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The axolotl limb: A model for bone development, regeneration and fracture healing

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Abstract

Among vertebrates, urodele amphibians (e.g., axolotls) have the unique ability to perfectly regenerate complex body parts after amputation. The limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. Hence, the limb has been chosen as a model to study the process of skeletogenesis during axolotl development, regeneration and to analyze this animal's ability to heal bone fractures. Extensive studies have allowed researchers to gain some knowledge of the mechanisms controlling growth and pattern formation in regenerating and developing limbs, offering an insight into how vertebrates are able to regenerate tissues. In this study, we report the cloning and characterization of two axolotl genes; *Cbfa-1*, a transcription factor that controls the remodeling of cartilage into bone and *PTHrP*, known for its involvement in the differentiation and maturation of chondrocytes. Whole-mount in situ hybridization and immunohistochemistry results show that *Cbfa-1*, *PTHrP* and type II collagen are expressed during limb development and regeneration. These genes are expressed during specific stages of limb development and regeneration which are consistent with the appearance of skeletal elements. The expression pattern for *Cbfa-1* in late limb development was similar to the expression pattern found in the late stages of limb regeneration (i.e. re-development phase) and it did not overlap with the expression of type II collagen. It has been reported that the molecular mechanisms involved in the re-development phase of limb regeneration are a recapitulation of those used in developing limbs; therefore the detection of *Cbfa-1* expression during regeneration supports this assertion. Conversely, *PTHrP* expression pattern was different during limb development and regeneration, by its intensity and by the localization of the signal. Finally, despite its unsurpassed abilities to regenerate, we tested whether the axolotl was able to regenerate non-union bone fractures. We show that while the axolotl is able to heal a non-stabilized union fracture, like other vertebrates, it is incapable of healing a bone gap of critical dimension. These results suggest that the axolotl does not use the regeneration process to repair bone fractures.

Keywords: Urodele/axolotl; Fracture healing; Limb regeneration; Osteogenesis; *Cbfa-1*/*PTHrP*/collagen

Introduction

The ability of an adult vertebrate to regenerate lost body parts is very limited; however, urodele amphibians such as the axolotl (*Ambystoma mexicanum*) are known to have exceptional abilities to regenerate multiple body parts throughout their life. The axolotl's capacity to regenerate its limbs as an adult offers the opportunity to conduct comparative studies of the genes expressed during development and regeneration in an identical genetic background. Among the complex structures able to regenerate, the limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. During limb development, the cells that form limb tissues are derived from undifferentiated progenitor cells [50]. This process is paralleled in the case of the regenerating limb, with the exception that differentiated cells become undifferentiated before subsequently reforming the missing parts after amputation [7,50]. It has been hypothesized that the process of limb regeneration is biphasic [8,24,60]. The first phase, known as the preparation phase, involves the events

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spanning from amputation to blastema formation whereas the second phase, known as the re-development phase, is characterized by the control of growth and pattern formation within the blastema and finally the re-differentiation of cells to reform the missing limb. Experimental data collected to date support the concept that the molecular mechanisms involved in limb development are recapitulated during the re-development phase of limb regeneration [48,58,59,71]. In addition to the aforementioned advantages, the complexity of the limb provides an excellent opportunity for studying many different tissue types including bone and cartilage. Bone represents approximately 50% of the exposed surface following limb amputation; however, as little as 2% of cells in a regenerated limb are derived from bone [48]. Previous limb regeneration studies also suggest that new bone is generated from dedifferentiated cells of the blastema and not derived from pre-existing bone [41,74]. Most skeletal bones, including those of the appendage, develop via an endochondral ossification process which involves the replacement of cartilage tissue by bone. This process can be divided into five stages: (i) the commitment of mesenchymal cells to cartilage cells; (ii) the subsequent condensation of these cells and their differentiation into chondrocytes; (iii) chondrocyte proliferation; (iv) chondrocyte hypertrophy and (v) chondrocyte apoptosis and replacement by osteoblasts [30]. Among the numerous genes potentially involved in the formation of skeletal elements during limb regeneration, two of them are of particular interest; *Core-binding factor α-1* (*Cbfa-1*), a transcription factor required for mesenchymal condensation, chondrocyte hypertrophy and osteoblast differentiation [34,53] and *Parathyroid hormone related peptide* (*PTHrP*), involved in the differentiation and maturation of chondrocytes [37]. The *Cbfa-1* gene, also known as Runx2/PEBP2αA/AML3/Osf2, is characterized by a Runt domain that binds DNA and heterodimerizes with Core-binding factor β (Cbfβ) [4,51]. Studies have shown *Cbfa-1* to be an essential factor for osteoblast maturation and normal ossification [34]. However, additional research has revealed a dual action for *Cbfa-1*: first as an osteoblast differentiation factor and also as a regulator of chondrocyte maturation and differentiation. Homozygous mutations in this gene are responsible for the skeletal malformation syndrome: cleidocranial dysplasia (CCD) [46]. This dysplasia is characterized by multiple skeletal malformations and the absence of ossification [46,53]. In fact, without *Cbfa-1*, osteoblasts are unable to differentiate and therefore abolish bone matrix deposition [46]. A more recent study by Kim et al. has revealed yet another function for *Cbfa-1* as a regulator of chondrocyte hypertrophy [33]. *PTHrP* is another important player to consider when characterizing skeletogenesis during the regeneration process. *PTHrP* is a small peptide first identified as the causative agent for humoral hypercalcemia malignancy [43,67]. This peptide uses the same transmembrane receptor as *parathyroid hormone* (*PTH*); consequently they share a well conserved PTH-like domain [54]. In addition to causing hypercalcemia, *PTHrP* is a major regulator of chondrocyte maturation, differentiation and proliferation [37]. *PTHrP* is known to be expressed in chondrocytes throughout the developing epiphyses, and in osteoblasts in metaphyseal bone [2]. *PTHrP*-deficient knockout mice die prematurely due to severe osteochondrodysplasia, characterized by shorter bones due to an early ossification. This can be explained primarily by reduced proliferation and increased apoptosis of immature chondrocytes as well as the precocious hypertrophy of chondrocytes [3,32,36].

Numerous studies have demonstrated the axolotl's ability to completely regenerate lost appendages in which every tissue including bone is regenerated, yet very few have concentrated on whether the axolotl can regenerate bone in a gap or how well they can heal a fracture [25]. The bone healing process has been well characterized in numerous vertebrates [6,19,61,65,72]. Most vertebrates are capable of healing a bone fracture through a callus formation process; however, all vertebrates studied to date are unable to heal bone defects of critical dimension [62]. These defects are gaps within which bone repair does not take place. The critical dimensions are dependent on the size of the animal. For example, the critical dimension in a rabbit ulna is 15 mm, comparatively; in a rat it is 4–5 mm [11,29]. Under this circumstance, bone formation must be assisted by inductive substances [57]. We are particularly interested in the axolotl's abilities to heal and repair bone since they can regenerate entire limbs including the skeletal elements. Does their unique ability to regenerate complex tissues also apply to bone defects or fractures of critical dimension? In this article, we describe the molecular cloning, sequence analysis and expression of *Cbfa-1* as well as *PTHrP* during limb development and regeneration in the axolotl. We also characterized the expression of type II collagen, a cartilage specific protein, during limb regeneration. *Cbfa-1* is an inhibitor of type II collagen and their expression should not overlap [20]. Both *Cbfa-1* and *PTHrP* regulate essential aspects of chondrogenesis and osteogenesis in vertebrate development (e.g. mammals and birds). Given that part of the regeneration process is a recapitulation of the developmental process, the regenerating axolotl limb offers an excellent model to analyze whether or not these genes are also required for bone regeneration. Our results show that *Cbfa-1* and *PTHrP* as well as type II collagen are expressed during limb development and regeneration. Additionally, we found that particular gene expression patterns during axolotl limb development are similar to those during regeneration. Finally, we show that while the axolotl is able to heal a non-stabilized fracture, like other vertebrates, the axolotl is incapable of healing or regenerating a bone gap of critical dimension.

Materials and methods

Cloning axolotl Cbfa-1 and PTHrP cDNA

Partial axolotl *Cbfa-1* and *PTHrP* cDNA sequences were cloned (5 and 3 clones for each gene, respectively) by RT-PCR using degenerate primers (see below). To obtain the full-length *Cbfa-1* gene, the SMART RACE cDNA Amplification kit (BD Biosciences, Clontech, USA) was used to construct 5′- and 3′-RACE ready cDNA from total RNA extracted from the axolotl hind limb during the medium bud regeneration stage using Trizol reagent (Invitrogen). Following the manufacturer's instructions, PCR products were obtained using 5′- and 3′-gene specific primers (GSPs) and nested gene specific primers (NGSPs)