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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.

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## 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, ~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.

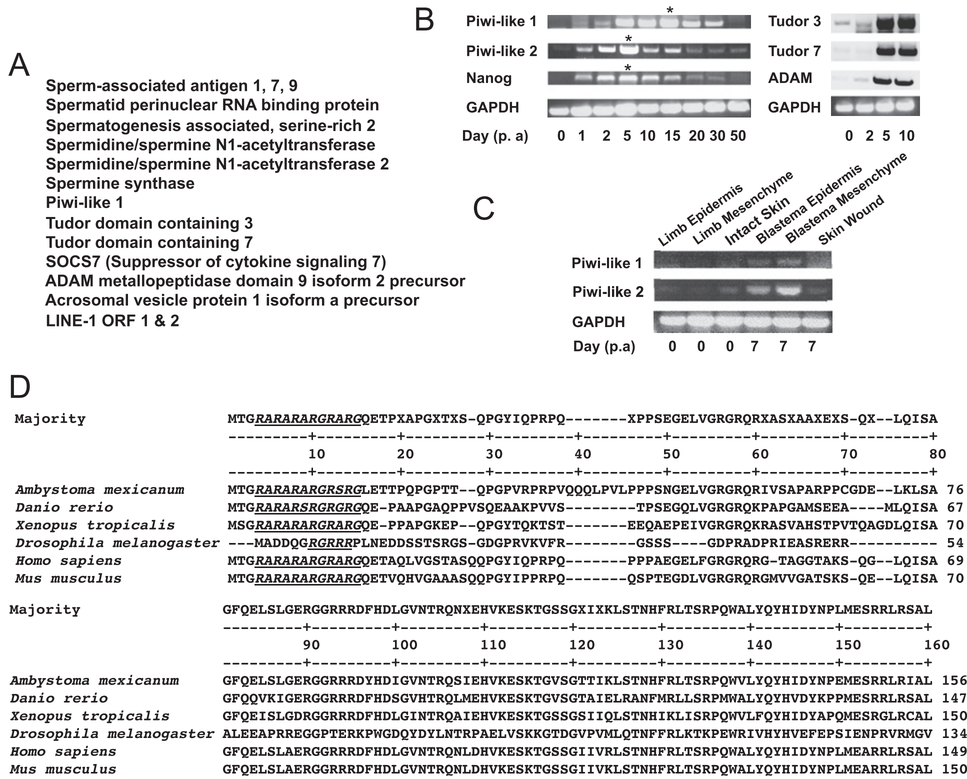


**Fig. 1.** Piwi-like 1 and 2 are specifically upregulated upon axolotl limb regeneration: (A) Listed here are the germline-specific genes found to be expressed in regenerating axolotl limbs upon limb amputation using Roche 454 cDNA sequencing. (B) RT–PCR time-course. Examination of the transcriptional activities of some of the indicated germline-specific genes during the process of axolotl limb regeneration. GAPDH was used as a control. The transcriptional activity peaks of PL1, PL2 and Nanog were labeled with asterisks. (C) RT–PCR analysis of PL1 and PL2 transcriptional activity in the intact limb epidermis or mesenchyme, and in a healing superficial limb skin wound and regenerating limb blastemal epidermis and mesenchyme. (D) Alignment of the N-terminal sequence of PL1 protein from several organisms, demonstrating that the arginine/alanine-rich motif (italic and underlined), a potential target for regulatory methylation, is evolutionarily conserved.

Conceivably, a basal level of PL2 activity in normal limbs could be sufficient to generate a small amount of primary piRNAs for this purpose. However, upon limb regeneration, as we found through Roche 454 cDNA sequencing, transposable elements, such as LINE-1, are significantly upregulated ( $>10$ fold on days 2 and 7 post amputation (pa), data not shown here), leading to a potentially huge surge of deleterious transposition activities. To neutralize the potential threat, in addition to the upregulation of the 'housekeeping' Piwi gene, PL2, a dramatic and sustained activation of PL1 would be critical to produce a large quantity of secondary piRNAs through the more efficient ping-pong amplification loop.

We cloned full-length cDNAs for axolotl PL1 and PL2 using degenerate RT-PCR, and 5′ and 3′ RACE to obtain their 5′ and 3′ UTR sequences. Amino acid sequence analysis indicated that axolotl PL1 and PL2 both have the PAZ domain nucleic acid-binding interface, as well as the PIWI domain that anchors the guide RNA and acts as the catalytic site for slicing, similar to their orthologs in other organisms. The N-terminal portions of Piwi-like proteins are much less conserved than their PAZ and PIWI domains, and axolotl PL1 and PL2 also differ significantly in their first $\sim$75 amino acids compared to those from other organisms (Fig. 1D). The function of the Piwi-like protein N-terminal region is not clear yet, although symmetrical arginine methylation of the RARA motif (RARARARGRSRG) immediately downstream of the first three amino acids at the PL1 N-terminus may initiate an interaction with other protein regulators involved in the piRNA-mediated silencing, such as Tudor domain-containing proteins (TDRDs). Interestingly, TDRD3 and TDRD7 were among the germline-specific genes we found expressed during limb regeneration, and they could act as regulators of Piwi-like proteins. Previous studies have demonstrated that deficiencies in TDRD proteins significantly impair piRNA biogenesis, especially the ping-pong amplification loop, which subsequently leads to TE overexpression. MILI and TDRD1 mutants share similar spermatogenic defects in the mouse (Wang et al., 2009). Interestingly, the RARA motif is absent in PL2s. Thus, although TDRD protein deficiency substantially impairs the ping-pong amplification loop, it might not entirely abolish the piRNA biogenesis pathway.

To decide whether or not the expression of PL1 and PL2 is merely an irrelevant episode during the regenerative process, we also examined the transcriptional activity of PL1 during superficial skin wound healing in the axolotl forelimb. In contrast to the transcriptional activation of PL1 and PL2 during forelimb regeneration at 7 dpa in both the regenerating epidermis and the blastemal mesenchyme, there was no detectable level of PL1 RNA in the forelimb superficial skin wound, and there was no significant change in the level of PL2 RNA (Fig. 1C), thus providing evidence that PL1 and PL2 expression is specifically upregulated upon amputation and is intimately associated with regeneration. In addition, we also examined the expression pattern of PL1 and PL2 during embryonic limb development (Fig. S1B). Interestingly, it seems that PL2 transcriptional activity appears undetectable in RT-PCR at embryonic limb bud stage 46, developmentally equivalent to the mid-blastemal phase during limb regeneration while both PL1 and PL2 are transcriptionally active at embryonic limb bud stages 44 and 48. Furthermore, tissue expression profiling using RT-PCR revealed that the germ cell tissues of both males and females exhibit high levels of PL1 and PL2 transcripts (Fig. S1A).

*Histological examination of PL1 and PL2 expression in the amputation-induced, regenerating axolotl forelimb*

Since expression of Piwi-like proteins is considered a hallmark for acquisition of cell stemness, it was important to localize PL1/2 expression. The cells in the limb blastema are believed to be a heterogeneous collection of dedifferentiated cells that have been reprogrammed to achieve varying levels of developmental potential exhibited by the cells involved in embryogenesis (Kragl et al., 2009). Accordingly, we were interested to know if there is differential positional expression of PL1 and PL2 in the blastema. We conducted *in situ* hybridization (ISH) of axolotl PL1 and PL2 RNA in the early-to-medium stage limb blastema, using sense probes as a control. Most of the blastemal cells stained positive for PL1 and PL2, with a small percentage of cells carrying a stronger signal than the others (Fig. 2A and C). In addition, a positive signal was also present in the regenerating wound epithelium (WE). However, the signal pattern was very different from that in the blastema mesenchyme. Whereas PL1 or PL2 transcripts were absent in most of the cells in the epidermis, the basal keratinocytes of the blastemal epidermis were stained positive. In contrast, sense RNA probes for PL1 and PL2 did not generate positive signals in the limb blastema or in the regenerating epidermis (Fig. 2B and D), suggesting that there was no detectable level of anti-sense PL1/2 transcripts in the limb blastema. The *buttonhead*-like zinc-finger transcription factor, *Sp9* is induced during limb regeneration in a pattern comparable to its expression in developing limb buds and is considered to be a valid marker for epidermis dedifferentiation (Satoh et al., 2008). The fact that the cells of the basal epidermal layer exhibited transcriptional activation of PL1 and PL2 agrees with the previous notion that those cells undergo reprogramming, reminiscent of the situation in embryonic limb development. Intriguingly, some cells in the thickened apical epithelial cap (AEC) of the regenerating epidermis had particularly strong signals for PL1. It is well known that AEC is the very center of the active outgrowth of the regenerating limb blastema. In conclusion, the distribution pattern of PL1 and PL2 appears to be paralleled by the activity profile of dedifferentiation and proliferation in the regenerating limb. We also performed PL1/2 ISH in regenerating limb blastemas collected at very early or late stage (data not shown). In both cases the signal is much weaker, in agreement with the RT-PCR analysis shown in Fig. 1B. The tissue distribution pattern of PL1/2 is similar to that demonstrated in the limb blastema at early-to-medium stage.

*PL1 and PL2 knockdown affects the progress of axolotl limb regeneration*

The striking PL1 and PL2 expression patterns in the axolotl limb blastema and wound epidermis prompted us to explore their roles in axolotl limb regeneration. To achieve this goal, we designed fluorescein-conjugated morpholino oligos (MOs) specifically targeting PL1 or PL2 protein translation (Fig. S2). As a control, MOs with inverted DNA sequences were used. To verify the efficacy and the specificity of the MOs, we performed axolotl PL1 and PL2 *in vitro* transcription and translation experiments. Since the ~900 bp fragment at the 5′ end of axolotl PL1 cDNA was found to be toxic to bacteria, as indicated by our failure to clone the full-length PL1 cDNA in a bacterial host, we replaced the 15-nucleotide fragment downstream of the start codon ATG in the axolotl PL1 cDNA with the corresponding fragment from the axolotl PL2 cDNA. Since the axolotl PL1 cDNA MO is designed to target the immediate 5′ UTR region, the start codon AUG and a few downstream nucleotides, the hybrid axolotl PL1-2 mRNAs should still be susceptible to the inhibitory effect of the PL1 MOs. As expected, the level of the full-length PL1/PL2 translation products from both the PL1-2 hybrid and PL2 RNAs was significantly reduced in the presence of the corresponding MOs when compared to the samples incubated with the control MOs. Consistent with the MOs causing specific inhibition of authentically initiated PL1/2 proteins, there was no effect of the specific MOs on the translation of the smaller products in the same reactions, which are presumably derived by internal initiation on shorter transcripts.

Having established the fidelity of the MOs, we tested whether MO injection into the blastema would have an impact on limb regeneration. MOs were injected into the early-stage limb blastemas 5 dpa followed by electroporation to increase cellular uptake. The specific MOs and the control MOs were transfected into the blastemas of the opposing forelimbs in the same animal. Transfection efficiency in the limb blastemas was examined 7 days after the first transfection by scoring for the level of fluorescein that was conjugated to the MOs. The length of the blastemas (from the amputation plane to the tip of the regenerating AEC) was measured. A second transfection was conducted

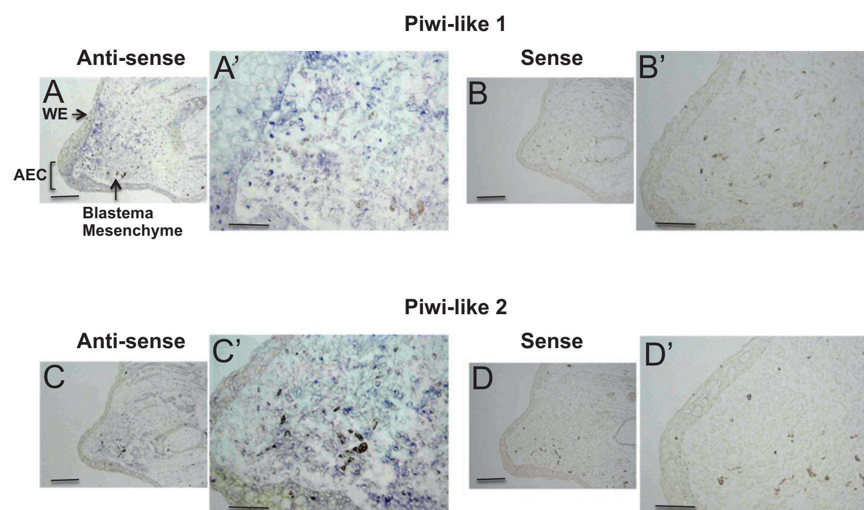


**Fig. 2.** *In situ* hybridization of axolotl PL1 and PL2 in regenerating axolotl forelimb blastemas at an early-to-medium stage. For both PL1 and PL2 anti-sense probes, the positive signal (blue) was spread through the entire region of blastema mesenchyme and was also present in the basal layer of the wound epidermis (WE) and the thickened apical epithelial cap (APC). Images A′, B′, C′ and D′ are higher magnification views of the corresponding images in A, B, C and D, respectively. Scale bar in images A–D is 500 μm, and for images A′–D′, 200 μm.

7 days after the first transfection to maintain the levels of the MOs. No further transfections were carried out to avoid excessive tissue damage elicited by the procedure.

We examined the progress of limb regeneration every week until the complete regrowth of lost limbs. Since PL1 and PL2 might have redundant functions, we first tested a combination of PL1 and PL2 MOs, and detected a significant retardation of limb regeneration. Since the MO transfection efficiency in limb blastemas was ~20–60% and since the MOs probably did not inhibit PL1 and PL2 translation completely, regeneration might be retarded to a greater extent if total inhibition of PL1/2 expression could be achieved. An inhibitory effect of PL1/2 depletion was observed both with respect to the size of the blastema and also the progress of regeneration compared to the control blastema (Fig. 3). The limb blastema transfected with PL1 and PL2 MOs was on average significantly smaller when compared to the contralateral limb blastema transfected with the control MOs and the pace of limb regeneration also seemed to be slower. At the end of late limb blastema formation and the onset of the palette stage (usually 3 weeks pa) during limb regeneration, cell differentiation and tissue repatterning begin to emerge as primary regenerating activities. Outgrowth of the limb blastema is much slower than in earlier stages and measurement of the length of the blastema would not be appropriate for the evaluation of regeneration progress. So we chose two time points, days 14 and 21 after the first transfection to decide whether there is a differential pace in the limb regeneration between the axolotls transfected with PL1 and PL2 MOs and the animals with control MOs. On day 14 after the first transfection, there was ~10% reduction in average blastema length for MO-transfected axolotls, whereas there was ~50% gain for the controls. On day 21 the average blastema outgrowth in the control animals was almost three fold greater than that in the MO group (181% versus 67%) (Fig. 4A).

In this study, we also explored the effect of individual PL1 and PL2 MOs on the regenerative process (Fig. 4B). In contrast to the combined PL1 and PL2 MOs, we did not observe a definite inhibitory effect in the case of PL1 MO. For PL2 MO, the inhibition appeared less (~2/3) than that of combined PL1 and PL2 MOs. On day 21, the average increase of blastema outgrowth in the control PL2 MO-transfected animals was approximatey two fold of that measured in the MO group (111% versus 55%). Thus, while it seems that PL1 and PL2 may share some overlapping roles in limb regeneration, PL2 does have unique functions and is indispensible during limb regeneration.

### *Examination of cell proliferation, cell death and retrotransposon LINE-1 transcriptional activity following PL1 and PL2 knockdown during limb regeneration*

To investigate how combined PL1 and PL2 depletion might mediate its effect on limb regeneration, we examined the levels of cell proliferation and cell death in the regenerating limbs. To measure proliferation, limb blastemas were transfected with MOs 7 dpa and EdU was administered for another 7 days after the transfection, 12–15 h after EdU intraperitoneal injection blastemas were collected and fixed. Analysis of EdU incorporation revealed that the percentage of proliferating cells in the PL1/2-transfected blastema was ~60% of that in the control (Fig. 5A and C). Similarly, TUNEL staining to detect apoptotic cells (Fig. 5B) showed that the number of apoptotic cells in the PL1/2 MO-transfected blastemas was ~3.5 fold more than that in the control (Fig. 5D). Since TUNEL is probably only visible/detectable for about 4 h before cells disintegration, the actual rate of cell death per day in the limb blastema is at least 6 × the TUNEL index, and thus in reality cell death is much more extensive than what is shown in Fig. 5D. Conclusively, this suggests that the pathways regulated by PL1 and PL2 in cells of the blastema promote proliferation and cell survival, perhaps similar to the roles of Piwi-like proteins in germ cell self-renewal and pre-cancerous stem cell survival. Due to the limit of the availability of experimental animals, the tissue sections used for quantitation analysis and for the images shown in Fig. 5A and B were obtained from two separate experiments.

In addition, we examined whether there was any change in the expression level of some of the genes reported to be upregulated during limb regeneration. FGF8, a member of the fibroblast growth factor (FGF) family, is highly expressed during embryonic limb development in vertebrates, and plays an essential role in morphogenesis (Goldfarb, 1996; Tanaka and Gann, 1995). In differentiated salamander limbs, the FGF8 expression level is

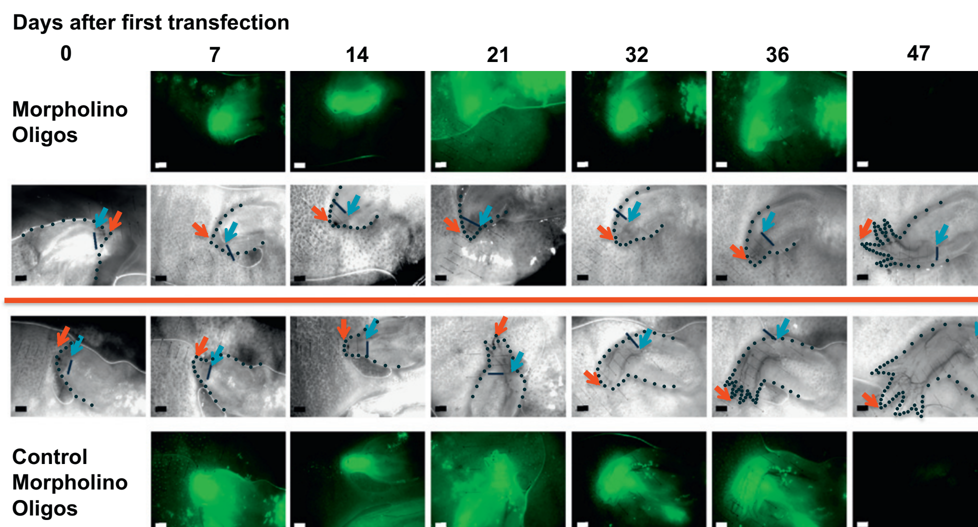


**Fig. 3.** Comparison of the regeneration progress in the right and left regenerating forelimbs from the same animal, with one transfected with mixed PL1 and PL2 MOs on day 5 pa and the other transfected with the mixed control MOs. Green fluorescence images demonstrate transfection efficiency in the limb regenerates. The blue arrows indicate the amputation planes and the red arrows indicate the tip of the limb regenerates. A microcaliper was used to measure the length of the blastema (from the amputation plane to the tip of the blastema) with the 0 mm line precisely marking the amputation plane. The 0 mm lines in the images were further strengthened in deep blue color. The microcaliper was placed perpendicularly to the blastemal AP axis. Deep blue dots were added along the outlines of regenerating limbs to make the regenerating limbs more visible. Scale bars = 1 mm.

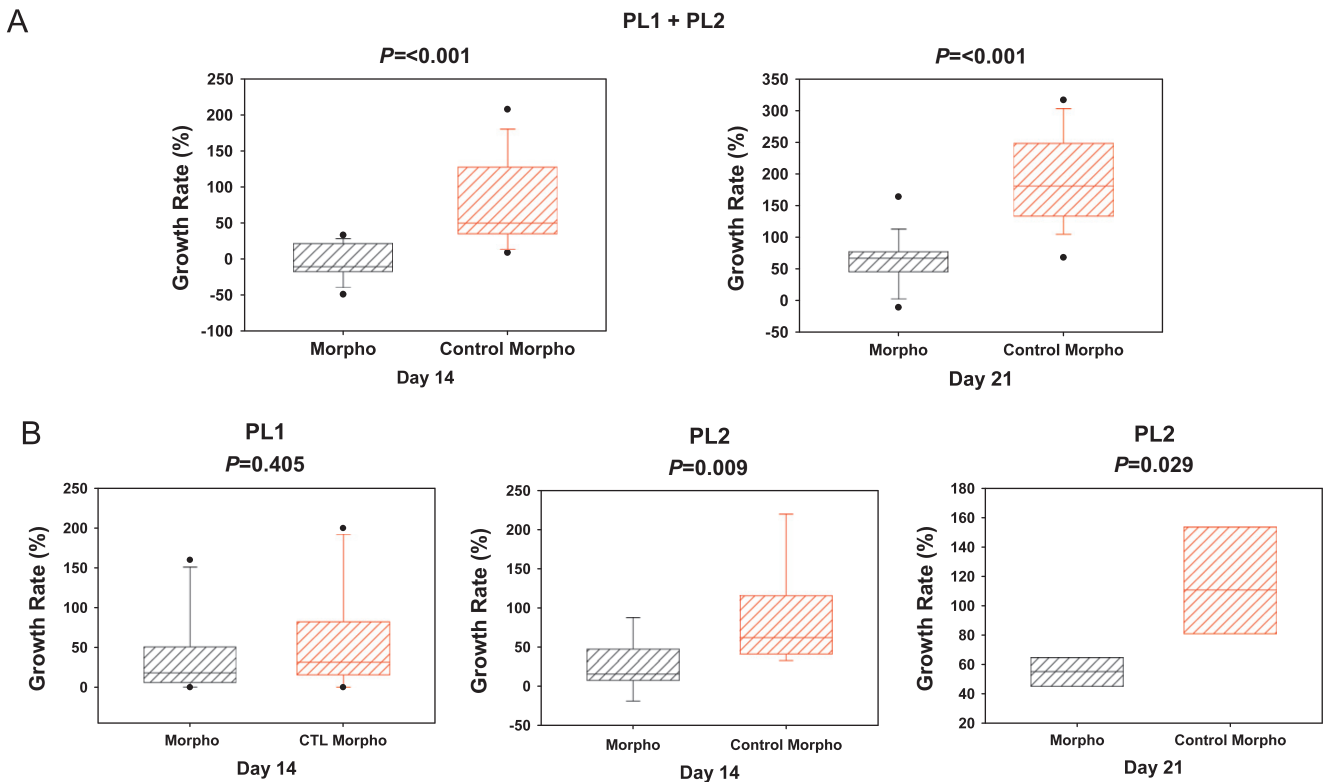


**Fig. 4.** Effect of PL1 and PL2 knockdown mediated by morpholino oligos (MO) on axolotl limb regeneration: (A) Evaluation of limb blastema growth rate by PL1 and PL2 MO transfection in limb regenerates. Length of limb blastemas was measured at days 14 and 21 after the first transfection to determine whether there was a differential pace of limb regeneration in the presence of PL1 and PL2 MOs. Results are compiled from four different experiments. 17 animals were used. *P* values are presented in the graph (PL1 and PL2 MOs, $n=17$; control MOs, $n=17$). (B) Effect of PL1 or PL2 individual knockdown in the growth rate of limb blastemas. The result for day 14 was obtained from two different experiments. In total 10 (for PL1) or 9 (for PL2) animals were used. The result for PL2 on day 21 was obtained from one experiment in which two animals were used. *P* values are presented in the graphs (PL1 and PL2 MO and control MO, $n=10$; PL2 day 14, MO and control MO, $n=9$; PL2 day 21, MO and control MO, $n=4$).

very low, but during limb regeneration in axolotls, FGF8 is re-expressed in the blastemal mesenchyme and the basal layer of the WE (Christensen et al., 2001, 2002; Han et al., 2001; Kawakami et al., 2004), reminiscent of the expression patterns of PL1 and PL2 shown here. FGF8 has many roles in vertebrate development, e.g. growth and patterning of limbs and midbrain, induction and posteriorization of the neuroectoderm through its effects on proliferation, differentiation, mesenchymal–epithelial cell interaction and the coordination of growth and differentiation during tissue patterning (Goldfarb, 1996). In recent studies of zebrafish embryo gastrulation, PL2 was reported to mediate FGF8 signaling responsible for patterning embryo and mesoderm formation (Chen et al., 2009). Since PL1 and PL2 depletion compromised cell proliferation and caused cell death in the limb blastema, we evaluated whether PL1 and PL2 might regulate FGF8 signaling during limb regeneration, and found that the expression level of FGF8 was significantly decreased in regenerating limbs transfected with PL1 and PL2 MOs (Fig. 5E) compared to control MOs, indicating that FGF8 might act as one of the downstream effectors of Piwi-like signaling cascade during vertebrate limb regeneration.

In addition to determining the effect of MO-mediated PL1/2 knockdown on cell proliferation and cell death in the regenerating limb blastema, we also examined its impact on the transcriptional activity of a retrotransposon, LINE-1. As discovered earlier, retrotransposons such as LINE-1, are dramatically upregulated upon limb amputation (Fig. S3A), potentially posing a threat to cell viability due to a surge of potentially harmful genomic transposition activities. Among the mechanisms combating increased transposition activity, PL1 and PL2 have been suggested to contribute by producing transposon-derived piRNAs that can cause silencing of transcriptional initiation or mRNA degradation (Siomi et al., 2011). If PL1 and PL2 can generate LINE-1-specific piRNAs and silence expression, one would predict that depletion of PL1 and PL2 might further increase LINE-1 RNA levels in regenerating tissue. To test this, we conducted Real Time qPCR (RT qPCR) using three different sets of primers (A09, D08 and 6–5) targeting either LINE-1 ORF-1 or ORF-2 transcripts to analyze the total RNAs extracted from PL1/2 MOs or control MOs-transfected limb regenerates. MO transfection was performed at 7 day pa with PL1/2 MOs injected in one forelimb of an animal and the control MOs in the other forelimb of the same animal. Limb regenerate tissues were collected 7 days after MO transfection. To provide a baseline control, total RNA extracted from limb tissues harvested on day 0 during limb amputation were also subjected to RT qPCR. The values for fold change in LINE-1 transcripts in limb regenerates transfected with PL1/2 MOs or control MOs when compared to the limb tissues on day 0 are shown in Fig. S3A. In all cases, the increase of LINE-1 transcriptional activity upon limb regeneration was consistently more significant in PL1/2 MO-transfected limb regenerates than in control MO transfected limb regenerates. To allow direct comparison of the effect of PL1/2 MOs on LINE-1 transcriptional activation, we normalized the fold-change values of the PL1/2 MOs-transfected samples by dividing by the values obtained with the control MOs, and thus obtained relative LINE-1 transcriptional activities (Fig. S3B). We found that the level of LINE-1 transcripts in PL1/2 MO-transfected limb blastemas was on average ~1.8 fold of that in control MO-transfected limb regenerates (Fig. S3B), and strongly supporting the idea

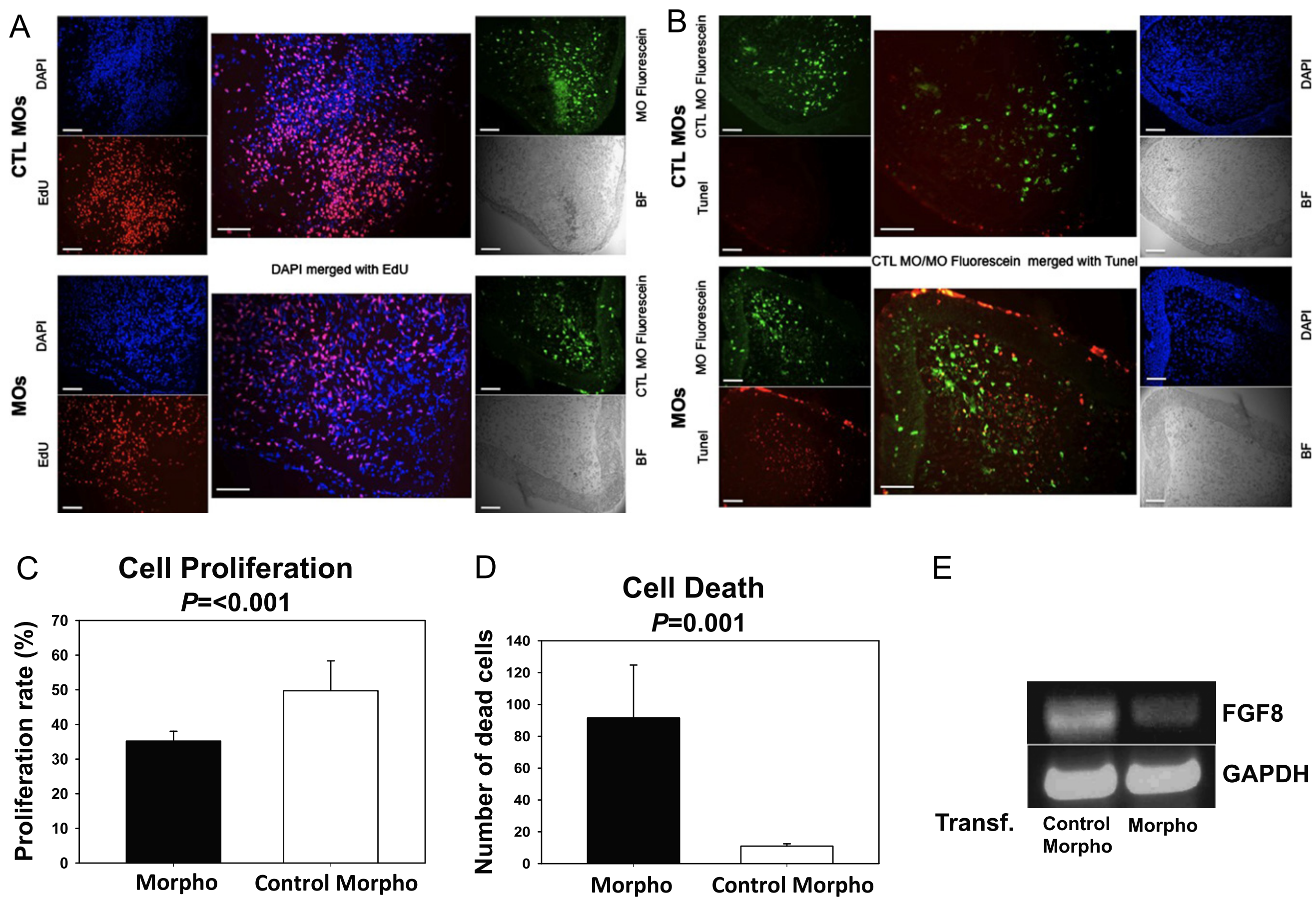


**Fig. 5.** Transfection of PL1 and PL2 MOs in the regenerating forelimb blastemas affects cell proliferation and cell death. MO transfection was performed at 7 day pa with PL1/2 MOs injected in one forelimb of an animal and the control MOs in the other forelimb of the same animal, respectively. Limb regenerate tissues were collected another 7 days after MO transfection. To provide a baseline control, the total RNA extracted from limb tissues harvested on day 0 during limb amputation was also subjected to RT qPCR: (A) Examination of proliferation by detection of EdU incorporated into genomic DNA. (B) TUNEL staining of limb blastemas. Scale bars=200 μm. (C, D) Statistical analysis of the effect of PL1 and PL2 knockdown on cell proliferation and cell death during limb regeneration. The percentage of proliferating cells was determined by the ratio of EdU-labeled cells in the population of DAPI-stained cells. We counted the absolute numbers of dead cells stained in red in the TUNEL assay to assess cell death rate. Error bars indicate the mean ± SD, $n=7$–8 per group ($n=7$–8 refers to tissue section slides, and the tissue sections were derived from three animals). (E) Axolotl PL1 and PL2 MO transfection led to decreased expression of FGF8 in the regenerating axolotl limb blastema. MOs were injected into one of the regenerating forelimb blastemas (5 dpa) while the control MOs were injected into the other forelimb blastema of the same animal. Seven days after transfection, limb blastemas were collected and total RNA was extracted for RT-PCR analysis. The tissue sections used for the quantitation analysis shown in panels C and D, and for the images shown in panels A and B were obtained from two separate experiments.

(reviewed by (Siomi et al., 2011) that Piwi-like proteins play a pivotal role in transposon silencing in the regenerating limb to preserve genomic integrity.

## Discussion

It is widely accepted that there are two distinct phases during epimorphic regeneration. While the second, late stage initiates re-differentiation and patterning of the limb blastema after blastema formation to re-grow morphologically and functionally intact limbs, the first, early phase is unique to regeneration and involves cellular dedifferentiation or reprogramming of the mature body cells to re-acquire the developmental potential of the embryonic limb bud cells, which eventually give rise to the regenerating limb blastema after the first wave of cellular proliferation of dedifferentiated cells (Yokoyama, 2008). Thus, it is crucial to understand the unique events occurring in the early stage of limb regeneration, particularly since this could enable development of strategies to enhance tissue and organ regeneration in less regenerative organisms, like humans.

Although the axolotl limb transcriptome has been delineated in the last couple of years, there is no characterization in regard to what kind of unique state is established in the limb blastema that confers on the blastema a developmental potential reminiscent of the embryonic limb bud. To define the unique state of the blastema, we set out to identify the genes in the regenerating limb that are usually active during embryonic development and are silent in the mature limbs. Through cDNA sequence analysis, we found a group of germline-like genes that are re-activated upon limb regeneration suggesting that a germline-like state exists in the limb blastema. Included among these germline-like genes are PL1 and PL2. Piwi-like gene knockout studies in mice and flies revealed that they are essential for the self-renewal of germ cells. As demonstrated using ISH, PL1 and PL2 are expressed in the blastema and the apical epithelial cap as well as in the basal layer of keratinocytes in the epidermis. We obtained evidence that PL1 and PL2 have a role in limb regeneration and blastemal cell proliferation and survival using MO-mediated knockdown of protein expression. Reduced expression of these two genes led to significantly delayed limb regeneration. Even greater defects might be observed if complete knockdown could be achieved by

using transgenic axolotls expressing PL1 and PL2 dominant-negative mutants.

Although our data suggest that there is a germline-like state in the limb blastema characterized by re-activation of germline-like genes, it has been reported that limb blastemal cells are very likely a collective of multipotent rather than pluripotent cells, which possess intrinsically different levels of developmental potential (Kragl et al., 2009). For instance, studies from Kragl in Tanaka's group, in which they grafted tissue between normal and GFP transgenic axolotls and tracked cell lineage fate of the major limb tissues in the limb regenerate showed that different tissues involved in limb regeneration produce progenitor cells with restricted potential at differential levels, with dermis as the most flexible tissue and muscle the least (Kragl et al., 2009). Thus, it is likely that the plasticity the blastemal cells acquire through reprogramming is not comparable to that in pluripotent stem cells. Although the cells at the amputation plane do not revert back to pluripotent state, there is a cell lineage switch in axolotl limb regeneration, and the fact that adult axolotl limb blastemas possess the unique capacity of reconstituting lost limbs, supports the notion that the reprogramming of limb blastemal cells may be limited to certain stages resembling post-limb developmental stages during a progressive, multistep process in embryogenesis, thus restricting the programming of dedifferentiated blastemal cells to specific developmental fates. Although blastemal cells appear to re-initiate embryonic programs of tissue formation, it is likely that the germline-like state in the limb blastema is not exactly the same as that described in germline cells. Instead, the blastemal cells are more likely a heterogeneous collective both in terms of the origin (e.g. epidermis, connective tissues, muscle, nerve system and blood) and the state of dedifferentiation. However, there could be an alternative explanation for the restricted cell lineage switch of the reprogrammed blastemal cells. As some blastemal cells still possess some of the inherent attributes unique to their original somatic cell identities, it is conceivable that even if a few pluripotent cells derived from dedifferentiation do exist in the blastema during certain windows of limb regeneration, they are likely to be guided or influenced by the partially reprogrammed blastemal cells carrying their original positional information and end up with limited developmental potentials as observed in axolotl limb regeneration (Tamura et al., 2010). So in this scenario, the presence of the blastemal cells that possess positional information rather than the absence of fully dedifferentiated, pluripotent cells, is responsible for the restricted developmental potentials of somatic tissues participating in the regeneration process. In the future, further characterization of the subpopulations of these reprogrammed cells with additional germline-specific markers might provide more insight into exactly how far cellular dedifferentiation can proceed and whether there are indeed a small number of cells that could be isolated before a certain developmental threshold and exhibit true pluripotency when isolated from the influence of the partially programmed blastemal cells in the proximity.

FGFs are pleiotropic signaling molecules that have been demonstrated to be involved in a broad variety of biological activities including cell proliferation, migration, differentiation and homeostasis in vertebrates (Goldfarb, 1996). In our effort to identify the downstream effectors of PL1 and PL2 signaling pathways during axolotl limb regeneration, we discovered that the knockdown of PL1 and PL2 by MOs led to a significant decrease of FGF8 expression in regenerating limb tissue. Indeed, Satoh reported in their studies of the accessory limb model (ALM) in axolotls that activation of MMP activity and simultaneous application of an FGF 2/8 supplement to a lateral skin wound were sufficient to induce a bump in the absence of a nerve, which appeared to be a regenerating limb blastema suggesting that FGF 2/8 may very well play a critical role in limb blastemal formation (Satoh et al., 2011). In the future, it would be interesting to examine whether PL1 and PL2 also modulate expression of FGFs other than FGF8 during regeneration. Like FGF8, FGF10 was also found to be upregulated in regenerating limbs, whereas FGF4 activity was undetectable by RT-PCR (Christensen et al., 2001). However, in regeneration-deficient denervated limbs, neither FGF8 nor FGF10 is upregulated, suggesting that these two FGFs are nerve-dependent. It would be worthwhile examining whether PL1 and PL2 are also neurotrophic factor-regulated genes during limb regeneration. Studies in zebrafish early embryonic development suggest that PL2 suppresses bone morphogenetic protein (BMP) signals by physically associating with Smad4 and preventing Smad2/3/4 and Smad1/5/9/4 complex formation (Sun et al., 2010). The BMPs are members of the TGF-$\beta$ family. FGF-regulated-BMP signaling has also been reported to play a crucial role in various biological activities. For example, in rats the oocyte factor, FGF8 enhances BMP signaling to regulate steroidogenesis through oocyte-granulosa cell communication (Kragl et al., 2009). During the development of oligodendrocyte precursors (OPCs), FGF can promote OPC biogenesis from embryonic neural precursor cells (NPCs) by counteracting BMP signaling, and Smad1 may be directly involved in the process (Bilican et al., 2008). In the estrogen receptor (ER)-expressing human breast cancer MCF-7 cells, FGF8 was shown to facilitate cell proliferation at least partially by increasing expression of inhibitory Smads leading to the suppression of BMP signaling (Masuda et al., 2011). Since BMP/TGF-$\beta$ signaling could be a downstream target of both Piwi-like proteins and FGFs, it is possible there is a crosstalk in some cases between these two protein families. Although we have not defined here whether Piwi-like proteins act directly or indirectly to alter FGF signaling during limb regeneration, we can conclude that PL1 and PL2 play multiple roles in tissue and organ regeneration, and that, in addition to their roles in the biogenesis of piRNAs to maintain tissue homeostasis, they may exert their influence on other aspects of regenerative activities via FGF signaling.

## Material and methods

### *Experimental animals*

Axolotls (*A. mexicanum*) were purchased from the Ambystoma Genetic Stock Center in University of Kentucky. For the isolation of RNAs and genomic DNAs, animals measuring 10–15 cm, from snout to tail tip, were used. For MO transfection in limb blastemas, smaller animals (6–10 cm, both male and female) were utilized.

### *Cloning of axolotl PL1 and PL2 cDNA*

Total RNAs were extracted from *A. mexicanum* from intact limbs or limb regenerates (5–10 dpa) using Trizol reagent. Reverse transcription was primed by random hexamers and oligo-dT. Gene-specific degenerate PCR and 5′ and 3′ RACE were performed to obtain full-length PL1 and PL2 cDNA sequences as well as their 5′ and 3′ UTRs. Genbank accession numbers of the submitted cDNAs are JN565976 (*A. mexicanum* PL1) and JN565976 (*A. mexicanum* PL2). The PCR primers for the other regeneration-activated germline-specific genes we examined by RT-PCR were derived from cDNA sequences available at www.ambystoma.org.

### *RT–PCR*

Axolotl intact limbs and limb regenerates from different stages of limb regeneration were collected for RNA extraction.

Reverse transcription was primed by random hexamers and oligo-dT. The DNA primers for the first-strand cDNA PCR in Fig. 1B are as follows: PL1 Forward: 5′CTGAATTTGCAGCAGATTGGACGC3′; Reverse: 5′GTAGTAGTCTATGTAACTTGTTTCACTTCCATCTGACTTC-TTGA3′; PL2 Forward: 5′ATGGATCCTTCCAGACCCCCTTTAAGAGG-TGCT3′; Reverse: 5′GGAGTCGGTAAGTATGACCGGCCAATTCCAATG3′; Tudor 3 Forward: 5′CCATAGCGCTTGGAGGTGAA3′; Reverse: 5′CT-GCCTCCACGTTGTTGCCA3′; Tudor 7 Forward: 5′CGCCTCAGGTT-TTGAAGT3′; Reverse: 5′ACATCCATCTGTTCACCA3′; ADAM Forward: 5′CGGAATGTGTCAGTTCTATGATGCA3′; Reverse: 5′CAGTAGCCCA-TCTCGCAATGA3′; GAPDH Forward: 5′GACAAGGCATCTGCTCACCT3′; Reverse: 5′ATGTTCTGGTTGGCACCTCT3′; Nanog Forward: 5′GCCC-GCCCACTGCATGACCCCGCAAAT3′; Reverse: 5′CGCCTGTGGTTGCCAG-CACCTCTTGTCCA3′; FGF8 Forward: 5′CTGCATCTTTTTGTCCTCTG-CA3′; Reverse: 5′CTATGAGGCTCCGTTGTTTGG3′. The identities of RT-PCR products were confirmed by DNA sequencing.

### *In situ hybridization*

The detailed protocol used here was described by Satoh et al. (2007a). The RNA probes were labeled with digoxigenin (DIG). Staining was developed with BCIP and NBT in an alkaline phosphatase buffer after *in situ* hybridization and anti-DIG alkaline phosphatase antibody incubation.

### *Morpholino oligos and transfection of morpholino oligos in regenerating limbs*

Fluorescein-conjugated morpholino oligos were purchased from Gene Tools. The sequence for the axolotl PL1 MO is 5′AG-CTCTACCAGTCATTTTCTTTCAA3′, and for PL2 MO it is 5′TCCAT-GTTTATGCTTACAATCCACC3′. The sequences are inverted so that the sequence of PL1 control MO is 5′AACTTTCTTTTACTGACCAT-CTCGA3′ and the sequence of PL2 control MO is 5′CCACCTAACAT-TCGTATTTGTACCT3′.

For transfection, the MOs were injected into the regenerating limbs at a concentration of 5 μg/μl. 0.1–0.5 μl of MOs in 1 × PBS solution was injected into the blastema of one of the regenerating forelimbs, while the control MOs were injected into the other forelimb blastema of the same animal. Electroporation of injected blastemas was performed immediately in the PBS solution using a BTX ECM 830 electroporator. The electroporation parameters: five pulses, 50 V with pulse length of 50 ms.

### *EdU incorporation detection*

Intraperitoneal injection of EdU in animals was conducted 12 h before tissue collection and fixation. EdU detection protocol (Click-it EdU cytometry assay, Invitrogen) was modified at the step of DNA exposure and denaturation such that tissue sections were treated with 0.8% pepsin in 0.2 N HCl at 37 °C for 10 min and after the PBS wash were incubated in methanol at −20 °C for 10 min.

### *TUNEL assay*

A Roche *in situ* cell death detection kit was utilized to assess cell death in tissue sections. For tissue permeabilization, we treated the sections with freshly prepared ice-cold ethanol and acetic acid (2:1) at −20 °C for 5 min.

### *Statistics*

A Student's *t*-test was performed on data obtained for the following experiments: cell proliferation analysis in limb blastemas. The nonparametric Mann–Whitney rank sum test for non-normally distributed values was conducted in the analysis of differential growth rates of limb blastemas and cell death (TUNEL assay). For each dataset, a normality test ($P > 0.05$) and an equal variance test ($P > 0.05$) were performed to identify normally distributed data. If either test failed, the nonparametric Mann–Whitney rank sum test was performed instead. All statistical analysis was done with the use of a SigmaPlot 11. A value of $P < 0.05$ was considered significant.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.07.021.

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