Mechanisms of Development 144 (2017) 182–190

Contents lists available at ScienceDirect

Mechanisms of Development

journal homepage: www.elsevier.com/locate/mod

Chemical activation of Wnt/β-catenin signalling inhibits innervation and causes skeletal tissue malformations during axolotl limb regeneration

Sabina Wischin [a], Cristina Castañeda-Patlán [b], Martha Robles-Flores [b], Jesús Chimal-Monroy [a,*]

[a] Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico DF 04510, Mexico
[b] Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico DF 04510, Mexico

ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form 14 November 2016
Accepted 30 January 2017
Available online 3 February 2017

Keywords:
Limb regeneration
Axolotl
Wnt/β-catenin signalling activation

ABSTRACT

Limb regeneration involves several interrelated physiological processes in which a particular signalling pathway may play a variety of functions. Blocking the function of Wnt/β-catenin signalling during limb regeneration inhibits regeneration in axolotls (*Ambystoma mexicanum*). Limb development shares many features with limb regeneration, and Wnt/β-catenin activation has different effects depending on the developmental stage. The aim of this study was to evaluate whether Wnt/β-catenin signalling activation during axolotl limb regeneration has different effects when activated at different stages of regeneration. To evaluate this hypothesis, we treated amputated axolotls with a Wnt agonist chemical at different stages of limb regeneration. The results showed that limb regeneration was inhibited when the treatment began before blastema formation. Under these conditions, blastema formation was hindered, possibly due to the lack of innervation. On the other hand, when axolotls were treated after blastema formation and immediately before the onset of morphogenesis, we observed structural disorganization in skeletal formation. In conclusion, we found that limb regeneration was differentially affected depending on the stage at which the Wnt signalling pathway was activated.

1. Introduction

The process of limb regeneration in urodeles may be divided into five stages that correspond to wound healing, dedifferentiation, blastema formation, morphogenesis and growth. During wound healing, the epithelial cells surrounding the wound migrate and cover the amputated surface; this epithelium later thickens to form the apical epidermal cap (AEC). Removal of this structure inhibits regeneration. In the phase of dedifferentiation, the cells of the distal end of the stump are released from their tissue organization and acquire a mesenchymal phenotype. These cells will proliferate and develop the blastema. The morphogenesis process begins in this tissue when cells start to redifferentiate into the corresponding tissues. Later on, the limb grows to reach the size of a complete limb, which is impossible to distinguish from one that was never amputated (Nye et al., 2003; Carlson, 2007; Stocum and Cameron, 2011).

In axolotl, *Xenopus* tadpole and zebrafish, limb regeneration is perturbed by inhibition of the Wnt/β-catenin signalling pathway. For instance, in adult axolotls, misexpression of the inhibitor of the Wnt/β-catenin pathway Axin1 results in spike-like limbs with no digits (Kawakami et al., 2006). Likewise, misexpression of the Wnt/β-catenin signalling inhibitor Dkk1 in axolotl larvae results in complete blockage of limb regeneration, as demonstrated in a few cases (Kawakami et al., 2006). In *Xenopus*, blocking the function of Wnt/β-catenin signalling by conditional expression of Dkk1 during early stages of tadpole limb regeneration inhibits this process, whereas inhibition of this signalling in froglet limb regeneration does not inhibit the usual spike formation (Yokoyama et al., 2007; Yokoyama et al., 2011). In contrast, Wnt/β-catenin signalling activation accelerates fin regeneration in zebrafish, presumably by promoting cell proliferation (Stoick-Cooper et al., 2007).

Limb regeneration has been described as a biological process that shares many similarities with limb development (Gardiner et al., 1998; Roy and Gardiner, 2002). Notably, during limb development, Wnt/β-catenin signalling controls limb bud initiation, outgrowth, patterning and morphogenesis (Geetha-Loganathan et al., 2008). It plays an essential role in establishing and maintaining the apical ectodermal ridge (AER) (Barrow et al., 2003; Hill et al., 2006), a structure very similar to the AEC (Mullen et al., 1996; Carlson et al., 1998; Christensen and Tassava, 2000), and, as with the AEC, its removal truncates limb development (Saunders, 1948; Tschumi, 1957). On the other hand, stabilization of β-catenin at early stages of limb development results in truncated limbs caused by early regression of the AER (Hill et al., 2006), while at later stages it gives rise to skeletal abnormalities (Akiyama et al., 2004). This suggests that proper limb development

* Corresponding author.
E-mail address: jchimal@unam.mx (J. Chimal-Monroy).

http://dx.doi.org/10.1016/j.mod.2017.01.005
0925-4773/

requires adequate spatial-temporal regulation of Wnt/β-catenin signalling.

Because limb regeneration is a complex process involving several physiological events at different regeneration stages, we speculated that Wnt/β-catenin activation may affect limb regeneration depending on the stage at which Wnt/β-catenin is activated. Hence, in this study, we evaluated the effect of chemical activation of Wnt/β-catenin signalling at different stages of limb regeneration. The results revealed two stages at which limb regeneration is affected by drug treatment. The first was when treatment was applied at the onset of limb regeneration before the formation of the blastema. This resulted in a lack of innervation and regeneration was inhibited. The second was when treatment was applied after blastema formation but before the onset of morphogenesis. This treatment led to skeletal disorganization. We also observed that Wnt agonist treatment increased cell proliferation at the blastema stage.

2. Experimental procedures

2.1. Animal maintenance and treatment

Axolotls (*Ambystoma mexicanum*) were obtained as small larvae or as eggs from the following investigators and laboratories: Cecilia Vanegas (Facultad de Ciencias, UNAM); Felipe Correa Sánchez (FES-Iztacala); Horacio Mena González and Luis Zambrano (Instituto de Biología, UNAM), Fernando Arana (Centro de Investigaciones Biológicas y Acuícolas de Cuemanco) and Enrique Godinez (PETMMAL). Hatched axolotls were fed with hatched artemia cysts; animals older than one month were fed with adult artemia. Axolotls measuring 5–6.5 cm were anaesthetized with 0.05% Tricaine (ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St. Louis, MO, USA), and one forelimb was amputated at mid zeugopod. One micromolar Wnt agonist (2-amino-4-(3,4-(methylenedioxy) benzylamino)-6-(3-methoxyphenyl) pyrimidine) purchased from Calbiochem (Billerica, MA, USA) was diluted in filtered tap water. The animals were placed in the solution on different days after amputation depending on the experiment. When required, the drug was replaced every other day until the end of the treatment. Control animals were treated with the vehicle dimethyl sulfoxide (DMSO). At the end of the experiment, limbs were amputated at the base of the arm and collected. For limb denervation experiments, brachial nerves were severed at 7 days post amputation (dpa). To determine the local effect of Wnt on mesenchymal cells, agonist compound AGI-×2 ionic exchange beads (Sigma-Aldrich) were soaked in 10 mM Wnt agonist and implanted with the help of a tungsten needle into the limb blastema. This research protocol was reviewed and approved by the Institutional Review Board for the Care and Use of Laboratory Animals of the Instituto de Investigaciones Biomedicas, UNAM. All experiments were carried out in accordance with the approved guidelines.

2.2. Tissue staining and skeletal preparation

For tissue staining, limbs were fixed in 4% paraformaldehyde at 4 °C overnight and then dehydrated and embedded in Paraplast (Sigma-Aldrich) using standard techniques. Tissue sections (7 μm) were stained using the Masson Trichrome technique. Limbs collected for skeleton staining were fixed in 95% ethanol and permeabilized with acetone overnight. Later, collected limbs were stained in an Alcian blue/Alizarin red solution for 3 days. Limb skeletons were cleared in 1% KOH/20% glycerol and stored in 50% ethanol/50% glycerol.

2.3. Immunohistochemistry

The following antibodies and dilutions were used: 1:400 acetylated tubulin (T7451, Sigma-Aldrich), 1:250 phospho-histone H3 (06-570, Millipore), 1:500 Cy3 anti-rabbit (111-16544, Jackson ImmunoResearch Laboratories, Inc., Sacramento, CA, USA), 1:500 anti-mouse Alexa 555 (A21426, Invitrogen, Carlsbad, CA, USA) and 1:500 anti-rabbit Alexa 488 (A21206, Invitrogen). Fluorescent immunostainings were analysed using a Fluoview FV1000 laser confocal system (Olympus) attached/interfaced to an Olympus IX81 inverted light microscope with a 20× glycerol-immersion objective. Anti-acetylated tubulin and anti-phospho-histone H3 were determined in Paraplast (Sigma-Aldrich)-embedded tissue sections at 10 μm and 7 μm, respectively.

2.4. Measurement of the mitotic index

To measure the mitotic index, we counted the number of positive cells for phospho-histone H3 out of 300 cells in the blastema and 150

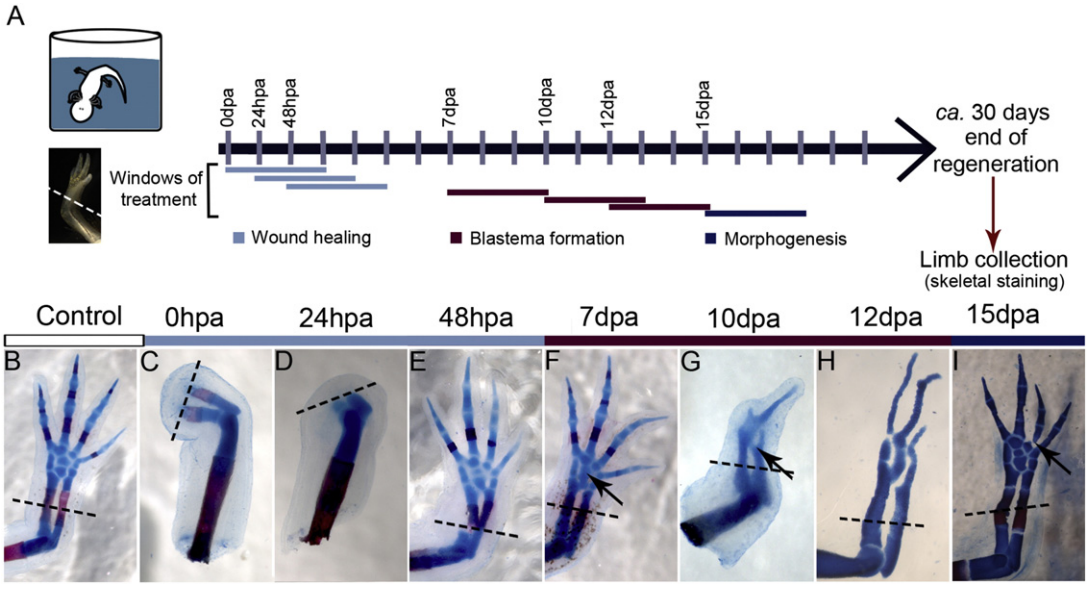

Fig. 1. Chemical activation of Wnt/β-catenin signalling affects limb regeneration at different limb regeneration stages. (A) Experimental strategy to evaluate the role of chemical activation of Wnt/β-catenin signalling at different limb regeneration stages. (B–I) Amputated animals were treated at 0, 1, 2, 7, 10, 12 or 15 days post amputation with the Wnt agonist for 3 days. Limbs were collected for Alcian blue/Alizarin red skeleton staining at approximately the same time at which controls completed limb regeneration. Arrow in F indicates fused carpal; arrow in G indicates a radius with a bifurcation. The dashed black line indicates the amputation plane.