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Activation of germline-specific genes is required for limb regeneration in the Mexican axolotl

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ABSTRACT

The capacity for tissue and organ regeneration in humans is dwarfed by comparison to that of salamanders. Emerging evidence suggests that mechanisms learned from the early phase of salamander limb regeneration—wound healing, cellular dedifferentiation and blastemal formation–will reveal therapeutic approaches for tissue regeneration in humans. Here we describe a unique transcriptional fingerprint of regenerating limb tissue in the Mexican axolotl (*Ambystoma mexicanum*) that is indicative of cellular reprogramming of differentiated cells to a germline-like state. Two genes that are required for self-renewal of germ cells in mice and flies, Piwi-like 1 (PL1) and Piwi-like 2 (PL2), are expressed in limb blastemal cells, the basal layer keratinocytes and the thickened apical epithelial cap in the wound epidermis in the regenerating limb. Depletion of PL1 and PL2 by morpholino oligonucleotides decreased cell proliferation and increased cell death in the blastema leading to a significant retardation of regeneration. Examination of key molecules that are known to be required for limb development or regeneration further revealed that FGF8 is transcriptionally downregulated in the presence of the morpholino oligos, indicating PL1 and PL2 might participate in FGF signaling during limb regeneration. Given the requirement for FGF signaling in limb development and regeneration, the results suggest that PL1 and PL2 function to establish a unique germline-like state that is associated with successful regeneration.

Introduction

The regeneration of body tissues and organs is a widespread phenomenon among urodele amphibians, but in mammals this capacity is limited to specific tissues and early stages of life (McCusker and Gardiner, 2010; Tanaka, 2003). The Mexican axolotl (*Ambystoma mexicanum*) has an unparalleled regenerative capacity among vertebrates. Among various vertebrate tissue/organ regeneration models in axolotls, limb regeneration is the most intensively studied (Brockes and Kumar, 2005). Axolotls regrow limbs with morphological and functional intactness, thus allowing us to achieve an understanding of the mechanistic bases of tissue and organ regeneration in adult vertebrates and to further explore potent regenerative medical practices in mammals that possess much less regenerative capacity, including humans (Muneoka et al., 2008; Stoick-Cooper et al., 2007; Tanaka, 2003).

In axolotl limb regeneration, immediately following wound healing a distinct structure, known as the regenerating blastema, which is a mass of proliferating mesenchymal cells beneath the wound epidermis, arises through epithelial–mesenchymal interactions, and will eventually regrow to replace the lost limb as regeneration progresses. Blastema formation is the hallmark for epimorphic regeneration phenomena in nature, and is the first recognizable lead in the regenerative scenarios characteristic of the re-activation of embryonic genes recapitulating the events that occurred during embryonic development (Tsonis, 2008; Yokoyama, 2008). Except for the resident stem cells, the blastemal cells are heterogeneous in their origin and have been demonstrated to be largely derived from somatic cells, including multinucleated muscle cells, epidermal basal keratinocytes and fibroblasts in the dermal and connective tissues. The re-acquisition of embryonic-like developmental potential of the blastemal cells is defined as dedifferentiation

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(Echeverri et al., 2001; Muneoka et al., 1986). It is hypothesized that a large-scale genomic reprogramming is involved in this process. However, other than a dramatic upregulation of oocyte-type linker histone B4 during lens regeneration in newts and a significant upregulation of three of the four mammalian stem cell pluripotency-inducing factors, Sox2, Klf4, and c-Myc in both lens and hindlimb regeneration in newts, no further evidence for reprogramming has been reported (Maki et al., 2010, 2009).

In addition to the initiation of cellular dedifferentiation, it has also been suggested that the first proliferation wave of dedifferentiated cells is crucial for blastemal formation (Satoh et al., 2007b). Since cell proliferation during embryonic development employs unique molecules and signaling pathways while engaging simultaneously highly conserved somatic signaling mechanisms, it is conceivable that the first proliferation wave of dedifferentiated cells in the limb blastema may re-capitulate some of the events occurring during embryogenesis. Indeed, recent progress has been made in defining the role of nerves during the early stage of axolotl limb regeneration (Kumar et al., 2007). Signals from nerves have been suggested to target the wound epithelium to induce keratinocyte dedifferentiation and formation of a regeneration epithelium (RE)/apical epithelial cap (AEC), with expression of the Sp9 transcription factor in a pattern similar to that observed during embryonic limb bud development (Satoh et al., 2008). The AEC corresponds functionally to the apical ectodermal ridge (AER) in embryonic development, a specialized epithelial structure essential for the outgrowth of developing limb buds of amniotes (Muneoka and Sassoon, 1992). Nevertheless, the extent to which regeneration truly re-capitulates embryogenesis remains unclear.

To approach these questions, we carried out transcriptome sequencing of innervated (NR) and denervated (DL) axolotl forelimbs on days 0, 5 and 14 post limb amputation (pa). We found that a group of germline-specific genes were expressed in innervated limb regenerates. Here we chose to focus on two genes, Piwi-like 1 (PL1) and Piwi-like 2 (PL2) that might play a role in this germline-like state. PL1 was initially identified to process piRNA precursors in *Drosophila* and was revealed to play an essential role in germline stem cell maintenance in flies. Subsequently, orthologous proteins have been found in other organisms, including worms and mammals, and shown to be required for biogenesis of piRNAs. In general, Piwi-like proteins are essential for the asymmetric division of germline stem cells (Klattenhoff and Theurkauf, 2008). In Piwi-like mutant flies, the defective phenotypes are exemplified by a reduced number of gametes and mutant gonads, while mutations of Piwi-like proteins in mice only affect the male gonads (Cox et al., 1998, 2000). Homozygous knockout mice of any of the three Piwi-like genes, MIWI (PL1), MILI (PL2) or MIWI2 (PL4) exhibit disrupted germ cell development leading to male sterility (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). In parallel with increased accumulation of γ-H2AX during zygotene, an impairment in repair of damaged genomic DNA causes loss of germ cells (Carmell et al., 2007). In planarians, RNAi depletion of either of the two Piwi-like genes, *smedwi*-2 or *smedwi*-3 compromises regeneration due to failure of homeostatic maintenance within the dividing pluripotent adult somatic stem cells, neoblasts (Palakodeti et al., 2008; Reddien et al., 2005). Pathophysiologically, PL2 has been suggested to play an important role in the development of pre-cancerous and cancer stem cells in studies of various types of cancers including prostate, breast, gastrointestinal, ovarian and endometrial cancer and also in breast tumors, rhabdomyosarcoma and medulloblastoma (Gao, 2008). PL2 seems to act as an oncogene by inhibiting apoptosis via the induction of high-level expression of the antiapoptotic gene Bcl-X(L) and by promoting proliferation via the Stat3/Bcl-X(L) signaling pathway (Lee et al., 2006).

In our studies of the role of PL1 and PL2 in axolotl limb regeneration, we found by reducing expression with morpholino oligonucleotides that PL1 and PL2 expression is required for limb regeneration by promoting cell proliferation and preventing cell death in the blastema. Moreover, our bioinformatics analysis of a small RNA sequence dataset from regenerating tissue samples also suggested that there are endogenous siRNAs and small RNAs that have some characteristics of piRNAs targeted against transposable elements in axolotl limb regenerates. In vertebrates, siRNAs have so far been exclusively identified in embryonic stem cells. Although there are emerging piRNA candidates present in somatic tissues from genomic regions depleted in transposons that may have a role in the regulation of target mRNAs, conventional piRNAs that are related to transposable elements (TEs) have been found much more frequently in germ cells and are considered to be vital for the transcriptional silencing of deleterious transposable elements and ultimately the genomic integrity of germ cells (Siomi et al., 2011). These two lines of evidence also support the suggestion that a germline-like state is established in the regenerating limb.

Results and Conclusions

Identification of transcriptionally activated germline-specific genes during axolotl limb regeneration and cloning of full-length cDNAs of axolotl Piwi-like 1 and 2 genes

Roche 454 sequencing of cDNA libraries generated at different stages of axolotl limb regeneration (9400 ESTs—www.ambystoma.org) (Monaghan et al., 2009) revealed that expression of a group of germline-specific genes is activated during limb regeneration (Fig. 1A). We decided to study the Piwi-like 1 (PL1) and Piwi-like 2 (PL2) genes because of their conserved role in germ cell development and their roles in generating piRNAs. Initially, we confirmed the cDNA sequencing results by conducting an RT-PCR time-course for PL1 expression and three other regeneration-induced germline-specific genes (Fig. 1B). In addition, we examined the transcriptional profile of PL2, a homolog of PL1 and another stem cell marker, Nanog during limb regeneration. We found that PL2 and Nanog were also expressed during limb regeneration. However, unlike PL2, which had a very low basal level of expression in intact limbs, PL1 and Nanog expression was not detected in normal limbs. Moreover, the transcription kinetics of PL1 did not parallel those of PL2 during limb regeneration. Expression of PL1 reached its peak about 15 days post amputation (dpa), and was maintained for another 10 days, whereas PL2 exhibited a short-term transcriptional upregulation with a peak at 5 dpa, followed by a decline to basal level 10–15 day later. Nanog showed the same profile as PL2. All the RT-PCR products were verified by DNA sequencing. The fact that we could detect transcriptional re-activation of PL2 and Nanog in the regenerating blastema by RT-PCR, but not cDNA sequencing, suggests there are likely to be additional regeneration-reactivated germline-like genes.

It has been suggested that PL1 and PL2 have distinct roles in the two steps of transposable element-associated piRNA biogenesis, with PL2 preferentially associating with primary piRNAs generated in the first step of piRNA biogenesis, the primary processing pathway, and PL1 associating with secondary piRNAs emerging in the second step, creating a ping-pong amplification loop (Siomi et al., 2011). Since the axolotl genome is huge, $\sim$10 times that of humans, and since the genome has a high percentage of repetitive elements, it is likely that the basal activity of TEs might be much higher than in most organisms. Therefore, the axolotl must have multiple defense mechanisms to maintain genomic integrity. Genomic homeostasis may be achieved via low level and constitutive expression of piRNAs in normal limbs.