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Wnt1 and Wnt2b Immunodetection of the Regenerating Tail and Comparative Ultrastructure of Tail Spinal Cord in the *Scincella tsinlingensis*

Inmunodetección Wnt1 y Wnt2b de la Cola en Regeneración y Ultraestructura Comparativa de la Médula Espinal de la Cola en *Scincella tsinlingensis*

Chun Yang[1]; Jinyu Sun[1]; Xin Wang[1]; Yongqin Yang[1]; Rui Chen[1] & Bo Liu[2]

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SUMMARY: The Wnt pathway is essential for the initiation of lizard tail regeneration. The regenerated lizard tails exhibit obvious morphological differences compared to the original ones. The expression of Wnt1 and Wnt2b proteins in the regenerating tail of *Scincella tsinlingensis* was detected by immunohistochemistry and then comparatively analyzed for ultrastructural changes in the original and regenerated spinal cord. The ependymal layer of the original spinal cord was pseudostratified with multiciliated cells and primary monociliated cells, while the cells of the ependymal layer of the regenerated spinal cord were organized in a monolayer with a few biciliated cells. Immunolocalization indicated that Wnt1 and Wnt2b were mainly distributed in the dermis near the original tail stump, spinal cord, and clot-positive migratory cells during Stage I, 0-1 days post-amputation (dpa). Wnt1 and Wnt2b were predominantly detected in the epaxial and hypaxial musculature near the original tail stump, wound epithelium, and spinal cord in the original tail during Stage II, 1-7 dpa. Mesenchymal cells and wound epithelium showed immunostaining during Stage III and IV, 7-15 dpa. The ependymal tubes contained these signaling proteins during Stage V and VI, 20-30 dpa. Labeling was mainly observed in nearby regenerative blood vessels, ependymal cells, epaxial and hypaxial musculature in the apical epithelial layer (AEC) after 45-160 dpa. These findings indicated that Wnt1 and Wnt2b proteins presented primarily in regenerating epidermis and nerve tissues were a critical signal for tail regeneration in *S. tsinlingensis*.

KEY WORDS: *Scincella tsinlingensis*; Regenerating tail; Ultrastructure; Immunohistochemistry.

INTRODUCTION

Different animal species have the ability to regenerate tissues and organs at different stages of growth and development, but this ability is gradually lost in vertebrate evolution. Understanding the similarities and the differences of appendage regeneration in zebrafish, salamander and lizard can provide important clues about effective regenerative strategies in mammals (Daponte *et al*., 2021). Tail regeneration and limb scarring in lizards provide a unique model to analysis of the cellular mechanisms of the regeneration barrier and the maintenance of regenerative repair ability of different organs in the same species. Wnt signaling is involved in almost every aspect of embryonic development, and controls self-renewal and homeostasis of many adult tissues (Clevers, 2006). Activating Wnt pathway to promote regeneration is essential in different animals. In other vertebrates, attempts to stimulate organ regeneration require inducing a highly hydrated, immune-depressed, hyaluronic acid-rich environment similar to the extracellular environment that occurs during development (Alibardi, 2017a).

The involvement of the Wnt signal pathways are identified in mouse fingertip and salamander limb (Knapp *et al*., 2013; Wu *et al*., 2013). Wnt positive and negative regulators of cell proliferation and noncoding RNAs dominate embryonic growth programs that are activated during tail regeneration (Alibardi, 2017b). In the tail blastema of *Anolis carolinensis* and *Podarcis muralis* (Hutchins *et al*., 2014; Vitulo *et al*., 2017), the exclusive expression of Wnt genes is the basis for tail regeneration, instead, scar formation precludes blastema formation in the limb (Alibardi,

[1] School of Life Sciences, Shanxi Normal University, No. 339, Taiyu Road, Xiaodian District, Taiyuan, 030031, Shanxi Province, P. R. China.
[2] Department of Intensive Care Medicine, Hanzhong Central Hospital, Hanzhong, 723000 Shaanxi Province, P.R. China.
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2020). The proliferation and distal growth of the blastema are regulated by Wnt1 in the regenerating tail of *P. muralis* (Alibardi, 2017b), and highly expressed Wnt2b in blastema suggests a key role of Wnts proteins for promoting tail regeneration (Alibardi, 2020).

The *S. tsinlingensis* is an endemic lizard species in China, and represents a conserved tail regeneration pattern as for many lizards. Histological events of regenerating tail consists of seven stages in the lizard (Yang *et al*., 2022). However, the distribution of Wnt proteins in the lizard remains unknown. To investigate whether Wnt1 and Wnt2b are required for regenerating tail in the lizard, the localization of these two proteins are detected in regenerating tail of *S. tsinlingensis* using light immunocytochemistry. Based on spinal cord injury without limb paralysis in lizard (Szarek *et al*., 2016), we observe the ultrastructural changes in the original and regenerated tail spinal cord by transmission electron microscopy.

MATERIAL AND METHOD

Animal treatment and tissue collection. Thirty healthy specimens of adult *S. tsinlingensis* (12 males, 18 females, SVL>57.08, BW 2.65 ± 0.47) were collected from Qiliyu forest region in Taiyue Mountain, Shanxi, China (36°21′-36°45′N, 110°40′-112°21′ E). These lizards were housed at a soil-filled terrarium under an ambient humidity of 40 %-60 %, photoperiod 12 h at 28 ºC daylight and 12 h at 20 ºC night. They were fed with mealworms daily and some tap water. Experiments pertinent followed the animal care guidelines of the ethical committee of Shanxi Normal University.

Autotomy was achieved at a position of 2 cm from the cloaca. Then, the autotomized lizards had a right to regenerate, were observed and recorded every day underthe same feeding condition as above. The tail samples were harvested at different regeneration stages, as previously indicated (Yang *et al*., 2022). The harvested tails (n=24) of *S. tsinlingensis* were fixed in 4 % paraformaldehyde for 1-3 days, decalcified for 1-3 days (Leica surgipath decalcifierI). After dehydrated with graded alcohols, the samples were cleared in xylenes and then embedded in paraffin wax.

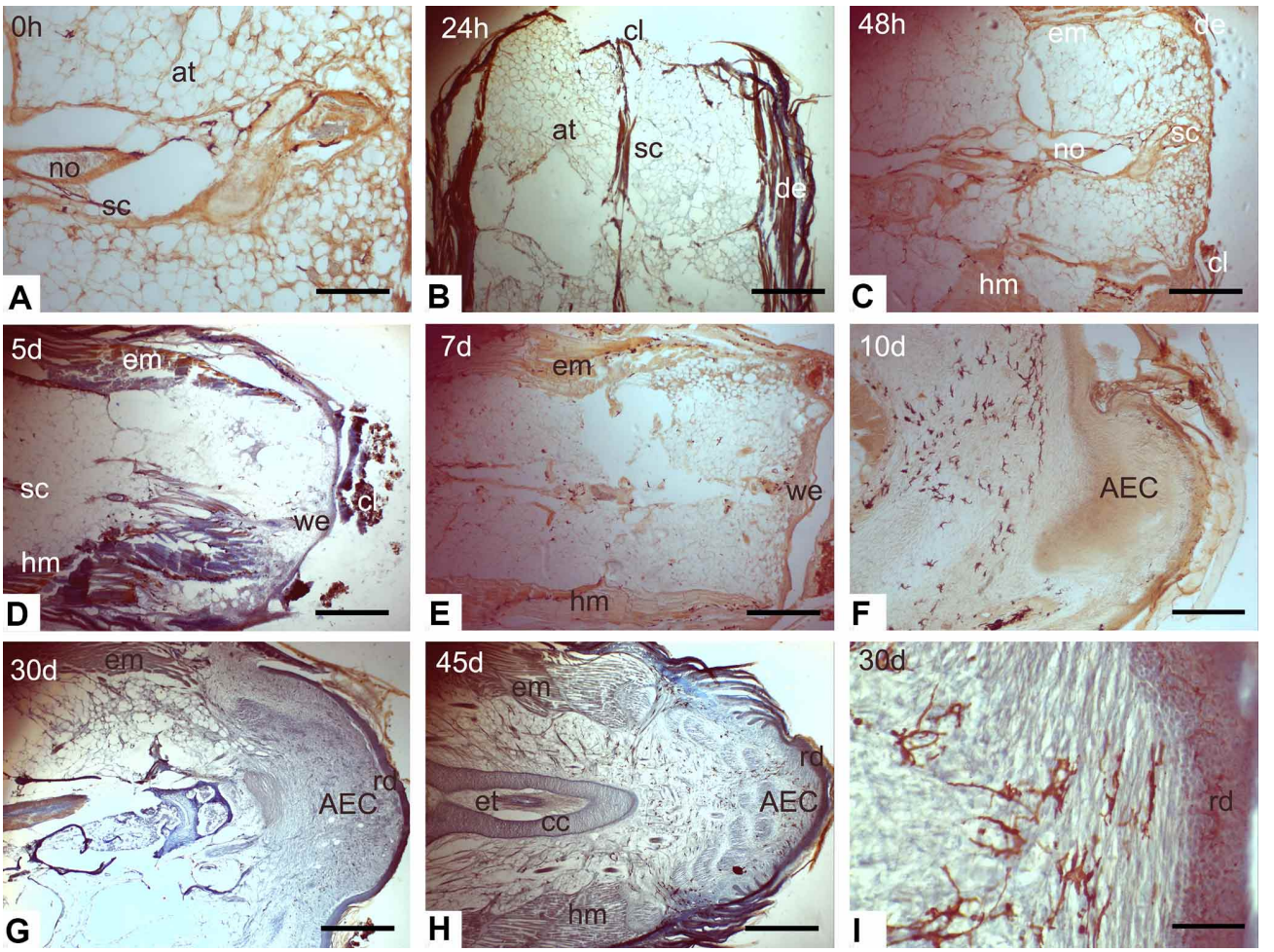
Immunohistochemistry. The sections were dewaxed with xylene, rehydrated, incubated in 3 % H2O2 for 30 min, rinsed three times with 0.1 M PBS (PH 7.4) and then blocked with 5 % BSA. Slices were incubated with primary antibodies rabbit anti-Wnt1 and Wnt2b (1:100, Boster) at 4 ºC overnight. Negative control slides replace original antibody with PBS. After rinsing with three changes in buffer, the slices were incubated with biotinylated goat anti-mouse/rabbit IgG (1:100, Boster) for 4 h at room temperature, followed by incubation with SABC and visualization using DAB. The samples were counterstained with Mayer's hematoxylin (Boster). All photos were obtained using the Olympus BX-51 digital imaging system and performed by Photoshop CS6.

Transmission electron microscopy. The dissected samples from original spinal cord (n=3) and the regenerated spinal cord (n=3) were fixed overnight at 4 ºC with 2.5 % glutaraldehyde solution. After rinsed in 0.1M PBS (PH 7.4), all samples were post-fixed in 1 % osmium tetroxide for 1h, dehydrated in a graded series of acetone, embedded in Epon 812 epoxy resin, cut into ultrathin sections, and double stained with uranyl acetate-lead citrate. In the end, these sections were observed with H-760 transmission electron microscope (Hitachi, Tokyo) and photographed at 80 kV (Yang *et al*., 2020).

RESULTS

Immunocytochemical localization of Wnt1 and Wnt2b in the regeneration tail. Weak immunolabeling Wnt1 was observed in dermis near the original tail stump and spinal cord (Figs. 1-A and B) during Stage I, 0-1 dpa. The positive Wnt1 wound epithelial cells spread across autotomy surface deep to clot (Figs. 1-C and D) during Stage II, 1-7dpa. The blastema and ependyma ampulla immunostained for Wnt1 (Figs. 1-E and F) during stage III, 5-10 dpa. An intense positive Wnt1 was detected in the apical region of the blastema, around the regenerating spinal cord, and the basal layer of the symmetric peg (Figs. 1-G and I) during Stage IV-VI, 15-30dpa. Close to the normal scales of the stump, a lowered labeling disappeared in differentiated scales, and then ependymal epithelium contained numerous Wnt1 labeled cells (Figs. 1-H) during VII, 45-160d.

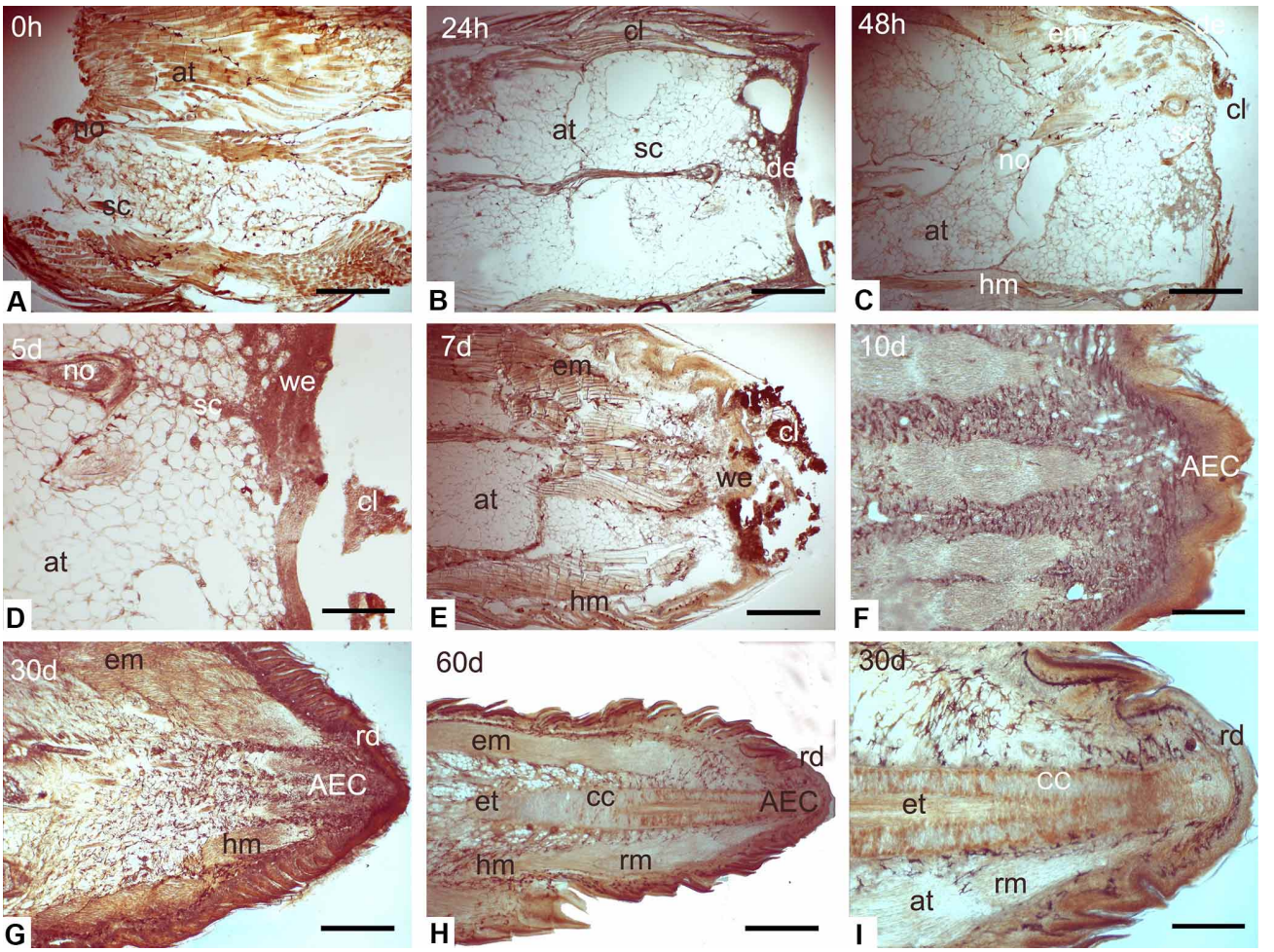
Immunolabeling for Wnt2b was mainly distributed in the dermis near the original tail stump and spinal cord (Figs. 2-A and B) during Stage I, 0-1 dpa. The positive Wnt2b proteins were mainly located in the epaxial and hypaxial musculature near the original tail stump, the wound epithelium and the spinal cord in the original tail (Figs. 2-C and D) during Stage II, 1-7 dpa. The wound healing period covers stage III (5-10 dpa) and IV (7-15 dpa), in which a higher Immunolabeling for Wnt2b was present in keratinocytes forming the AEC and surrounding wound epidermis, while mesenchyme and extracellular matrix showed weaker labeling (Figs. 2-E and F). Wnt2b was



Figs. 1. Immunohistochemical localization of Wnt1 at different stages of regenerating tail in *S. tsinlingensis*. Note: Figs A-H indicated the longitudinal section of the regenerating tail during 0h-45 dpa. The immunopositive tissues for Wnt1 showed brown to dark brown. Hypaxial musculature (hm), epaxial musculature (Em), wound blood clot (cl), blastema (bl), dermis (de), ependymal tube (et), adipose tissue (at), notochord (no), apical epithelial cap (AEC), spinal cord (sc), wound epithelium (we), regenerated dermis (rd), cartilage tube (cc). Scale bars in (F, I)=50 µm, Scale bars in (A-E,G-H)=20 µm.

detected in regenerative ependymal tubes (Figs. 2-G and I), and immunohistochemistry for Wnt2b was high in the forming pegs (Figs. 2-I) during stage V (10-25 dpa) and VI (20-30 dpa). The wound healing period covers stage VII (45-160 dpa), in which Wnt2b were distributed in the wound epithelium, nearby regenerative blood vessels, ependymal tube layer cells, epaxial and hypaxial musculature in AEC (Figs. 2-H). The differentiated segmental muscles in more proximal regions showed weaker immunolabeling for Wnt2b than in the wound epidermis (Figs. 2-H).

Ultrastructural features of ependymal layer cells in the original and regenerate spinal cords. The external morphology of the regenerative tail in *S. tsinlingensis* was similar to the original tail, but its internal structure was significantly different from the original tail. The main distinction was that the rigid hyaline cartilage tube in regenerative tail replaces the endoskeleton formed by the vertebrae in original tail. Regenerated muscle bundles with a high connective tissue content were loosely organized radially around the cartilage tube, but the original tail muscle tissue with the regular arrangement was divided into four quadrants. The regenerative tail had a limited regenerative spinal cord, but could support axonal growth. The pattern of ependymal layer cells (ELCs) ciliation for both original and regenerate spinal cords were observed using transmission electron microscopy (Figs. 3). The ependymal layer of the orginal spinal cord was pseudostratified, one to three cells deep, with ciliated cells, surrounding the central canal (*). Although most of ELCs of the orginal spinal cord were multiciliated, there were also a large number of primary monociliated cells.



Figs. 2. Immunohistochemical localization of Wnt2b at different stages of tail regeneration in *S. tsinlingensis*. Note: Figs A-H indicated the longitudinal section of the regenerating tail during 0h-60 dpa. The immunopositive tissues for Wnt2b showed brown to dark brown. Hypaxial musculature (hm), epaxial musculature (Em), wound blood clot (cl), blastema (bl), dermis (de), ependymal tube (et), adipose tissue (at), notochord (no), apical epithelial cap (AEC), spinal cord (sc), wound epithelium (we), regenerated dermis (rd), cartilage tube (cc). Scale bars in (F, I)=50 μm, Scale bars in (A-E,G-H)=20 μm.

DISCUSSION

Lizards provided a unique opportunity to investigate functional tissue replacement in the absence of structural replication (Gilbert & Vickaryous, 2018). The tail regeneration process of *S. tsinlingensis* tail was similar to other lizards (Cox, 1969; McLean & Vickaryous, 2011). Based on gross anatomy and histology, the process of the tail regeneration in *S. tsinlingensis* contained four stages, i.e., wound healing, blastema formation, cell differentiation and tail growth. The external morphology of the regenerative tail in *S. tsinlingensis* was similar to the original tail, but its internal structure was significantly different from the original tail. The main distinction was that the rigid hyaline cartilage tube in regenerated tail replaced the endoskeleton formed by the vertebrae in original tail. Regenerated muscle bundles with a high connective tissue content were loosely organized radially around the cartilage tube, but the original tail muscle tissue with the regular arrangement was divided into four quadrants (Yang *et al*., 2022).

Reptiles provided a model for the study of the mechanisms leading to successful functional recovery following spinal cord injury. During spinal cord regeneration, the ELCs played a crucial role (Gilbert & Vickaryous, 2018). Among amniotes, uniciliated, biciliated, and multiciliated cells were identified lining the ventricular lumen. Biciliated and uniciliated cells represented the proliferative pools,

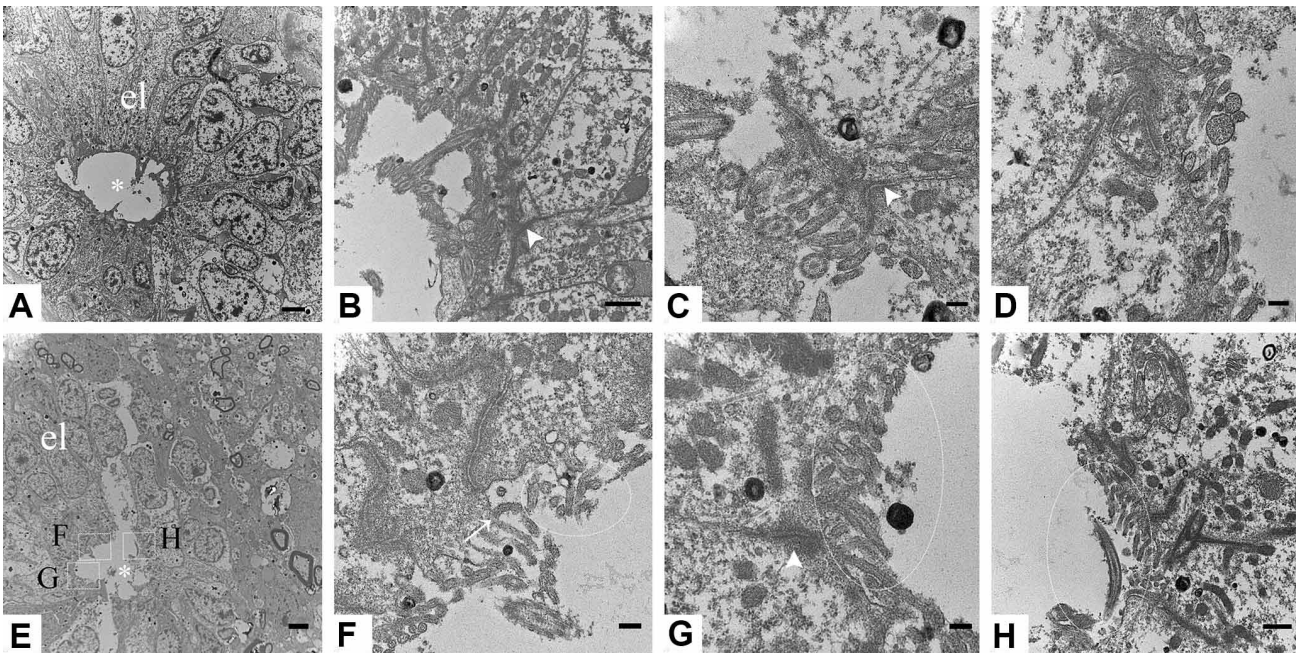


Fig. 3. TEM images of the ependymal layer in *S. tsinlingensis* containing different cilia-bearing cells. Note: The original spinal cord (A–D) and regenerate spinal cord (E–H). The original ependymal layer was pseudostratified (A), with multiciliated cells (B) and flanked by tight junctions (C and D, white triangle). E. The arrangement of ELCs in the regenerate spinal cord were organized into monolayer (E), including biciliated (F, white arrows), multiciliated (G), and monociliated (H) cells. Scale bars in (A) and (E) =2 μm; Scale bars in (B) and (H) =0.5 mm; scale bars in (C, D, E, G)=0.2 μm.

which were linked to neurogenesis in the mammalian brain (Tong *et al*., 2014). The ependymal layer of the original spinal cord was pseudostratified with multiciliated cells and primary monociliated cells, but the ELCs were organized into monolayer with a few of bicilicated cells in the regenerated spinal cord of *S. tsinlingensis*. TEM data from the leopard gecko (*Eublepharis macularius*) revealed the presence of ciliated ependymal layer cells, including multiciliated and uniciliated cells in the original spinal cord, and uni-, bi- and multi-ciliated in the regenerated spinal cord (Gilbert & Vickaryous, 2018). The uniciliated Type B (with one or two cilia) and multiciliated Type E cells (with one or two cilia), as observed in the mammalian spinal cord, were observed within neurogenic niches of the lizard brain (Gilbert & Vickaryous, 2018).

Wnt pathway involved in the whole process of lizard tail regeneration. During the wound healing period, both Wnt1 and Wnt2b positive proteins appeared in the dermis, spinal cord, and migrating cells of clots near the tail stump, which demonstrated that the Wnt signaling pathway may be highly activated in proliferating cell populations. Wnts protein secreted from the apical wound epidermis and the ependyma could influence cell proliferation in the blastema and the distal-most growing center of the new tail (Alibardi, 2017c). High levels of the Wnt proteins was in the apical region of the regenerative tail, in particular the apical blastema where major mesenchymal cells and blood cells were present, the apical wound epidermis along with AEC, and in the regenerating ependymal tip. These three regions contained active proliferating cells, which maintained the caudal tip growth area for regeneration. Immunolabeling also occured in the ependymal tubes, but at a lower intensity than in the epidermis. In other studies, the Wnt pathway was shown to be necessary for the formation of AER/AEC (apical epidermal ridge/cap) in the developmental and regenerative limbs of amphibians and in the fins of fish (Kawakami *et al*., 2006; Hodgkinson *et al*., 2009). In vertebrates, the main signaling factors involved in blastema were Wnts, fibroblast growth factor 8 (FGF8), and FGF10, similar to the signaling factors involved in the growth of the regenerative limbs of larval Xenopus laevis (Yokoyama *et al*., 2011). Wnt activated FGF8, which in turn activated FGF10, forming a positive feedback pathway during AEC formation that promotes cell proliferation and the reconstruction of limb morphology (Easterling *et al*., 2019). A distal to proximal gradient of Wnt proteins was not detected using immunolabeling in *S. tsinlingensis* and P. muralis (Hutchins *et al*., 2014).

Various forms of Wnt proteins showed a slightly different expression in the same type of tissue. Both

members of the canonical Wnt-pathway (Wnt1l, Wnt2b, Wnt 10) and noncanonical Wnt-pathway (Wnt5a,b, Wnt6) were active in the blastema of lizard *P. muralis* (Vitulo *et al*., 2017). Transcriptome analysis of regenerating tails in lizards had indeed shown the up-regulation of Wnt members, including Wnt1, in the apical regenerating blastema (Hutchins *et al*., 2014; Vitulo *et al*., 2017), together with grem, fgfr4, edgfd6 and msx2, stimulated the proliferation of regenerated tail tip cells, supporting the immunolocalization in *S. tsinlingensis*. While, signaling genes, e.g., hhipl2, gas1 and DKK2, inhibit the Wnt pathway, not forming proliferative zones in the appendages, but stimulating cell differentiation, tissue repair, and eventually scarring (Hutchins *et al*., 2014). Wnt1 which was labeled in the basal-most layers of the forming scales could act with beta-catenin to promote cell proliferation and lead to the epidermal layers, especially the corneous beta-layer formation (Wu *et al*., 2013). Low labeled for Wnt1 in differentiating muscles and the axial cartilage of *S. tsinlingensis* suggested that other signaling pathways could be more important for their differentiation (Gilbert & Vickaryous, 2018).

In conclusion, the Wnt1 and Wnt2b in the regenerating epidermis, nervous tissues and the AEC were involved in the Wnt pathway to direct the process of tail regeneration in *S. tsinlingensis*.

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YANG, C.; SUN, J.; WANG, X.; YANG, Y.; CHEN, R. & LIU, B. Inmunodetección Wnt1 y Wnt2b de la cola en regeneración y ultraestructura comparativa de la médula espinal de la cola en *Scincella tsinlingensis*. *Int. J. Morphol., 40(5)*:1202-1208, 2022.

RESUMEN: La vía Wnt es esencial para el inicio de la regeneración de la cola del lagarto. Las colas de lagarto regeneradas exhiben diferencias morfológicas obvias en comparación con las originales. La expresión de las proteínas Wnt1 y Wnt2b en la cola en regeneración de *Scincella tsinlingensis* se detectó mediante inmunohistoquímica y luego se analizaron comparativamente los cambios ultraestructurales en la médula espinal original y regenerada. La capa ependimaria de la médula espinal original se pseudoestratificó con células multiciliadas y células monociliadas primarias, mientras que las células de la capa ependimaria de la médula espinal regenerada se organizaron en monocapa con algunas células bicilicadas. La inmunolocalización indicó que Wnt1 y Wnt2b se distribuyeron principalmente en la dermis cerca del muñón de la cola original, la médula espinal y las células migratorias positivas en el coágulo durante la Etapa I, 0-1 días después de la amputación (dpa). Wnt1 y Wnt2b se detectaron predominantemente en la musculatura epaxial e hipaxial cerca del muñón de la cola original, el epitelio de la herida y la médula espinal en la cola original durante la Etapa II, 1-7 dpa. Las células mesenquimales y el epitelio de la herida mostraron inmunomarcaje durante la Etapa III y IV, 7-15 dpa. Los tubos ependimarios contenían estas proteínas de señalización durante la Etapa V y VI, 20-30 dpa. El marcaje se observó principalmente en vasos sanguíneos regenerativos cercanos, células ependimarias, musculatura epaxial e hipaxial en la capa epitelial apical (AEC) después de 45-160 dpa. Estos hallazgos indicaron que las proteínas Wnt1 y Wnt2b están presentes principalmente en la epidermis en regeneración y en los tejidos nerviosos y eran una señal crítica para la regeneración de la cola en *S. tsinlingensis*.

PALABRAS CLAVE: *Scincella tsinlingensis*; Cola regeneradora; Ultraestructura; Inmunohistoquímica.

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Corresponding author:
Chun Yang, Assoc. Prof.
School of Life Sciences
Shanxi Normal University
No. 339, Taiyu Road
Xiaodian District
Taiyuan 030031
Shanxi Province
PR CHINA

E-mail:yangchun774@163.com