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Expression of Zfhep/δEF1 protein in palate, neural progenitors, and differentiated neurons

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Abstract

Zfhep/δEF1 is essential for embryonic development. We have investigated the expression pattern of Zfhep protein during mouse embryogenesis. We show expression of Zfhep in the mesenchyme of the palatal shelves, establishing concordance of expression with the reported cleft palate of the δEF1-null mice. Zfhep protein is strongly expressed in proliferating progenitors of the nervous system. In most regions of the brain, post-mitotic cells stop expressing Zfhep when they migrate out of the ventricular zone and differentiate. However, in the hindbrain, Zfhep protein is also highly expressed in post-mitotic migratory neuronal cells of the precerebellar extramural stream that arise from the neuroepithelium adjacent to the lower rhombic lip. Also, Zfhep is expressed as cells migrate from a narrow region of the pons ventricular zone towards the trigeminal nucleus. Co-expression with Islet1 shows that Zfhep is expressed in motor neurons of the trigeminal nucleus of the pons, but not in the inferior olive motor neurons at E12.5. Therefore, Zfhep is strongly expressed in a tightly regulated pattern in proliferating neural stem cells and a subset of neurons. Zfhep protein is also strongly expressed in trigeminal ganglia, and is moderately expressed in other cranial ganglia. In vitro studies have implicated Zfhep as a repressor of myogenesis, however, we find that Zfhep protein expression increases during muscle differentiation.

Keywords

AREB6; brain nuclei; cleft; cleft palate; cranial ganglia; delta EF-1; ganglion; heart; hindbrain; medulla; muscle; myogenesis; migration; neurogenesis; neurogenin; pons; pontine nucleus; precerebellar; rhombic lip; trigeminal; ventricular zone; ZEB; Zfhx1a

1. Results and Discussion

Zfhep (δEF1; ZEB; AREB6; TCF8; Zfhx1a) is a member of an unusual family of transcription factors having multiple zinc fingers and also having one or more homeodomains (Hashimoto et al., 1992; Funahashi et al., 1993; Watanabe et al., 1993; Genetta et al., 1994; Cabanillas and Darling, 1996; Darling et al., 1998). Zfhep can either

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repress (Postigo and Dean, 1997) or activate (Dillner and Sanders, 2002) target genes. The phenotype of the Zfhep/δEF1$^{null(LacZ)}$ mice is perinatal lethality due to respiratory failure, with defects of craniofacial development (e.g., cleft palate), skeletal patterning, and severe T cell deficiency (Takagi et al., 1998). The defect in skeletal patterning is relatively mild, involving fusions of the wrists, ankles, ribs, and defects of vertebrae. Zfhep represses the osteocalcin (Sooy and Demay, 2002), alpha1-collagen (Terraz et al., 2001), and collagen type II genes (Murray et al., 2000), suggesting a direct role as a modifier during chondrogenesis. In addition, Zfhep has been suggested to be one of the repressors of myogenesis (Krempler and Brenig, 1999) due to antagonism of bHLH factors such as myogenin and MyoD. Zfhep effectively blocks MyoD-induced myogenesis or transcriptional activation in vitro (Sekido et al., 1994; Postigo and Dean, 1997). However, the cellular expression of Zfhep during myogenesis is unknown. Similarly, Zfhep-null mice have cleft secondary palate, but it is unknown whether Zfhep is expressed in the developing palate. Also, the cause of respiratory failure of Zfhep/δEF1-null mice is not known.

We sought to define the expression pattern of Zfhep protein during embryogenesis of palate and muscle. Given the essential role of bHLH factors in development of the nervous system, and the interaction of Zfhep and certain bHLH factors, we also sought to define the pattern of Zfhep expression in the nervous system.

1.1. Expression of Zfhep in the developing palate

Embryonic development of the secondary palate involves bilateral outgrowths of the maxillary mesenchyme that grow downward along each side of the tongue from embryonic day 11 (E11) through E13. The palatal shelves elevate to a horizontal position above the tongue and their tips adhere by E14.5. For fusion, the medial edge epithelium forms an "epithelial seam", and these cells subsequently transform into mesenchyme. Cleft palate can be caused by disruption of any of these steps, including proliferation of mesenchymal cells, adherence of the epithelium, or epithelial-mesenchymal transformation (Kaartinen et al., 1997; Zhang et al., 2002). Alternatively, cleft palate can be the result of pleiotropic effects of changes in craniofacial development, as with the chondrodysplasia gene (Barrow and Capecchi, 1999; Lavrin et al., 2001).

Targeted disruption of the Zfhep/δEF1 gene causes cleft palate with 100% penetrance. However, it is unclear whether this is due to a cellular defect of the palatal mesenchyme or epithelium, or is a secondary effect of altered growth rates of craniofacial bones or tissues. Expression of Zfhep during palatogenesis has not been described. We examined both mRNA and protein expression during palatogenesis. Reverse transcriptase-PCR (RT-PCR) of RNA from E12.5 palatal shelves shows specific amplification of the expected 597 bp Zfhep amplicon (Fig. 1A). The control reactions confirm that the amplicon is not due to genomic DNA contamination of the RNA sample. These data demonstrate that Zfhep mRNA is expressed in the palate.

Cryostat sections were used for immunohistochemistry (IHC) to determine the cell type and timing of Zfhep expression in the palatal shelves. Our antiserum to the central homeodomain region of Zfhep protein identifies transfected and native Zfhep in western blot analysis of nuclear extracts (Cabanillas et al., 2001; Yen et al., 2001). Immunocytochemistry with this antiserum shows a direct correlation with western analysis (Costantino et al., 2002), showing strong Zfhep protein expression in nuclei of COS-7 cells, but not in JEG3 cells (not shown), consistent with specific labeling of Zfhep. Anti-Zfhep labeled the nuclei of mesenchyme of the growing palatal shelves, but not the epithelium (Fig. 1B,C,E,F). Use of preimmune serum from the same rabbit did not label adjacent sections, demonstrating antibody specificity. Matched exposures of palates from E11.5, E12.5, E13.5 and E14.5 embryos show Zfhep protein expression at the early stages, and a decrease of expression in the older

embryos prior to fusion (Fig. 1). At early stages, the layers of cells near the epithelium were labeled more strongly than mesenchyme in the center of the palatal shelves. The intensity of labeling with anti-Zfhep was similar to that observed in the perichondrium around developing cartilage (Fig. 1G). Our demonstration of Zfhep protein in the mesenchyme of the palatal shelves establishes concordance of expression with the mutant cleft phenotype.

1.2 Expression of Zfhep during myogenesis

Skeletal muscle differentiation is mediated by interactions of the MyoD family (including myogenin and MRF-4), the E protein family, and the myocyte enhancer factor 2 family. Zfhep mRNA is expressed in lateral and medial myotome at E11.5 (Takagi et al., 1998). The presence of Zfhep (by transfection) effectively blocks MyoD-induced myogenesis or transcriptional activation in vitro (Sekido et al., 1994; Postigo and Dean, 1997; Postigo and Dean, 1999). Similarly, Zfhep blocks myogenic differentiation of C_2C_{12} cells (Postigo and Dean, 1997). However, the physiological significance of this is unclear since the Zfhep/δEF1-null mice have no muscle defect that was detectable by histological analysis or northern analysis of myogenin, MyoD, muscle creatine kinase, or desmin (Takagi et al., 1998).

The pattern of Zfhep protein expression has not been examined at the cellular level during muscle development. We investigated the expression of Zfhep protein during differentiation of striated muscle, using double-labeling with anti-myogenin to identify myoblasts, and either anti-myosin or anti-troponin T to identify myotubes that express markers of terminal differentiation. We used myosin and troponin as markers to allow direct comparison to in vitro studies in which Zfhep blocked expression of these proteins (Sekido et al., 1994; Postigo and Dean, 1997). At E14.5, anti-myosin labeled skeletal muscles of the body wall and limbs (Fig. 2A). Individual muscle fibers are labeled with anti-myosin (green), and contain nuclei that are strongly positive for Zfhep (red) (Fig. 2B–D). Similarly, anti-troponin and anti-Zfhep are expressed in the same skeletal muscle fibers (Fig. 2E).

Development of the striated muscles of the tongue is necessary for swallowing activity during embryogenesis. The tongue must be capable of moving to allow the palatal shelves to elevate on the morning of E14 (Barrow and Capecchi, 1999). MyoD and myogenin mRNAs are expressed in the tongue on E11 and their expression peaks on E13, which is earlier than in other muscles (Yamane et al., 2000). We find that Zfhep is broadly expressed in cells of the tongue at E13.5, with the strongest expression of Zfhep in cells forming a pattern of striations similar to developing muscles (Fig. 2G,H). Anti-myogenin identifies a subset of cells in the tongue as myoblasts, all of which express Zfhep protein (Fig. 2J–L). As myotubes form and cells express terminal differentiation markers such as myosin, Zfhep continues to be strongly expressed (Fig. 2M–O). Therefore, the expression of Zfhep per se does not block myogenesis in vivo. In fact, Zfhep expression increases during striated muscle differentiation. These data are consistent with the observation that transfected MyoD strongly activates the endogenous Zfhep gene (Bergstrom et al., 2002).

1.3 Expression of Zfhep in the forebrain

Anti-Zfhep antiserum specifically labels cells in the ventricular zone (VZ) of the lateral ventricle (Fig. 3A–C) of the telencephalon. This matches the Zfhep mRNA expression pattern (Yen et al., 2001), and demonstrates that Zfhep protein expression is lost as cells around the telencephalon migrate out of the VZ and differentiate. The loss of Zfhep expression prior to differentiation in the forebrain is confirmed by double-label IHC that shows no overlap of Zfhep and neurofilament immunoreactivity (Fig. 3D). This indicates that Zfhep protein expression is tightly regulated during the process of neurogenesis.

Zfhep expression is observed in the entire VZ of the lateral ventricle, and in the thinner VZ of the 3rd ventricle (Fig. 3A). Zfhep is not expressed in the mantle of the cerebral cortex or in the thalamus, but is expressed in some isolated cells of the SVZ of the ganglionic eminence. The observation of Zfhep in the VZ and some cells of the SVZ suggests that Zfhep is expressed in proliferating progenitors. To address this possibility, we used double-label IHC with antibodies to Proliferating Cell Nuclear Antigen (PCNA), and to Zfhep. PCNA is strongly expressed during S phase of mitosis, and therefore labels nuclei at the outer margin of the VZ (Takahashi and Caviness, 1993). Expression of Zfhep in the proliferative cells of the VZ was confirmed (Fig. 3E–G). In the forebrain, all PCNA-positive areas also expressed Zfhep. Comparison of reported expression patterns with our results indicates that Zfhep expression likely overlaps Ngn1 and Ngn2 in the dorsal VZ of the telencephalon (Fode et al., 2000), and overlaps Mash1 and Dlx expression in the ventral VZ (Wilson and Rubenstein, 2000). Conversely, Zfhep is likely not coexpressed with the p73 gene, which is limited to cells outside the VZ of the neocortex, consistent with reports that Zfhep directly represses the p73 promoter (Fontemaggi et al., 2001).

1.4 Expression of Zfhep in the embryonic hindbrain

Analysis of the developing pons and medulla oblongata shows a very different pattern of expression than observed in the forebrain. In the hindbrain, Zfhep is specifically expressed in a post-mitotic population of migratory cells and certain brain nuclei, as well as in the proliferative neuroepithelium. During development, the lower rhombic lip generates the precerebellar neuroepithelium (PCN), which produces three post-mitotic migratory streams of cells. These migratory cells are destined to become the neurons in specific precerebellar nuclei within the pons and medulla (Altman and Bayer, 1997; Engelkamp et al., 1999). Zfhep and PCNA are both strongly expressed in the highly proliferative precerebellar neuroepithelium (Fig. 4A,B; yellow cells in the PCN). The neuronal precursors that form the anterior precerebellar extramural migratory stream (PEMS) are post-mitotic, and as expected do not express PCNA , however, cells of the PEMS continue to express Zfhep strongly (Fig. 4B; red cells). The Zfhep-positive migratory stream can be traced posteriorly to the base of the pons. Migration of the Zfhep-positive cells occurs on the surface of the pons, external to nerve tracts as shown by double-labeling of Zfhep and neurofilament (Fig. 4C), consistent with identification of these cells as the anterior PEMS (Altman and Bayer, 1997). The anterior PEMS also expresses proneural transcription factors such as Math1 and Pax6 (Engelkamp et al., 1999).

Within the hindbrain, Zfhep is also expressed in a cluster of neurons (Fig. 5). Serial transverse and sagittal sections of paraffin-embedded embryos clearly identifies the nucleus in Fig. 5D as the trigeminal nucleus (Vn) of the pons (Jacobowitz and Abbott, 1998; Widmer et al., 1998). Double-label IHC shows that the Zfhep-positive trigeminal cells are closely associated with neurofilament, indicating that cells in this area are differentiated (not shown). To further identify these Zfhep-positive cells, transverse sections were labeled with anti-Islet1 and anti-Zfhep (Fig. 5D–F). Islet1 is expressed in early post-mitotic motor neurons in the vertebrate hindbrain. Islet1 and Zfhep are co-expressed in the trigeminal nucleus, identifying these neurons as part of the trigeminal motor nucleus (Fig. 5). The characterization of these Zfhep-positive trigeminal cells as motor neurons is supported by our observation that Brn3, a marker for sensory neurons, is not co-expressed with Zfhep in this region (not shown). In contrast to the observed expression of Zfhep in the trigeminal nucleus, anti-Islet1 labels the inferior olive nucleus of the medulla oblongata, but these neurons do not express Zfhep (Fig. 5G–I). Cells of the inferior olive are not derived from the adjacent ventricular zone, but rather from the posterior PCN via an intramural migratory stream (Altman and Bayer, 1997). Similarly, the small Islet-positive nucleus at the lateral edge of the pons (Fig. 5K, red box) shows no expression of Zfhep at any level of the pons.

Therefore, Zfhep expression is associated with just specific brain nuclei. This pattern of Zfhep expression is similar to Phox2b, Math1, and Pax6, which are in the PEMS and certain differentiated hindbrain nuclei (Engelkamp et al., 1999).

Formation of the trigeminal motor nucleus is not well described. Transverse sections through the pons at E12.5 indicate that Zfhep-positive cells migrate out of a restricted area of the basal plate ventricular zone (Fig. 5J, K). These cells continue to express Zfhep as they differentiate, as evidenced by their distinctive shape and co-expression of Islet1 on E12.5 (Fig. 5L). The stream of Zfhep-positive cells from the VZ to the trigeminal nucleus (Fig. 5K), suggests the presence of a migratory stream arising from a discrete region of the VZ.

Parasagittal sections through this area demonstrate that Zfhep is co-expressed with Islet1 throughout the trigeminal nucleus on E13.5 (Fig. 5M–O). The size and columnar morphology of the Zfhep-positive Vm matches that observed by DiI-labeling of the trigeminal nerve at the masseter muscle (Widmer et al., 1998), emphasizing that these are differentiated neurons. In the mouse, Vm neurons are born on E9 and E10 (Taber Pierce, 1973), and axon pathfinding of Vm neurons occurs from E9.5 to E13.5 (Zhang et al., 2000), indicating that the Zfhep-positive Vm cells at E13.5 (Fig. 5O) are terminally differentiated. These data are the first demonstration of Zfhep expression in differentiated neurons of the CNS.

1.5 Expression of Zfhep in cranial ganglia

We find high levels of Zfhep expression in some cranial ganglia. The trigeminal ganglion (Vg) forms in part from cells of the trigeminal placode, and in part from neural crest cells that migrate into the ganglion. Fig. 6A shows strong expression of Zfhep protein in the trigeminal ganglion of an E11.5 embryo. Co-expression of Zfhep and Brn3a in the trigeminal ganglion identifies these cells as neural progenitors or neurons (Fig. 6B–D). This is consistent with the suggestion that a Zfhep-binding site is necessary for expression of the TrkA promoter within the trigeminal ganglion (Ma et al., 2000). Zfhep is also expressed in the facial (VII) and vestibulocochlear (VIII) ganglia (Fig. 4), and in the X/XI ganglia complex (Fig. 6E–H).

1.6 Expression of Zfhep in the spinal cord

Tagaki et al. (Takagi et al., 1998) reported Zfhep/δEF1 mRNA in the VZ of the spinal cord at E11.5. We observed little or no expression in the neural tube at E10.5, followed by strong expression of Zfhep protein in the spinal cord VZ from E11.5 through E13.5 (Fig. 7A–D). Zfhep is not strongly expressed in the floor plate, and expression in the VZ decreases in a ventral to dorsal wave. By E14.5 isolated Zfhep-positive cells are present in the spinal cord, and in a layer around the central canal. Weakly Zfhep-positive cells are present in the postnatal spinal cord, scattered in the gray and white matter (Fig. 7F). Expression of Zfhep is strong in the dorsal root ganglia of the spine (Fig. 7C).

1.7 Summary

In summary, the domains with the strongest embryonic expression of Zfhep protein are the central nervous system and striated muscle. Expression in the nervous system is widespread in proliferative cells of the ventricular zone. Interestingly, Zfhep is observed in two specific streams of migratory cells; the anterior PCEM, and the cells migrating to form the trigeminal motor nucleus. Zfhep continues to be expressed as the neurons of the trigeminal nucleus differentiate, but this was not observed in other brain nuclei examined.

2. Materials and Methods

2.1 Immunohistochemistry

Mouse embryos or heads were either frozen in OCT, or fixed in 4% paraformaldehyde for 2 to 4 hours and embedded in paraffin. Frozen embryos were sectioned in a Reichert-Jung Frigocut 2800N cryostat, thaw-mounted onto gelatin-coated microscope slides, and stored at -80C. Slides were warmed to room temperature and fixed in fresh 4% paraformaldehyde, 0.1 % Triton X-100, 0.1 M phosphate buffered saline, pH 7.4, for 30 minutes, and blocked in 10% calf serum, 0.1 % Triton, in PBS for 1 hour. Immunohistochemistry was done with a rabbit anti-Zfhep antiserum (1:400 for frozen sections) raised to the central homeodomain region of Zfhep (Cabanillas et al., 2001; Costantino et al., 2002). Additional primary antibodies were; mouse anti-neurofilament 165 (1:10), mouse anti-myogenin (F5D, neat), mouse anti-myosin (F1.652, 1:5), mouse anti-Islet1 (40.2D6, neat), and mouse anti-troponin T (CT3, 1:100) (all from Developmental Studies Hybridoma Bank), and mouse anti-PCNA (1:200; Labvision), or mouse anti-Brn3a (1:20; Chemicon). Antibodies were diluted in 4% calf serum, 0.1 % Triton, in PBS and incubated on the sections for 1 hour. Secondary antibodies were sheep anti-rabbit IgG conjugated to FITC (Chemicon), donkey anti-rabbit IgG-rhodamine (Chemicon), goat anti-mouse IgG-FITC (Sigma), donkey anti-mouse IgG-rhodamine (Chemicon), or goat anti-rabbit IgG, or anti-mouse IgG, each labeled with Alexafluor 488, or Alexafluor 594 (Molecular Probes). Sections were viewed and imaged with a Nikon E600 fluorescence microscope with a SPOT-RT digital camera and images processed with Adobe Photoshop 6.

Paraffin sections were treated with heat-induced epitope retrieval by incubating at 98 °C in 10 mM citrate buffer, pH 6.0, for 10 minutes. Blocking and antibody incubations were as described above, except that for paraffin sections, the anti-Zfhep antibody was used at 1:1000 dilution.

2.2 RT-PCR of embryonic palate

E12.5 mouse embryos were dissected to obtain palatal tissue (Warner et al., 2003). Total RNA was amplified by RT-PCR by standard methods using random hexamer primers with Superscript II reverse transcriptase (Invitrogen), and Expand High Fidelity Taq/Pwo DNA polymerases (Roche). PCR primers (21 bases long) with 5’-ends located at nucleotide 294 (in exon 3) and 890 (in exon 7) of the Zfhep cDNA were used. PCR reactions were analyzed on a 1% agarose gel.

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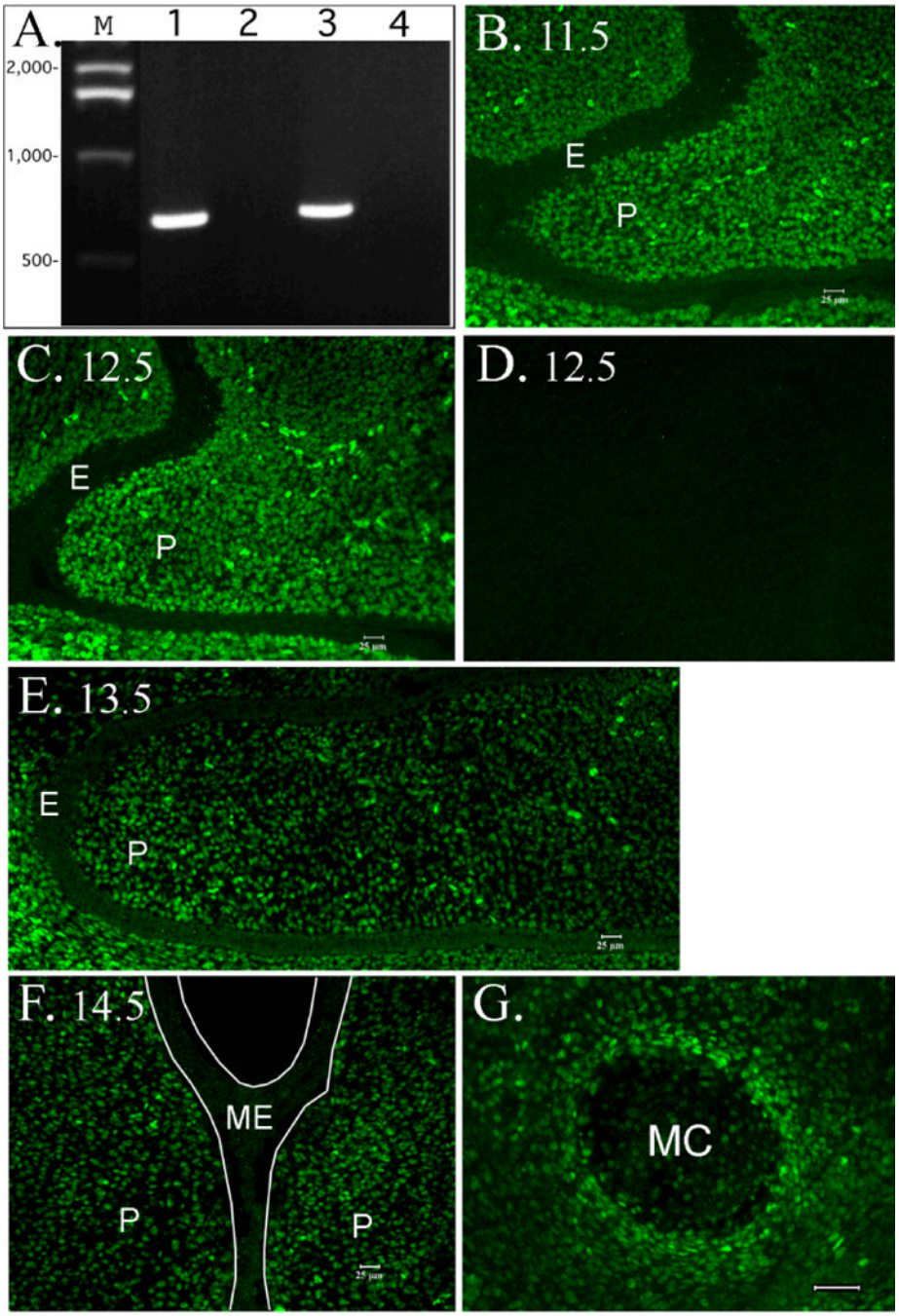

Fig. 1.

Expression of Zfhep in palatal shelf mesenchyme. (A) Agarose gel showing RT-PCR amplification of Zfhep mRNA from E12.5 mouse palate. Lanes 1 and 3 are separate RT-PCR amplifications with 1 μMolar (lane 1) or 2 μMolar (lane 3) of each primer. Lanes 2 and 4 are control PCR reactions done without the reverse transcriptase enzyme. M; Markers. (B-G) Immunohistochemistry of transverse frozen sections probed with anti-Zfhep or preimmune serum (both 1:400), and a FITC-conjugated secondary antibody. Matched exposures are shown to compare the different embryonic ages. (B) E11.5, anti-Zfhep; (C) E12.5, anti-Zfhep; (D) E12.5, preimmune; (E) E13.5 anti-Zfhep; (F) The fused tips of the horizontal palatal shelves at E14.5, labeled with anti-Zfhep. The white lines in F outline the

medial edge epithelium. The orientation matches Kaufman (Kaufman, 1992) plate 30d-c. (G) Meckel's cartilage of an E13.5 embryo labeled with anti-Zfhep showing expression in the perichondrium. The tongue is along the lower edge of the picture in B, C, and E. The bar is 25 microns for B, C, E, and F, and 50 microns for G. E, epithelium; P, tip of the palatal shelf; ME, medial edge epithelium; MC, Meckel's cartilage.

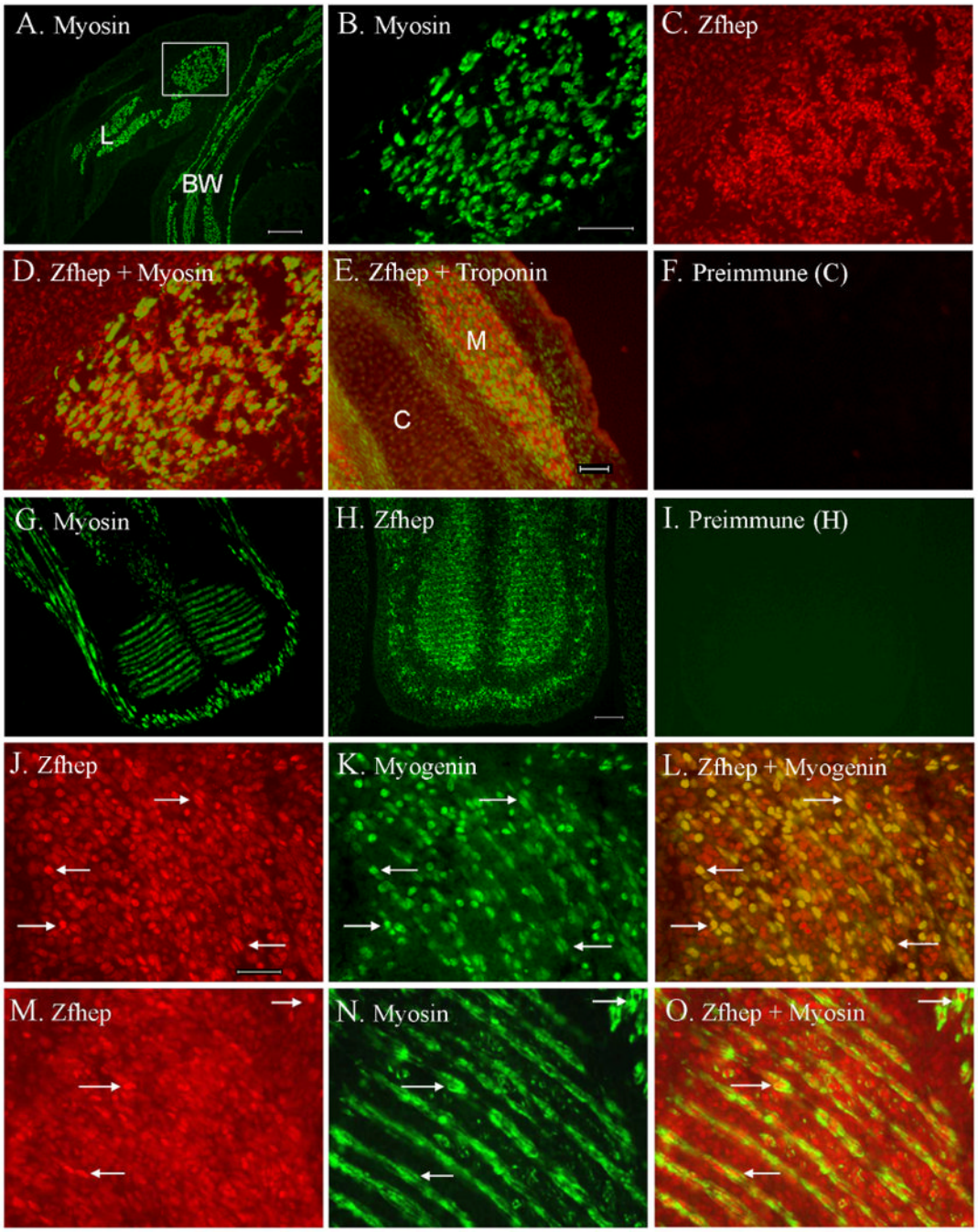

Fig. 2.

Expression of Zfhep throughout muscle differentiation. (A) IHC of an E14.5 embryo frozen section. Anti-myosin labels the muscles of the body wall and forelimb. The boxed muscle is shown in panels B, C, and D. The bar is 300 microns. (B) Limb muscle labeled with anti-myosin and FITC-secondary antibody. The bar is 100 microns. (C) Anti-Zfhep and rhodamine-conjugated secondary. (D) Combination of panels B and C. The nuclei within the myofibers (green) are Zfhep-positive (red). (E) IHC of E13.5 limb muscle labeled with anti-Zfhep and FITC-conjugated secondary, and anti-troponin and a rhodamine-secondary. The limb muscle is identified by troponin-positive (red) cells, which contain Zfhep-positive (green) nuclei. (F) Negative control for panel C, showing the lack of labeling with

preimmune serum. (G-O) Expression of Zfhep during tongue striated muscle differentiation. Immunofluorescence of E13.5 embryo frozen sections. (G) Anti-myosin and FITC-secondary antibody labels the muscles of the tongue. (H) Anti-Zfhep and FITC-secondary antibody show a similar pattern as myosin. The bar is 100 microns. (I) Matched control for H using preimmune serum and FITC-secondary antibody. (J) Anti-Zfhep and rhodamine-conjugated secondary antibody labeled tongue cell nuclei. Bar is 40 microns. (K) The same section labeled with anti-myogenin and FITC-conjugated secondary. (L) Combination of panels J and K shows expression of both Zfhep and myogenin in the same nuclei (yellow). (M) Anti-Zfhep and rhodamine secondary antibody labeled tongue cell nuclei. (N) Anti-myosin and FITC-conjugated secondary. (O) Combination of panels M and N. Arrows in J, K, and L, or in M, N, and O, identify the same cells in related panels. BW, body wall; C, cartilage; L, limb; M, muscle.

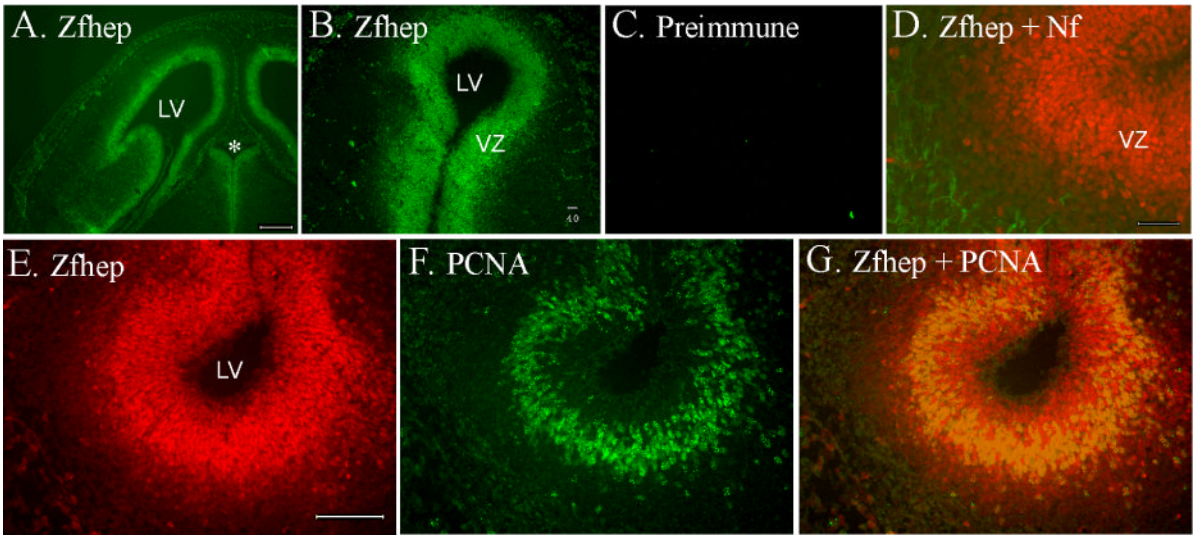

Fig. 3.

Zfhep expression in the telencephalon. (A) Anti-Zfhep (1:400 dilution) and goat FITC-anti-rabbit IgG secondary antibody immunofluorescence of a transverse cryosection of the forebrain of an E14.5 embryo. The bar is 300 microns. (B) E13.5 mouse embryo brain labeled with anti-Zfhep showing the anterior portion of the superior horn of a lateral ventricle (LV) with Zfhep expression in cells of the ventricular zone (VZ). The bar is 40 microns. (C) Matched exposure of a section adjacent to B, and incubated with preimmune serum and FITC-secondary Ab, indicating the specificity of the anti-Zfhep antiserum. (D) Transverse cryostat section of lateral ventricle of E13.5 embryo double-labeled with monoclonal anti-neurofilament 165 (detected with FITC-secondary Ab) and anti-Zfhep (detected with donkey anti-rabbit IgG-rhodamine). The VZ contains Zfhep-positive (red) nuclei, however, cells that have differentiated are neurofilament-positive (green neurites) and Zfhep-negative. Bar is 40 microns. (E) LV of an E14.5 brain labeled with anti-Zfhep and rhodamine. Bar is 100 microns. (F) The same section labeled with anti-PCNA and FITC. (G) Combination of F and G showing co-expression of Zfhep and PCNA. *, 3rd ventricle.

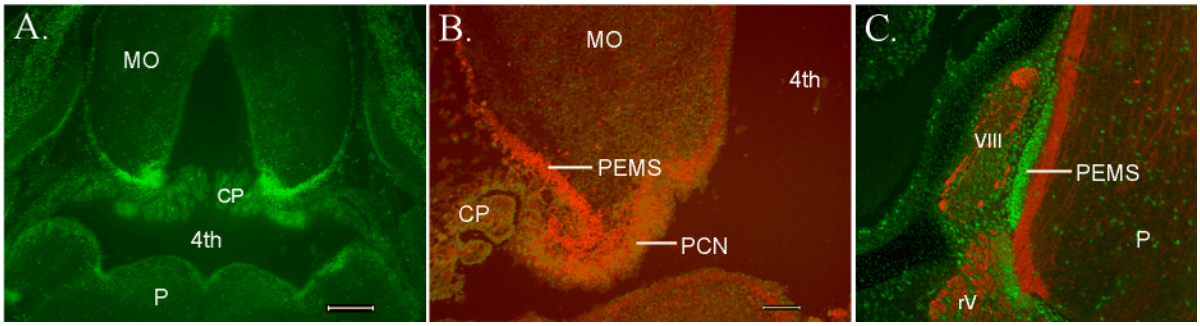

Fig. 4.

Zfhep in the anterior PEMS. Immunofluorescence of transverse cryostat sections of an E14.5 embryo. Dorsal is up. (A) Hindbrain section labeled with anti-Zfhep (1:400 dilution) and FITC-secondary antibody. The orientation of the section matches (Kaufman, 1992) plate 32b-a. Zfhep is strongly expressed in cells of the PCN and PEMS. Bar is 300 microns. (B) The edge of the MO labeled with anti-Zfhep detected with donkey anti-rabbit IgG-rhodamine, and anti-PCNA detected with goat anti-mouse IgG-FITC. Yellow-green cells of the PCN express both antigens. Zfhep (red) is strongly expressed after the cells exit mitosis and migrate across the medulla. The bar is 100 microns. (C) A section at a lower level, through the base of the pons (similar to (Kaufman, 1992), plate 32b-c) labeled with anti-Zfhep (detected with FITC) and anti-neurofilament 165 (detected with anti-mouse IgG-rhodamine). The labyrinth of the inner ear is to the left. Labeled neurofilament (red) shows differentiation of cells in the pons and in the vestibulocochlear (VIII) ganglion. Zfhep (green) strongly labels the anterior PEMS. 4^{th}, 4^{th} ventricle; CP, choroid plexus; MO, medulla oblongata; P, pons; PEMS, anterior precerebellar extramural migratory stream; PCN, precerebellar neuroepithelium; rV, proximal rootlets of the trigeminal nerve.

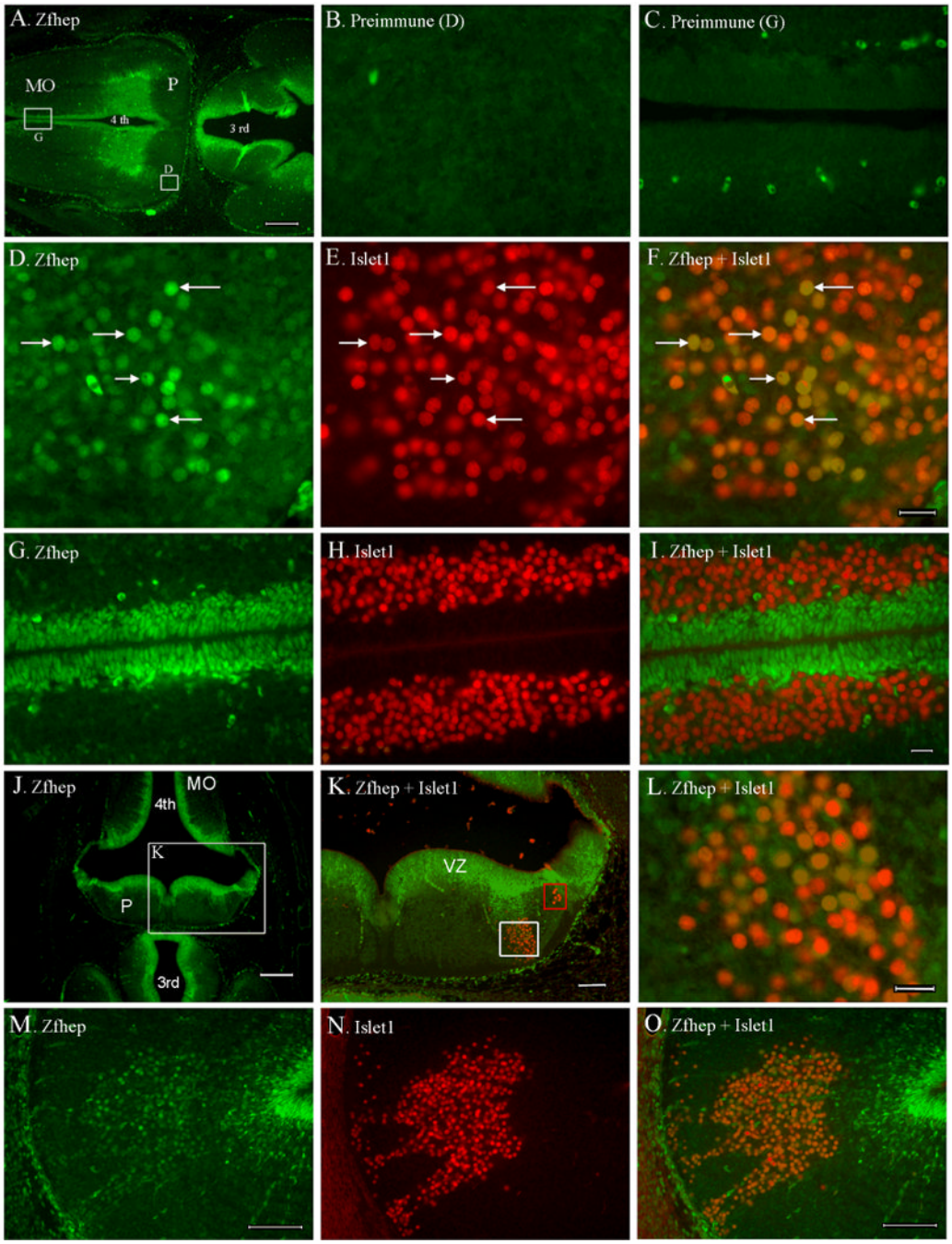

Fig. 5.

Expression of Zfhep in a hindbrain nucleus. (A) Transverse section through the medulla and pons of an E12.5 paraffin-embedded embryo labeled with anti-Zfhep. Dorsal is to the left. This section is similar to (Kaufman, 1992) plate 28a-h. Bar is 300 microns. (B) Image of preimmune serum control matched to image D. (C) Preimmune serum control matched to image G. Autofluorescence of blood cells is detected with paraffin sections, however, no cells in the ventricular zone are labeled. (D) Area D in image A, labeled with anti-Zfhep and Alexafluor 488 conjugated secondary antibody (green). The cell nuclei are positive for Zfhep, to a variable degree (compare to C). (E) Anti-Islet labeled area D. (F) Combined images D and E. Orange and yellow-green nuclei express both antigens. Arrows identify

individual nuclei in the three images. Bar is 20 microns for D, E, and F. (G) Area G in image A labeled with anti-Zfhep. (H) The inferior olive nucleus of the medulla (in area G) labeled with anti-Islet1. (I) Combined images G and H. The absence of yellow or orange nuclei shows that the two antigens are not co-expressed in the inferior olive nucleus. Bar is 20 microns for G, H, and I. (J-O) Formation of Vm within the pons. (J) Transverse section of an E12.5 hindbrain labeled with anti-Zfhep and Alexafluor 488 conjugated anti-rabbit IgG secondary antibody. Similar to (Kaufman, 1992) plate 28a-f. Dorsal is up. Bar is 300 microns. (K) Section of the pons labeled with anti-Zfhep (green) and anti-Islet (red, Alexafluor 594) from the box in image J. The stream of Zfhep-positive cells from a restricted region of the basal plate of the pons is shown above the white box. Bar is 100 microns. (L) Area of the white box in K double-labeled with anti-Zfhep and anti-islet1. Individual nuclei are yellow-green or orange indicating expression of both antigens. Bar is 20 microns. (M) Parasagittal section of the pons of an E13.5 embryo labeled with anti-Zfhep and Alexafluor 488 secondary antibody. Dorsal is to the right. Bar is 100 microns. (N) The same section as D, labeled with anti-islet and Alexafluor 594 to identify the trigeminal motor nucleus. (O) Combined images D and E. Orange and yellow-green nuclei, expressing both antigens, are throughout the trigeminal motor nucleus. Bar is 100 microns. 3rd; third ventricle, 4th; fourth ventricle, MO; medulla oblongata, P; pons, VZ; ventricular zone.

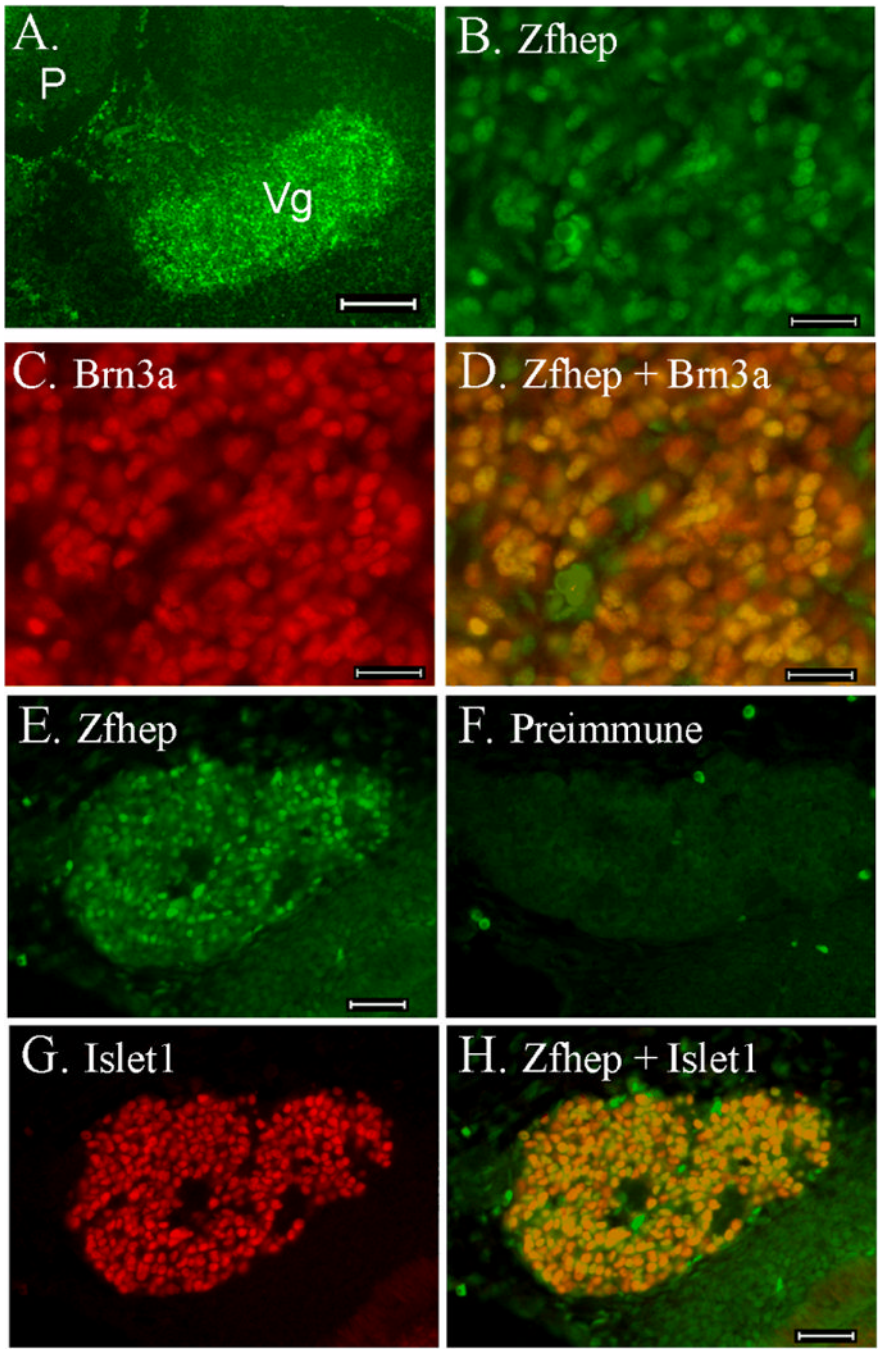

Fig. 6.

Expression of Zfhep in cranial ganglia. (A) The early trigeminal ganglion is labeled by IHC of an E11.5 embryo with anti-Zfhep and Alexafluor 488 secondary antibody. Bar is 200 microns. (B-D) Trigeminal ganglion of an E12.5 embryo labeled with (B) anti-Zfhep, or (C) anti-Brn3a, or (D) both. Bar is 20 microns. (E-H) The complex of the X and XI ganglia labeled with (E) anti-Zfhep, or (F) preimmune serum, or (G) anti-Islet1, or (H) both anti-Zfhep and anti-Islet1. The bar is 40 microns for E and H. P, pons; Vg, trigeminal ganglion.

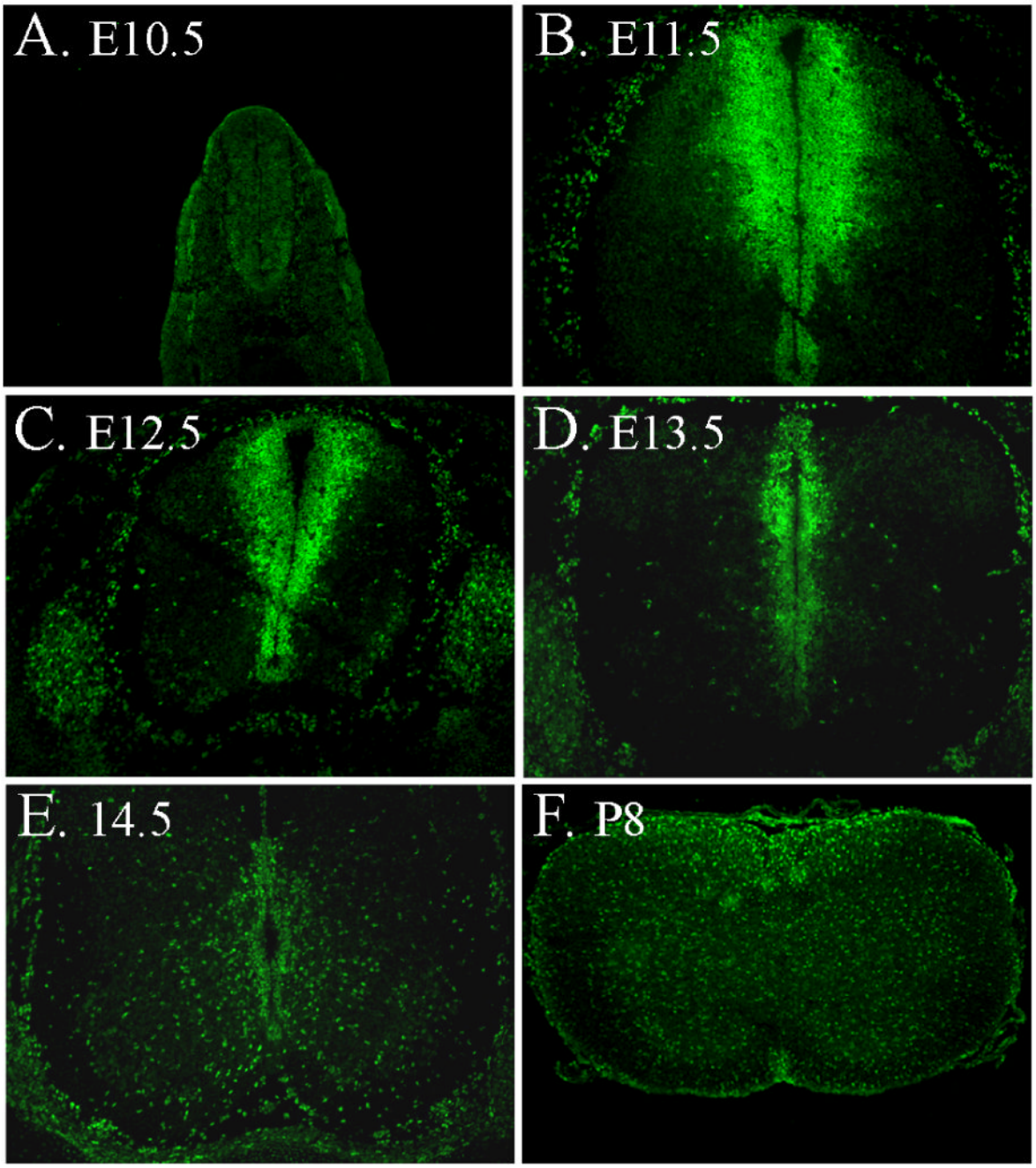

Fig. 7.

Zfhep protein expression in the spinal cord. Cryosections of spinal cord in the cervical region were labeled with anti-Zfhep and FITC. Matched preimmune serum images are blank (not shown). (A) E10.5. (B) E11.5. (C) E12.5. (D) E13.5. (E) E14.5. (F) postnatal day 8.