

## Expression of the tyrosine kinase receptor EphA5 and its ligand ephrin-A5 during mouse spinal cord development

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**Abstract: Objectives** To study the expression patterns of two Eph family molecules, the receptor EphA5, and the ligand ephrin-A5, during spinal cord development. **Methods** The receptor expression was analyzed using beta-galactosidase knockin mice, and affinity ligand probe binding. The ligand expression was assessed using two different affinity probes, and knockout mouse tissues as controls. **Results** EphA5 was expressed in the ventral spinal cord, while ephrin-A5 was located in the dorsolateral regions of the spinal cord throughout development. **Conclusions** These results show that EphA5 and ephrin-A5 are expressed over broad developmental stages and may play important roles in establishing the dorsoventral organization of the spinal cord.

**Keywords:** axon guidance; embryogenesis; dorsal root ganglion; histochemistry; alkaline phosphatase affinity probe;  $\beta$ -galactosidase

### 1 Introduction

Cell migration and differentiation are tightly regulated by various environmental cues during development. Receptor tyrosine kinases are transmembrane proteins, which, as key components in the transduction of certain extracellular signals across the cell membrane, regulate cell growth, differentiation, survival and migration<sup>[1]</sup>. These receptors play critical roles in various aspects of embryonic development, such as tissue morphogenesis and the formation of neuronal circuits. The Eph family is the largest group of receptor tyrosine kinases known, consisting of 13 different genes in mammals<sup>[2,3]</sup>. According to sequence homology and specificity, these receptors can be classified into EphA, which contains eight members (EphA1 through EphA8), and EphB, which consists of five members (EphB1-EphB4, EphB6). EphA and EphB receptors have three major structural domains: extracellular, transmembrane,

and an intracellular domain with tyrosine kinase activity, and play multiple roles in cellular differentiation during development<sup>[3]</sup>. There are eight ephrins, the ligands that bind to the Eph receptors, which can be divided into two subclasses: glycosylphosphatidylinositol (GPI)-anchored ephrin-A (ephrin-A1 to ephrin-A5) and ephrin-B (ephrin-B1 to ephrin-B3) proteins that have a transmembrane and a cytoplasmic domain. In general, ephrin-A ligands interact with EphA receptors, and ephrin-B ligands bind to EphB receptors. However, exceptions to this general rule do exist. For example, EphA4 also binds to ephrin-B ligands, and ephrin-A5 can activate EphB2<sup>[2,4]</sup>.

Several Eph family members have been shown to be expressed in the spinal cord during development<sup>[5-8]</sup>. Our investigation of EphA receptors and ephrin-A ligands revealed that several Eph receptors (EphA3, A4 and A5) and ephrin-A5 are present in the embryonic mouse spinal cord<sup>[8]</sup>. We observed that ephrin-A5 and EphA5 transcripts are expressed in mutually exclusive patterns at embryonic day 14. Here we examined patterns of expression of ephrin-A5 and its receptor, EphA5, in the mouse spinal cord at different developmental stages. Our results show that ephrin-A5 and EphA5 maintain complimentary expression patterns throughout the developing spinal cord with very little overlap.

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## 2 Materials and methods

**2.1 Animal care and genotyping** Mice were maintained in the animal facility at Rutgers, The State University of New Jersey and used in accordance with protocols approved by the Rutgers University Animal Care and Use Committee. Wild-type mice were from the C57BL/6 strain. EphA5-lacZ and ephrin-A5 mutations were genotyped as previously described<sup>9,101</sup>.

**2.2 EphA5- $\beta$ gal Histochemistry** For whole-mount staining, embryos were collected in 0.1 mol/L PBS (pH 7.3), incubated at room temperature for 30 min in fresh lacZ fix buffer (2% glutaraldehyde, 5 mmol/L EGTA, 2 mmol/L MgCl<sub>2</sub> in PBS), rinsed several times in wash buffer (2 mmol/L MgCl<sub>2</sub>, 0.02% NP-40 in PBS), and incubated at 37 °C overnight in lacZ staining buffer (wash buffer containing 1 mg/mL X-gal, 2.12 mg/mL potassium ferrocyanide, and 1.64 mg/mL potassium ferricyanide). Embryos (day 11 and 13) were then rinsed in wash buffer, postfixed in formalin, dehydrated in an ethanol series, and cleared in benzyl alcohol:benzyl benzoate (1:2) immediately prior to observation and photography. For histochemical analysis, embryos were dehydrated in ethanol, embedded in paraffin and sectioned at 6  $\mu$ m.

For later stages of development (E15 and later), unfixed specimens were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Inc., CA, USA) and immediately cryosectioned at 15  $\mu$ m. Air-dried sections were immersed in lacZ fix buffer for 10 min at room temperature, rinsed through multiple changes in wash buffer for 20 min, incubated at 37 °C overnight in lacZ staining buffer, rinsed in wash buffer, and postfixed in formalin. Bright-field photography was carried out with a Nikon microscope.

**2.3 Alkaline phosphatase (AP) affinity probe binding** The affinity probe EphA5-AP and ephrin-A2-AP were created as described in previous reports<sup>11,121</sup>. Frozen tissue sections (14  $\mu$ m) of mouse tissues at various stages (E11, E13, E15, E17, P0, and P7) were mounted on slides coated with 3-aminopropyltrimethoxy silane (Sigma). Coated slides were prepared by washing first in acetone for 5 min, followed with 100% ethanol. The slides were then dried at room temperature and coated by dipping in 2% 3-aminopropyltrimethoxy silane in acetone for 15 s. After coating, the slides were washed in acetone and distilled H<sub>2</sub>O and dried at room temperature overnight. Ligand detection was performed as described by Cheng *et al.*<sup>121</sup>. Briefly, frozen sections on the coated slides were incubated with EphA5-AP-

containing tissue culture media for 90 min at room temperature and washed five times with Hanks' balanced salt solution (HBSS, Sigma). The sections were then fixed for 30 s in 60% acetone, 3% formaldehyde, 20 mmol/L HEPES, pH 7.5, and washed twice with 150 mmol/L NaCl, 20 mmol/L HEPES, pH 7.5. Because the human placental alkaline phosphatase in the probe is heat-stable, the slides were heated at 65 °C for 15 min to inactivate endogenous phosphatases. After heat inactivation, the slides were rinsed with 100 mmol/L Tris-HCl (pH 9.5), 100 mmol/L NaCl, and 5 mmol/L MgCl<sub>2</sub>, and stained for 24 h in the same buffer containing 10 mmol/L L-homoarginine, 0.17 mg/mL BCIP, and 0.33 mg/mL NBT. After staining, the slides were mounted with coverslips and photographed.

**2.4 EphA5-Fc-ligand binding** EphA5-Fc fusion protein was also used to determine the binding patterns for ephrin-A5 in mouse spinal cord at various stages (E11, E13, E15, E17, P0, and P7). For this procedure, mounted sections were preincubated in blocking solution containing 0.1 mol/L PBS, 10% goat serum, 2% BSA, and 0.02% sodium azide for 30 min at room temperature. The sections were then incubated for 1-2 h at room temperature with EphA5-Fc fusion protein (R&D Systems) at a concentration of 2  $\mu$ g/mL. Sections were rinsed three times for 10 min each in PBS, fixed in 4% buffered paraformaldehyde for 5 min, and rinsed again in PBS. Secondary antibodies (goat anti-human IgG conjugated to biotin; Chemicon) were added at a 1:500 dilution and incubated for 1 h at room temperature. Fusion protein binding sites were then visualized using the Vectastain elite ABC kit (Vector Laboratories) per the manufacturer's instructions.

## 3 Results

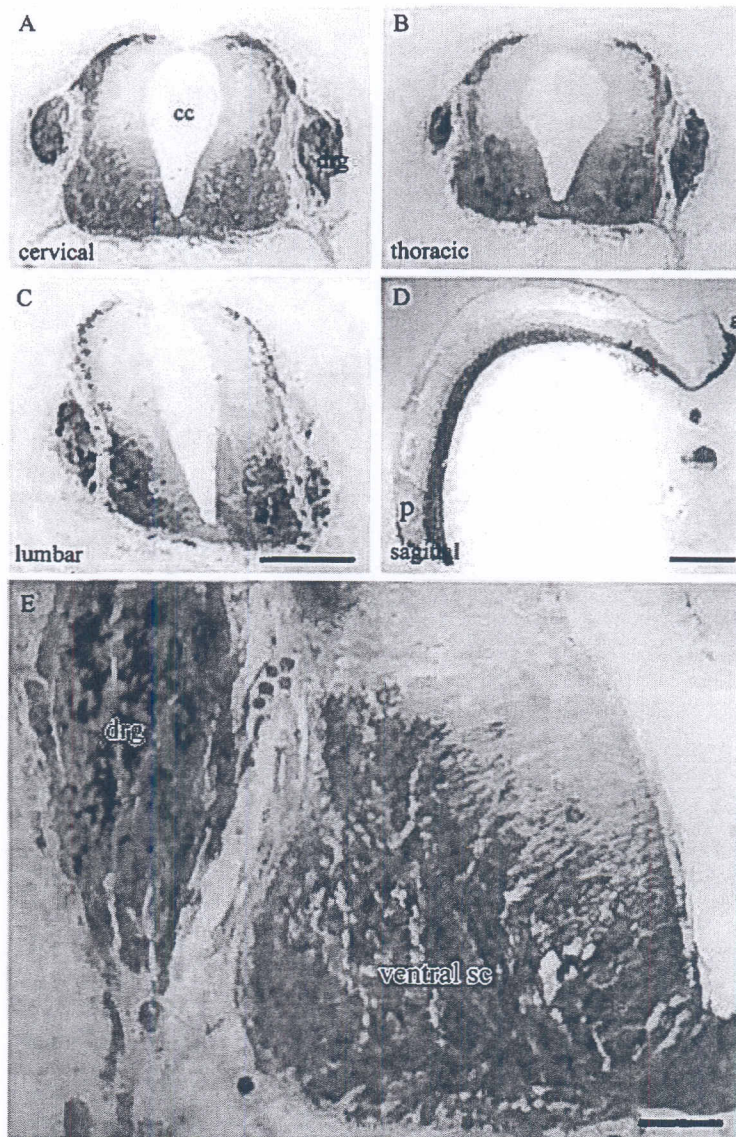
In our initial studies, we have focused on the potential roles of the EphA/ephrin-A subclass molecules in the development of the spinal cord neural circuits. We showed that ephrin-A5 and a member of the EphA-type receptors, EphA5, are expressed at high levels at embryonic day 14 in the developing spinal cord, and that neurons from different spinal cord regions respond differently to ephrin-A5<sup>81</sup>. To better understand the function of EphA5 and ephrin-A5 in spinal cord development, we extended our previous studies by examining the expression of EphA5 and ephrin-A5 at earlier and later stages of development. Commercially available antibodies did not work well on mouse spinal cord cryosections. We thus employed the affinity probe tech-

nique to define the developmental expression<sup>[12,13]</sup>.

### 3.1 Expression of EphA5 in embryonic mouse spinal cord

To observe the expression of EphA5 proteins, we utilized a lacZ knock-in mouse line in which the kinase domain of EphA5 was replaced with beta-galactosidase<sup>[10]</sup>. We performed beta-galactosidase histochemistry on spinal cord sections from E11, E13, E15, E17, P0, P7 and 1 month old mouse spinal cord sections to examine the expression of EphA5. EphA5, as revealed through LacZ reactivity, was

expressed throughout the ventral and dorsolateral portion of the E11 spinal cord (Fig. 1). This expression pattern was consistent throughout all levels of the embryonic spinal cord. At this stage, we found that the dorsal root ganglion, dorsal root entry zone, and ventral motor neuron rootlets also contain EphA5-LacZ reactivity (Fig. 1A, B, C; 2A). The highest levels of expression were found on the cell membrane (Fig. 1E). At E13, which coincides with the period when sensory afferents enter the spinal cord (Fig. 2B),



**Fig. 1** Expression of EphA5 in the embryonic day 11 (E11) mouse spinal cord. EphA5 expression was revealed using beta-galactosidase (beta-gal) staining of the EphA5-lacZ fusion protein. EphA5 labeling was widely distributed throughout the ventral horn and dorsal root ganglion in the cervical (A), thoracic (B) and lumbar (C) sections of the E11 mouse spinal cord. The image in D shows the presence of EphA5 in a sagittal section of the E11 mouse spinal cord. EphA5 protein is expressed at highest level on the cell membrane (E). cc, central canal; a, anterior of the embryo; p, posterior of the embryo; sc, spinal cord. Scale bar: 100  $\mu$ m in C (for A-C); 500  $\mu$ m in D; 20  $\mu$ m in E.

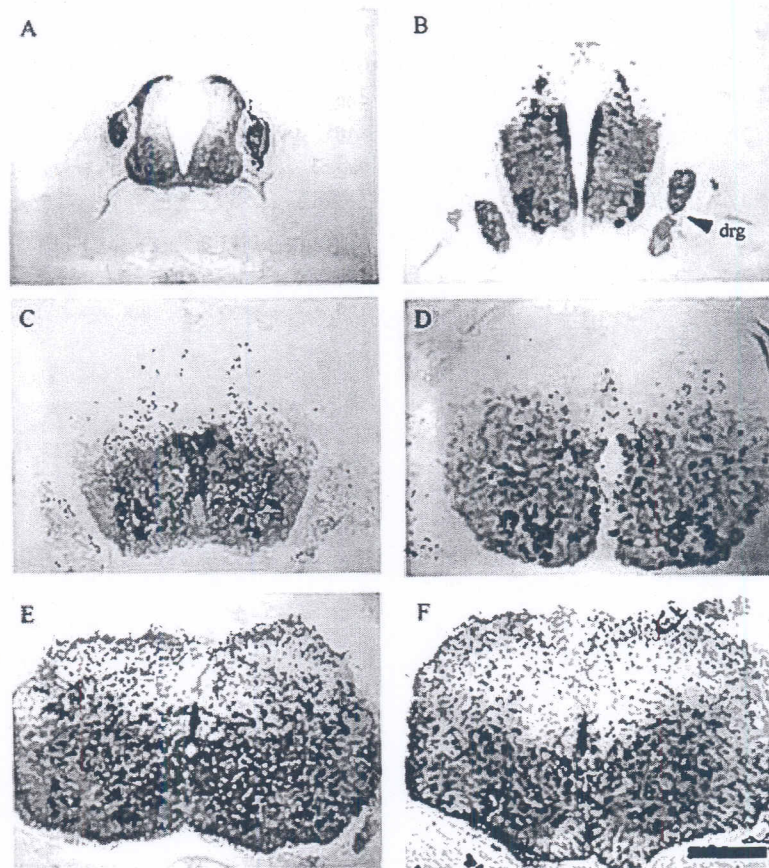


Fig. 2 Expression of EphA5 in the developing spinal cord. Spinal cord sections from E11 (A), E13 (B), E15 (C), E17 (D), postnatal day 0 (E), and P7(F). EphA5<sup>lacZ/+</sup> heterozygous mice were processed for lacZ histochemistry. Scale bar: 250  $\mu$ m.

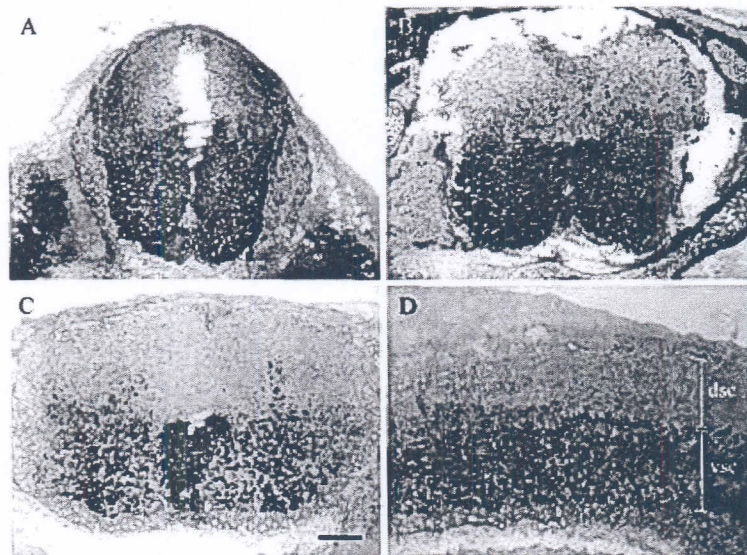
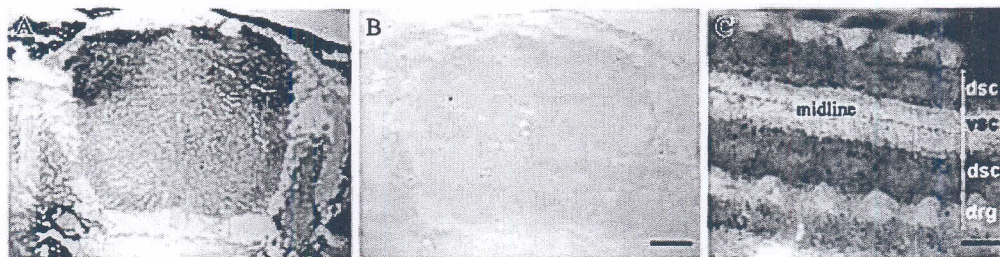
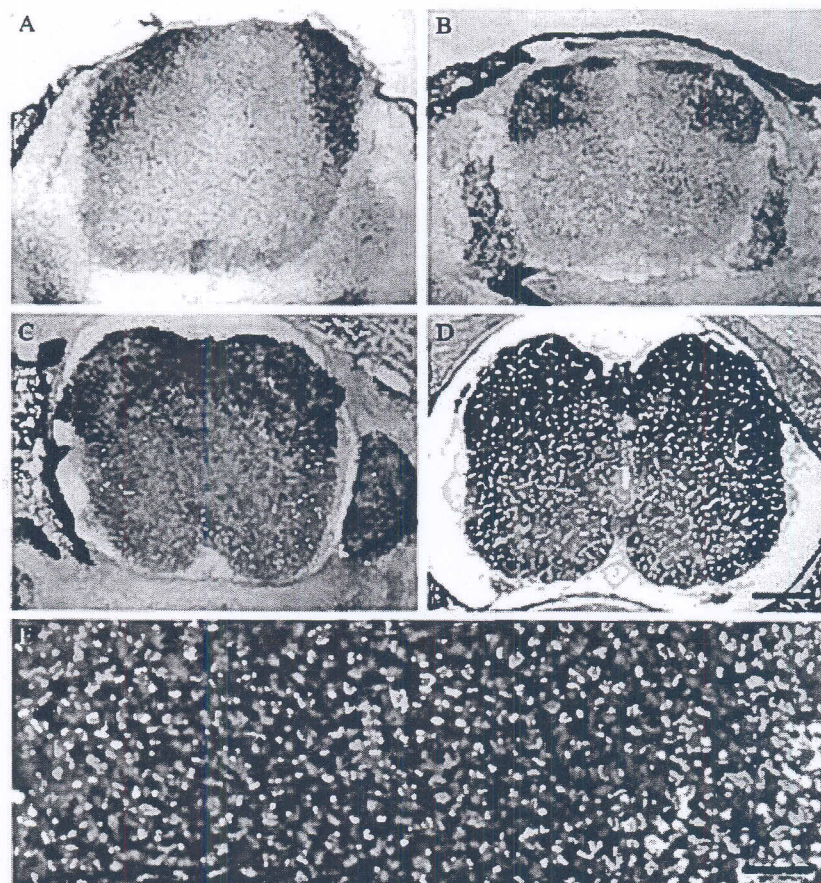


Fig. 3 Ephrin-A5-AP labeling reveals presence of EphA receptors in the developing spinal cord of the mouse. Ephrin-A5-AP labeling was present in the ventral regions of the E11 (A), E13 (B), E15 (C) cross sections of the spinal cord. A sagittal section from an E15 mouse spinal cord also shows the ventral expression of EphA receptors (D). dsc, dorsal spinal cord; vsc, ventral spinal cord. Scale bar: 100  $\mu$ m.



**Fig. 4** Ephrin-A5 expression in the E13 wild type mouse spinal cord. Expression of ephrin-A5 in E13 mouse spinal cord was detected using affinity probe EphA5-Fc. The bound probe was imaged with anti-human Fc secondary antibody, in conjunction with DAB-Ni method. Expression of the ligand was found in the wild type (A), but not in the ephrin-A5<sup>-/-</sup> (B) dorsolateral spinal cord. Staining of whole-mount E13 mouse spinal cord also shows ligand expression in the dorsal portion of the spinal cord (C). Scale bar: 100  $\mu$ m in B (for A and B), 200  $\mu$ m in C.



**Fig. 5** Developmental expression of ephrin-A5 throughout the embryonic mouse spinal cord as revealed by EphA5-AP binding. Spinal cord sections from E11, E13, E15 and E17 wild-type embryos were labeled with EphA5-AP. In E11 embryos (A), ephrin-A5 was expressed in the dorsolateral portion of the spinal cord. At E13 (B), ephrin-A5 labeling extends towards the dorsal roof plate. By E15 (C), ephrin-A5 expression is seen throughout the dorsal spinal cord, including the roof plate and continues to the ventrolateral portion of the spinal cord at E17 (D). Ephrin-A5 is also most expressed on the cell membrane (E, from E15 dorsal spinal cord). Scale bar: 100  $\mu$ m in D (for A-D); 20  $\mu$ m in E.

EphA5 signal was localized in the ventral ventricular zone [region where oligodendrocyte progenitors (oligo1/2) are expressed], motoneurons and the roof plate, but not the dorsolateral portion of spinal cord. The developmental expression of EphA5 in the ventral spinal cord was detected

in E11 (Fig. 2A) and maintained in E13 (Fig. 2B), E15 (Fig. 2C), E17 (Fig. 2D), P0 (Fig. 2E) and P7 (Fig. 2F), but is absent in the adult (data not shown).

To examine whether lacZ signals accurately reflect the wild type protein expression, endogenous EphA5 protein

was examined using a ligand affinity probe, ephrin-A2 tagged alkaline phosphatase (ephrin-A2-AP)<sup>[12]</sup>. Since ephrin-A2-AP also binds to other EphA receptors, the patterns observed should reflect expressions of multiple EphA receptors. However, ephrin-A2-AP binding patterns closely reflect the patterns of the lacZ reactivity, suggesting that the major Eph receptor expressed in the developing spinal cord is EphA5. At E11, the ventral ventricular zone, floor plate and dorsolateral portion of the spinal cord contained EphA5 (Fig. 3A). A few days later around E13 to E15, EphA5 labeling was prominently expressed in the ventral ventricular zone, motoneurons and the floor plate, but was absent in the dorsolateral regions of the mouse spinal cord (Fig. 3B, C). A sagittal section taken at E15 also showed expression of EphA5 in the ventral spinal cord (Fig. 3D).

**3.2 Expression of ephrin-A5 as revealed by EphA5-AP binding** To examine the presence of ephrin-A5 in the developing spinal cord, we used EphA5-Fc, which consists of the extracellular domain of EphA5 fused onto the Fc region of human IgG. This fusion protein binds well with the ephrin-A ligands since the ligand binding domain has been preserved. Cryosections taken from the wild type E13 mouse spinal cord were incubated with EphA5-Fc and the bound probe was revealed with the DAB-Nickel method (Vector Laboratories). Prominent binding was found only in the dorsal spinal cord regions (Fig. 4A, C). Since EphA5-Fc may bind to other A-type ephrins, we further determined whether the signal observed is due to expression of ephrin-A5 by performing EphA5-Fc binding of spinal cord sections from E13 ephrin-A5-null mice. Analysis of the mutant sections showed no significant staining, indicating that signals detected with EphA5-Fc affinity probe is largely due to ephrin-A5 expression.

We also performed similar experiments with a different affinity probe, EphA5-AP, the alkaline phosphatase-tagged EphA5 extracellular domain<sup>[11,13]</sup>, and examined ephrin-A5 expression throughout embryonic development (Fig. 5). At E11, ephrin-A5 was observed only in the dorsolateral region of the spinal cord-this area corresponds to the dorsal root entry zone (DREZ) for sensory afferents (Fig. 5A). Ephrin-A5 protein was found at the highest level on the cell membrane (Fig. 5E). By E13, the expression of ephrin-A5 extended towards, but not in, the roof plate and was more prominently present in the dorsal root ganglion (Fig. 5B). At E15, ephrin-A5 was strongly expressed in the roof plate and throughout the dorsal spinal cord (Fig. 5C). Two

days later (E17), ephrin-A5 was also observed in the ventrolateral portion (Fig. 5D). Staining of ephrin-A5-null spinal cord sections with EphA5-AP also yielded no specific signals (not shown).

#### 4 Discussion

The present study has examined the expression of EphA5 and ephrin-A5 during mouse spinal cord development. Our results showed that ephrin-A5 and EphA5 were expressed in mutually exclusive regions of the developing spinal cord, with the possible exception of embryonic day 11. Throughout embryonic development, ephrin-A5 was present in the dorsal spinal cord while its receptor, EphA5, was expressed in the entire ventral spinal cord. These expression patterns are also consistent with our previous results that showed complimentary expression of ephrin-A5 and EphA5 mRNAs in the E14 mouse spinal cord<sup>[8]</sup>.

The significance of EphA5 and ephrin-A5 expression pattern throughout spinal cord development is not known at present. Their complimentary expression pattern suggests that the interaction between ephrin-A5 and Eph receptors may help segregate dorsal and ventral spinal cord neurons during development. Our previous study showed that ephrin-A5 may regulate the formation of spinal cord neural circuitry by restricting the growth of the ventral spinal cord neurites<sup>[8]</sup>. Also, it has been shown that ephrins and their receptors are expressed alternatively in rhombomeres and regulate rhombomere boundaries<sup>[14,15]</sup>. Future studies using ephrin-A5 and EphA5 knock-out mice will provide a better understanding of their role during spinal cord development.

Another possible function of ephrin/Eph interaction may involve the regeneration of spinal cord axons following injury. Several Eph family receptors and ligands have been shown to be upregulated during spinal cord injury<sup>[16-19]</sup>. Ephrin-B2 and its receptor, EphB2, are upregulated in astrocytes and meningeal fibroblasts, respectively, following spinal cord lesions in the adult rat<sup>[20]</sup>. In addition, Ephrin-B3 is expressed in myelinating oligodendrocytes and inhibits neurite outgrowth<sup>[21]</sup>. Finally, adult mice lacking EphA4<sup>-/-</sup> display increased axonal regeneration and a reduction in astrocytic gliosis after spinal cord lesion<sup>[22]</sup>. These data suggest that the expression of Eph family members may result in an inhibitory environment for axon regeneration following spinal cord injury.

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## 酪氨酸激酶受体 EphA5 及其配体 ephrin-A5 在小鼠脊髓发育过程中的表达

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**摘要:** 目的 研究两个 Eph 家族分子, EphA5 受体及其配体 ephrin-A5, 在脊髓发育过程中的表达方式。方法  $\beta$ -半乳糖苷酶基因敲入小鼠和配体亲和探针分析受体的表达, 两种不同的亲和探针分析配体的表达, 基因敲除小鼠作为对照。结果 在发育过程中, EphA5 表达于脊髓腹侧, 而 ephrin-A5 表达于脊髓背侧。结论 EphA5 和 ephrin-A5 在多个脊髓发育阶段都有表达, 他们可能在脊髓背腹侧组织结构的建立过程中发挥重要作用。

**关键词:** 突触引导; 胚胎产生; 背根神经节; 组织化学; 碱性磷酸酶亲和探针;  $\beta$ -半乳糖苷酶