilated ventricle, reduced tissue mass and defective organization in the midbrain (Fig. 4B) and hindbrain (Fig. 4D) and poorly organized retina and underdeveloped lens in both eyes (Fig. 4F). These results suggest that nestin is required for normal brain and eye development in zebrafish.

**Figure 3. Dose-dependent induction of hydrocephalus and eye size changes by nestin MO1.** A. Hydrocephalus was visualized at 48 hpf after injecting increasing doses of nestin MO1 or 10 ng 5-mis nestin MO and classified as “mild” and “severe” as illustrated in Fig. 2M and 2N. Number of embryos (n) for each dose of nestin MO1: n = 125 for 0 group, n = 96 for 3.5 ng group, n = 125 for 7 ng group and n = 142 for 10 ng group. n = 118 for 5-mis MO group. B. The diameter of eyes was measured at 48 hpf after nestin MO1 or 5-mis MO injection. Each bar represents mean ± SD. * denotes p<0.001.

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Reduction of NPCs by Nestin MO Treatment

To identify neural cells affected by nestin MO, we performed (1) WMISH to detect ascl1b, a marker of NPCs; and otx2, a marker of midbrain neurons and (2) confocal microscopy to detect Elavl3/4, a marker of mature neurons. Ascl1b transcript was detected in the brain of zebrafish embryos at 25 hpf (Fig. 5A), and the transcript level was reduced in nestin morphant (Fig. 5A). Elavl3/4 was also detected in the brain and its expression pattern was not significantly altered by nestin MO treatment (Fig. 5B). Otx2 was detected only in the midbrain and its expression was not different between nestin morphant and control (Fig. 5C). These results suggest that the effect of nestin knockdown was limited to NPCs.

We next analyzed the expression pattern of motor neuron marker, isl1, using in situ hybridization and a transgenic fish line Tg(isl1:GFP), glial lineage marker, glial fibrillary acidic protein (gfap) [15], using heterozygous Tg(gfap:GFP), and axon marker, acetylated tubulin (AcTub), using immunofluorescence microscopy. Compared to control, isl1 transcript in nestin morphant was reduced primarily in the forebrain (Fig. 6A) and increased slightly in the spinal cord (Fig. 6A). The results were highly reproducible in multiple experiments (Supplementary Fig. S1A and S1B). Analysis of cranial nerve isl1 expression in isl1-GFP transgenic fish reveals that motor nerve III and IV were poorly detectable, and V and VII were reduced in size and disorganized in nestin morphants (Fig. 6B). There was a striking abnormality in nerve X morphology.

Figure 4. Examination of brain tissues and eyes by H&E staining. Embryos at 48 hpf after control or nestin MO injection were sectioned at midbrain (A and B) and hindbrain (C and D) levels. Tissues were stained with H&E. Eyes at the midbrain level were visualized and shown in E and F. The scale bar is 50 μm.
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and positioning. Immunofluorescent microscopic analysis of AcTub expression reveals abnormal axon distribution (Fig. 6C). To evaluate the effect of *nestin* MO on *gfap* expression, we compared GFP intensity in heterozygous *Tg(gfap:GFP)* treated with *nestin* MO vs. control. There was a consistent reduction of GFP expression in *nestin* MO-treated embryos (Fig. 6D). These findings indicate the importance of nestin in motor neuron, axon and glial cell development.

Figure 5. Effects of *nestin* MO injection on *ascl1b* (marker of NPC), Elavl3/4 (marker of mature neurons) and *otx2* (marker of midbrain tissue). A. Ascl1b expression was analyzed by WMISH at 25 hpf. Upper panel shows dorsal view with deletion of yolk. Lower panel shows lateral view, head to the left. B. Elavl3/4 was analyzed by confocal microscopy at 25 hpf. C. Otx2 was analyzed by WMISH at 25 hpf. Scale bar: 200 μm. doi:10.1371/journal.pone.0009318.g005
Increased Apoptosis in Nestin MO-Treated Embryos

The developmental defects in brain and eyes could be due to several possible mechanisms such as increased cell death and reduced cell proliferation. To assess cell death as a possible mechanism, we analyzed apoptotic cells in embryos by acridine orange staining and TUNEL assay. Acridine orange staining of living embryos revealed increased staining of brain cells especially the hindbrain region of nestin MO treated embryos at 29 hpf (Fig. 7A). The staining became more intense and extended to the midbrain and eyes at 53 hpf (Fig. 7B). Acridine orange staining was minimal in embryos treated

Figure 6. Effects of nestin MO injection on isl1, acetylated tubulin (AcTub) and gfap:GFP expression. A. Isl1 expression was analyzed by WMISH at 25 hpf. Arrow indicates for brain and arrow head, spinal cord. B. Transgenic Tg(isl1:GFP) embryos were injected with nestin MO. GFP expression was examined under confocal microscopy at 49 hpf. Uninjected WT (no inject) was used as a control. C. AcTub expression (axon marker) was examined by confocal microscopy at 49 hpf. The upper panels show lateral view and the lower panels, dorsal view. D. Heterozygous Tg(gfap:GFP) embryos were injected with nestin MO and GFP was visualized under fluorescent microscopy at 49 hpf. Scale bars: 200 μm.
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with 5-mis nestin MO control (Fig. 7A and 7B). TUNEL-positive cells were examined by confocal microscopy. Analysis of z-stack images (Fig. 8A and 8B) revealed that TUNEL-positive cells were increased in the hindbrain of embryos treated with nestin MO for 25 hours (Fig. 8B, top panel) as compared to 5-mis nestin MO control (Fig. 8A, top panel). TUNEL-positive cells detected outside the brain are considered to be yolk debris. TUNEL-positive cells were noted to extend to the midbrain of embryos treated with nestin MO for 49 hours (Supplementary Fig. S2). To determine whether the apoptotic signals are localized to NPCs, we examined by fluorescence in situ hybridization ascl1b expression and merged the image with TUNEL. Analysis of the merged images shows increased numbers of cells in the hindbrain exhibiting colocalization of TUNEL with ascl1b as indicated by increased yellow dots in Fig. 8B vs. Fig. 8A. To ascertain colocalization of TUNEL with ascl1b, we analyzed high magnification confocal images of a single z-plane (Fig. 8C and 8D). The results confirmed increased numbers of apoptotic ascl1b-positive cells in the hindbrain of embryos treated with nestin MO for 25 h (Fig. 8D vs. 8C). These results suggest that nestin MO increased apoptosis of ascl1b-positive cells.

To determine whether knockdown of nestin reduces cell proliferation in brain and eyes, we analyzed BrdU uptake at 25 hpf. BrdU uptake was not significantly different between nestin MO and its 5-mis nestin MO control (Fig. 9A). Expression of proliferative markers such as penm and mom5 was also not different between nestin MO and control (Fig. 9B and 9C).

Discussion

Nestin is a hallmark of NPCs. Its expression in zebrafish embryos was first detected about 13 hpf and spread all over the brain and the spinal cord by 24 hpf. Nestin expression during this period of zebrafish embryo development may be critical for brain development. The findings reported here indicate that nestin is indeed essential for zebrafish brain and eye development. Knockdown of nestin in zebrafish embryo by morpholino injection causes distinct morphological changes characterized by small head, small eyes and overt hydrocephalus. These changes become more obvious with time. They are relatively mild at 24 hpf but become prominent at 48 hpf and thereafter. Histological examination of the brain and eyes of developing embryos confirms shrinkage of the hindbrain and the midbrain and the consequent enlargement of ventricles, as well as defective retina structure and underdeveloped lens. Severe suppression of Nestin protein expression at 24 hpf by nestin MO results in fewer NPCs in the brain as analyzed by ascl1b, a marker of NPCs in mammals and zebrafish [16,17,18]. However, nestin suppression does not reduce the density of mature neuron markers including Elavl3/4 and otx2. These results suggest that Nestin is required for the normal NPC function during embryo development.

Our results show developmental defects of motor neurons, axons and glial cells in nestin MO-treated embryos, indicating an important role of nestin in motor nerve and glial development. The underlying mechanism is not entirely clear. It was reported that nestin is expressed in a population of human parenchymal brain cells, co-expressing glial (gfap) and neuronal (betaIII-tubulin) markers. They differentiate into cells of the neuronal or the glial lineages through asymmetric regulation of gene expression. In neuronal cell differentiation, nestin and gfap are down-regulated while in glial cell differentiation, betaIII-tubulin and nestin are down-regulated [15]. Taken together, the results suggest that NPC nestin plays a pivotal role in determining the neuronal and glial cell specification.
Our data provide evidence that Nestin controls the survival of neuronal progenitor cells (NPC). We showed that loss of Nestin is associated with increased apoptosis of hindbrain and midbrain cells. Colocalization experiments revealed that TUNEL-positive cells coincide with ascl1b expression. These results suggest that Nestin suppression enhances NPC apoptosis.

The use of MO in studying development may be limited by undesirable off-target effects that produce CNS developmental defects and apoptosis [19]. To minimize the chance of off-target effects, we have performed experiments such as dose-response, time course and use of multiple controls including a 5-nucleotide mutated nestin MO. The 5-mis MO control and a standard MO

Figure 8. Colocalization of TUNEL signals with ascl1b expression. A and B, z-stack images and C and D, single z-plane images. Embryos were injected with (A) and (C) 5-mis nestin MO or (B) and (D) nestin MO and images were analyzed by confocal microscopy at 25 hpf. Top panels show TUNEL and middle panels, ascl1b stained by fluorescence in situ hybridization. The lower panels show merged images. Yellow dots as denoted by white arrows indicate colocalization of ascl1b with TUNEL. Bar scale: 200 μm.

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control up to 10 ng did not cause morphological abnormality and apoptotic changes. The results indicate that the defects in brain and eyes are unlikely due to off-target effects of MO as there is consistent dose-response and time-dependent effects.

Nestin is a selective NPC intermediate filament involved in cytoskeletal functions such as maintaining cell shape and regulating cell motility. Recent studies suggest that it is involved in other cellular functions [6]. Its role in apoptosis is less well characterized. However, several studies have indicated that the actin cytoskeleton regulates apoptosis and its defects are associated with neurodegenerative diseases [20]. Furthermore, filamentous aggregates of neuronal intermediate filament proteins are considered to be neuropathological signatures of neurodegenerative diseases [21]. It has been reported that deletion of the hepatocyte intermediate filament proteins, keratin 8/keratin 18 heterodimers, increases apoptosis [22]. Our results suggest that Nestin plays an important role in NPC survival in zebrafish embryo development. The mechanism by which nestin protects cell survival is unclear. Studies from murine cells have suggested that nestin may act as a scaffold to control cell functions including apoptosis [23]. It has been reported that in a murine NPC line, Nestin interacts with and sequesters cyclin-dependent kinase 5 (Cdk5), which is constitutively expressed in NPCs. Reduced Nestin protein levels lead to enhanced Cdk5 activity, promoting NPC apoptosis [23]. Further studies are needed to clarify whether control of NPC survival by Nestin in zebrafish is also mediated by this mechanism.

Our results show that the cranial motor nerve development is disrupted by suppression of nestin in Tg(isl1:GFP) fish which express GFP under the control of a motor neuron-specific islet1 enhancer [12]. This model is well suited to detect changes in cranial motor nerves III, V, VII and X [24]. Nestin MO injection is accompanied by disappearance of III and IV, reduction of V and VII and aberrant positioning of X. These results indicate that the cranial motor neuron development is very vulnerable to nestin defects. Cranial axons are reduced or even abolished in nestin MO-treated embryos, most prominently in the tectum.
Nestin MO injection also has a profound effect on eye development. Both eyes are small and the retina and lens are underdeveloped in nestin knockdown fish. These developmental defects are correlated with increased retinal apoptosis. It has been shown that Nestin is expressed in murine retinal progenitor cells [25]. It is also reported in zebrafish that Nestin is expressed in the retinal ganglion cell layer and ciliary marginal zone, which constitutes the retinal proliferation zone [6]. Our whole mount in situ hybridization results are consistent with expression of nestin in embryonic eyes, presumably in the retinal progenitor cells. It is reasonable to assume that, like its role in neuronal development, Nestin is essential for the retinal development primarily by controlling the retinal progenitor cell survival. Lens development may require Nestin for similar reasons, Nestin was reported to be expressed in the epithelium of lens vesicle [26]. Nestin MO may induce apoptosis of the epithelial progenitor cells, resulting in defective lens development and organization.

In summary, nestin is widely expressed in developing zebrafish brain and eyes, especially in the neural, retinal and lens epithelial progenitor cells. It plays a crucial role in protecting progenitor cells from apoptosis thereby facilitating progenitor cell development into neurons, glial cells, motor nerves, retina and lens. Its deficiency leads to enhanced progenitor cell apoptosis and defective brain, eye and cranial nerve development.

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