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Inhibition of Gecko GSK-3β Promotes Elongation of Neurites and Oligodendrocyte Processes But Decreases the Proliferation of Blastemal Cells

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ABSTRACT

GSK-3β signaling is involved in regulation of both neuronal and glial cell functions, and interference of the signaling affects central nervous system (CNS) development and regeneration. Thus, GSK-3β was proposed to be an important therapeutic target for promoting functional recovery of adult CNS injuries. To further clarify the regulatory function of the kinase on the CNS regeneration, we characterized gecko GSK-3β and determined the effects of GSK-3β inactivation on the neuronal and glial cell lines, as well as on the gecko tail (including spinal cord) regeneration. Gecko GSK-3β shares 91.7–96.7% identity with those of other vertebrates, and presented higher expression abundance in brain and spinal cord. The kinase strongly colocalized with the oligodendrocytes while less colocalized with neurons in the spinal cord. Phosphorylated GSK-3β (pGSK-3β) levels decreased gradually during the normally regenerating spinal cord ranging from L13 to the 6th caudal vertebra. Lithium injection increased the pGSK-3β levels of the corresponding spinal cord segments, and in vitro experiments on neurons and oligodendrocyte cell line revealed that the elevation of pGSK-3β promoted elongation of neurites and oligodendrocyte processes. In the normally regenerate tails, pGSK-3β kept stable in 2 weeks, whereas decreased at 4 weeks. Injection of lithium led to the elevation of pGSK-3β levels time-dependently, however destructed the regeneration of the tail including spinal cord. Bromodeoxyuridine (BrdU) staining demonstrated that inactivation of GSK-3β decreased the proliferation of blastemal cells. Our results suggested that species-specific regulation of GSK-3β was indispensable for the complete regeneration of CNS. J. Cell. Biochem. 113: 1842–1851, 2012.

KEY WORDS: GECKO; GSK-3β; BLASTEMA; REGENERATION; CNS

Glycogen synthase kinase-3 (GSK3) is a broadly expressed and highly conserved serine/threonine protein kinase encoded by two genes, *GSK*-3α and *GSK*-3β. GSK3 regulates multiple cellular processes ranging from cell signaling, gene transcription, embryonic development, cell proliferation and adhesion, to neuronal death and apoptosis [Chen et al., 2007; Wu and Pan, 2010]. Although in some signal pathways the two kinases have shown to be functionally redundant [Wu and Pan, 2010], they actually play independent roles evidenced from the GSK-3β deletion in mice [Hoeflich et al., 2000]. Over the last few years, GSK-3β has been highlighted for its key roles in many fundamental processes during central nervous system (CNS) development and regeneration [Dill et al., 2008; Alabed et al., 2010; Hur and Zhou, 2010], aside from its mediation of immune and inflammatory responses [Park et al., 2011], bone remodeling [Li et al., 2011], carcinogenesis [Korur et al., 2009], and dorsal–ventral axis formation [Dominguez et al., 1995; Wu and Pan, 2010]. In developing and regenerating CNS, emerging evidence points to GSK-3β as a key regulator of neurogenesis, neuronal polarization, axon growth, and differentiation and myelination of glial cells [Dill et al., 2008; Hur and Zhou, 2010; Azim and Butt, 2011]. The regulatory mechanisms are mainly attributed to its activity and subcellular distribution, which affect the cell signaling cascades.

GSK-3β plays central roles in diverse signaling pathways, including those activated by Wnts, hedgehog, growth factors, cytokines, and G-protein-coupled ligands [Eickholt et al., 2002;

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Kockeritz et al., 2006; Park et al., 2011]. By activating or inactivating its substrates including β-catenin, Axin, Tau, cyclin D1, AP-1, C/EBP, p53, and NF-κB, the protein exerts distinct functions in specific tissues or organs. The activity of GSK-3β has been associated with site-specific phosphorylation of the amino acids. The phosphorylated residue of Tyr216 increases the activity while Ser9 inhibits it [Bijur and Jope, 2001; Gross et al., 2004]. It is widely accepted that Akt is the major family that mediates Ser9 phosphorylation and the subsequent inactivation of GSK-3β [Cross et al., 1995; Hur and Zhou, 2010]. However, in the developing nervous system, there likely exist alternative pathways regulating GSK-3β activity [McManus et al., 2005]. Lithium ion has been proved to be an effective inhibitor of GSK-3β through activation of phosphatidylinositol 3 (PI3) kinase and its downstream effector Akt, which in turn leads to the inactivation of the kinase [Chalecka-Franaszek and Chuang, 1999]. Additionally, lithium ions substitute the essential magnesium ions at the GSK-3β active site and directly inhibit its activity [Zhang et al., 2003]. Lithium inhibition of GSK-3β is assumed to mimic the effects of Akt activation.

Gekko japonicus has a remarkable ability to regenerate their tails, including major axial structures, such as spinal cord, cartilage, and spinal nerves. Therefore, the animal has become the perfect amniote model to investigate the regeneration of spinal cord, which fails to succeed in the adult mammals for the reduced intrinsic growth capacity of adult CNS neurons and the poor environment for axon extension (Tanaka and Ferretti, 2009). Several studies have revealed multifaceted roles of GSK-3β in association with spinal cord recovery following injury. Inactivation of GSK-3β was supposed to overcome the growth suppression of CNS inhibitory substrates and promotes axonal growth and recovery in the CNS [Dill et al., 2008]. However, another evidence indicated that overexpression of GSK-3β inhibits formation of a L-CRMP4–RhoA complex and may be protective in the context of myelin inhibition [Alabed et al., 2010]. The insights of the GSK-3β regulating the regenerating spinal cord might be helpful to clarify the critical functions of this kinase in injured CNS, and to provide an important strategy for promoting the spinal cord regeneration. In this report, we focus on the expression changes of the GSK-3β in the regenerating spinal cord, and the roles of GSK-3β inactivation on neurites, oligodendrocyte processes as well as blastemal cells. We demonstrate that lithium inhibition of GSK-3β can stimulate neurites and oligodendrocyte processes elongation, but decrease the proliferation of blastemal cells.

MATERIALS AND METHODS

ANIMALS

Adult *Gekko japonicus* were used as described by Wang et al. [2011]. Briefly, adult animals were fed freely with mealworms and housed in an air-conditioned room under controlled temperature (22–25°C) and saturated humidity. Anesthesia was induced by cooling the animals on ice prior to tail amputation. Amputation was performed at the 6th caudal vertebra, identified based on the special anatomical structure present at that position [Zhenkun and Guang, 1990], by placing a slipknot of nylon thread and pulling gently until the tail was detached, thus mimicking the autotomy undergoing for natural defense.

For inactivation of GSK-3β, LiCl was injected intraperitoneally (3 mmol/kg/day) post-amputation according to the protocol of Dill et al. [2008]. Control experiments were performed by the injection, after amputation, of an equivalent amount of 0.9% saline. Bromodeoxyuridine (BrdU) was injected intraperitoneally 1 day before tissues were collected. Cell proliferation was assayed following manufacturer's instructions (5-bromo-2′-deoxy-uridine labeling and detection kit; Roche Molecular Biochemicals, Mannheim, Germany).

All experiments were conducted in accordance with guidelines established by the NIH, found in *Guide for the Care and Use of Laboratory Animal* (1985), and by the Society for Neuroscience, found in *Guidelines for the Use of Animals in Neuroscience Research*. The experiments were approved according to the *Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee* (Approval ID: SYXK(SU)2007-0021). All geckos were anaesthetized on ice prior to euthanatizing, and the animal carcasses were disposed together by the animal center.

CLONING AND ANALYSIS OF *GSK*-3β

A cDNA library of the brain and spinal cord from *Gekko japonicus* was constructed according to methods described previously [Liu et al., 2006]. In a large-scale sequencing of the cDNA library, more than 5,000 clones were analyzed for coding probability with the DNATools program [Rehm, 2001]. To obtain the full length of gecko *GSK*-3β, anti-sense primer 5′-GGCATATTCCAAAGGAATGGATA-TAGGC-3′ and sense primer 5′-CAGGGTCCAGACAGGCCACAA-GAAGT-3′ were designed according to the partial cDNA sequence, and 5′-RACE and 3′-RACE were performed using the BD SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. Comparison against the GenBank protein database was performed using the PSI-BLAST network server at the National Center for Biotechnology Information [Altschul et al., 1997]. Multiple protein sequences were aligned using the MegAlign program by the CLUSTAL method in the DNASTAR software package [Burland, 2000].

RNA ISOLATION AND POLYMERASE CHAIN REACTION (PCR)

Total RNA was prepared with Trizol (Gibco) from different tissues, including the brain, spinal cord, heart, liver, lung, kidney, and ovary of adult geckos. For semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), the first-strand cDNA was synthesized using the Omniscript Reverse Transcription Kit (Qiagen) in a 20 μl reaction system containing 2 μg total RNA, 0.2 U/μl M-MLV reverse transcriptase, 0.5 mM dNTP mix, 1 μM Oligo-dT primer. A 1 μl aliquot from the synthesized first-strand cDNA was amplified with anti-sense primer 5′-GGCATATTCCAAAGGAATG-GATATAGGC-3′ and sense primer 5′-CAGGGTCCAGACAGGCCA-CAAGAAGT-3′ designed to investigate the expression of *GSK*-3β. The PCR amplification reaction was performed in 1.5 mM $MgCl_2$, 200 μM of each dNTPs, with 20 pmol of each primer and 2 U of Taq polymerase (MBI Fermentas) in a final volume of 50 μl. After the cDNA/primer denaturation at 94°C for 5 min, the PCR amplification was carried out in 26 cycles using the following parameters:

denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The reaction was continued for a final extension at 72°C for 10 min. Normalization was carried out simultaneously by amplification of *EF-1α* using an antisense primer 5′-CTGGCTGTAAGGTGGCTCAG-3′ and a sense primer 5′-CATGTC-GATTCTGGCAAGTC-3′ under the same conditions described above. A negative control without the first-strand cDNA was also performed. The expression levels were assessed by an image analysis system.

WESTERN BLOT

To determine GSK-3β expression levels, vertebra segments containing a region of spinal cord extending from L13 to the 6th caudal vertebra were collected at 1 day, 3 days, 1 week, and 2 weeks following amputation. And the regenerate tails were also cut and collected at 1, 2, and 4 weeks, respectively. Samples of vertebra segments (n = 15) or the regenerate tails (n = 10) were lysed in a buffer containing 1% NP-40, 50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L PMSF, 10 mg/ml aprotinin, and 1 mg/ml leupeptin. After centrifugation at 13,000 rpm for 30 min at 4°C, 20 μg of total protein of each sample was loaded into a 12% SDS–PAGE gel and transferred to PVDF membranes (Millipore). The membrane was then blocked with 5% nonfat dry milk in TBS containing 0.05% Tween-20 (TBS-T) for 1 h and incubated with monoclonal mouse anti-human GSK-3β IgG (1:1,500; Abcam) or polyclonal rabbit anti-mouse phospho-GSK-3β (Ser9) IgG (1:1,000; Abcam). After reaction with the second antibody, goat anti-mouse IgG-IRDye or goat anti-rabbit IgG-IRDye (1:10,000), the membrane was scanned with an Odyssey Infrared imager (LiCor, Lincoln, NE). The data were analyzed using PDQuest 7.2.0 software (Bio-Rad). β-Actin (1:5,000) was used as an internal control.

TISSUE IMMUNOHISTOCHEMISTRY

The vertebra segments and corresponding regenerate tails at different stages were harvested, post-fixed, and sectioned. Sections were allowed to incubate with monoclonal mouse anti-human GSK-3β antibody (1:200 dilution; Abcam), polyclonal rabbit anti-mouse phospho-GSK-3β (Ser9) antibody (1:200 dilution; Abcam), polyclonal rabbit anti-bovine galactocerebroside antibody (1:200 dilution; Millipore), or polyclonal rabbit anti-human neuron-specific enolase (NSE) antibody (1:200 dilution; Abcam) at 4°C for 36 h. The sections were further reacted with the FITC-labeled secondary antibody goat anti-mouse IgG (1:400 dilution; Gibco), or the TRITC-labeled secondary antibody donkey anti-rabbit IgG (1:400 dilution; Gibco) at 4°C overnight, followed by observation under a confocal laser scanning microscope (Leica, Heidelberg, Germany).

CELL CULTURE, LiCL TREATMENT, GSK-3β ASSAYS, AND IMMUNOFLUORESCENCE STAINING

The culture of oligodendrocyte cell line, Gsn3, was referred to the methods by Liu et al. [2010]. The cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum in a 30°C humidified incubator with 5% CO_2. The cells with 95% confluency were pretreated with 0, 1, 5, and 10 mM LiCl for 0.5 h so as to select the optional concentration. In the subsequent experiments, cells were treated with 10 mM LiCl for 24, 48, and 72 h, respectively.

For the culture of gecko primary spinal cord neurons, spinal cords from adult geckos were dissected out, cleaned of their meningeal membranes, and minced into small pieces in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The tissues of approximately 1 mm^2 were incubated in 0.25% trypsin (Boehringer, France) for 15–25 min at 30°C, and dissociated by repeated trituration for 20 times using a Pasteur pipette with a fire-polished tip. Then, they were added DMEM medium (Gibco) containing 1% penicillin/streptomycin (Invitrogen) and F12 supplemented with 10% fetal bovine serum to stop trypsination. The tissue was further centrifuged at 1,000 rpm for 5 min, and the supernatant was carefully discarded. Following resuspended in the above medium, the cells were dissociated by repeated trituration, and plated onto 24-well plastic plates previously coated overnight with poly-L-lysine (10 μg/ml in borate buffer pH 8.4). The cultures were incubated at 30°C in a humidified atmosphere of 5% CO_2. Four days later, the medium was half changed, and further subjected to maintain in Neurobasal-A/B27 medium (Invitrogen) after 10 days culture.

For GSK-3β assays, protein was extracted from cells after LiCl treatment with a buffer containing 1% SDS, 100 mM Tris–HCl, 1 mM PMSF, and 0.1 mM β-mercaptoethanol. Protein concentration of each sample was detected by the Bradford method to maintain the same loads. Western blots were performed as described above.

Gecko primary neurons or Gsn3 cells were grown on round coverslips in multiwell culture plates and exposed to LiCl (10 mM) for indicated times. For the cell staining, the permeabilized neurons were incubated with polyclonal rabbit anti-human NSE antibody (1:200 dilution, Abcam), while Gsn3 cells with polyclonal rabbit anti-mouse phospho-GSK-3β (Ser9) IgG (1:200; Abcam), for 36 h at 4°C. After washing, the cells were stained with the TRITC-labeled secondary antibody donkey anti-rabbit IgG (1:400 dilution; Gibco) overnight at 4°C, then they were counterstained with the Hoechst 33342 (1 μg/ml) for 10 min at 37°C and mounted on slide glasses with mounting medium. Images were captured on a Nikon Diaphot microscope. For the length quantification of neurites and Gsn3 process, the length of axons or process in each neuron or oligodendrocyte was traced manually and measured with Photoshop and NIH Image software. Thirty to 50 neurons or oligodendrocytes were used from each coverslip. The mean values were reported from 5 to 6 separate experiments for the length assay experiments.

RESULTS

ANALYSIS OF GECKO GSK-3β REVEALS A HIGHLY CONSERVED PROTEIN IN THE PHYLOGENY

The cDNA clone (GenBank accession number JN225462) obtained from the brain and spinal cord cDNA library and by 5′ RACE and 3′ RACE amplification is 1,485 bp long, and its longest open reading frame codes for a protein of 420 amino acid residues with a predicted molecular mass of 46.7 kDa (data not shown). There are two initiator methionine codons at the 5′-end and a stop codon at the 3′-end. In addition, the 3′-untranslated region (UTR) contains a polyadenylation tail. Therefore, the cDNA encodes a full-length sequence protein.

An alignment showed that the encoded protein had the highly conserved kinase domain with 11 serine/threonine kinase subdomains, characteristic of GSK-3 proteins [Hanks and Quinn, 1991]. The protein shares 95.2%, 95.5%, 95.5%, 96.7%, 92.4%, and 91.7% identity with GSK-3β proteins of human, rat, mouse, chicken, frog, and zebrafish, respectively, and the N-terminal region of this protein lacked the N-terminal feature of the α-form. These results suggested that the encoded protein was a member of GSK-3β family with a high degree of evolutionary conservation (Fig. 1).

GSK-3β ACTIVITY CHANGES IN RESPONSE TO THE SPINAL CORD REGENERATION

We first analyzed the tissue expression of gecko *GSK*-3β by RT-PCR. The results demonstrated that *GSK*-3β was ubiquitously expressed in all tissues including the brain, spinal cord, heart, liver, lung, kidney, and ovary, with a higher abundance in the CNS (Fig. 2A). In order to get further insights into the role of GSK-3β on spinal cord regeneration, much attention was then directed to the GSK-3β expression in the spinal cord and blastemal cells. Western blots revealed that the GSK-3β levels in the segment ranging from L13 to the 6th caudal vertebra decreased markedly at 1 and 3 days than control values, and then returned to control levels at 1 and 2 weeks following tail amputation (Fig. 2B). Tissue immunochemistry detected that GSK-3β distributed along the boundary of gray matter and white matter of the spinal cord, with a strong colocalization with the galactocerebroside-positive cells (Fig. 2F–H), while a less colocalization with NSE-positive cells (Fig. 2C–E). The results indicated that most of the gecko oligodendrocytes in the spinal cord expressed GSK-3β, comparing with a small number of neurons.

It is interesting that inactivation of GSK-3β may promoted the axonal growth and recovery in the CNS of rat, by decreasing the effects of the major CNS inhibiting substrates [Dill et al., 2008]. The spontaneous regeneration of gecko spinal cord might provide related information on the roles of GSK-3β in the CNS. We further examined the expression levels of phosphorylated GSK-3β at Ser9 (pGSK-3β) in the regenerating spinal cord by using the specific antibody, in attempt to observe an increased pGSK-3β level.

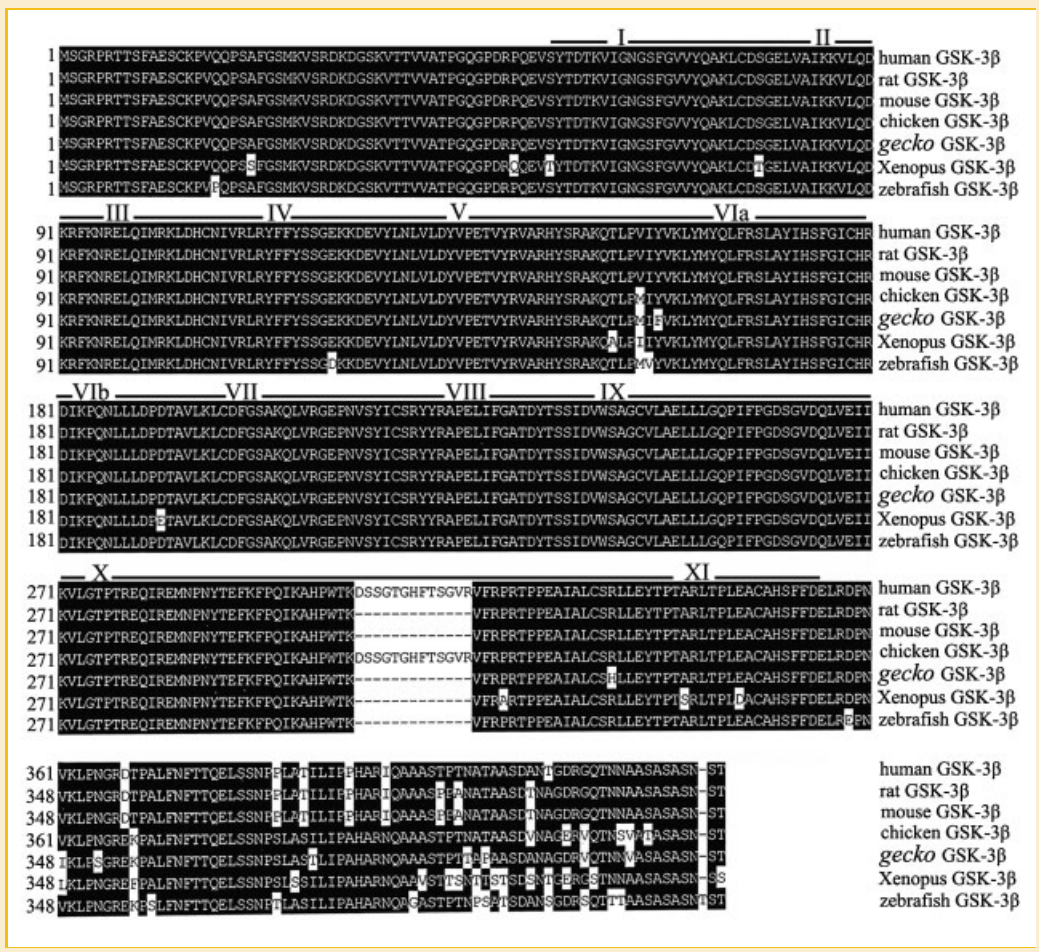

Fig. 1. The deduced amino acid sequence of gecko GSK-3β aligned with those of several vertebrates. Shaded (with solid black) residues are the amino acids that match the consensus sequence. Gaps introduced into sequences to optimize alignment are represented by dashes. The 11 serine/threonine kinase subdomains are marked with roman numerals. Sequences obtained from GenBank or SwissProt are gecko (JN225462), human (NM_002093), mouse (NM_019827), rat (NM_032080), chicken (XM_416557), Xenopus (NP_001083752), zebrafish (NP_571456).

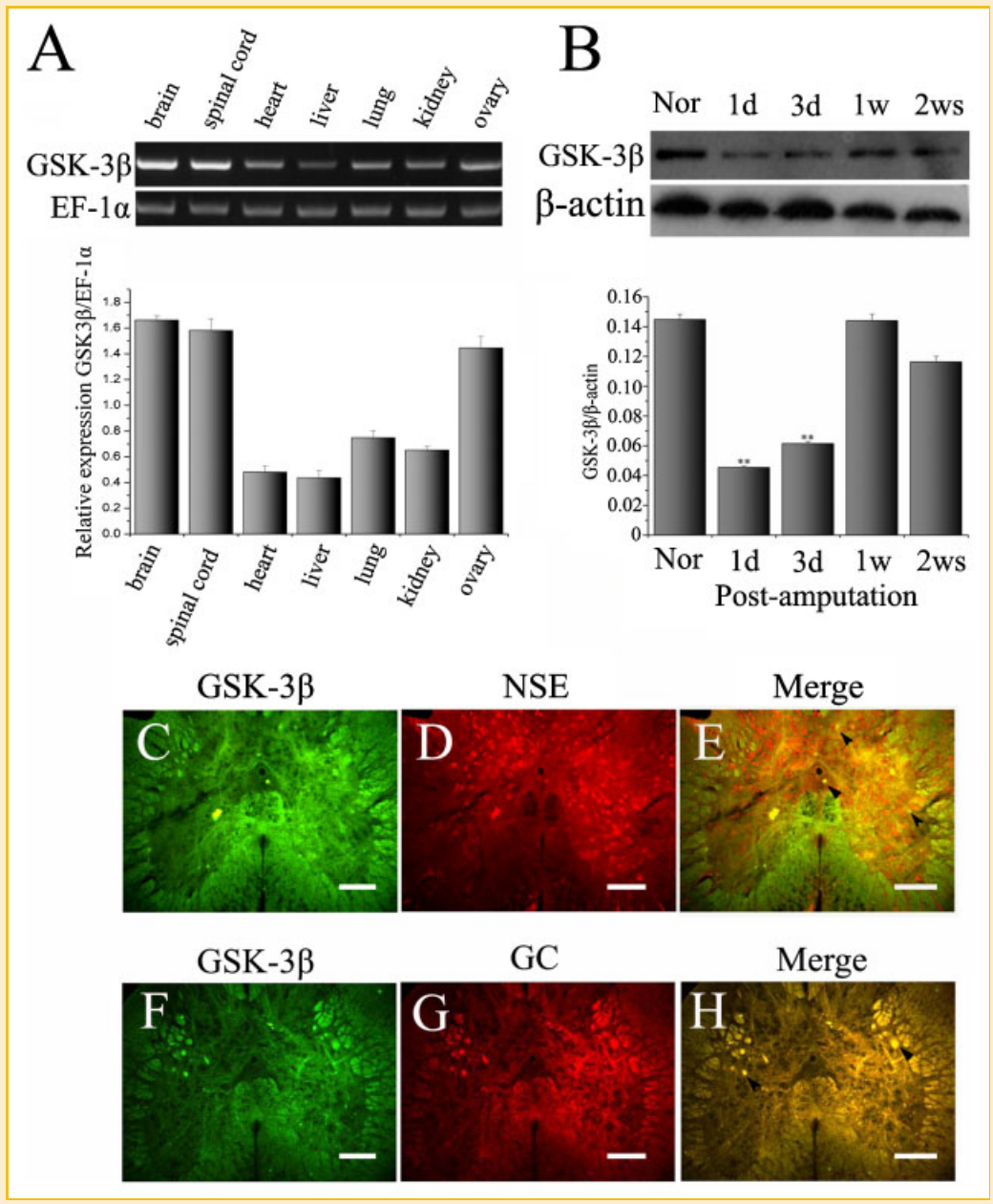

Fig. 2. Tissue expression of gecko GSK-3β. A: Semi-quantitative RT-PCR analysis of *GSK*-3β transcripts in different gecko tissues. Gecko *EF-1α* was used for normalization. B: Western blots analysis of GSK-3β in the spinal cord from L13 to the 6th caudal vertebra following tail amputation at 1 day, 3 days, 1 week, and 2 weeks, the Nor indicates control group. Quantities were normalized to endogenous β-actin. Error bars represent the standard deviation ($P < 0.01$). C–H: Distribution of GSK-3β in the spinal cord detected by immunohistochemistry. C–E: Less colocalization of GSK-3β with NSE-positive cells (arrowhead). F–H: Strong colocalization with the galactocerebroside-positive cells (arrowhead). Scale bars, 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Unexpectedly, similar reductions of pGSK-3β levels were present in the regenerating spinal cord of gecko following tail amputation (Fig. 3A). Lithium is a direct and indirect noncompetitive inhibitor of GSK-3β [Stambolic et al., 1996; Ryves and Harwood, 2001], therefore we injected gecko intraperitoneally with 3 mmol/kg/day LiCl to examine its effects on the regeneration of spinal cord. pGSK-3β levels were significantly elevated during the first and second weeks post-amputation (Fig. 3B), confirming that LiCl mimics the effects of Akt activation. The unsuccessful cross-reactions of Akt and β-catenin antibodies from other species restricted us to detect the relative signaling pathways.

GSK-3β INACTIVATION PROMOTES ELONGATION OF NEURITES AND OLIGODENDROCYTE PROCESSES

As GSK-3β inactivation showed a significantly negative effect on the successful regeneration of spinal cord in the regenerative reptiles, we next examined the effects of GSK-3β inactivation on both neurons and oligodendrocytes in vitro. Gecko oligodendrocyte

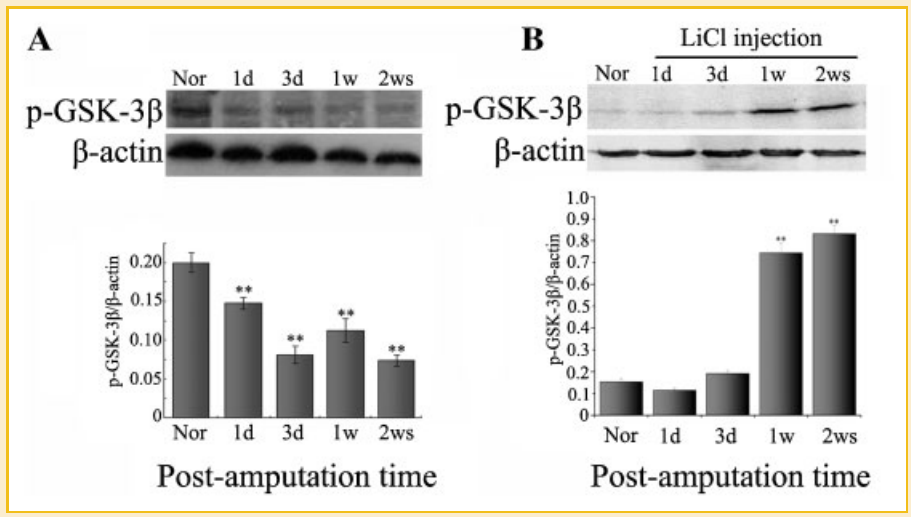

Fig. 3. Western blots analysis of phosphorylated GSK-3β (pGSK-3β) in the regenerating spinal cord. A: pGSK-3β levels of the spinal cord from L13 to the 6th caudal vertebra for the controls (Nor) and following tail amputation at 1 day, 3 days, 1 week, and 2 weeks. B: pGSK-3β levels of the spinal cord from L13 to the 6th caudal vertebra for the controls (Nor) and following tail amputation at 1 day, 3 days, 1 week, and 2 weeks after injection of 3 mmol/kg/d LiCl intraperitoneally. Quantities were normalized to endogenous β-actin. Error bars represent the standard deviation ($P < 0.01$).

cell line Gsn3 [Liu et al., 2010] was treated with 1, 5, and 10 mM LiCl for 0.5 h, and results showed that total GSK-3β levels presented no obvious changes (Fig. 4A,B). But pGSK-3β increased significantly when treated with 5 and 10 mM LiCl (Fig. 4A,B). Gsn3 cells were thus treated with 10 mM LiCl for 1, 2, and 3 days, which greatly suppressed the activation of the GSK-3β. The length of the processes increased time-dependently (Fig. 4C,D). Also, the parallel experiments on the culturing primary spinal cord neurons of gecko

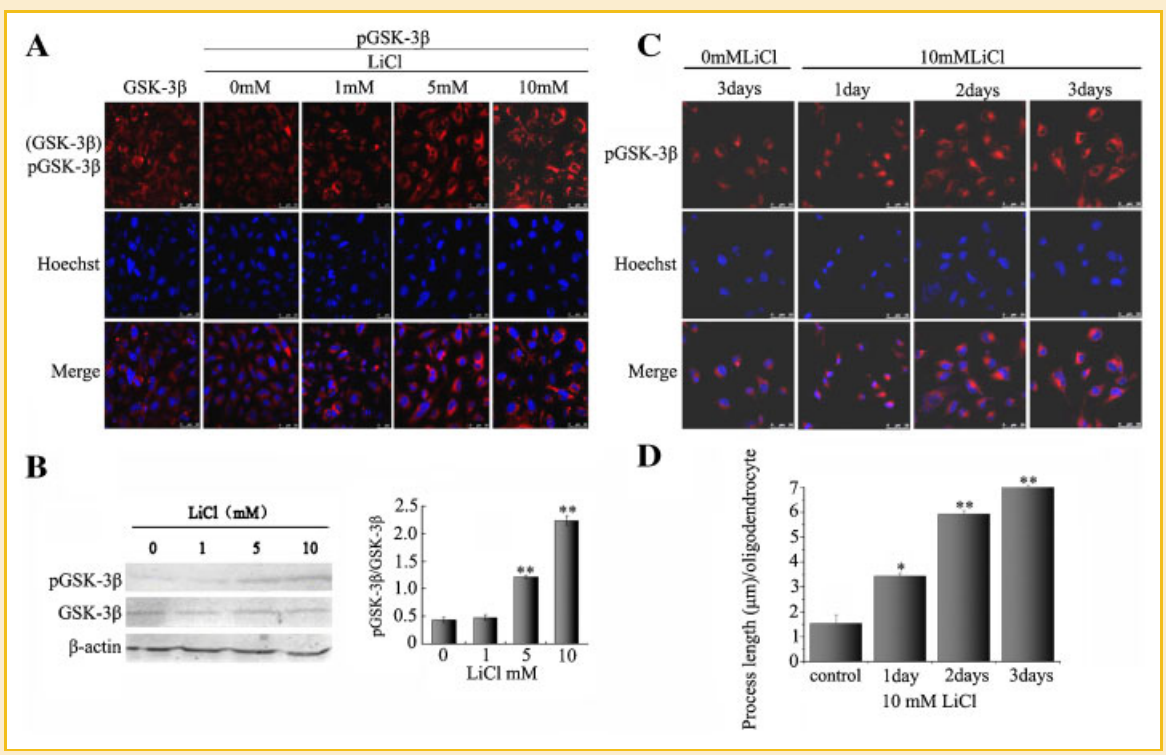

Fig. 4. LiCl increases the pGSK-3β levels of oligodendrocyte Gsn3 cells and stimulates the elongation of processes. A,B: pGSK-3β levels elevated in a range of 0–10 mM LiCl. C,D: 10 mM LiCl stimulates the elongation of Gsn3 cells processes after treatment for 1, 2, and 3 days. Control means the cells were cultured for 3 days without treatment with LiCl. Error bars represent the standard deviation ($P < 0.01$). Scale bars, 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

demonstrated that the neurite length increased significantly (Fig. 5A,B). Our results suggest that GSK-3β inhibition is effective in promoting elongation of neurites and oligodendrocyte processes.

INACTIVATION OF GSK-3β DESTRUCTS THE TAIL REGENERATION

Injection of 3 mmol/kg/day LiCl resulted in the failure of the tail regeneration, rather than enhanced the regenerative ability (Fig. 6A). During the tail regeneration, pGSK-3β levels in regenerate tails decreased sharply at 4 weeks, while the total levels of the GSK-3β kept stable (Fig. 6B). Injection of LiCl led to the time-dependent elevation of pGSK-3β levels in the regenerate tails, in comparison with the stability of total GSK-3β (Fig. 6B). Immunochemistry staining of 2 weeks' regenerate tail found that GSK-3β distributed in all forming tissues including promuscle, ependymal tube, and chondroblasts [Wang et al., 2011] (Fig. 6C). Brdu staining revealed that inactivation of GSK-3β decreased the proliferating numbers of blastemal cells (Fig. 6D) in the regenerate tails at 1 and 2 weeks. These findings indicated that inactivation of GSK-3β destructed the tail regeneration (including spinal cord) by decreasing the proliferating numbers of blastemal cells.

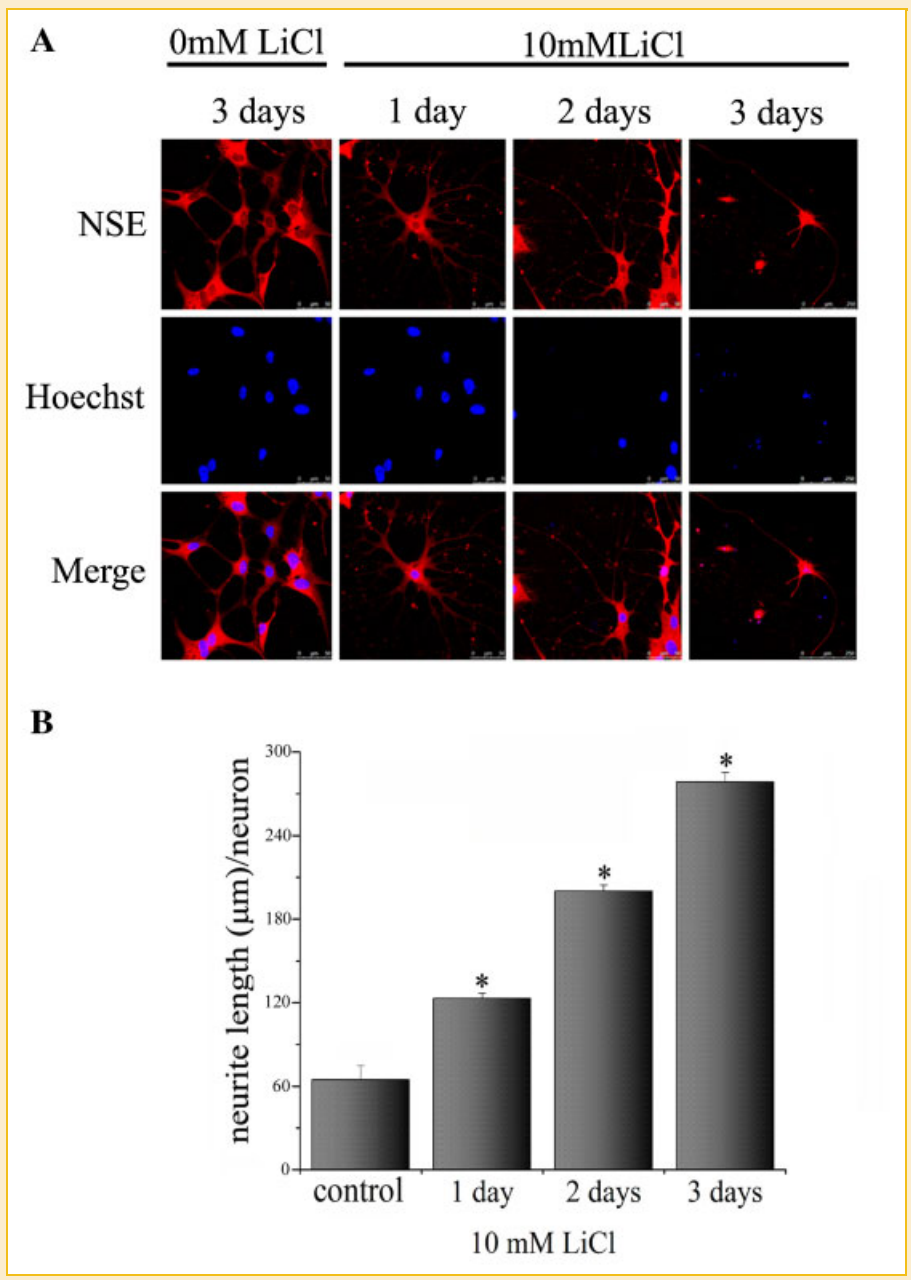

Fig. 5. LiCl stimulates the elongation of neurites of primary spinal cord neurons. A: The cells were treated with 10 mM LiCl for 1, 2, and 3 days. B: Statistical analysis of neurite lengths. Control means the cells were cultured for 3 days without treatment with LiCl. Error bars represent the standard deviation ($P < 0.01$). Scale bars, 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

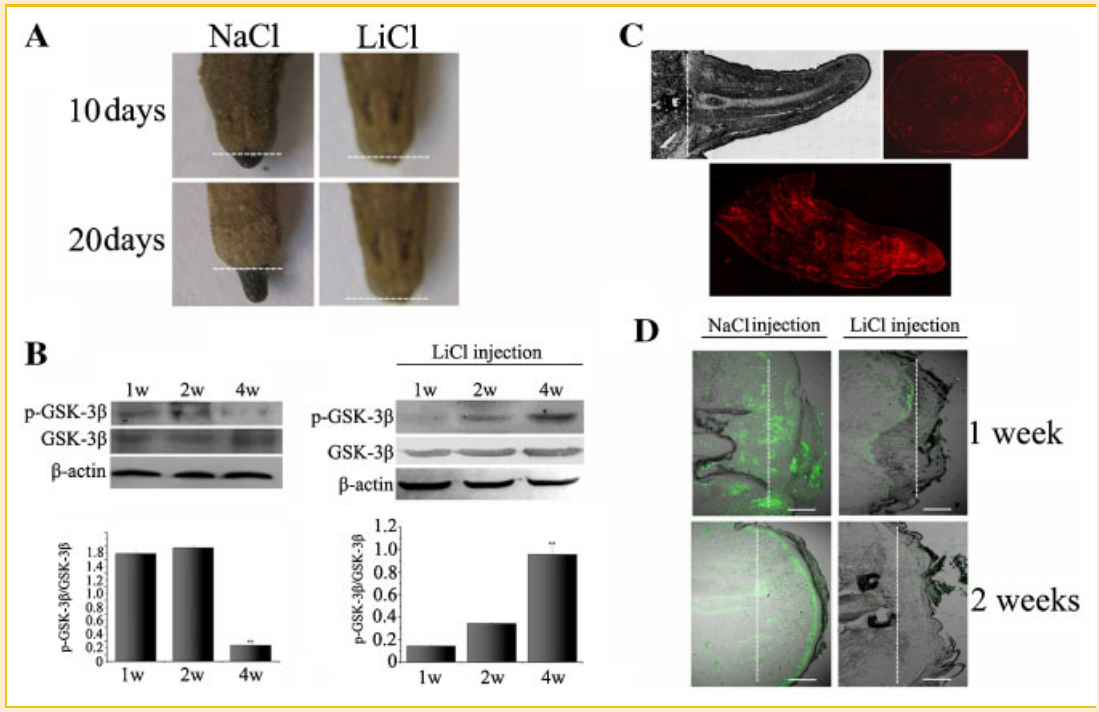

Fig. 6. GSK-3β inhibitor destructs the normal regeneration of the gecko tail. A: A comparison of tail regenerates at 10 and 20 days after injection of 3 mmol/kg/day LiCl and equivalent NaCl intraperitoneally. B: Determination of pGSK-3β in the regenerate tails at 1, 2, and 4 weeks post-amputation after injection of 3 mmol/kg/day LiCl and equivalent NaCl intraperitoneally. C: Distribution of GSK-3β in the 2 weeks' regenerate tail. D: Brdu staining of regenerate tail at 1 and 2 weeks after injection of 3 mmol/kg/day LiCl and equivalent NaCl intraperitoneally. Dash lines indicate the amputation sites. Scale bars, 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

The molecular basis of adult CNS regeneration, particularly of the intrinsic growth capacity of neurons and inhibition of myelin-associated inhibitors, is the subject of extensive research, focusing on signaling pathways, and gene interactions already described in diverse injured or developmental models [Yiu and He, 2006]. GSK-3β is a candidate to play a role in these processes [Adell et al., 2008; Fancy et al., 2009; Hur and Zhou, 2010; Azim and Butt, 2011]. The kinase regulates axon outgrowth through inactivation of myelin-associated inhibitors [Alabed et al., 2010], and also be able to negatively regulate oligodendrocyte differentiation and myelination [Fancy et al., 2009; Azim and Butt, 2011]. Therefore, GSK-3β signal was proposed to be an important therapeutic target for promoting functional recovery of adult CNS injuries [Dill et al., 2008]. However, the contradictory conclusions from Dill et al. [2008] and Alabed et al. [2010] with respect to the positive or negative regulation of GSK-3β on the axon outgrowth, remind us to clarify the specific functions of the kinase in the CNS. We chose the regenerative gecko as a model and examined the expression of pGSK-3β in the regenerating spinal cord. The reduction of pGSK-3β in the normally regenerating spinal cord suggested that the appropriate elevation of GSK-3β activity might be necessary for the complete regeneration of CNS in the most striking example of epimorphic regeneration. However, these cannot preclude the promoting effects of GSK-3β inhibition on the injured CNS recovery in other species. Kim et al. [2006] found that GSK3 activity toward primed or nonprimed substrates was able to result in distinct neuronal phenotypes. Our in vitro experiments on both primary spinal cord neurons and oligodendrocyte cell line Gsn3 also demonstrated growth-promoting roles of GSK-3β inhibitor. The specific upregulation of GSK-3β in the regenerating spinal cord of gecko was possibly associated with the nonprimed substrates in vivo.

Treatment of 10 mM LiCl on both culturing primary spinal cord neurons and gecko Gsn3 promotes the elongation of neurites or oligodendrocyte processes, indicating a safe-threshold of the concentration for the inactivation of GSK-3β in vivo. It can be ruled out that the elevation of pGSK-3β in both spinal cord and regenerate tails were caused by the toxicity of less than 10 mM LiCl. GSK-3β signaling has also been shown to be implicated in the neurogenesis and neuronal polarization [Hur and Zhou, 2010]. We failed to detect the neuronal differentiation, axonal formation, and structure following LiCl treatment due to those unidentified precursor cells, which should be marked by species-specific antibodies. However, the similar effects of GSK-3β inactivation on CNS neuron cannot be excluded. Additionally, inhibition of GSK-3β stimulates oligodendrocyte differentiation and myelination in vivo via the canonical Wnt signaling pathway [Fancy et al., 2009; Feigenson et al., 2009; Azim and Butt, 2011], but we herein firstly demonstrated that GSK-3β negatively regulated the elongation of the processes.

It is interesting to note that inhibition of GSK-3β resulted in the failure of tail (including spinal cord) regeneration. In contrast, gain of function experiments of the Wnt/β-catenin pathway promotes the limb regeneration of axolotls, *Xenopus laevis*, and zebrafish [Kawakami et al., 2006]. The process of gecko tail regeneration is a very complex and continuous one [Wang et al., 2011]. A hard wound scab formed by days 1 and 2 following amputation. At the margins of the broken surface underneath the scab and beyond the open end of the central canal, lymphocytes, macrophages, and microglial elements of the spinal cord accumulated. By day 3, melanocytes were found on the dorsal surface of the spinal cord. On the 4th day, an outgrowth of the ependyma appeared as a sac or vesicle. Wound epithelium became much thicker to form a thick cap, and a cone of blastemal cells developed on the 5th day. Two days later, within the blastema, the promuscle aggregates appeared. At 10 and 11 days, a wide space was still observed between the regenerating myoblasts and the original musculature, occupied by a loose mesenchyme. By days 11–14, the ependyma approached the epithelium and the regenerate began to enlarge. When the regenerate became 1 mm in length, the apical region, occupied by blastemal cells, began to form an ordered aggregate around the outgrowing ependymal tube. Over the next few days, chondroblasts formed the cartilaginous tube, and other tissues including scale began to develop. It is still uncertain about the origins of the blastemal cells which are responsible for the formation of different tissues. During the normal regeneration, pGSK-3β kept stable in 2 weeks, whereas decreased at 4 weeks, suggesting that formation, proliferation, and differentiation of blastemal cells are involved in a GSK-3β-independent mechanism. However, the growth of differentiated tissues after 4 weeks of tail amputation is dependent on the activation of the GSK-3β. Inactivation of GSK-3β, which decreased the proliferation of blastemal cells, eventually destructed the tail regeneration. As to what types of blastemal cells affected, it deserves further study.

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