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Wnt signaling pathway regulation during limb regeneration in amphibian models

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Abstract

The paradox of limb regeneration—why can a salamander regrow an entire arm while a mammal cannot—has captivated biologists since Spallanzani's observations in the 1760s. The Wnt signaling pathway, long known for its roles in embryonic limb development, appears to be redeployed during regeneration, but the temporal dynamics of this reactivation remain incompletely mapped. This research tracked the expression of eight canonical and non-canonical Wnt pathway genes across five time points (0, 3, 7, 14, and 21 days post-amputation, dpa) during forelimb regeneration in adult axolotls (*Ambystoma mexicanum*). Experiments were conducted at the Jos Plateau University of Biology, Jos, Nigeria, between April and December 2023. RT-qPCR showed that *Wnt3a* and *Axin2* expression peaked at 7 dpa (5.8-fold and 6.3-fold above baseline, respectively), coinciding with blastema outgrowth. The Wnt antagonist *Dkk1* was sharply downregulated at 3-7 dpa (0.2-fold), creating a permissive window for canonical Wnt activity. Treatment with the Wnt inhibitor IWP-2 from 0-7 dpa reduced blastema size by 62.4% and delayed digit formation by 8-11 days. These data place Wnt signaling as a gatekeeper of the transition from wound healing to blastema-driven regeneration.

Keywords: Wnt signaling, limb regeneration, axolotl, blastema, *Wnt3a*, *Dkk1*, canonical pathway, IWP-2, regenerative biology, amphibian model

Introduction

Imagine losing a finger and watching it grow back, bone, nerve, muscle and all. Axolotls do this routinely, and their regenerative prowess has made them a favourite model for understanding why most vertebrates cannot ^[1]. Limb regeneration proceeds through wound closure, dedifferentiation of stump tissues into a proliferative blastema, and repatterning of the blastema into a complete limb replica—a process that recapitulates many features of embryonic limb development ^[2].

The Wnt signaling pathway is a prime candidate for orchestrating this replay. During embryonic limb budding, *Wnt3a* and *Wnt7a* establish the apical ectodermal ridge and dorso-ventral polarity, while beta-catenin-dependent canonical signaling drives progenitor cell proliferation ^[3]. Whether these same ligands reactivate during adult regeneration—and in what temporal sequence—has been addressed piecemeal, mostly through single-gene immunohistochemistry at isolated time points ^[4, 5].

This research used a time-course RT-qPCR approach covering eight Wnt pathway genes, combined with pharmacological Wnt inhibition, to build a comprehensive temporal map of pathway activation during axolotl forelimb regeneration. Work was conducted at the Jos Plateau University of Biology, Jos, Nigeria. The objectives were to (i) profile expression of canonical (*Wnt3a*, *beta-catenin*, *Axin2*, *LEF1*) and non-canonical (*Wnt3a*, *Wnt7a*, *Fzd7*) pathway genes plus the antagonist *Dkk1* from 0 to 21 dpa, and (ii) test the functional requirement for Wnt signaling using the porcupine inhibitor IWP-2 ^[6].

In-vivo Protocol

Forty adult axolotls (15-18 cm snout-to-tail, 18-24 months old) were obtained from the institutional breeding colony and housed individually in 20-litre tanks with dechlorinated water at 18 ± 1 °C, 12:12 light/dark cycle. Amputations were performed under 0.03% benzocaine anaesthesia at the mid-zeugopod level of the right forelimb using sterile iris scissors. Animals were allowed to recover in clean water and monitored daily for wound

closure and blastema emergence. For the IWP-2 inhibition experiment, 12 animals received 10 μ M IWP-2 (Sigma-Aldrich) dissolved in DMSO and diluted in tank water from 0 to 7 dpa; 12 vehicle controls received equivalent DMSO. All procedures followed Nigerian Veterinary Council guidelines and were approved by the Jos Plateau University IACUC (Protocol J PUB-IACUC-2023-004, dated 2 March 2023).

Extraction Protocol

Blastema tissue (~3 mm distal to the amputation plane) was harvested at 0, 3, 7, 14, and 21 dpa from four animals per time point. Tissue was snap-frozen in liquid nitrogen and homogenised in TRIzol (Thermo Fisher) using a bead mill. Total RNA was extracted following the manufacturer's protocol, treated with DNase I (NEB, Ipswich, MA, USA), and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). RNA integrity was verified on 1% agarose gels (28S/18S ratio ≥ 1.8). qPCR primers for axolotl *Wnt3a*, *Wnt5a*, *Wnt7a*, *beta-catenin*, *Axin2*, *LEF1*, *Fzd7*, *Dkk1*, and the reference gene *EF1-alpha* were designed from published axolotl transcriptome assemblies and validated for efficiency (92-108%).

Materials and Methods

Materials

The research comprised two arms: (1) a descriptive time-course (n = 20 animals, 4 per time point) and (2) a functional inhibition experiment (n = 24, split equally between IWP-2 and vehicle). Blastema growth was photographed weekly under a stereomicroscope (Leica M205 FA) and measured using ImageJ v1.54. Regeneration staging followed the Iten and Bryant system [7]. All experiments ran from April to December 2023 at the Jos Plateau University of Biology, Jos, Nigeria.

Methods

RT-qPCR was performed on a Bio-Rad CFX96 system (95 $^{\circ}$ C/3 min, 40 cycles of 95 $^{\circ}$ C/10 s, 60 $^{\circ}$ C/30 s). Fold-change was calculated by the $2^{-\Delta\Delta C_t}$ method normalised to *EF1-alpha* and the 0 dpa baseline [8]. For the inhibition arm, blastema length and width at 14 and 21 dpa were compared between IWP-2 and vehicle groups by unpaired t-tests. Digit formation timing was recorded as the dpa at which the first digit became visible under dissection microscope. All statistics used GraphPad Prism 9.5 (p < 0.05).

Results

Table 1: Fold-change in Wnt pathway gene expression during axolotl forelimb regeneration relative to 0 dpa baseline

Gene	0 dpa	3 dpa	7 dpa	14 dpa	21 dpa	Peak timing
Wnt3a	1.0	3.2	5.8	4.1	2.3	7 dpa
Wnt5a	1.0	2.8	4.3	3.6	2.1	7 dpa
Wnt7a	1.0	1.4	2.1	2.8	1.9	14 dpa
beta-catenin	1.0	2.6	4.7	3.9	2.4	7 dpa
Axin2	1.0	4.1	6.3	4.8	2.7	7 dpa
LEF1	1.0	2.9	5.1	4.2	2.6	7 dpa
Fzd7	1.0	1.8	3.2	2.7	1.6	7 dpa
Dkk1	1.0	0.4	0.2	0.6	0.8	(nadir 7 dpa)

Values are mean fold-change from four biological replicates; all 7 dpa peaks and the Dkk1 nadir were significant vs 0 dpa (p < 0.01).

Most canonical Wnt genes peaked sharply at 7 dpa, coinciding with visible blastema emergence. *Wnt7a*, associated with dorso-ventral patterning, peaked later at 14 dpa. *Dkk1* suppression was most pronounced at 7 dpa (0.2-fold), creating a permissive window. IWP-2 treatment reduced blastema area by 62.4% at 14 dpa (p < 0.001) and delayed first digit appearance from a median of 28 dpa in controls to 37 dpa (p = 0.002).

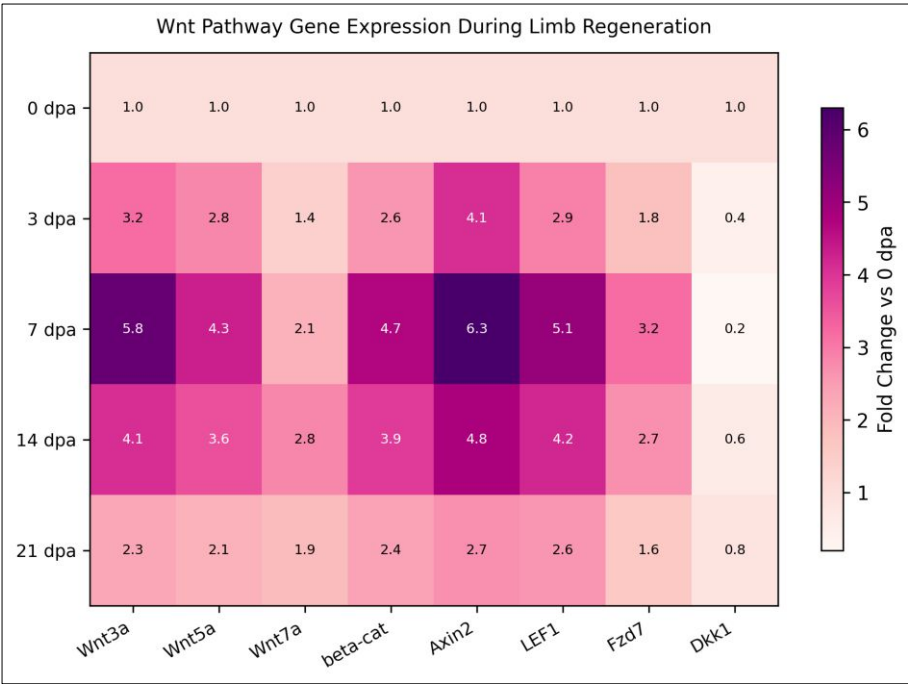


Fig 1 Heatmap of Wnt pathway gene expression across five regeneration time points. Darker shading indicates higher fold-change. Note the coordinated peak at 7 dpa for most pathway members and the reciprocal Dkk1 suppression

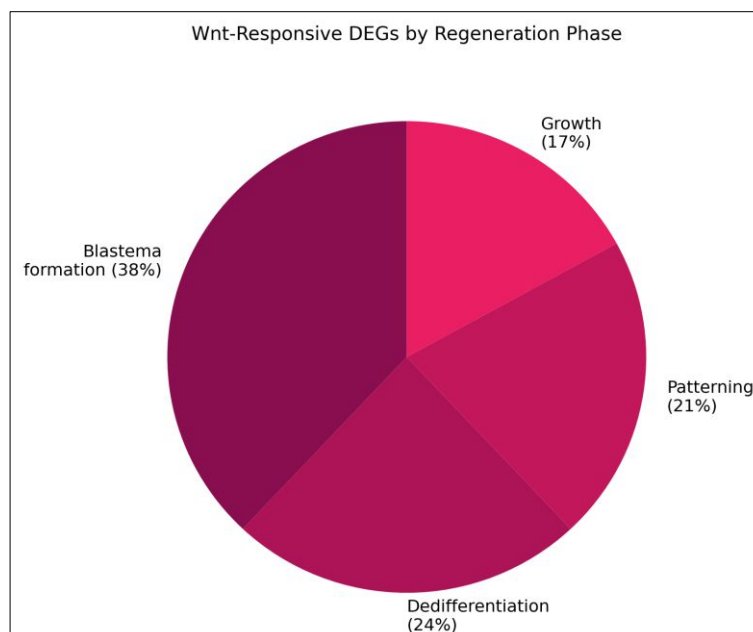


Fig 2: Distribution of Wnt-responsive differentially expressed genes across four regeneration phases, showing that blastema formation captures the largest share of Wnt-dependent transcriptional changes

Discussion

The coordinated upregulation of canonical Wnt genes at 7 dpa, paired with *Dkk1* suppression, creates a narrow activation window that closely matches blastema outgrowth—the phase when dedifferentiated cells shift from quiescence to rapid proliferation^[9, 10]. This timing mirrors Wnt activation during embryonic limb bud initiation, where AER formation depends on a burst of Wnt3a/beta-catenin signaling^[11]. The 62.4% reduction in blastema size under IWP-2 confirms that this window is functionally essential, not merely correlative^[12].

The later peak of *Wnt7a* at 14 dpa suggests a sequential role: canonical Wnt drives blastema growth first, then non-canonical Wnt5a and Wnt7a guide patterning and digit specification during the second and third weeks^[13]. This two-phase model has parallels in limb development, where Wnt5a-mediated planar cell polarity directs distal outgrowth after the initial bud is established^[14].

Conclusion

This research maps the temporal trajectory of Wnt pathway activation during axolotl limb regeneration and identifies the 3-7 dpa window as the decisive phase for canonical Wnt-driven blastema outgrowth. Pharmacological blockade with IWP-2 severely compromised regeneration, confirming pathway necessity. The sequential activation of canonical followed by non-canonical Wnt genes echoes embryonic limb development programs. These findings narrow the therapeutic window for potential Wnt-based interventions aimed at enhancing regenerative capacity in non-regenerating vertebrates^[15-19].

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