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ORIGINAL ARTICLE

Gene Expression of *Spag6* in Chick Central Nervous System

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Summary

Using a differential display method, we identified *sperm-associated antigen 6* (*Spag6*) as a gene with a dynamic expression profile within the chick embryonic spinal cord. The expression of *Spag6* gradually decreased along with spinal cord development. *Spag6* expression was detected adjacent to the ventricular zone of the spinal cord at embryonic day (E) 4. At E6, *Spag6* was apparent in the ventral ventricular zone adjacent to floor plate and the surrounding region close to the ventricular zone, with additional weak expression at the adjacent region to the ventral horn. At E10, the *Spag6* mRNA can be detected slightly in the ventral ventricular zone and surrounding region of dorsal ventricular zone. In the E6 hindbrain, *Spag6* was detected in the roof, the ventricular zone adjacent to floor plate and the surrounding regions of the ventricular zones. In the E6 caudal diencephalon, *Spag6* expression was detected adjacent to the ventricular zone. As *Spag6* was expressed in areas containing ependymal progenitor cells and in the borders of undifferentiated regions, *Spag6* may be involved in the development of ependymal cells and in the differentiation process of neuronal cells in chick neural organs.

Introduction

To understand the functions of the nervous system in maturity, it is important to determine the mechanisms by which it is formed. During neural development, cells differentiate to specific types of neurons and undergo migration, synapse formation and cell death precisely, with respect to time and location (Edelman, 1983; Reichardt, 1984; Goodman and Shatz, 1993; Tanabe and Jessell, 1996). Accumulating evidence indicates that morphological differentiation and cell motility are based on many kinds of molecules, which are located on cell surfaces, secreted from cells and within the cells, such as cytoskeletal molecules (Edelman, 1983; Riederer, 1990; Tanabe and Jessell, 1996). Therefore, one of the approaches to reveal the mechanisms underlying neural differentiation is to identify molecules, the expression of which changes during development.

The development of the spinal cord has been extensively studied morphologically using chick embryos (Hamburger, 1948). It is well known that developmentally important phenomena occur in the spinal cord from E4 to E10, examples of which include natural occurring cell death of motoneurons, and glial cell differentiation and migration (Hamburger, 1975; Miller et al., 1997).

The present study was conducted to isolate molecules related to differentiation of the spinal cord in chick embryos. To realize this aim, using the differential display procedure, we isolated several genes, expressions of which changed drastically from E4 to E10. Among them, in this

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article, we report the expression pattern of *sperm-associated antigen 6* (*Spag6*) (Neilson et al., 1999; Sapiro et al., 2000) in chick spinal cord. Our data indicated that the expression levels of *Spag6* decreased according to spinal cord development. Furthermore, *in situ* hybridization showed that *Spag6* was expressed in areas containing ependymal progenitor cells and in the borders of undifferentiated neural regions.

Methods

Animals

Fertilized white leghorn chicken eggs were purchased from a local poultry (Miyake Eggs, Shizuoka, Japan). The eggs were incubated at 37°C in a humidified egg incubator.

RNA isolation, differential display and cDNA cloning

Preparation of total RNA from chick organs, for differential display, molecular cloning and Northern blotting analysis, was performed by LiCl precipitation method. The differential display method was performed using the RNAimage kit (GenHunter, Nashville, TN, USA) with Ampli Taq Gold (Perkin-Elmer, Waltham, MA, USA) and [α-^{35}S]dATP (NEN). Several differentially expressed cDNA fragments were obtained and their full-length open reading frames were identified using 5′-rapid amplification of the cDNA ends using the 5′-RACE System, version 2.0 (Invitrogen, Carlsband, USA) and cDNA library screening (Hamada et al., 2000).

Reverse transcription (RT)-PCR

RT-PCR was performed according to the method described in Sambrook and Russell, 2001. The paired primers used (sense/antisense) were as follows: for *Spag6*, GAAGACATA CTCCTGAGCAT/ACACAGCTTGCTGGAAATAC [583-base pairs (bp) PCR product]; for Glyceraldehyde phosphate dehydrogenase (GAPDH), GGCTGCTAAGGCTGTGGGGA/ TATCAGCCTCTCCCACCTCC [546-bp PCR product].

Northern blotting analysis

Total RNAs (20 μg) were run on a denaturing formaldehyde-agarose gel and transferred to nylon membrane. RNAs blotted onto the membrane was hybridized with a [^{32}P]-labelled *Spag6* cDNA at 65°C in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA. The membrane was washed twice by immersing in a 0.2 × SSC and 0.1% SDS solution for 40 min at 65°C.

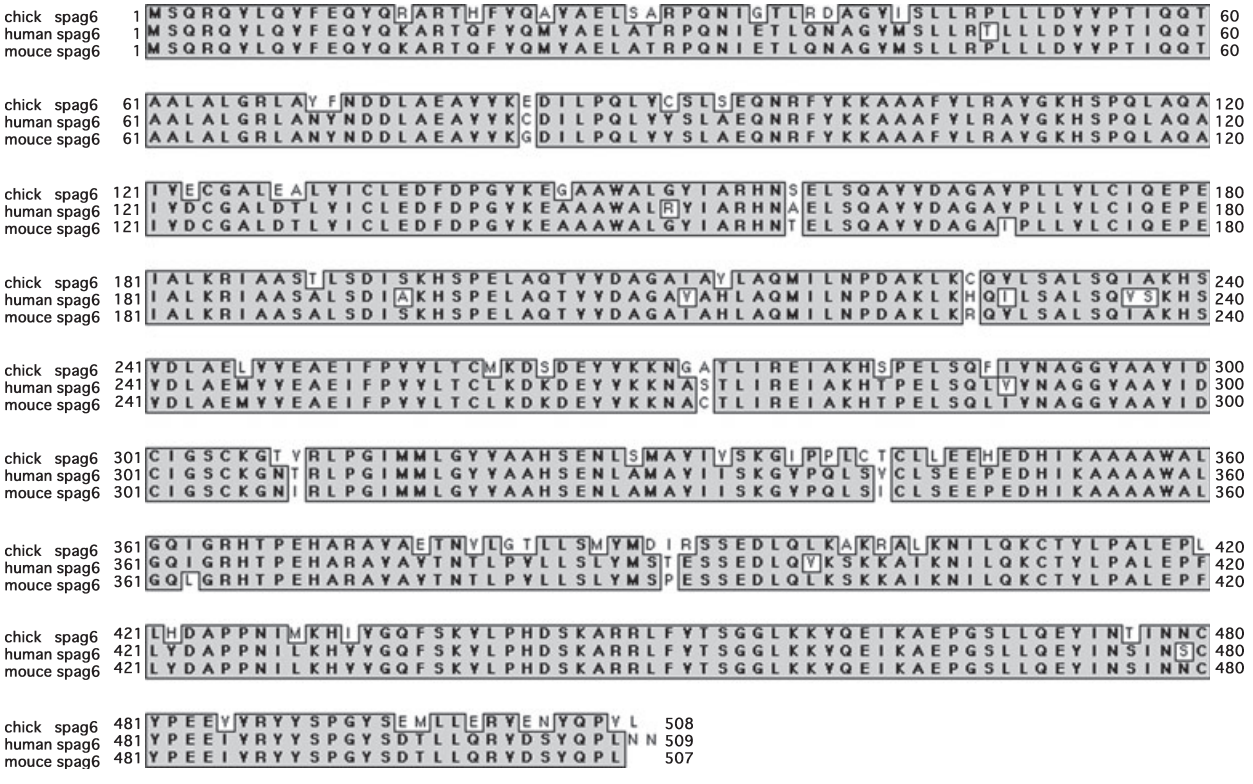

Fig. 1. Deduced amino acid alignments of chick, human and mouse Spag6 (Neilson et al., 1999; Sapiro et al., 2000). Identical amino acids are boxed. Numbers on the right indicate amino acid positions relative to the initiator methionine residue.

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The membrane was analysed using a BAS2000 system (Fuji, Tokyo, Japan) and exposed to an X-ray film.

In situ hybridization

Chick embryos were fixed in paraformaldehyde. Frozen specimens were sectioned with a cryostat at 20 μm thickness and mounted onto silanize-coated slides. *In situ* hybridization was performed with RI- single strand *Spag6* RNA according to a previous report (Yoshida et al., 1993). Briefly, sections were dried for 0.5–1 h and immersed in 1% Triton X-100 in 50 mM Tris/HCl buffer (pH 8.0) containing 25 mM EDTA for 30 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine. The sections were dehydrated in an ascending ethanol series and air-dried. The probe (1×10^6 cpm/ml) was dissolved in a buffer containing 50% formamide, 10% dextran, 1× Denhart's solution (0.02% each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin), 12 mM EDTA (pH 8.0), 10 mM Tris/HCl (pH 8.0), 30 mM NaCl, 0.5 mg/ml yeast tRNA, and 10 mM dithiothreitol, and 100 μl of probe solution was applied to each slide. The slides were coverslipped and incubated at 65°C overnight. The coverslips were then removed and the slides were rinsed in 4× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na-citrate, pH7.5), digested with RNAse A (20 mg/ml) for 30 min at 37°C, and rinsed sequentially in 2× SSC, 1× SSC, 0.5× SSC, then in 0.1× SSC for 30 min at 65°C. The sections were exposed to X-ray films (Kodak BioMax, Rochester, NY, USA) for 3–4 days, then dipped in NTB2 nuclear emulsion (1:1 with water; Kodak), and then exposed for 1–3 weeks. Counter staining was performed with thionin.

Results

One approach to understand the mechanisms underlying neural development is to search for a molecule(s), the expression of which dramatically changes over certain developmental windows. To identify such molecules, we employed a differential display method using the chick spinal cord from embryonic day (E) 4 to E10. One of the genes the expression of which gradually decreased from E4 to E10 was chick sperm-associated antigen 6 (Spag6) (GenBank accession no. XM418607). The chick Spag6 has an open reading frame of 1527 nucleotides encoding a putative protein composed of 508 amino acids (Fig. 1). The amino acid sequence of chick Spag6 showed 87.4 and 85.8% homology to mouse and human Spag6 (Neilson et al., 1999; Sapiro et al., 2000) respectively (Fig. 1).

We analysed the developmental change of *Spag6* expression in chick spinal cord by RT-PCR. *Spag6* expression was apparent until E6 and decreased gradually subsequently

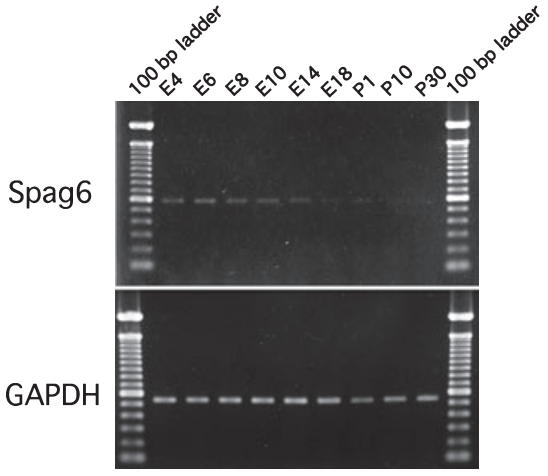

Fig. 2. Reverse transcription-PCR analysis of the change in chick *Spag6* expression according to spinal cord development. Developmental stages are indicated in each lane. GAPDH was used as control.

(Fig. 2). Weak *Spag6* expression could be detected until postnatal day (P) 1 and was not apparent thereafter.

Next, we examined the expression of *Spag6* mRNA at E10 in neural organs, i.e. the spinal cord and brain, and in several other organs by Northern blotting analysis using chick *Spag6* cDNA as a probe. Northern blot analysis against total RNAs from spinal cord and brain of E10

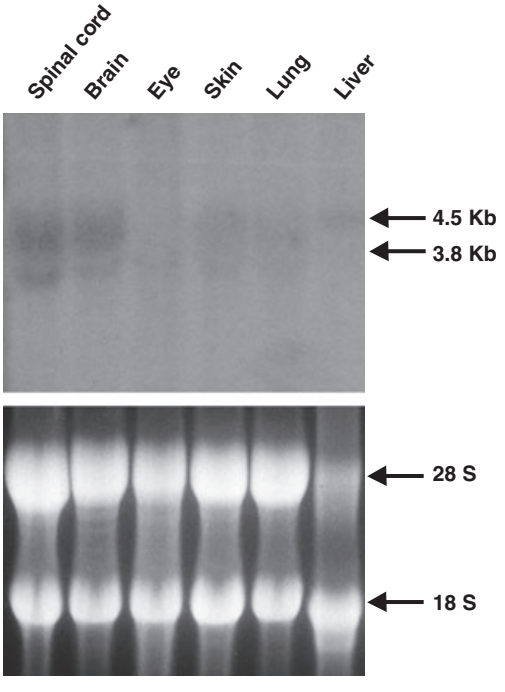

Fig. 3. Northern blot analysis of *Spag6* mRNA expression in various chick tissues at E10. *Spag6* bands are indicated by arrows. The lower panel shows ethidium bromide-stained 18S and 28S rRNAs as control.

chick detected two bands (4.5 and 3.8 kb) of *Spag6* mRNAs, indicating that *Spag6* may have alternative splicing variants, transcripts with different poly (A) lengths or two poly (A) additional sites. At E10, *Spag6* expression was also detected in the kidney, gut and heart (data not shown) (Fig. 3).

We next examined the detailed expression pattern of *Spag6* in the chick embryonic central nervous system by *in situ* hybridization. In the E4 spinal cord, *Spag6* mRNA was detected in the region adjacent to the ventricular zone (Fig. 4a,b). At E6, *Spag6* was expressed in the ventral ventricular zone adjacent to the floor plate and in the area surrounding the dorsal ventricular zone. Weak expression in the region adjacent to the ventral horn was observed (Fig. 4c,d). At E10, *Spag6* mRNA can be detected in the ventral ventricular zone and surrounding region of the dorsal ventricular zone (Fig. 4e,f). In the E6 hindbrain, *Spag6* signals were also detected in the ventricular zone adjacent to the floor plate and in the surrounding regions of the ventricular zone and roof (Fig. 5a,b). In the fore- and midbrain, expression of *Spag6* was detected clearly in a region adjacent to the ventricular zone of the caudal diencephalon (Fig. 5c,d). The retina appeared whitish because of the reflection of pigmentation under dark field optics.

Discussion

This is the first report documenting *Spag6* expression in embryonic neural organs. *Spag6* is found in the testis, specifically in the sperm flagella and cells in spermatogenesis process and has an important role in sperm motility (Neilson et al., 1999; Sapiro et al., 2000). Recently, *Spag6* expression was reported in tumour and undifferentiated proliferating cells *in vitro* (Shen et al., 2006; Steinbach et al., 2006). However, there are no reports documenting the expression of *Spag6* in embryonic tissues in detail.

In postnatal mouse, Spag6 localizes to the surface of the brain ventricles, and *Spag6* knockout mice phenotypes have hydrocephalus, arising secondary to defects in motility of the ependymal cell cilia, which line the lumen of the neural tube and form from the ventricular cells (Sapiro

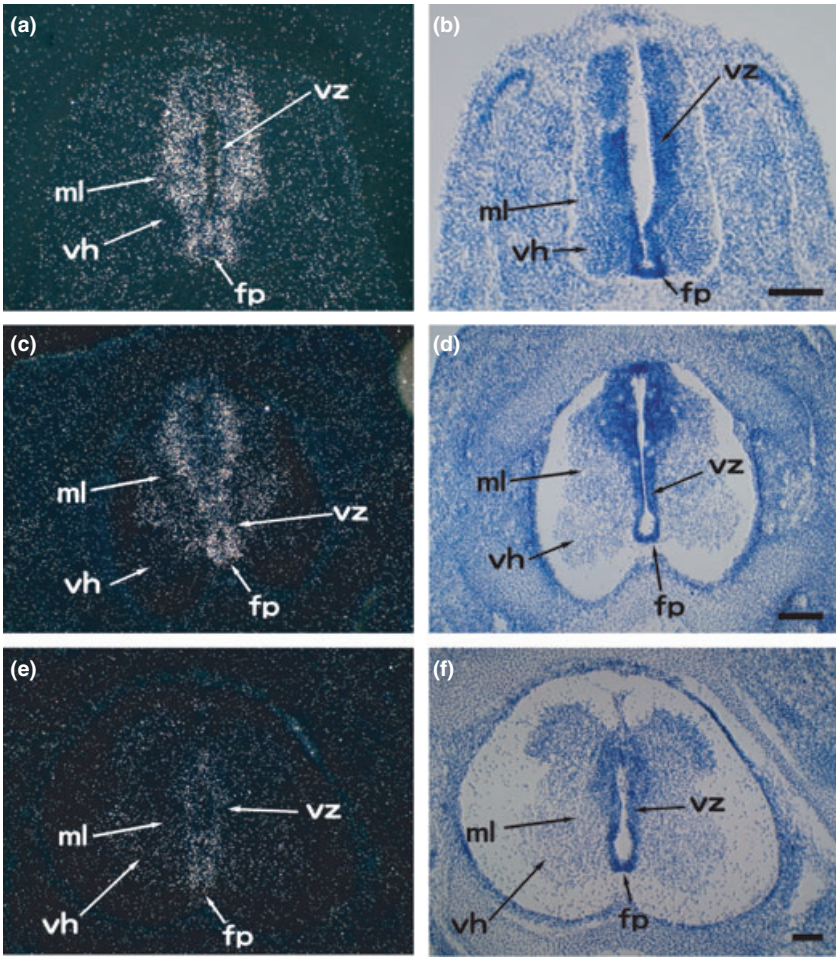

Fig. 4. *In situ* hybridization analysis of chick *Spag6* mRNA in the embryonic spinal cord at E4 (a and b), E6 (c and d) and E10 (e and f). (a, c and e) are dark field and (b, d and f) are bright field photomicrographs. (a) and (b, c and d), and (e and f) were taken from the same slice. fp, floor plate; ml, mantle layer; vh, ventral horn; vz, ventricular zone. Scale bar = 100 μm.

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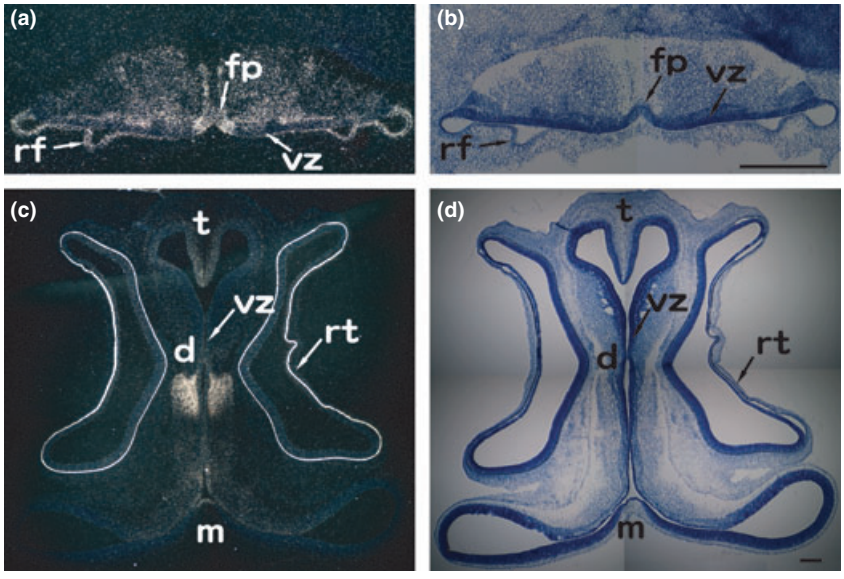

Fig. 5. *In situ* hybridization analysis of chick *Spag6* mRNA in the embryonic brain at E6. (a) and (c) are dark field and (b) and (d) are bright field photomicrographs. (a) and (b) (hindbrain), (c) and (d) (fore- and midbrain) were taken from the same slice. fp, floor plate; vz, ventricular zone; rf, roof; rt, retina; t, telencephalon; d, diencephalon; m, mesencephalon. Scale bar = 250 μm.

et al., 2002; Zhang et al., 2007). In addition, our results showed that *Spag6* was expressed in the roof of the hindbrain and in the spinal ventral ventricular zone adjacent to the floor plate at E6 (Figs 4 and 5), which contains ependymal progenitor cells (Fu et al., 2003). Our data suggest that *Spag6* is expressed from the early stages of ependymal cell development and may play a role in axoneme formation of ependymal cell cilia, such as in sperm flagella formation during spermatogenesis. However, we observed that *Spag6* was not only expressed in ependymal progenitor cells but also in areas other than the ventricular cells of embryonic spinal cord and brain. Furthermore, it was reported that *Spag6* is expressed in an artificial *in vitro* culture of undifferentiated human neural stem cells, the expression of which decreases according to the number of cell passages (Shen et al., 2006). This cited report corroborates our result. Moreover, at E4, *Spag6* was expressed in the surrounding region of the ventricular zone, but not in the ventricular zone itself or in the lateral mantle layer (Fig. 4). In the embryonic spinal cord, the ventricular zone contains undifferentiated neural cells, whereas the lateral mantle layer has differentiated cells. Our data suggest that *Spag6* might act in the differentiation process of neural cells in chick embryonic spinal cord as *Spag6* is mainly expressed in the intermediate region of the abovementioned areas, and expression decreased according to progression of spinal cord development. Furthermore, as the role of microtubules in neural development and differentiation is well known (Riederer, 1990) and the interaction between Spag6 and microtubules has been previously documented (Sapiro et al., 2000), Spag6 might play roles associated with microtubules for neural differentiation.

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