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ORIGINAL ARTICLE

# Sonic hedgehog overexpression regulates the neuroepithelial cells proliferation in the spinal cord of dorsal regions during chicken embryo development

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**Abstract**

**OBJECTIVE:** Sonic hedgehog(SHH) is early expressed in the floor plate and notochord during the development of chicken embryo, which is required for the establishment of dorsal axis and the formation of spinal cord, but the mechanism of SHH affecting the patterns of spinal development is still unclear.
**METHODS:** In this study using *in vivo* electroporation ectopic expression of SHH in spinal cord of dorsal regions during chicken embryo development. Besides, the expression of NF, TAG, Pax-7 and N-Cadherin was examined by the fluorescent immunohistochemistry.
**RESULTS:** The result showed that the pattern of spinal cord development changed such as the distortion was observed during the chicken embryo development, and the location of dorsal root transforms from the original site to the roof plate, and neuroepithelial cell layer at the roof plate creased. Furthermore, the expression of nuclear protein Pax-7 was inhibited at the site of SHH ectopic and the expression site of neurofilament(NF) and TAG-1 changed, while the expression of SC-1 was down-regulated.
**CONCLUSIONS:** This study demonstrated that SHH may be directly required for the formation of spinal patterns or affect the formation of spinal cord through regulating the associated proteins and more important is SHH promote the neuroepithelial cells proliferation and then lead to neural plate to form the neural tube. This study could provide reliable references for the research on SHH determining the formation of spinal cord during the development of chicken embryo.

## INTRODUCTION

The hedgehog (HH) family encodes a secreted glycoprotein which is responsible for developmental patterning in a variety of systems, including the neural tube, limbs and somites. As one of HH family, Sonic hedgehog (SHH), which is essential for maintenance of notochord and prechordal mesoderm (Chiang *et al.* 1996) as well as the induction of floorplate and ventral neuronal populations that form at different positions along the anterior-posterior (AP) axis of the neural

tube (Echelard *et al.* 1993; Marti *et al.* 1995a; Marti *et al.* 1995b). Within the neural tube SHH are proposed to function as a ventral patterning influence, with the capability of inducing floor plate and motor neurons (Ekker *et al.* 1995). Shh null mutant animals do not develop many ventral structures of the nervous system (Chiang *et al.* 1996). In contrast to its roles in neural patterning and differentiation, recent studies have implicated the Hedgehog signaling pathway in proliferation and tumorigenesis (Rubin *et al.* 2006; Xie *et al.* 2013; Wan *et al.* 2014). Accumulating evidence has shown that the Shh signaling pathway is activated in several cancer types, including neuroblastoma, hepatoma cancer, and small cell lung cancer (Jeng *et al.* 2012; Xu *et al.* 2012; Watkins *et al.* 2003; Zheng *et al.* 2013). SHH has been shown to be associated with the proliferative capacity of endogenous neural precursor cells during embryonic development. It has also been shown to regulate the proliferative capacity of neural stem cells in the adult subventricular zone (Bambakidis *et al.* 2012).

In the present work, we asked whether SHH expression in the spinal cord of dorsal region can lead to neuroepithelial cells proliferation change during the chicken embryo development. Moreover, the dorsal region of the SHH expression whether lead to change of spinal cord developmental patterning. We also asked whether SHH expression can lead to change of associated proteins expression in the spinal cord.

## MATERIALS AND METHODS

*Embryos and tissue preparation*

Fertilized eggs of Sea blue brown were obtained from a local farm and incubated in a constant temperature and humidity incubator (HWS-150, Jing Hong, China) at 37.8 °C with 65% humidity. Chicken embryos were staged according to Hamburger and Hamilton (Hamburger & Hamilton 1992). Embryos were studied at stage18 (E3)-stage29 (E6) (at least 3 embryos at each stage). For immunohistochemistry, spinal cords were fixed in 4% formaldehyde solution on ice for 6 to 24 h, depending on the size of the spinal cords. After fixation, the tissues were immersed in 18% sucrose solution. Then, specimen were embedded in Tissue-Tec O.C.T. compound (Sakura Finetek USA), frozen in liquid nitrogen and stored at –80 °C. The samples were adjacently sectioned with 20μm thickness on the Poly-L-lysine-coated slides with frozen sections (Leica, Germany).

*In ovo electroporation*

The protocol of *in ovo* electroporation was performed as followings. All steps are performed with the assistance of a stereomicroscope. Fertilized eggs were incubated at stage18 (E3). About 3–4 ml of albumin was removed from the egg without disrupting the yolk, then, the shell is cut carefully with a pair of curved scissors to get one 1–2 cm diameter window without touching the embryo. Plasmid (containing 2 μg/μl pCAGGS-SHH and 0.25 μg/μl pCAGGS-GFP plasmid, 0.25 μg/μl pCAGGS-GFP plasmid as control)/Fast Green dye (0.1%) was injected and loaded into the neural tube lumen(cavity) with a (mouth) pipette until the dye fills the entire space. The electrodes are immediately placed and paralleled on two sides of the neural tube in the embryo. The parameter of electroporation is at 18 volts lasting for 60 ms, pausing for 100 ms of each pulse, totally 6 pulses. Bubbles will be seen near the electrodes if it works properly. After electroporation (CUY-21; Nepa Gene, Japan), the electrodes are carefully removed and the egg are well sealed with tape. Then, window side up, the operated eggs are placed back into the incubator, and incubated until the desired stage for collecting samples, fixation and analysis.

*Antibodies and immunohistochemistry*

The primary rabbit monoclonal antibodies of mouse anti SHH, mouse anti neurofilament (Redies laboratory gift), mouse anti Tag-1(DSHB, American), mouse anti Pax-7 (DSHB, American), rabbit anti chicken N-Cadherin (Redies laboratory gift) were used for immunostaining of sections in combination with appropriate Cy3-labeled secondary antibodies. Goat anti rabbit Cy3 labeled (Molecular Probes); Goat anti mouse Cy3 labeled (Jackson Immuno Research); mounting medium with DAPI (4',6-diamidino-2-phenylindole, DAPI, Roche, Germany).

For immunofluorescent staining, the sections were first blocked with primary antibody solution (2% sheep serum, 4% bovine serum albumin, 0.3% Triton X-100, and 0.1% sodium azide in Tris-buffered saline, TBS) at room temperature for 60 min and then incubated with the primary antibody at 4 °C overnight. In turn, an appropriate Cy3-labeled secondary antibody against species IgG was added at room temperature for 2h. Finally, the dye DAPI was used for staining cell nuclei. The sections were visualized and photographed under a fluorescence microscope (Nikon ECLIPSE 80i, Japan) equipped with a digital camera (LEICA DFC300FX, Germany).

## RESULTS

*The influence of SHH ectopic expression to the embryonic development during the early development of spinal cord*

The embryo was co-transfected with pCAGGS-SHH and pCAGGS-GFP at E3 by *in vivo* electroporation. Then we observed the embryo at stage24–stage29(E4–E6) while regarding the one transfected with pCAGGS-GFP alone as the control group. The Figure 1 shows that the morphology of developmental embryo in the control (Figure 1 A–B) be compared to the group of SHH ectopic expression (Figure 1 C–D) changed at stage 24(E4) and the distortion also occurred at the site transfected with SHH in the spinal cord. Besides, there was an apparently difference between experiment group

and control group (Figure 1 E–F) at stage27(E5), with the physical distortion (Figure1 G–H). At stage 29(E6), there also existed a transformation of physical structure compared with the control. Next, for further analyzing the mechanism of transforming the morphology of embryonic development, the embryo was cut into slices to examine the expression of associated proteins.

*The ectopic expression of SHH at the early spinal development*

The Figure 2 shows that there was SHH ectopic expression at stage24–stage27(E4–E5) (Figure 2 A–L) and the structure of spinal cord obviously changed after ectopic expression at the dorsal horn while the spinal cord bent (Figure 2 A–D) and formed crease (Figure 2 E–H, I–L) at the SHH ectopic expression site, whereas in the tissue of the control group transfected with pCAGGS-GFP alone the spinal growth was symmetric. This study therefore indicated that the ectopic expression of SHH has a significant effect on the formation of spinal patterns during the embryonic development and it may promote the neuroepithelial cells proliferation lead to formed crease. Furthermore, we examined the expression of specific proteins in spinal cord.

*The effect of SHH ectopic expression on specific proteins expression of spinal cord during the early development of spinal cord*

We observed from the tissue sections that the SHH ectopic expression led to the developmental difference at two side of spinal cord. At one side of spinal cord expressing SHH, physical change occurred. For further exploring its reason and result, we examine the spinal cord-related proteins in the embryonic sections transfected at stage29(E6). At stage29(E6) of embryonic development, there was a significant difference of NF expressed at one side of SHH expression position with that expressed at the other side and the control group. For example, the dorsal root and dorsal root ganglia marked with NF were transformed toward dorsal horn, especially dorsal root to the roof plate (Figure 3 C), and the transformation of two side structure of spinal cord was apparently observed from the result marked with TAG-1 (Figure 3 G). The part using "▲" labled is the abnormal area, and the one using "→" labled is the ectopic expression area (Figure 3 G), which suggest the formation of dorsal root and dorsal root nerves after SHH ectopic expression changed. The result obtained from the examination to the expression of nuclear proteins Pax7 showed that the Pax-7 expression was inhibited at one side of SHH ectopic expression position (Figure 3 K), which suggests that the early expressed SHH inhibited Pax7 expression, and Pax7 is one of important transcription factor, would affect a series of transcription process. After staining the SC-1, the result showed its expression at the dorsal root reduced and was deviated to the roof plate (Figure 3 O); and the result after staining N-Cadherin showed there was no significant variance (Figure 3 S).

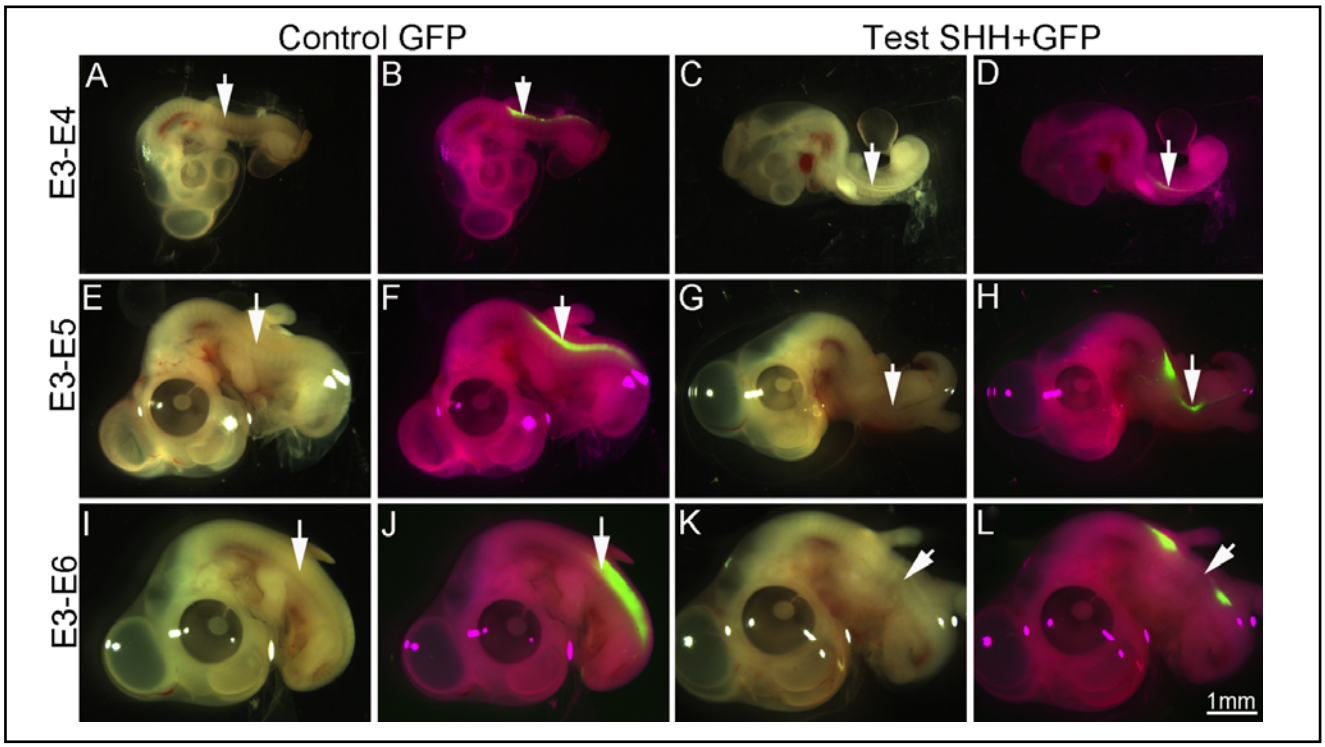


**Fig. 1.** Whole embryo compare of ectopic expression of SHH and control. A,B,E,F,I,J:control group only electroporation pCAGGS-GFP plasmid in spinal cord and observed at different stage. C,D,G,H,K,L: test group of co-electroporation pCAGGS-GFP and pCAGGS-SHH plasmid and observed at different stage.

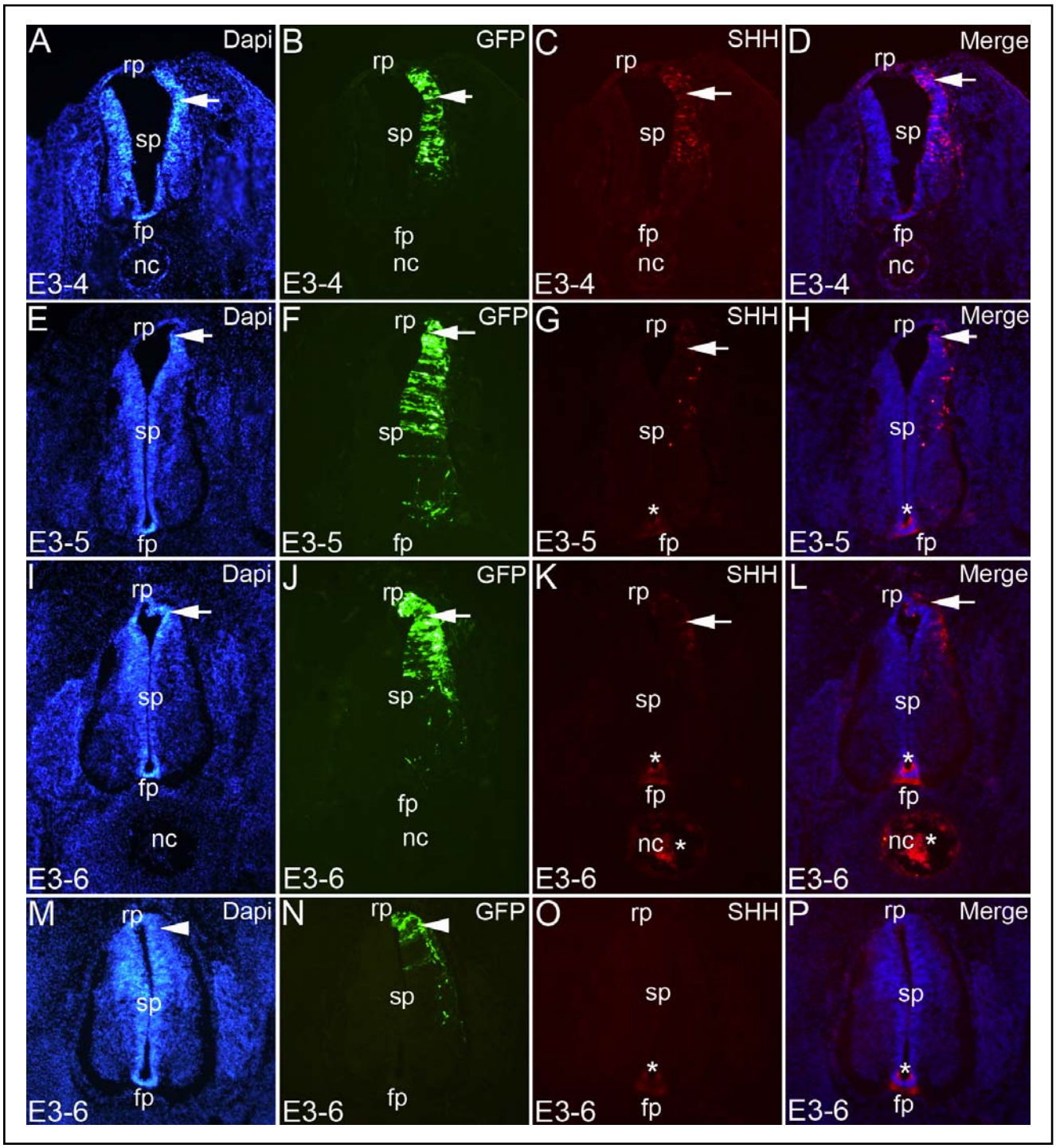


**Fig. 2.** The result of SHH ectopic expression on slice by immunohistochemistry. A,E,I,M: DAPI staining at different stage; B,F,J,N: GFP expression result at different stage; C,G,K,O: SHH expression by immunohistochemistry(red); D,H,L,P: merged of DAPI(blue) and SHH(red).

## DISCUSSION

SHH plays a critical role at the formation of central nervous system during the embryonic development. The majority of SHH was expressed at the floor plate of spinal cord during the early embryonic development to induce the formation of floor plate and motor neurons. Additionally, SHH also cooperates with Bmp and Fgf molecules in the control of diverse neuronal cell fates in the brain (Litingtun *et al.* 2000). As for the mechanism of SHH affecting the formation of floor plate, it is still unclear. By the technique of chicken embryonic *in vivo* electroporation we obtained the ectopic expression of SHH which was over-expressed at one side of spinal cord during chicken embryonic development for further analyzing the affect of

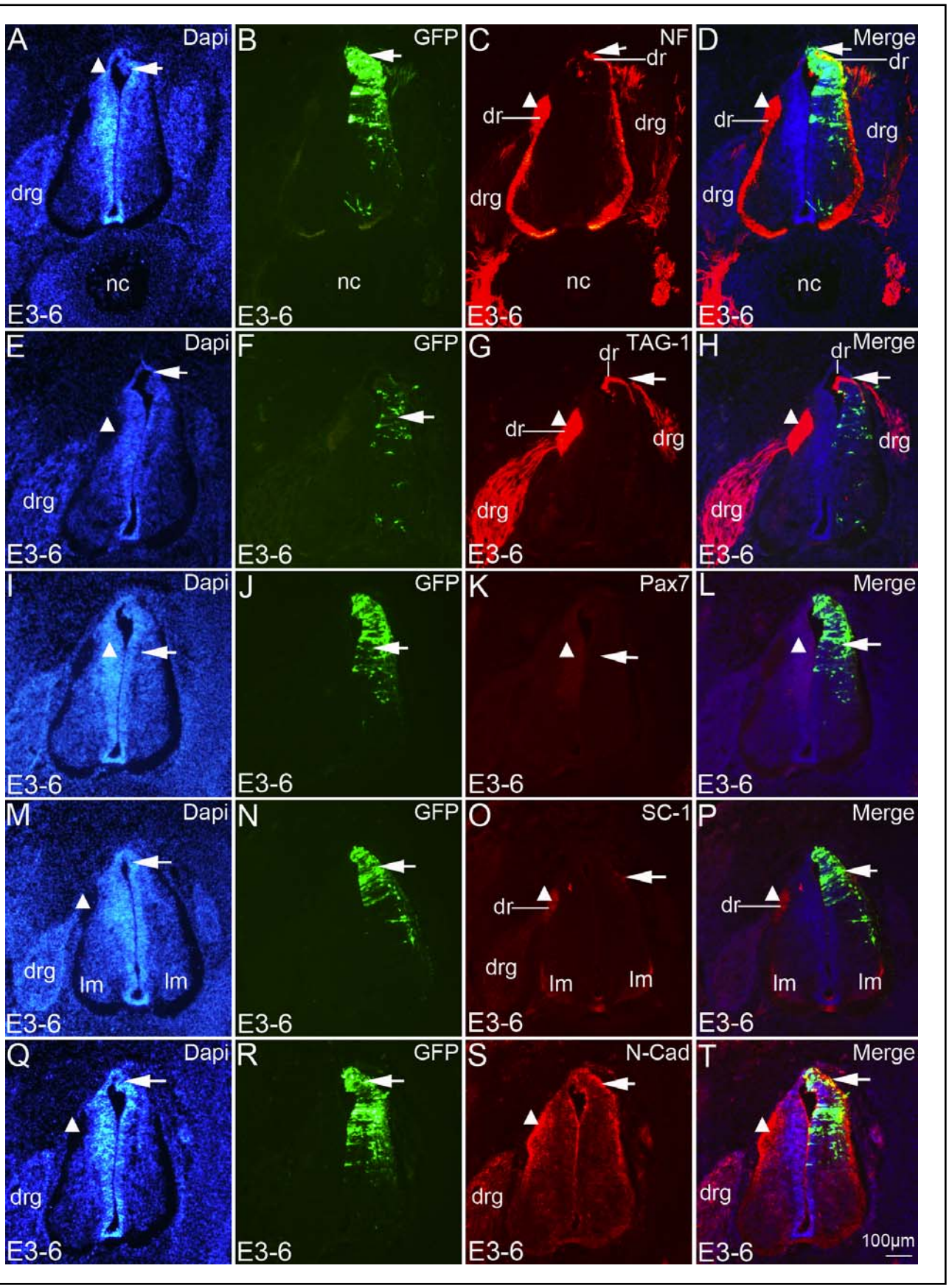


**Fig. 3.** Effect of SHH ectopic expression on association protein in the spinal cord. A,E,I,M,Q: DAPI staining result at stage29(E6); B,F,J,N,R: GFP expression result at stage29(E6) represent SHH ectopic expression area; C: NF expression result at stage29; D: Merge of B and C; G: TAG-1 expression result at stage29; H: Merge of F and G; K: Pax-7 expression result at stage29; L: Merge of J and K; O: SC-1 expression result at stage29; P: Merge of N and O; S: N-Cadherin expression result at stage29; T: Merge of R and S.

SHH abnormal expression to the formation of spinal cord and the neuroepithelial cells proliferation. The result shows that the morphology of chicken embryo changed and the physical distortion of spinal cord occurred. It was reported that the abnormal expression of SHH at the central nervous system not only led to abnormalities of many organelles (Ekker *et al.* 1995; Rowitch *et al.* 1999), but also caused the variation of protein translation during the development of spinal cord (Pituello *et al.* 1999), thus it indicates that the mechanism of SHH affecting the formation of spinal cord probably is by the direct action or achieved by affecting the expression of associate-proteins. In our experiment the developmental pattern of spinal cord after the SHH ectopic expression changed and a crease was formed at one side of SHH expression position after 3 day, which indicates that SHH could directly promote the proliferation of epithelial cells and then result that epithelial cells bent, besides, during the formation of nervous tube the expression of associate-proteins in notochord influenced the floor plate and epithelial cells formed a hollow for proliferation and then into a nervous tube. It was reported that Sonic hedgehog is a pleiotropic factor in the developing central nervous system, driving proliferation, specification, and axonal targeting in multiple sites within the forebrain, hindbrain, and spinal cord (Álvarez-Buylla *et al.* 2014) as well as our result in spinal cord. Through the examination of associate-proteins in spinal cord after SHH ectopic expression, the result shows that SHH expression could inhibit some associate-proteins expression, such as Pax, which was inhibited by SHH expression at the dorsal part of spinal cord, while the expression of N-Cadherin hardly got influenced by the examination to N-Cadherin, that is, SHH may affect the developmental pattern of spinal cord through promote the neuroepithelial cells proliferation. Numerous surveys indicate that no matter ectopic expression or deletion of SHH both could influence the embryonic development (Bennett *et al.* 1998; Ekker *et al.* 1995).

We observed that the dorsal root and dorsal root nerve emigrated toward the roof plate. There are two possibilities causing that result. One is that the over-proliferation of epithelial cells led to excursion. By the examination of NF and TAG-1, the result shows that both proteins emigrated, which could be presumed that cell proliferation causes excursion, while the result obtained from SC-1 examination shows that its expression obviously reduced compared with the offside and the structure of dorsal root wasn't observed, which supports that the dorsal root can't be generated at the original position. The other one is SHH may change the expression of association protein and then lead to parterns of spinal cord changed. As for the study on SHH during the embryonic development, there are tons of work to take on but it is for sure that SHH can induce the formation of nervous tube at floor plate and also determine the formation of dorsal axis.

## CONCLUSION

This study demonstrates that the SHH ectopic expression at the dorsal region of spinal cord could lead to the physical change of spinal cord and the excursion of dorsal root during chicken embryonic development while the SHH expression inhibits the expression of Pax-7 and change the expression site of NF and TAG-1 and reduce the SC-1 expression but shows no effect on the expression of N-Cadherin.

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