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Lizard tail regeneration: regulation of two distinct cartilage regions by Indian hedgehog

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ABSTRACT

Lizards capable of caudal autotomy exhibit the remarkable ability to "drop" and then regenerate their tails. However, the regenerated lizard tail (RLT) is known as an "imperfect replicate" due to several key anatomical differences compared to the original tail. Most striking of these "imperfections" concerns the skeleton; instead of the vertebrae of the original tail, the skeleton of the RLT takes the form of an unsegmented cartilage tube (CT). Here we have performed the first detailed staging of skeletal development of the RLT CT, identifying two distinct mineralization events. CTs isolated from RLTs of various ages were analyzed by micro-computed tomography to characterize mineralization, and to correlate skeletal development with expression of endochondral ossification markers evaluated by histology and immunohistochemistry. During early tail regeneration, shortly after CT formation, the extreme proximal CT in direct contact with the most terminal vertebra of the original tail develops a growth plate-like region that undergoes endochondral ossification. Proximal CT chondrocytes enlarge, express hypertrophic markers, including Indian hedgehog (Ihh), apoptose, and are replaced by bone. During later stages of tail regeneration, the distal CT mineralizes without endochondral ossification. The sub-perichondrium of the distal CT expresses Ihh, and the perichondrium directly calcifies without cartilage growth plate formation. The calcified CT perichondrium also contains a population of stem/progenitor cells that forms new cartilage in response to TGF-β stimulation. Treatment with the Ihh inhibitor cyclopamine inhibited both proximal CT ossification and distal CT calcification. Thus, while the two mineralization events are spatially, temporally, and mechanistically very different, they both involve Ihh. Taken together, these results suggest that Ihh regulates CT mineralization during two distinct stages of lizard tail regeneration.

Introduction

As reptiles, lizards are positioned evolutionarily between amphibians and mammals, and their regenerative potential reflects this. Amphibians, specifically the urodeles (newts and salamanders), are able to regenerate tails and limbs. Lizards are able to regenerate their tails only, and mammals can regenerate neither tails nor limbs. (Note: Not all lizards are able to regenerate their tails. Here, lizards will refer to autotomous, regenerative lizards.) Furthermore, lizards are more closely related to mammals, both of which are classified as amniotes, and, therefore, fundamentally different in terms of life cycle and development from the anamniote, amphibian urodeles. Thus, the lizard is an amniote with developmental processes similar to that of mammals, and, hence, represents a more suitable model than the salamander model to investigate the biology of tissue regeneration (Alibardi, 2010).

Lizard tail regeneration is especially interesting in terms of cartilage regeneration. Urodeles regenerate tissues as near perfect replicas of the originals. The regenerated lizard tail (RLT), however, is known as an "imperfect replicate" due to several key anatomical differences (Bellairs and Bryant, 1985; Alibardi, 2010; Fisher et al., 2012). Most striking of these "imperfections" concerns the skeleton, which is cartilaginous in the RLT. The spinal column and vertebrae of the original tail are regenerated as a single, unsegmented cartilage tube (CT). Unique in the animal kingdom, the lizard CT is an adult organ that only exists in regenerated tissues and is maintained for the duration of the lizard's life without transitioning to bone.

The ability of the CT to resist ossification becomes even more interesting and counterintuitive considering the role cartilage typically plays in bone development. During vertebrate limb development, and during tail and limb regeneration in urodeles, bone is formed from cartilage intermediates in a process known as endochondral ossification (Iten and Bryant, 1976; Kronenberg,

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2003; Mackie et al., 2008). This well-studied process begins with mesenchymal cells condensing to from a cartilage anlage or template. As the mesenchymal cells differentiate into chondrocytes, they proliferate and begin to deposit cartilage extracellular matrix high in collagen type II (Col2) and sulfated glycosaminoglycans (GAGs) (DeLise et al., 2000; Tsang et al., 2014). Stratification of chondrocytes in various states of maturity causes formation of the growth plate, in which the most mature cells cease proliferating and undergo hypertrophy. This critical milestone in the process of endochondral ossification is typified by characteristic changes in chondrocyte morphology, including dramatic increases in cell volume, and a defined gene expression profile (Man-Ger Sun and Beier, 2014). Hypertrophic chondrocytes begin to produce a very specialized matrix consisting of collagen type X (Col10) and express alkaline phosphatase (Alk Phos), and matrix calcification is initiated (Adams et al., 2007; van der Eerden et al., 2003; Anderson et al., 2004). The hypertrophic chondrocytes also begin secreting growth factors such as bone morphogenetic protein 6 (BMP-6) and vascular endothelial growth factor (VEGF) (Zelzer et al., 2001), which induces blood vessels to sprout from the surrounding tissues. The hypertrophic chondrocytes then undergo apoptosis and are replaced by mesenchymal cells and pre-osteoblasts brought into the cartilage template through invading capillaries (Dirckx et al., 2013; Mackie et al., 2008). The remnant cartilage matrix is further cleared by invading cathepsin K-positive osteoclasts and replaced with bone matrix as mesenchymal cells differentiate into osteoblasts. Endochondral ossification concludes when the growth plate closes and the cartilage template is replaced by bone.

The behavior of growth cartilage chondrocytes is exquisitely regulated by environmental signals as they proceed through differentiation, maturation, hypertrophy, and apoptosis. One of the most important factors in regulating endochondral ossification is the molecule Indian hedgehog (Ihh), which has been called a master regulator of embryonic skeletal development (Kronenberg, 2003). Ihh is secreted by pre-hypertrophic chondrocytes as they leave the proliferative pool and, upon binding to its receptor Patched-1 (PTCH-1), activates signaling cascades that activate the expression of a number of factors, including parathyroid hormone-related peptide (PTHrP) (Adams et al., 2007; Chung et al., 2001; Lanske and Kronenberg, 1998; Vortkamp et al., 1996; St-Jacques et al., 1999). PTHrP signals through its receptor, parathyroid hormone receptor-1 (PTHR1), which is expressed at higher levels by hypertrophic chondrocytes than in proliferating chondrocytes. PTHrP signaling prevents chondrocytes from undergoing hypertrophy by keeping them in the proliferative pool (Lee et al., 1996; Kronenberg, 2006; Ohba and Chung, 2014). As the bone develops and lengthens, PTHrP produced at the ends of bones no longer reaches distant chondrocytes, which stop proliferating, undergo hypertrophy, and begin secreting Ihh. Thus, a feedback loop arises in which Ihh secreted in the hypertrophic zone signals back to apical cartilage regions to express PTHrP, thereby controlling the regions in which chondrocytes undergo hypertrophy and terminal differentiation.

The roles of these molecules in lizard tail regeneration have not been studied, and lizards are predisposed to forming cartilage that resists complete calcification and ossification. The peculiarities in skeletal development make the lizard CT without equivalent among animal models. Here we have performed the first detailed staging of skeletal development of the lizard (*Anolis sagrei*) regenerate, with special attention paid to factors known to regulate endochondral ossification. To elucidate the roles and mechanisms of several of these factors, lizard CTs were manipulated with treatments known to be either stimulatory or inhibitory to embryonic cartilage development. The cellular and molecular features here support developing the regenerated lizard tail as a novel experimental model of cartilage regeneration.

Results

The terminal vertebra of the original tail-stump is remodeled prior to autotomy

To analyze mineralization within the RLT, it became critical to accurately distinguish between original and regenerated tail skeletal elements. Thus, we began our study by characterizing the changes that occur within the original tail-stump following autotomy. Each vertebra of the original tail is separated from one another by dorsal zygapophysial joints (Z-joints) (Fig. 1A, B) and ventral intervertebral pads (IPs) (Fig. 1A, C). Like the tails of most autotomous lizards, the original *Anolis* tail exhibits intravertebral fracture planes, preformed breaks in the bone approximately halfway through each vertebrae (Fig. 1A, D, E). During autotomy, the tail separates along a fracture plane, and the tail-stump of a recently autotomized lizard tail ends with a half vertebra (Fig. 2A). A clot immediately forms over the stump, and, by two days post-autotomy (DPA), the stump tissues contract, causing the distal portion of the autotomized half-vertebra to protrude (Fig. 2B). Cathepsin K-positive osteoclasts begin to accumulate at

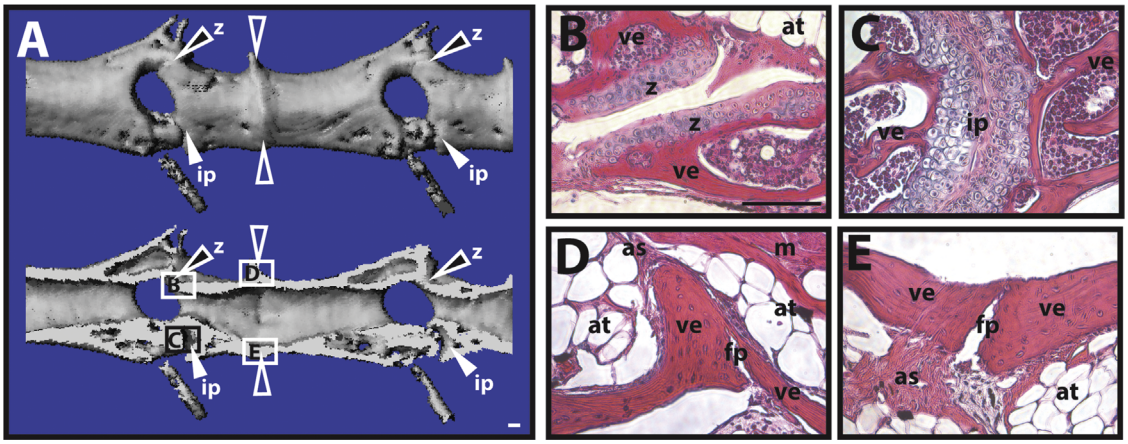

Fig. 1. The vertebrae of the original tail contain fracture planes. (A) Unsectioned (top) and sagittally sectioned (bottom) views of the same portion of original lizard tail analyzed by microCT. Open arrowheads denote intravertebral fracture plane. White-filled arrowheads mark intervertebral pads (ip). Black-filled arrowheads identify Z-joints (z). (B-E) Histological analysis (H&E) of (B) Z-joint, (C) intervertebral pad, and (D, E) fracture plane regions identified in Panel A. at, adipose tissue; as, autotomy septum; fp, fracture plane; m, muscle; ve, vertebra. Bar = 100 μm.