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cadherin-6 Message Expression in the Nervous System of Developing Zebrafish

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Cadherins are cell surface adhesion molecules that play important roles in development of a variety of tissues including the nervous system. In this study, we analyzed expression pattern of cadherin-6, a member of the type II cadherin subfamily, in the embryonic zebrafish nervous system using in situ hybridization methods. *cadherin-6* message is first expressed by the neural keel, then by restricted regions in the brain and spinal cord. *cadherin-6* expression in the brain transiently delineates specific brain regions. In the peripheral nervous system, *cadherin-6* message is expressed by the neurogenic placodes and the dorsal root ganglia. As development proceeds, *cadherin-6* expression domain and/or expression levels increased in the embryonic nervous system. Our results show that *cadherin-6* expression in the zebrafish developing nervous system is both spatially and temporally regulated, implicating a role for cadherin-6 in the formation of these nervous structures. *Developmental Dynamics 235:272–278, 2006.* © 2005 Wiley-Liss, Inc.

Key words: zebrafish; development; cell adhesion molecules; brain, cranial ganglia, lateral line system

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INTRODUCTION

Zebrafish has become an important model system to study animal development due to its many experimental advantages, including ease of care and maintenance, external embryonic development, routine production of large clutches of eggs from single females, transparency of embryos, and its demonstrated utility as a genetic model. Early embryogenesis of the zebrafish central nervous system (CNS), similar to other vertebrates, involves the partitioning of the anterior portion of the neural tube into three vesicles called the forebrain, midbrain, and hindbrain, and subsequent development further divides these vesicles into smaller units: the telencephalon, diencephalon, midbrain, hindbrain, and spinal cord (reviewed by Kimmel, 1993).

The zebrafish cranial ganglia derive from neurogenic neural crest precursors and cranial epidermal thickenings called placodes (Metcalfe, 1985; Andermann et al., 2002). The cranial ganglia, located lateral to the hindbrain, include the trigeminal, facial, statoacoustic, glossopharyngeal, and vagal ganglia; and anterodorsal, anteroventral, medial, and posterior lateral line ganglia (Metcalfe, 1985, 1989; Raible and Kruse, 2000). The molecular mechanisms underlying regionalization of the brain and formation of the cranial ganglia are largely unknown, although specific regulatory genes (Puelles and Rubenstein, 1993; Macdonald et al., 1994; Mastick et al., 1997; Andermann et al., 2002) and adhesion molecules including members of the cadherin superfamily (Inoue et al., 1997, 1998; Redies, 2000; Kerstetter et al., 2004) have been implicated in the formation of these structures.

Cadherins are Ca^{++}-dependent cell surface molecules that mediate cell adhesion mainly through homophilic interactions (Takeichi, 1991; Gumbiner, 1996). Most cadherins exhibit unique expression patterns, and functional studies of several cadherins (e.g., cadherin-1 and cadherin-2, also called E-cadherin and N-cadherin, respectively) have shown that these molecules play essential roles in development of vertebrate tissues and organs, including zebrafish CNS (Yagi and Takeichi, 2000; Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003). Cadherin-6 (called K-cadherin previously) is a member of the type-II cadherin subfamily (Redies, 1995; Nollet et al., 2000). The majority of

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studies on cadherin-6 have focused on its expression and function in normal renal development and/or renal carcinoma formation (Xiang et al., 1994; Cho et al., 1998; Mah et al., 2000; Paul et al., 2004). Cadherin-6 expression in the nervous system has been analyzed in a variety of vertebrate species including *Xenopus* (David and Wedlich, 2000), chick (Nakagawa and Takeichi, 1995), and mouse (Inoue et al., 1997). To the best of our knowledge, there is no information on cadherin-6 expression in fish nervous system. Zebrafish *cadherin-6* cDNA sequence and complete amino acid sequence were published in GenBank (accession no. AB193290) by Kubota and Murakami, but there is no published report on cadherin-6 expression in developing zebrafish nervous system.

RESULTS AND DISCUSSION

cadherin-6 Expression in the Central Nervous System

Compared with other cadherin amino acid sequences, the zebrafish cadherin-6 was most similar to cadherin-6 from human (66.8%) and mouse (66.0%); less similar to zebrafish cadherin-11 (46.7%), another type II cadherin; and showed even a lower degree of orthology to zebrafish cadherin-2 (33.9%), a type I cadherin.

The expression pattern of *cadherin-6* message in the developing zebrafish CNS was examined by wholemount in situ hybridization methods. At 12 hours postfertilization (hpf), shortly after the zebrafish neural anlage becomes distinctive (Kimmel, 1993), *cadherin-6* expression was detected in specific regions of the neural keel (Fig. 1A–C). *cadherin-6* message was confined to a patch near the dorsal surface of the anterior neural keel (Fig. 1A,B). *cadherin-6* expression became faint in regions immediately posterior and posterolateral to the anterior patch (Fig. 1B). In the posterior half of the neural keel, weak *cadherin-6* expression was found in three longitudinal thin bands, with one along the midline (middle band) and the other two at the lateral edge of the neural keel (lateral bands, Fig. 1C). The middle band, not present in the anterior neural keel (Fig. 1B), became wider as it approached the posterior end of the neural keel (Fig. 1C). This *cadherin-6* expression in the lateral edge of the neural keel resembles staining patterns of zebrafish early neural crest-specific markers such as *foxd3* and *ap2* (Nguyen et al., 1998; Odenthal and Nusslein-Volhard, 1998); therefore, the *cadherin-6*-expressing cells in the lateral bands are likely early neural crest cells. By 18 hpf when the telencephalon and diencephanlon become recognizable, *cadherin-6* message was observed in the dorsal telencephalon and in distinct regions of the diencephalon (Fig. 2A–C). The *cadherin-6* expression domain in the telencephalon was confined to a longitudinal band extending the entire dorsal telencephalon (Fig. 2B,C). In the diencephalon, *cadherin-6* message was detected in the presumptive epiphysis and in the ventral diencephalon where its expression domain was a patch parallel to the optic recess (Fig. 2B,C). *cadherin-6* expression was also observed in the dorsal region of the entire spinal cord (Fig. 2E,F).

The major divisions of the zebrafish brain become readily identifiable by 24 hpf (Kimmel, 1993; Ross et al., 1992). At this stage, *cadherin-6* expression pattern in the telencephalon, diencephalon (Fig. 3A–D), and the spinal cord (Fig. 3H) remained the same as in 18 hpf embryos. *cadherin-6* message was also found in the cerebellum and hindbrain where its expression domains were mainly confined to the dorsal regions (Fig. 3E–G). *cadherin-6* expression in the telencephalon and the dorsal diencephalon remained the same at 34 hpf (Fig. 4B) as in the earlier stages. By this time, the ventral region of the diencephalon has developed into the hypothalamus, where *cadherin-6* expression was confined to a vertical band extending the entire width of the hypothalamus (Fig. 4B,C). This expression domain appeared to demarcate the anterior and posterior hypothalamus. It is likely developed from the ventral diencephalic *cadherin-6* expression domain of the younger stages, because this vertical band continued to be parallel to the optic recess, and their change in orientation (from rostrocaudal to dorsoventral) is due to continued ventral bending of the longitudinal axis of the embryonic zebrafish (Hauptmann and Gerster, 2000). *cadherin-6* message was also detected in the thalamus (Fig. 4C) and in the optic nerve (Fig. 4D). *cadherin-6* expression in the cerebellum, hindbrain, and spinal cord (Fig. 4E–J) remained the same as in the 24 hpf embryos.

By 46–48 hpf, in addition to the above-mentioned regions in the CNS, *cadherin-6* expression was also detected in the central telencephalon, the central diencephalon, the neural retina, the posterior hypothalamus, the anterior cerebellum, and in the anterolateral and ventral hindbrain (Fig. 5A–F). In the central diencephalon, *cadherin-6* expression, weaker than the hypothalamic band, was confined somewhat to two perpendicular bands, with the first band oriented parallel to the optic recess, whereas the second band, in the thalamus, perpendicular to the optic recess (Fig. 5B). In the posterior hypothalamus *cadherin-6* expression, again weaker than the hypothalamic band, was located at the boundary between the diencephalon and the hindbrain (Fig. 5B). In the neural retina, *cadherin-6*-positive cells were located in the retinal ganglion cell layer and in the inner nuclear layer (Fig. 5D). The majority of the *cadherin-6*-expressing cells in the inner nuclear layer were located in the inner most region of the layer, where amacrine cells reside. The optic nerve continued expressing *cadherin-6* message (Fig. 5D). Scattered cells in the optic tectum were also *cadherin-6* positive (Fig. 5C). In the anterior cerebellum, a vertical band of *cadherin-6*–expressing domain delineated the anterior boundary of the cerebellum (Fig. 5A,B). In the dorsal cerebellum, *cadherin-6* expression domain was confined to the midline region (Fig. 5F), much more restricted than at younger stages (Fig. 4G). *cadherin-6* expression was also found in the anterolateral cerebellum and in clusters in the ventral hindbrain immediately dorsal to the statoacoustic ganglion (Fig. 5E–G).

cadherin-6 expression in the embryonic zebrafish brain is similar to the situation in the mouse brain in that *cadherin-6* message is located in restricted areas (Inoue et al., 1997, 1998). In both species, *cadherin-6* was detected in confined regions in the anterodorsal telencephalon, diencephalon, dorsal cerebellum, anterior hind-

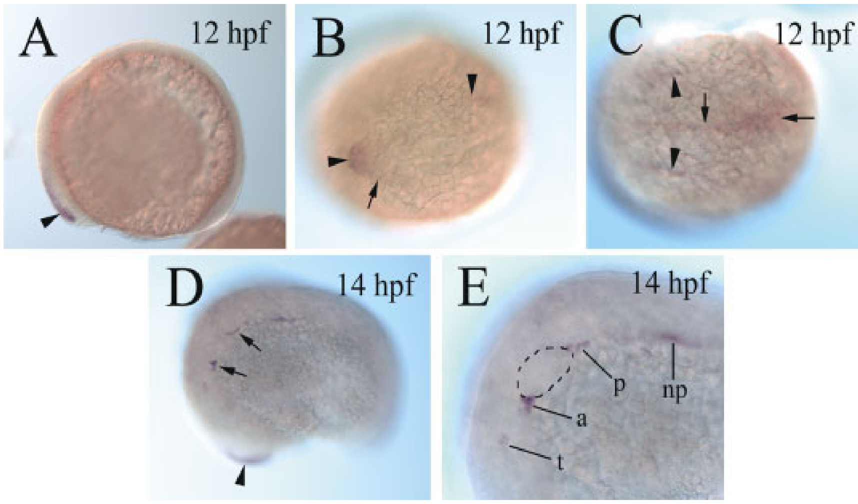


Fig. 1.

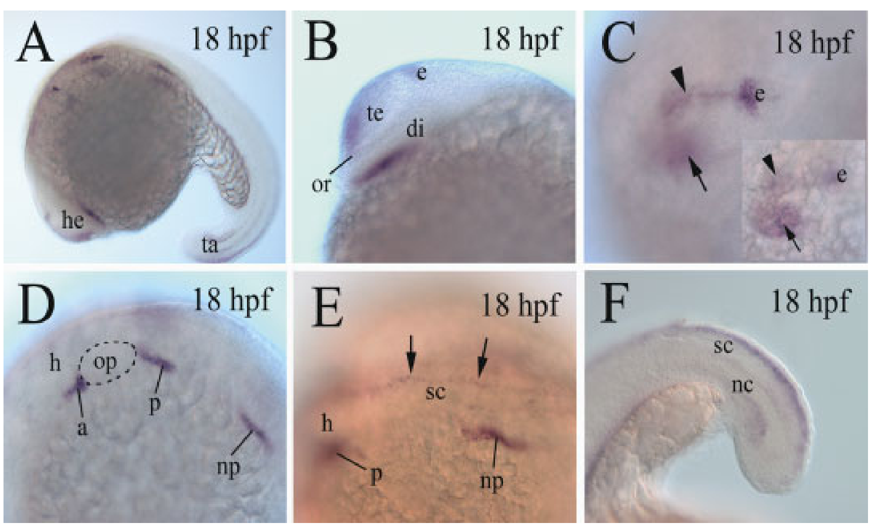


Fig. 2.

brain, and the spinal cord; and *cadherin-6* expression in the brain increases as development proceeds. Moreover, *cadherin-6* expression in both species transiently delineates specific brain subdivisions. The anterior cerebellum/hindbrain *cadherin-6* expression domain marks the boundary between the midbrain and hindbrain in both species. Another example is *cadherin-6* expression in the diencephalon, where in the mouse a *cadherin-6* expression domain is located along the boundary between the dorsal and ventral thalamus (Inoue et al., 1997), whereas in the zebrafish, the thalamic *cadherin-6* expression domain appears to divide the diencephalon into a dorsal and ventral compartments.

cadherin-6 Expression in the Peripheral Nervous System

Using molecular markers *neurogenin1* and *neuroD*, Andermann et al. (2002) showed that the trigeminal placode/ganglion was the first cranial placode/ganglion in zebrafish to become recognizable at 9 hpf. The anterior lateral line placode area and the posterior lateral line placode/ganglion are recognizable at 14 hpf, when the otic placode becomes readily identified by differential interference contrast (DIC) optics. All these three placodes/ganglia expressed *cadherin-6* message by 14 hpf (Fig. 1E). The size of the *cadherin-6*-expressing trigeminal pla-

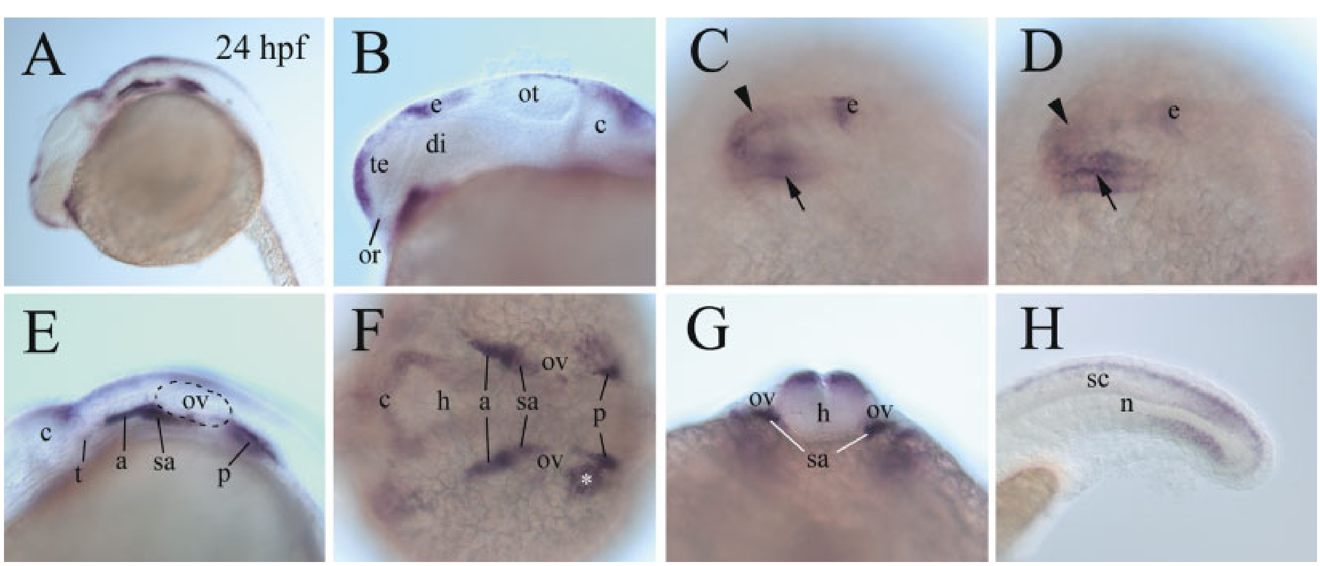


Fig. 3.

code/ganglion was much smaller than *neuroD*-expressing trigeminal placode/ganglion of the same stage (Andermann et al., 2002), suggesting that only a subset of cells in this placode/ganglion contained *cadherin-6* message. Both the anterior lateral line placode area and the posterior lateral line placode/ganglion continued expressing *cadherin-6* message at 18 hpf (Fig. 2D), while its expression in the trigeminal placode/ganglion was weak (data not shown).

By 24 hpf, in addition to these *cadherin-6*–expressing cranial ganglia, the newly formed statoacoustic ganglion (Haddon and Lewis, 1996) became intensely labeled with the *cadherin-6* cRNA probe (Fig. 3E,F), while the trigeminal ganglion continued expressing low levels of *cadherin-6* message (Fig. 3E). At 34 hpf, after the anterior lateral line placode area has given rise to the anterodorsal and anteroventral lateral line ganglia (Andermann et al., 2002), *cadherin-6* expression in the former was much weaker than in the latter (Fig. 4E). At this stage, the medial lateral line ganglion and the vagal placode/ganglion also expressed *cadherin-6* message, while the statoacoustic ganglion and the posterior lateral line ganglion continued expressing high levels of *cadherin-6* message (Fig. 4E,G). At this stage *cadherin-6* expression in the trigeminal ganglion became undetectable (Fig. 4E). In the trunk region, *cadherin-6* expression was detected in small clusters ventrolateral to the spinal cord (Fig. 4H). These clusters, bilaterally and segmentally localized, are likely the newly formed dorsal root ganglia (An et al., 2002). *cadherin-6* expression in older embryos (46–48 hpf) was similar to that of the younger ones in that the statoacoustic ganglion, the posterolateral line, and medial lateral line ganglia expressed high levels of *cadherin-6* message, while there was little or no *cadherin-6* expression in the trigeminal and the anterodorsal lateral line ganglia (Fig. 5E,G,H). The anteroventral and facial ganglia contained little or no *cadherin-6* message (Fig. 5E,H).

cadherin-6 expression in the developing zebrafish peripheral nervous system was similar to that in the mouse (Inoue et al., 1997, 1998) and *Xenopus* (David and Wedlich, 2000) in that cadherin-6 is expressed by neural crest cells and many of their derivatives such as the neurogenic cranial placode/ganglia and dorsal root ganglia. We previously showed that several type I classic cadherins (e.g., cadherin-2 and cadherin-4) exhibited unique expression patterns in the developing zebrafish cranial ganglia (Liu et al., 2003), and development of the cranial ganglia was severely disrupted in zebrafish embryos with reduced or no cadherin-2 activity (Kerstetter et al., 2004). The *cadherin-6* expression pattern in the cranial ganglia shown in this study is distinct from the other cadherins. For example, cadherin-2 is expressed by all cranial ganglia during the first 2 days of development, whereas cadherin-4 is not expressed by the trigeminal ganglion of embryos younger than 30 hpf but is expressed by this ganglion of 32–72 hpf embryos (Liu et al., 2003). Together with the current findings, our results suggest that normal formation of the zebrafish cranial ganglia require combined activities of multiple cadherins. Finally, the early expression of *cadherin-6* message by the zebrafish cranial ganglia and dorsal root ganglia makes this molecule a useful early marker for the zebrafish peripheral nervous system differentiation.

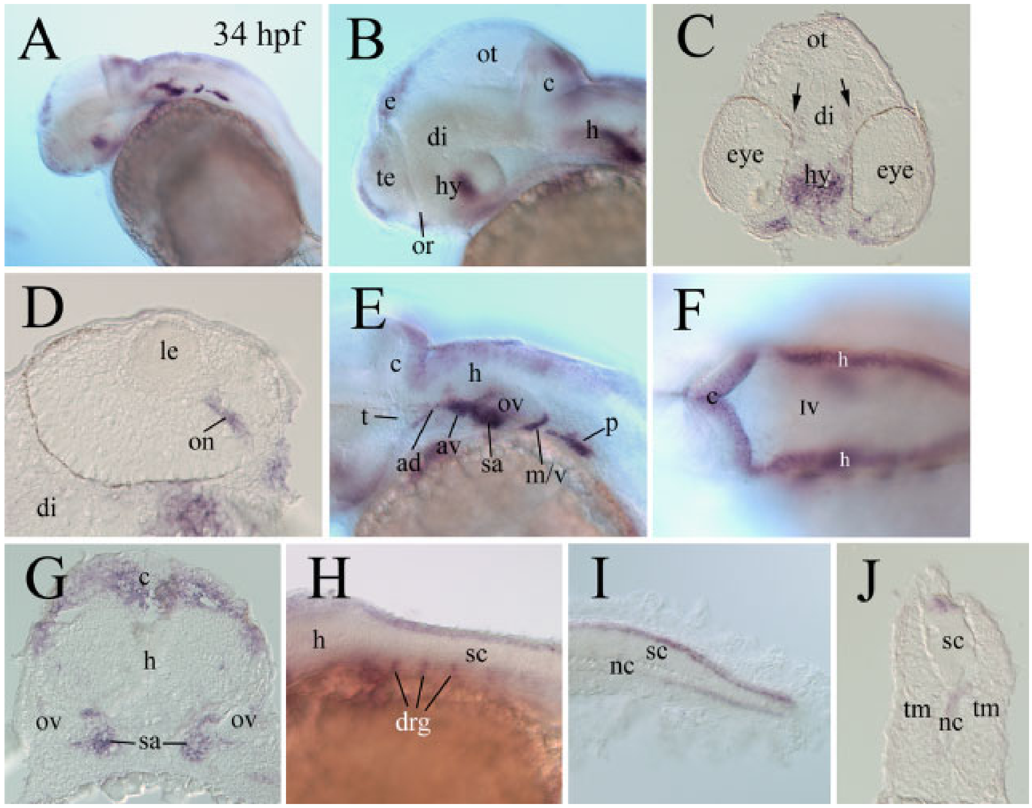
EXPERIMENTAL PROCEDURES

Zebrafish embryos were maintained at 28°C as described in the Zebrafish Book (Westerfield, 1995). Embryos for whole-mount in situ hybridization were raised in PTU (1-phenyl-2-thio-

Fig. 1. *cadherin-6* expression in 12 and 14 hours postfertilization (hpf) zebrafish embryos. **A:** A lateral view of a whole-mount embryo, showing *cadherin-6* expression (arrowhead) in the anterodorsal neural keel. B,C: Dorsal views of a whole-mount embryo (anterior to the left). **B:** The horizontal arrowhead indicates the anterodorsal *cadherin-6* expression, whereas the vertical arrowhead points to the anterior limit of the *cadherin-6* expression at the midline. The arrow indicates the lateral edge of the expression domain. **C:** The arrowheads point to cadherin-6 expression at the edge of the neural keel, whereas the vertical arrow indicates the cadherin-6 expression domain at the midline. *cadherin-6* expression near the posterior end of the neural keel is indicated by the horizontal arrow. **D:** The arrowhead indicates the cadherin-6 expression domain in the head region (out of focus). The two arrows point to two of the *cadherin-6*–expressing regions. **E:** A higher magnification view of the labeled regions indicated by the arrows in D. The otic placode (op) is outlined with dashed lines. a, anterior lateral line placode area; np, nephric duct; p, posterolateral line placode/ganglion; t, trigeminal placode/ganglion.

Fig. 2 *cadherin-6* expression in 18 hours postfertilization (hpf) embryos. **A,B:** B is a higher magnification image of the head region of the embryo in A, with anterior to the left and dorsal up. **C:** A higher magnification image of a dorsolateral view of the forebrain (anterior to the left), focusing on the *cadherin-6* expression domain (arrowhead) in the dorsal forebrain, whereas the inset shows the same brain, focusing on *cadherin-6* expression domain (arrow) in the ventral diencephalon. **D:** A higher magnification of the hindbrain region (anterior to the left and dorsal up) of the embryo in A. **E:** A higher magnification of a dorsolateral view of the anterior spinal cord (sc) region (with anterior to the left) of the embryo in A. The two arrows indicate *cadherin-6* expression in the dorsal spinal cord. **F:** A lateral view (anterior to the left and dorsal up) of the tail region of an embryo showing *cadherin-6* expression in the dorsal spinal cord. The otic placode in D is outlined with dashed lines. di, diencephalon; e, epiphysis; h, hindbrain; nc, notochord; or, optic recess. The remaining abbreviations are the same as in Figure 1.

Fig. 3. Expression of *cadherin-6* message in 24 hours postfertilization (hpf) embryos. **A,B:** B is a higher magnification of the fore- and midbrain regions of the embryo in A, with anterior to the left and dorsal up. **C,D:** Higher magnification dorsolateral views (anterior to the left) of the forebrain region of the same whole-mount embryo, with C focusing on *cadherin-6* expression in the dorsal forebrain (arrowhead) and D focusing on *cadherin-6* expression in the ventral diencephalon (arrow). **E:** A higher magnification of the hindbrain region of the embryo in A (anterior to the left and dorsal up). **F:** A higher magnification dorsal view (anterior to the left) of the hindbrain region of a whole-mount embryo. The asterisk indicates *cadherin-6* expression outside the nervous tissue. **G:** A frontal view of the hindbrain region (dorsal up) at the level of otic vesicle (ov), of a whole-mount embryo. **H:** A higher magnification lateral view (anterior to the left and dorsal up) of a whole-mount tail region. c, cerebellum; sa, statoacoustic ganglion. The remaining abbreviations are the same as in Figures 1 and 2.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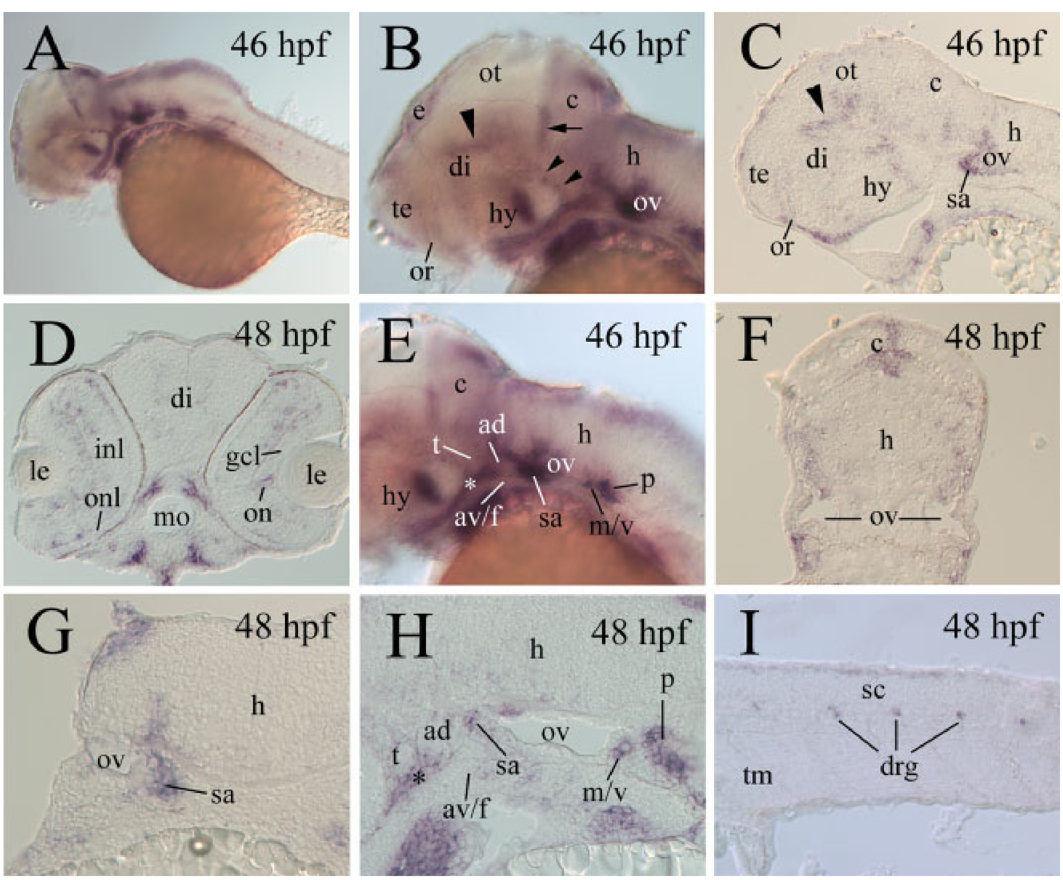
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Fig. 5.

Fig. 4. *cadherin-6* expression in 34 hours postfertilization (hpf) embryos. **A:** A lateral view (anterior to the left and dorsal up) of a whole-mount embryo. **B,E:** Higher magnifications of the anterior half and posterior half, respectively, of the embryo in A. **C,D,G,J:** Cross-sections of embryos processed for whole-mount in situ hybridization. C (dorsal up) shows a frontal view of the *cadherin-6* expression in the hypothalamus (hy) and the pretectal area (arrows). D: A higher magnification of an eye section (dorsal to the left). **F:** A dorsal view of the hindbrain region (anterior to the left). **G:** A cross-section (dorsal up) through the cerebellum and anterior hindbrain region. **H,I** Lateral views of the anterior spinal cord and tail regions (anterior to the left and dorsal up), respectively, whole-mount embryos. **J:** A cross-section (dorsal up) through the midtrunk region. ad, anterodorsal lateral line ganglion; av, anteroventral lateral line ganglion; IV, the fourth ventricle; le, lens; m/v, medial lateral line ganglion and vagal ganglion; tm, trunk muscles. The remaining abbreviations are the same as in the previous figures.

Fig. 5. *cadherin-6* expression in 46–48 hpf embryos. **A,B,E:** Lateral views of whole-mount embryos (anterior to the left and dorsal up), whereas the remaining panels are sections from whole-mount embryos processed for in situ hybridization. **C,H,J:** Parasagittal sections (anterior to the left and dorsal up). **D,F,G:** Cross-sections (dorsal up). B,E: Higher magnification images of the anterior half and posterior half, respectively, of the head region of the embryo in A. B: The large arrowhead and two smaller arrowheads point to the *cadherin-6* expression domains in the dorsal thalamic and the posterior hypothalamic regions, respectively, whereas the arrow indicates *cadherin-6* expression in the anterior cerebellum. E: The asterisk indicates *cadherin-6* expression outside the nervous system. C: The arrowhead indicates the dorsal thalamic *cadherin-6* expression. G: A higher magnification of the hindbrain at the level of the otic vesicle. H: A higher magnification image of the hindbrain, with the asterisk indicating *cadherin-6* expression outside the nervous system. av/f, anteroventral lateral line and facial ganglia; gcl, ganglion cell layer; inl, inner nuclear layer; onl; outer nuclear layer. Other abbreviations are the same as in the previous figures.

urea, 0.003%) and fixed in phosphate buffered 4% paraformaldehyde.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using total RNA isolated from 50 hpf whole zebrafish embryos and zebrafish *cadherin-6*–specific primers (forward primer, 5′-GCGGAAAAGAT-GAGGACTTG-3′; reverse primer, 5′-CATCCACATCCTCGACACTG-3′) to obtain a cDNA fragment corresponding to the nucleotides 228-1359 of the zebrafish *cadherin-6* (GeneBank accession no. AB193290, F. Kubota F. and T. Murakami). This cDNA fragment, cloned into the PCRII-TOPO vector (Invitrogene, Carlsbad, CA), was verified using restriction mapping and used as a template for the synthesis of digoxigenin-labeled zebrafish *cadherin-6* RNA sense or antisense probes for in situ hybridization. Detailed procedures for the probe synthesis and zebrafish whole-mount in situ hybridization were described previously (Liu et al., 1999). There was no staining in zebrafish embryos at 46 hpf and 72 hpf using the sense *cadherin-6* probe (data not shown).

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