Hedgehog and Wnt Signaling Pathways Regulate Tail Regeneration

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Urodele amphibians have a tremendous capacity for the regeneration of appendages, including limb and tail, following injury. While studies have focused on the cellular and morphological changes during appendicular regeneration, the signaling mechanisms that govern these cytoarchitectural changes during the regenerative response are unclear. In this study, we describe the essential role of hedgehog (Hh) and Wnt signaling pathways following tail amputation in the newt. Quantitative PCR studies revealed that members of both the Hh and Wnt signaling pathways, including the following: *shh*, *ihh*, *ptc-1*, *wnt-3a*, β -*catenin*, *axin2*, *frizzled (frzd)-1*, and *frzd-2* transcripts, were induced following injury. Continuous pharmacological-mediated inhibition of Hh signaling resulted in spike-like regenerates with no evidence of tissue patterning, whereas activation of Hh signaling enhanced the regenerative process. Pharmacological-mediated temporal inhibition experiments demonstrated that the Hh-mediated patterning of the regenerating tail occurs early during regeneration and Hh signals are continuously required for proliferation of the blastemal progenitors. BrdU incorporation and PCNA immunohistochemical studies demonstrated that Hh signaling regulates the cellular proliferation of the blastemal cells following amputation. Similarly, Wnt inhibition resulted in perturbed regeneration, whereas its activation promoted tail regeneration. Using an inhibitor-activator strategy, we demonstrated that the Wnt pathway is likely to be upstream of the Hh pathway and together these signaling pathways function in a coordinated manner to facilitate tail regeneration. Mechanistically, the Wnt signaling pathway activated the Hh signaling pathway that included *ihh* and *ptc-1* during the tail regenerative process. Collectively, our results demonstrate the absolute requirement of signaling pathways that are essential in the regulation of tail regeneration.

Keywords: newt, tail regeneration, proliferation, sonic hedgehog, Wnt signaling pathways

Introduction

MAMMALS HAVE A LIMITED CAPACITY for complete regeneration of tissues and appendages [1]. This limited regenerative capacity in mammalian tissues ultimately results in the replacement of viable tissue as well as scar formation [2–4]. In contrast, urodele amphibians have the unique potential to completely regenerate the injured/amputated tissue, including appendages [5–9]. The regenerative response in the newt proceeds with the formation of a blastema, cellular proliferation, patterning, and differentiation to ultimately restore the cellular architecture [6,10]. Each of these phases is regulated by a distinct molecular and signaling network [11,12]. While several signaling and regulatory factors have been identified, the regenerative program remains largely undefined.

The regenerative response in the amphibian progresses through a cascade of events that are closely interlinked, including the formation of wound epithelium, blastemal proliferation followed by outgrowth, and patterning [13–15]. Transcription factors, small heat shock proteins (sHSPs), microRNAs (miRNAs), as well as signaling networks play a

crucial role in regulating each of these steps during both development and regeneration [6,11,16–20]. For example, c-Myc and SP1 have been shown to be an integral part of the limb regeneration network [21,22]. Similarly, SP9 is activated in the regenerating blastema in an FGF7-dependent manner, suggestive of a coordinated interaction between signaling pathways and transcription factors during regeneration. Multiple studies have shown that a set of conserved miRNAs is differentially regulated during appendage regeneration [23]. Collectively, these studies underscore the role of developmental pathways and mechanisms that may also govern the regeneration program following injury. Previous studies in appendage regeneration have shown that the blastema (consisting of a heterogeneous cell population) is derived from the dedifferentiation of mature cells following injury [8,24,25]. A study by Echeverri et al. [24] has shown that endogenous multinucleated muscle fibers dedifferentiate into mononucleated cells and form a blastema during tail regeneration. These blastemal progenitors proliferate, migrate, and differentiate to regenerate the damaged or absent lineages following injury [6,10,11]. Animals

lacking a blastema (devoid of blastema) have an incomplete regenerative potential, thus emphasizing the importance of blastema formation during regeneration [26]. Currently, the signaling pathways that regulate the blastemal proliferation and differentiation during regeneration are not completely known. An elegant study by Knopf et al. [27] has demonstrated the dispensable nature of FGF signaling in osteoblast dedifferentiation during zebrafish fin regeneration. In addition, our laboratory has previously shown that hedgehog (Hh) signaling does not impact the dedifferentiation process during amphibian limb regeneration [6,28]. Whether a combination of transcription and signaling factors are required to regulate these processes still remains unclear.

Studies focused on regeneration indicate the involvement of multiple signaling pathways that include Notch, FGF, Shh, Wnt, and BMP, and their essential role during regeneration [6,12,29–32]. While the importance of these pathways has been demonstrated in isolation, their interactions and hierarchy are unclear. For example, both Wnt and FGF signaling are required for regeneration and Wnt signaling is upstream of FGF signaling [17,33]. These findings suggest that a coordinated interaction between signaling pathways is necessary for regeneration, but the details regarding the hierarchical relationship between pathways are not currently known. Our laboratory has previously demonstrated the coordinated interaction between Hh and Wnt signaling pathways during newt limb regeneration [6]. Whether a similar signaling mechanism regulates the regeneration of other appendages such as the tail is unknown as they differ in tissue architecture and anatomy. Hh signaling is known to regulate the tissue expansion and patterning during regeneration of other tissues [6,11]; however, whether it functions in a coordinated manner with other signaling pathways remains unclear.

In this study, we have defined the essential role of Hh signaling during tissue patterning and expansion following tail amputation. We demonstrate that continuous activation of Hh signaling is crucial for regeneration, and, Hh signaling is key in regulating tissue patterning during the first phase of tail regeneration. Furthermore, we provide evidence that both Hh and Wnt signaling are critical for regeneration and function in a coordinated manner. Overall, these studies provide insight into the mechanisms that govern tail regeneration.

Experimental Procedures

Animals and surgery

All experiments were performed according to Institutional Animal Care and Use Committee (IACUC) guidelines, University of Minnesota. Adult red-spotted newts, *Notophthalmus viridescens*, were obtained from Connecticut Valley Biological, maintained in an aquarium at 18°C–20°C, and fed on red blood worm slurry twice a week. For the tail regeneration studies, animals were anesthetized in a solution of 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS-222), pH 7.2, for 10 min. Following tail amputation, each animal was treated overnight in an aqueous solution of 0.5% sulfamerazine solution for recovery and subsequently placed in a specified aqueous environment. At distinct time periods, animals were sacrificed and tissues were collected for further analysis.

Inhibitor/activator treatment and imaging

Following the recovery period from anesthesia, the animals with amputated tails were exposed to dimethyl sulfoxide (DMSO), cyclopamine (CyA; 2 µg/mL), an Shh agonist (SAG; 5 µg/mL), a Wnt activator (BIO; 150 nM), or a Wnt inhibitor (IWR-1-endo; 2.5 µM) alone or in combination. These doses have been shown by our laboratory and others to promote and antagonize the respective pathways [6]. The aquarium water containing activators or inhibitors was changed daily throughout the experimental procedures. The stock solutions of the respective activators or inhibitors were diluted in aquarium water to prepare working solutions; animals were placed in the respective aquariums containing small molecules or DMSO alone. For imaging and photography, animals from each treatment group were anesthetized at specified time points and placed under a 0.5× objective (Zeiss, SteREO, Discovery 2.0), and imaged using AxioVision Rel 4.8 software. Regenerating tail images obtained were arranged and processed using Adobe photoshop software CS6.

RNA isolation and quantitative real-time PCR

Regenerating tissues were collected at specified time points and total RNA was isolated using the mirVANA RNA Isolation Kit (Thermo Fisher Scientific). RNA samples were treated with proteinase K and DNase 1 digestion before the reverse transcription step for complementary DNA preparation using Superscript III first-strand Synthesis SuperMix Kit (Invitrogen). The transcript levels were analyzed using SYBR Green PCR Master Mix (Applied Biosystems, USA). Lists of primers used are provided in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Histological analysis

To undertake a histological analysis, animals were euthanized at specified time periods and tissues were immersion fixed in 4% paraformaldehyde, decalcified, infiltrated, and embedded in paraffin blocks. Tissue sections (10 µm thick) were obtained and hematoxylin and eosin staining was performed as previously described [34]. Stained sections were imaged using light microscopy (Leica, AxioPlan2) with AxioVision Rel 4.8 software.

Skeletal analysis

Alcian blue and alizarin red staining to evaluate cartilage and bone were performed as previously described [35]. Briefly, the animals were immersion fixed in 10% formalin, followed by evisceration using 0.5% potassium hydroxide (KOH) and several changes of distilled water for 48 h at room temperature. Cartilage was stained using 0.1% Alcian blue solution for 24 h, followed by washing and dehydration using absolute alcohol. The animals were then macerated in 1.0% KOH solution for at least 48 h, followed by staining in Alizarin red (0.1%) in 1.0% KOH solution for 24 h, cleared using graded series of glycerin-1% KOH solution (3:1, 1:1, 1:3), and stored in 100% glycerol.

Antibodies and reagents

Rabbit polyclonal anti-PTCH1 (1:200, Abbiotech; 200138), rabbit polyclonal anti-Smoothed (Smo) (1:200, Abcam; ab38686), mouse monoclonal anti- β -catenin (1:200, SantaCruz Biotechnology; sc-7963), rat monoclonal anti-BrdU (1:200, Abcam, Cambridge; ab6326), and goat polyclonal anti-PCNA (1:100, SantaCruz Biotechnology; sc-9857) sera were used as primary antibodies. Alexa-488- (1:300, Molecular Probes; anti-Rat 488: A-11006) and Cy3-conjugated (1:300, Jackson ImmunoResearch laboratories; anti-mouse Cy3: 715-165-150; anti-goat Cy3: 705-165-147, and anti-rabbit Cy3: 711-165-152) sera were used as secondary antibodies for immunofluorescence studies. Vectashield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories. Sulfamerazine, ethyl 3-aminobenzoate methanesulfonate salt (MS-222), was purchased from Sigma-Aldrich, CyA was purchased from LC laboratories, and purmorphamine (Shh agonist) and IWR-1-endo (Wnt inhibitor) were purchased from EMB Biosciences, Inc. BIO (Wnt activator) was purchased from Tocris.

Immunohistochemistry

Tissues were processed for immunohistochemical analysis as described previously [6,36–39]. The harvested tissues were snap frozen in isopentane supercooled with liquid nitrogen. Frozen sections (10 μ m thick) were thawed at room temperature and immediately fixed in acetone/methanol (1:1) for 10 min at -20°C . Sections were blocked with 20% normal goat serum in phosphate-buffered saline with 0.1% Triton X-100 (PBST) for 1 h at room temperature. For β -catenin staining, frozen sections were washed with PBS for 5 min followed by incubation with antigen retrieval solution (DAKO) for 20 min at 95°C . These sections were cooled to room temperature for further processing. Subsequently, sections were incubated with specific primary antibodies at 4°C overnight and then incubated with the appropriate secondary antibodies for 1 h at room temperature. The immunostained sections were co-stained with DAPI (nuclear stain) and coverslipped with Vectashield mounting medium (Vector laboratories).

Statistical analysis

Data represent the average from multiple animals and shown as mean $\pm$ standard error of the mean. Student's *t*-test (unpaired and two-tailed variants) and one-way analysis of variance (multiple comparison) were used to determine the statistical significance of individual datasets using Excel and GraphPad Prism6 software. Differences were considered significant with $P < 0.05$.

Results

Members of the Hh and Wnt signaling pathways are upregulated following tail amputation

Following resection (or severe injury), the adult newt has a remarkable capacity to regenerate and completely restore the injured or amputated tail [9,28]. To enhance our understanding of the mechanisms that govern regeneration, we

examined the newt tail following resection and analyzed the signaling mechanisms involved in this process. We amputated the tail and allowed it to regenerate for a defined period of time. The day of amputation was considered 0 day postamputation (dpa). Our morphological analyses revealed that newts could successfully mount a regenerative response following amputation and restore the cellular architecture of the appendage (Fig. 1A, B). Bone and cartilage staining revealed the presence of a segmental skeletal architecture of the regenerating tail at 60 dpa (Fig. 1A). A number of signaling pathways in various tissues have been previously described during the regenerative process in lower organisms [4,5,29,40]. We directly interrogated the expression of Hh signaling and Wnt signaling members to evaluate their role during regeneration. Both of these pathways have been documented during limb and fin regeneration [28,29]; however, their functional role(s) have not been completely examined in the regenerating tail following amputation. Using quantitative PCR (qPCR), we observed increased expression of Hh signaling members such as *sonic hedgehog* (*shh*), *Indian hedgehog* (*ihh*), and their cognate effectors, *patched1* (*ptc-1*) (Fig. 1C–E), in the regenerating tissue. Similarly, we found an increased expression of canonical Wnt signaling pathways, including *wnt3a* morphogens, their receptors *frizzled-1* (*frzd-1*) and *frzd-2*, and effectors such as β -catenin and *axin2* transcripts in the regenerating tail (Fig. 1F–L). The levels of these transcripts were induced as early as 7 dpa and remained elevated until 21 dpa, after which their levels showed a significant decrease at 60 dpa of regeneration. qPCR analysis for the noncanonical Wnt signaling pathway such as *wnt5b* transcripts showed no change until 21 dpa, after which its level increased at 60 dpa in the regenerating tail tissue (Fig. 1I). Furthermore, our quantitative data revealed that the levels of canonical Wnt signaling morphogens such as *wnt3a* and their effector β -catenin were higher than the levels of canonical Hh signaling morphogens *shh* and *ihh*, and their effector *ptc-1* transcripts at both 7 dpa and 21 dpa (Fig. 1C–G). Next, we performed immunohistochemical analysis for Smo and β -catenin using the regenerating tail tissue. Consistent with the qPCR results, our confocal microscopic analysis revealed robust expression of these factors in the regenerating tail following injury (Fig. 2A–H). These results supported an active role for Hh and Wnt signaling pathways throughout the regeneration process.

Hh signaling plays an important role in tissue patterning during the early stages of tail regeneration

To evaluate the requirement of Hh signaling during tail regeneration, we ablated Hh signaling using the potent chemical inhibitor, CyA. CyA blocks Hh signaling by antagonizing the Hh receptor Smo [41]. Previously, we demonstrated that CyA effectively blocks limb regeneration in the newt [6]. Under similar settings, we demonstrated that, while the untreated newts regenerated the amputated tail, continuous inhibition of Hh signaling using CyA (2 $\mu\text{g/mL}$) resulted in retarded regeneration with spike-like structure formation (perturbed patterning) (Fig. 3A, B). To further validate these findings, we treated the newts following tail amputation with an Hh agonist (SAG) to determine whether

FIG. 1. Hh and Wnt signaling are upregulated during tail regeneration. (A) Whole mount image of the regenerating newt tail following amputation at multiple time points. Skeleton staining revealed the bone and cartilage in the regenerates. (B) Quantitative analysis of the regenerating tail between 7 and 60 dpa. (C–L) Quantitative real-time PCR analysis of *shh*, *ihh*, *ptc-1*, *wnt3a*, β -catenin, *axin2*, *wnt5b*, *frizzled-1* (*frzd-1*), *frzd-2*, and *frzd-5* transcripts using RNA isolated from the uninjured and regenerating tail tissue at 7, 21, and 60 dpa. *ef1 α* was used as a loading control. Note the increased abundance of *shh*, *ihh*, *ptc-1*, *wnt3a*, β -catenin, *axin2*, *frzd-1*, and *frzd-2* transcripts in the regenerates compared to the uninjured tissue. Dotted lines indicate the point of amputation. Error bars indicate SEM ($n=10$; $*P<0.05$). Scale bar: 1000 μ m. dpa, day postamputation; Hh, hedgehog; SEM, standard error of the mean.

activation of Hh signaling could promote tail regeneration. As hypothesized, treatment with SAG resulted in significant enhancement of tail regeneration, when compared to control, with the presence of bone and cartilaginous tissue at 60 dpa (Fig. 3A, C). These results indicated that continuous activity of the Hh signaling pathway is crucial for tail regeneration.

Hh signaling regulates blastema patterning at the initial stages of regeneration

Hh signaling is known to be involved in tissue patterning during embryonic development [42]. We and others have previously shown that the *shh* gene is transcribed early during limb regeneration and regulates anterior–posterior (AP) patterning [6,32]. Based on these reports, we investigated whether Hh signals could regulate the distinct regenerative stages during tail regeneration. Our data indicated that inhibition of Hh signals with CyA from only 0–21 days (0–21 dpa) re-

sulted in spike-like structure formation (Fig. 4A, B; Table 1). While the regenerative growth of the 0–21 dpa newts was not significantly different from the untreated control group, the formation of a spike-like structure demonstrated a perturbed patterning. On the other hand, inhibition of Hh signaling between 21 and 60 dpa resulted in significant growth reduction, but had no effect on the patterning outcome (Fig. 4A, B; Table 1). Importantly, our skeletal analysis revealed the presence of cartilage (blue), but not bone (red) structures in the regenerating tissue in both 0–21 dpa and 21–60 dpa treatment conditions. Next, we inactivated the Hh signaling for distinct time periods followed by no treatment to define its requirement during the early phase of regeneration. Our data indicated that inhibition of Hh signaling between 0 to 7 dpa (0–7 days) and 0 to 14 dpa (0–14 days) did not alter the regenerative process and resulted in complete regeneration (Table 1). Since inhibition of Hh signaling between 0 and 21 dpa resulted in patterning defects without growth attenuation, and since inhibition of Hh signaling between 0 and 60 dpa

FIG. 2. Members of Hh and Wnt signaling pathways are upregulated in the regenerating tail. (A–D) Longitudinal sections of the regenerating tail tissues stained with anti-Smo antibody (red) showing induced expression of Smo following injury (C, D, D') compared to uninjured animals (A, B, B'). The box area is shown in (B') and (D'). (E–H) Longitudinal sections of the regenerating tail tissues stained with anti- β -catenin antibody (red) showing induced β -catenin expression following injury (G, H, H') compared to uninjured animals (E, F, F'). The box area is shown in (F') and (H'). Nuclei were stained with DAPI (blue). Dotted lines indicate the point of amputation. Scale bar: 200 μ m. Smo, smoothed.

results in growth retardation, we proposed that Hh signaling is important for tissue patterning during early time periods, and it is required for tissue expansion throughout the regenerating process. These findings showed that Hh signals are required for the tissue patterning at the early stages of regeneration.

Hh signaling modulates the proliferative response during regeneration

Having established the role of Hh signaling during the early stages of regeneration, we examined whether the perturbed regeneration was due to cell death or defective cellular proliferation. We performed the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using control and CyA-treated samples to evaluate cell death. We did not find any difference between the control newts and continuously CyA-treated samples at 21 dpa, suggesting that cell death was not associated with perturbed regeneration (Supplementary Fig. S1). To monitor the impact of the Hh signaling

pathway on cellular proliferation, we performed a PCNA immunostaining assay using the amputee from control and CyA-treated newts. We found a robust proliferative response at the site of injury at 21 dpa in the control tissues (Fig. 5A, A', C). In contrast, we observed that the PCNA-positive cells were reduced in CyA-treated tissues (Fig. 5B, B', C). We then tested the proliferation index by pulsing with BrdU (100 μ g/gm) intraperitoneally for 48 h before sacrifice and performed immunohistochemistry in the regenerating tissues. Similar to PCNA incorporation studies, the analysis of BrdU-pulsed regenerates revealed decreased cellular proliferation (BrdU-positive nuclei) in the CyA-treated tissue (Fig. 5E, E') compared to the control tissue (Fig. 5D, D'). Furthermore, continuous inhibition of Hh signaling resulted in significantly reduced BrdU incorporation from $11 \pm 3\%$ to $5 \pm 2\%$ ($P < 0.05$) at 21 dpa and $16 \pm 3\%$ to $7 \pm 2\%$ ($P < 0.05$) at 42 dpa (Fig. 5F). Our results demonstrated that Hh signaling has a crucial role in cellular proliferation during regeneration and Hh signaling is continually required throughout the tail regeneration process for regenerative growth.

FIG. 3. Hh signaling is essential for tail regeneration. (A) Morphological and skeletal analysis from the regenerates treated with control, CyA and SAG. Blue and red staining (skeleton) revealed the presence of bone and cartilage in the regenerates. (B) Quantification of the tail regenerates from control (black) and CyA (white)-treated animals. Note the significant perturbation of regeneration upon inhibition of Hh signaling. Note the spike-like structure formation in CyA-treated animals. (C) Quantification of the tail regenerates from control (black) and SAG (gray)-treated animals. The boxed area is magnified and shown in the right panel. Note the significant enhancement in the regenerates upon activation of Hh signaling. Dotted lines indicate the point of amputation. Error bars indicate SEM ($n=7$; $*P<0.05$). Scale bar: 1000 μm . CyA, cyclopamine; SAG, shh agonist.

Hh and Wnt signaling function in a coordinated manner to regulate tail regeneration

Multiple studies have demonstrated the functional role of the Wnt signaling pathway in the regulation of cellular proliferation [17]. Having established the role of Hh signaling during regeneration, we next modulated the Wnt signaling pathway using the small molecule activator (BIO) and inhibitor (IWR-1-endo [WI]) to define its role and interaction with Hh signaling during the regenerative response. To monitor the effectiveness of these small molecule-

mediated activation/inhibition, we performed qPCR analysis for Wnt signaling members at 7 dpa following the continuous treatment of regenerating animals with DMSO, BIO, or IWR-1-endo. qPCR analysis revealed that BIO-mediated activation of Wnt signaling resulted in increased expression of Wnt signaling members, including *wnt2b*, *wnt3a*, and β -catenin transcripts, whereas IWR-1-endo-mediated inhibition resulted in decreased expression of Wnt signaling effectors, including *axin2* and β -catenin transcripts, relative to the control tissues (Supplementary Fig. S2A–E). In these settings, our morphological analysis demonstrated that selective activation of

FIG. 4. Temporal inhibition of Hh signals affects dorsoventral patterning during tail regeneration. (A) Morphological and skeletal analysis from the regenerates treated with CyA from 0 to 21 days (0–21 dpa) followed by no treatment (top panel) and the bottom panel represents the regenerates treated with CyA from 21 to 60 days (21–60 dpa). Blue and red staining revealed the presence of bone and cartilage in the regenerates. (B) Quantification of the average length of the regenerating tail from the different treatment groups. Note the presence of defective patterning in the animals treated with CyA from 0 to 21 dpa. Dotted lines indicate the point of amputation. Error bars indicate SEM ($n=6$; $*P<0.05$). Scale bar: 1000 μm .

TABLE 1. REQUIREMENT OF HEDGEHOG SIGNALS DURING TAIL REGENERATION

	Regeneration status				n	% Regeneration
	Regenerating bud	Partial regeneration	Complete regeneration	No regeneration		
Control	10	0	10	0	10	100
SAG	7	0	7	0	7	100
CyA						
0–60 dpa	9	6 (Spike)	0	3	9	66.6
0–7 dpa	6	0	6	0	6	100
0–14 dpa	7	1	6	0	7	100
0–21 dpa	6	5 (Spike)	1	0	6	100
21–60 dpa	6	0	6	0	6	100

CyA, cyclopamine; dpa, day postamputation; SAG, shh agonist.

β -catenin-dependent Wnt signaling led to enhanced tissue regeneration at 60 dpa (Fig. 6R) compared to the control (Fig. 6O) at 60 dpa, whereas IWR-1-endo-mediated inhibition of β -catenin-dependent Wnt signaling resulted in perturbed tissue regeneration (Fig. 6S) relative to control (Fig. 6O) at 60 dpa. Since we found that both Hh and Wnt signaling were critical for regeneration, we next examined their interaction and the hierarchical positioning between the two pathways during regeneration. To examine their hierarchical positioning, we utilized an inhibitor–activator combinatorial approach, whereby, we pharmacologically blocked one pathway, while the other was activated. We treated the

regenerating newts with CyA alone (Fig. 6C, J, Q) or in combination with BIO to activate the Wnt pathway in the background of Hh inhibition (Fig. 6G, N, U). As previously described, morphological analysis revealed that both control and BIO-treated groups regenerated completely (Fig. 6O, R; Table 2), whereas CyA alone resulted in retarded regeneration with spike formation (Fig. 6Q; Table 2) at 60 dpa. Interestingly, activation of Wnt signaling in the background of Hh inhibition was unable to rescue the regenerative response of the CyA-treated amputated tail (Fig. 6Q, U, V). Next, we undertook an activator–inhibitor approach using SAG alone or in combination with IWR-1-endo (WI) to inhibit

FIG. 5. Hh signaling is required for cellular proliferation during regeneration. (A, B) Longitudinal sections of the regenerating tail tissues at 21 dpa stained with anti-PCNA antibody (red) showing reduced cellular proliferation in CyA-treated animals (B, B') compared to control animals (A, A'). The box area is shown in (A') and (B'). (C) Quantitative analysis of the percentage of the PCNA-positive nuclei in control (white bar) and CyA-treated (black bar) tissues at 7, 21, and 42 days of regeneration. (D, E) Longitudinal sections of the regenerating tail tissues at 21 dpa stained with anti-BrdU antibody (green) illustrating reduced proliferation in CyA-treated animals (E) compared to control animals (D). The box area is shown in (D') and (E'). (F) Quantitative analysis of the percentage of the BrdU-positive nuclei in control (white bar) and CyA-treated (black bar) tissues at 7, 21, and 42 days of regeneration. Nuclei were stained with DAPI (blue). Dotted lines indicate the point of amputation. Error bars indicate SEM ($n=4$; $*P<0.05$). Scale bar: 200 μ m. DAPI, 4', 6-diamidino-2-phenylindole.

FIG. 6. Wnt signaling functions upstream of Hh signaling during tail regeneration. (A–U) Whole mount analysis of the regenerating tails at 21, 42, and 60 dpa from control (A, H, O), SAG (B, I, P), CyA (C, J, Q), BIO (D, K, R), WI (E, L, S), SAG+WI (F, M, T), and CyA+BIO (G, N, U). (V) Quantitative analysis of the regenerating tails from control and CyA-, SAG-, BIO-, WI-, SAG+WI-, and CyA+BIO-treated regenerates at 21 and 60 dpa. Dotted lines indicate the point of amputation. Error bars indicate SEM ($n=6$; $*P<0.05$). #Shows the significance between control and IWR-1-endo (WI)-treated animals. Scale bar: 1000 μ m.

the Wnt pathway in the background of Hh activation. As described previously, treatment with SAG alone (Fig. 6P) resulted in significant promotion in regeneration relative to control (Fig. 6O) at 60 dpa, whereas IWR-1-endo treatment (Fig. 6S) led to a significantly attenuated regenerative response compared to the control (Fig. 6O). Interestingly, we found that in these settings, there was partial rescue of tail

regeneration in the newts treated with SAG and IWR-1-endo (SAG+WI) (Fig. 6T, V) relative to IWR-1-endo alone treatment (Fig. 6S, T, V) at 60 dpa. The regenerative ability of these newts was higher compared with IWR-1-endo alone treated group (Fig. 6V). To examine whether the activation of Wnt signaling could modulate the Hh signaling pathway, we performed qPCR analysis using

TABLE 2. HIERARCHICAL POSITIONING OF HEDGEHOG AND WNT SIGNALING DURING TAIL REGENERATION

	Regeneration status				n	% Regeneration
	Regeneration bud	Partial regeneration	Complete regeneration	No regeneration		
Control	10	0	10	0	10	100
CyA	9	6 (Spike)	0	3	9	66.6
SAG	7	0	7	0	7	100
BIO	6	0	6	0	6	100
IWR-1-endo (WI)	7	5	0	2	7	71.4
CyA+BIO	6	4	1	1	6	83.3
SAG+WI	6	5	0	1	6	83.3

FIG. 7. Wnt signaling modulates Hh signaling pathway during tail regeneration. (A, B) Quantitative PCR analysis of *ihh* and *ptc-1* transcripts using RNA isolated from the DMSO- and BIO-treated regenerating animals at 7 dpa. *RPL27* was used as a normalization control. Note the increased abundance of *ihh* and *ptc-1* transcripts in BIO-treated animals compared to controls. (C) Longitudinal sections of the regenerating tail tissues stained with anti-Ptc-1 antibody (red) showing induced expression of Ptc-1 in BIO-treated animals compared to controls. (D) Schematic depiction of the convergence of Hh and Wnt signaling during tail regeneration. Both Hh and Wnt signaling pathways are activated in response to amputation injury. Based on our data, we hypothesize that the activation of Hh signaling could partially rescue the Wnt inhibition phenotype. However, other unknown upstream regulators of the Hh signaling pathway cannot be ruled out. Data points represent average lengths of the regenerate from all experiments. Error bars indicate SEM (* $P < 0.05$). Scale bar: 200 μ m. DMSO, dimethyl sulfoxide.

RNA isolated from DMSO- and BIO-treated regenerating tissue. We found an increased expression of *ihh* and *ptc-1* transcripts in the BIO-treated animals compared to control animals (Fig. 7A, B). To verify these findings, we performed immunohistochemical analysis for Ptc-1 using the DMSO- and BIO-treated regenerating tissue. Our fluorescence microscopic analysis revealed an enhanced expression of Ptc-1 following BIO treatment compared to control tissue (Fig. 7C). Collectively, these results support the notion that Wnt signaling acts upstream of Hh signaling during tail regeneration.

Discussion

Mammals have a limited capacity to regenerate owing to a reduced proliferative capacity of the mature differentiated cells [1,43]. Multiple attempts have been made to enhance the mammalian regenerative potential, but limited successes have been achieved to date [44–47]. In contrast, lower vertebrates such as the newt can regenerate efficiently in response to severe injury [48]. The definition of factors and signaling

pathways that promote regeneration in these lower organisms may serve as a platform for regenerative therapies following injury in mammalian tissues. In this study, we defined the essential role of Hh and Wnt signaling pathways during tail regeneration. We further established the interaction between the Hh and Wnt signaling pathways during the regenerative process.

During regeneration, a variety of signaling pathways, including FGF, Notch, Wnt, and BMP signals, are activated [6,12,17,30,31]. All of these pathways have been shown to regulate the regenerative response in various tissues in both zebrafish and newt [29,48]. How these signals interact with each other during this process remains unclear. Multiple lines of evidence indicate that dedifferentiation of the pre-existing cells is the major cellular source that promotes regeneration following injury [24]. Whether these pathways have any role during the dedifferentiation process is unknown. We and others have shown the dispensable nature of Hh and FGF signaling for dedifferentiation [6,27]. Therefore, the identification of additional transcriptional or

signaling factors may facilitate our ability to induce dedifferentiation in mammalian lineages. In this study, we have made several fundamental discoveries using the newt as a model organism during tail regeneration. First, we have demonstrated the necessity of both Hh and Wnt signaling during tail regeneration. During embryonic development, Hh signals are known to modulate the AP patterning in the limb [42]. A similar role for Hh signaling in AP patterning is also documented during limb regeneration [6]. However, in this study, we found that Hh signaling functions differently for tail regeneration. This differential role may be a result of distinct tissue types, architecture, and anatomy. For example, continuous inhibition of Hh signaling completely abolished the limb regeneration [6], but during tail amputation injury, Hh inactivation led to spike-like structure formation and defective dorsoventral (DV) patterning. The DV patterning defect was confined to the early stage of regeneration, as evident from the temporal inhibition (0–21 days) of Hh signaling. We observed that both early and late CyA treatment appears to have normal cartilaginous tissue, but diminished bone formation. This could be due to an already established role for Hh signaling in the regulation of bone formation and osteogenesis during development and regeneration. An elegant study by Cordero et al. [49] demonstrated that the craniofacial malformations in avian embryos due to CyA-mediated temporal inhibition of Hh signaling resulted in multiple defects, including hypotelorism, midfacial hypoplasia, and facial cleft. These defects were associated with molecular reprogramming of an organizing center whose activity controls outgrowth and patterning of midface and upper face [49]. The defective bone formation in the early (0–21 dpa) CyA treatment could be due to blockade or delayed formation of the bone organizing center in the regenerating tail tissue. Furthermore, we demonstrated that the treatment of tail-amputated newts with the Hh agonist (SAG) resulted in significant enhancement of tail regeneration compared to control, with the presence of bone and cartilaginous tissue at 60 dpa. However, we further noticed that these regenerates did not reach a level of unamputated tails (1.5 cm) even after SAG treatment for 60 dpa. Nonetheless, our studies provide a clear understanding about the initial cues and signaling mechanism in the initiation of the regeneration process in these model organisms. Based on these findings, we believe that Hh signaling is crucial for cellular proliferation throughout the regenerative period, and Shh morphogens are required early on and transiently for patterning.

Genetic mouse models as well as pharmacological inhibition/activation studies have demonstrated the important developmental role of Hh signaling [42,50]. In the developing limb bud, Shh-Gremlin1-FGF feedback loop controls the proliferative response of the mesenchymal cells [51]. We found a conserved role of Hh signals in the regulation of cellular proliferation during tail regeneration. Whether the proliferative defect upon inhibition of Hh signals was due to failure to activate the proliferative program of the dedifferentiated cells or recruitment of resident stem cell progenitors is not clear from this study. Future experiments are required to completely interrogate the mechanistic details that regulate tail regeneration.

Previous studies in zebrafish and *Xenopus* have shown remarkable similarities in the requirement of FGF and Wnt signaling during appendage regeneration [17]. Despite structural and species differences, these signaling pathways have

conserved function during the regenerative response. As previously mentioned, other signaling pathways, including retinoic acid, Notch, Hh, and BMP signaling, are activated following injury [29,48]. Whether all these factors have a common focal point and function in a coordinated manner are unclear. In this study, we examined the roles, relationship, and point of convergence of Hh and Wnt pathways during tail regeneration. However, unlike zebrafish and *Xenopus* studies [30], we found that Wnt signaling inactivation partially blocks the regenerative response in the newt. Our data suggested that a member of the non-canonical Wnt signaling pathway, *wnt5b*, was unaltered at the earlier stages of regeneration and was enhanced at the later stage of regeneration. A study by Stoick-Cooper et al. [52], has shown that noncanonical Wnt signaling impairs proliferation of the progenitors and inhibits fin regeneration in zebrafish. Based on our data and others, we propose that the noncanonical Wnt signaling pathway may have an inhibitory role in the regenerative process following injury. We have previously shown that the Hh signaling pathway functions upstream of the Wnt/ β -catenin canonical pathway during limb regeneration [6]. To our surprise, in this study, we found the canonical Wnt signaling pathway to be upstream of the Shh pathway, where it functioned in tandem to regulate tail regeneration (Fig. 7D). Based on our data, we propose that modulation of the levels of Hh signaling members may be one of the mechanisms whereby the canonical Wnt signaling pathway impacts tail regeneration. However, the possibility of other Hh signaling regulatory mechanisms during tail regeneration cannot be ruled out. Collectively, our results defined the importance of signaling pathways during tail regeneration and determined the interaction between Hh-Wnt cascades in the modulation of tail regeneration. Furthermore, these studies emphasize the importance and value of using regenerative animal models to define regenerative mechanisms following injury.

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Author Disclosure Statement

The authors declare that they have no conflict of interests with the contents of this article.

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