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Nerve-mediated expression of histone deacetylases regulates limb regeneration in axolotls

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ABSTRACT

Axolotls have amazing abilities to regenerate their lost limbs. Nerve and wound epidermis have great impacts on this regeneration. Histone deacetylases (HDACs) have been shown to play roles in the regeneration of amphibian tails and limbs. In this study, a bi-phasic up-regulation of HDAC1 was noted before early differentiation stage of axolotl limb regeneration. Limb regeneration was delayed in larvae incubated with an HDAC inhibitor MS-275. Local injection of MS-275 or TSA, another HDAC inhibitor, into amputation sites of the juveniles did not interfere with wound healing but more profoundly inhibited local HDAC activities and blastema formation/limb regeneration. Elevation of HDAC1 expression was more apparent in wound epidermis than in mesenchyme. Prior denervation prohibited this elevation and limb regeneration. Supplementation of nerve factors BMP7, FGF2, and FGF8 in the stump ends after amputation on denervated limbs not only enabled HDAC1 up-regulation but also led to more extent of limb regeneration. In conclusion, nerve-mediated HDAC1 expression is required for blastema formation and limb regeneration.

1. Introduction

Axolotl limb regeneration is one of the best investigated models of regeneration on complex structures in vertebrates (Carlson, 2007). In the first phase of response to amputation, epidermal cells from the edge of the stump rapidly migrate to cover the wound. This wound epithelium/epidermis (WE) then thickens and forms an apical epidermal cap (AEC). Stump cells beneath the AEC dedifferentiate and form blastema containing undifferentiated and proliferating cells. Non-regenerative animals consistently fail to show blastema formation after limb amputation (Tassava and Mescher, 1975; Brockes and Kumar, 2008; Makanae et al., 2014). Undifferentiated blastema cells differentiate into various cell types and grow within the newly formed limbs. Once patterning and growth are completed, a functional new limb is regenerated. Thus blastema formation is the most critical event leading to successful limb regeneration.

Successful blastema formation depends on both WE/AEC and nerves (Singer, 1974; Wallace, 1981; McCusker et al., 2015). It has been shown that both WE and AEC serve as information centers to influence underlying mesenchymal tissues to form blastema during regeneration (Singer and Salpeter, 1961; Tassava and Mescher, 1975; Boilly and Albert, 1990; Globus and Vethamany-Globus, 1985). Antecedently, nerve signaling regulates WE to form keratinocytes and promotes WE to transform into AEC during the early stages of limb regeneration (Satoh et al., 2010). Absence of WE formation or denervation of the limbs result in failure of blastema formation (Todd, 1823; Singer, 1952; Goss, 1956; Mescher, 1976; Tassava and Garling, 1979; Loyd and Tassava, 1980). All these evidences suggest both WE/AEC and nerve signals play critical roles in blastema formation.

It's interesting and critical to understand the molecules and signals driving the influences of WE/AEC and nerve to blastema formation. Recent studies have indicated that many molecules and signal pathways

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activated during development are also activated during regeneration (Beck, 2009). Many changes of epigenetic modifications are found on the regulatory domains of tissue-specific genes (Shaw and Martin, 2009). One of the important epigenetic regulations is through histone acetylation and deacetylation which are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HDACs are a group of enzymes that remove acetyl group from lysine residues within the N-terminal tails protruding from the histone cores, leading to more condensed chromatin structure and gene silencing (Bolden et al., 2006; Haberland et al., 2009). There are four classes of HDACs, of which class I and class II have been described as important regulators for tissue regeneration (Spallotta et al., 2013). Class I HDACs (HDAC1, 2, 3, and 8) show a prevalent intranuclear distribution and they predominantly target chromatins. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) shuttle between cytoplasm and nucleus. When phosphorylated, these molecules are exported from nucleus into cytoplasm and become inactivated (de Ruijter et al., 2003; Delcuve et al., 2012). Together, they play key roles in the regulation of numerous cellular functions, including microtubule dynamics, aging and differentiation (Hu et al., 2003; Hess-Stumpp et al., 2007). HDACs have been shown to be involved in development and stem cell pluripotency (Hoxha et al., 2011). Recent studies also revealed the importance of HDACs in tadpole tail regeneration and demonstrated HDAC1 expression in the regenerating buds up to two days after amputation (Tseng et al., 2011). Thus, it's rational to hypothesize that HDACs

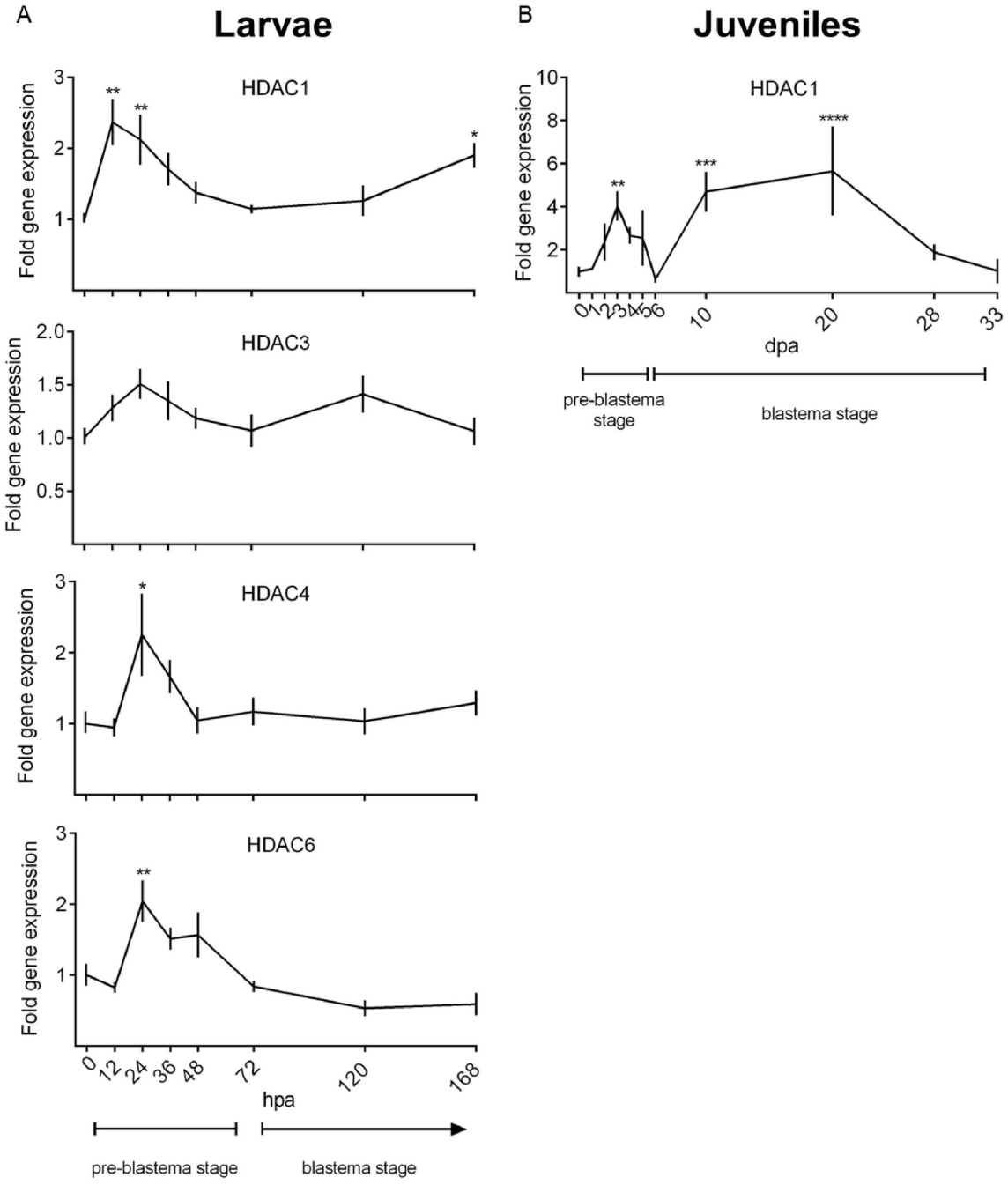


Fig. 1. Bi-phasic up-regulation of HDACs during axolotl limb regeneration. (A) Time course of HDAC expression in larvae. Expression of HDAC1 in the stump ends and regenerating buds showed two waves of significant elevation at 24 and 168 hpa, respectively. HDAC4 and HDAC6 expression is elevated only at 24 hpa. (B) Time course of HDAC1 expression of the juveniles. Bi-phasic up-regulation of HDAC1 in the pre-blastema stage and blastema stage is clearly shown in both larvae and juveniles. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

play roles in the WE/AEC-nerve axis during axolotl limb regeneration.

In the present study, we showed a bi-phasic up-regulation of HDACs during axolotl limb regeneration. Inhibition of HDAC activities impeded limb regeneration. Denervation of axolotl limbs abolish the up-regulation of HDAC1 and also inhibited limb regeneration. Supplementation of three nerve factors, BMP7, FGF2, and FGF8, into the stumps partially rescued denervation-induced non-regeneration. These data demonstrated a critical role of nerve-mediated HDACs in axolotl limb regeneration.

2. Results

2.1. Bi-phasic up-regulation of HDACs at pre-blastema and blastema stage of axolotl limb regeneration

To evaluate whether HDACs play a role in limb regeneration, HDACs expression in the stump ends and regenerating buds of 3–4 cm larval axolotls were examined during the early stage of regeneration. Quantitative polymerase chain reaction (Q-PCR) showed a trend of rapid and significant up-regulation of HDAC1, 4, and 6 peaked at around 12–24 h post-amputation (hpa). The elevation returned to basal levels at 72 hpa corresponding to the time point of initiation of blastema formation. Second wave of up-regulation, more obviously in HDAC1, began since 120 hpa and became statistically significant at 168 hpa corresponding to mid bud stage (Fig. 1A).

To more confirm the bi-phasic up-regulation of HDAC1 in the larvae, we used 10–12 cm juvenile axolotls to serially examine HDAC1 expression in the stump ends and regenerating buds up to 33 days post-amputation (dpa), a time point of initiation of early differentiation stage. Consistent with the HDAC1 expression pattern in larvae, it showed a rapid up-regulation peaked at 3 dpa and returned to the basal level at 6 dpa (the pre-blastema stage). A second wave of up-regulation during blastema stage began since 10 dpa (early bud stage), 20 dpa (mid bud stage) and returned to basal level at 33 dpa of late bud stage (Fig. 1B).

2.2. Delayed progression of limb regeneration in axolotl larvae incubated in HDAC inhibitor MS-275-containing solution

As up-regulation of HDACs was demonstrated during early phase of limb regeneration, we then tried to evaluate whether incubation of axolotl larvae in Holtfreter's solution containing MS-275, a synthetic inhibitor of class I HDACs, would affect limb regeneration. To determine an optimal concentration for the experiment, escalating MS-275 concentrations (6.25, 12.5, 25, and 50 μM) in Holtfreter's solution were used to incubate the larvae. When the concentration of MS-275 was escalated to 50 μM, around half of the larvae died (data not shown). Thus, only the concentrations of MS-275 at 6.25, 12.5, and 25 μM were used in the remaining experiments. The HDAC activities in the stump ends were dose-dependently decreased post treatment by MS-275 at 6.25, 12.5, and 25 μM from 12 to 48 hpa (Fig. 2A). However, inhibition of HDAC activities at the concentration of 25 μM from 12 to 48 hpa was only mild to modest (between 14.7 and 34.8%), suggesting the incubation method of MS-275 treatment in the larvae was not effective enough to reduce regional HDAC activity at the amputation site. Fig. 2B showed MS-275 treatments (6.25–25 μM) in a dose-dependent delay of regeneration at 15 dpa. Control subjects reached the "near complete regeneration (NCR)" stage by 21.8 ± 1.2 days, whereas MS-275-treated groups spent significantly longer period in a dose-dependent manner (25.5 ± 2.2 days at 6.25 μM, 30.3 ± 1.8 days at 12.5 μM, and 38.0 ± 2.6 days at 25 μM of MS-275, respectively. Fig. 2B) to reach NCR stage.

In order to better dissect the requirement of HDAC at different stages of limb regeneration, we performed MS-275 treatment at different timing

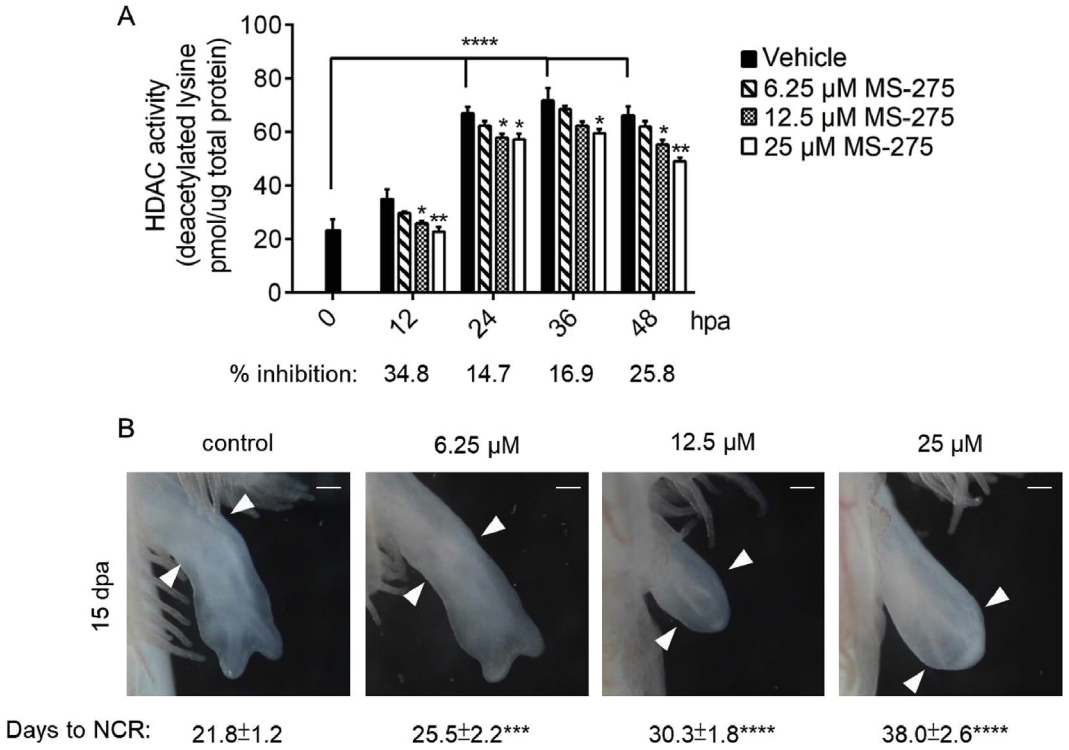


Fig. 2. Dose-dependent inhibitory effects of MS-275 on HDAC activities and limb regeneration in larvae axolotls incubated in MS-275-containing solution. (A) Soon after amputation, the larvae were respectively incubated in Holtfreter's solution containing 6.25, 12.5, or 25 μM of MS-275 till 48 hpa. HDAC activities in the stump ends significantly and dose-dependently reduced at all time points. However, the percentage of inhibition was only between 14.7 and 34.8%, even at the highest 25 μM concentration. (B) Representative photos of the regenerating limbs at 15 dpa under 0, 6.25, 12.5, or 25 μM of MS-275 showed dose-dependent inhibition of regeneration. White arrowheads indicate the plane of amputation. The duration to near complete regeneration (NCR) stage were also prolonged dose-dependently. % inhibition= (activity in control – activity in 25 μM MS-275)/activity in control × 100%. Scale bars = 1 mm *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and duration post amputation. We examined the treated axolotl larvae under microscope every day and carefully recorded the limb regeneration stages associated with each treatment protocol (Fig. 3). Treatment through the initial 3 days after amputation (0–3 dpa group) did not affect the speed of regeneration compared with the vehicle control group. However, treatments for the initial 9 and 13 dpa (0–9 dpa group and 0–13 dpa group, respectively) showed a treatment duration-dependent delay of regeneration. Notably, the delay by treatment through the initial 13 and 17 days (0–13 dpa group and 0–17 dpa group) was similar to the group treated for the whole course of 37 days (whole course group), indicating that treatment for the initial 13 days had already reached the maximal effect. However, treatment between 0 and 3 days (0–3 dpa group), 3–9 days (3–9 dpa group) or between 9 and 17 days (9–17 dpa group) did not affect regeneration speed compared with vehicle controls, indicating treatment in the initial 3 days and between 3 dpa and 9 dpa was critical to delay the progression. In correlation with the bi-phasic pattern of HDAC1 up-regulation (Fig. 1), both pre-blastema and blastema waves of HDAC1 expression were indispensable for the regeneration.

2.3. Significant reduction of cell proliferation and profound inhibition of limb regeneration by local injection of HDAC inhibitors MS-275 and TSA in juvenile axolotls

As the incubating larval axolotls in higher concentrations of MS-275 may cause lethal systemic effects and in lower concentrations only resulted in mild delay in regeneration progression (Fig. 3), we explored more effective method of MS-275 administration to further clarify the requirement of HDAC1 for limb regeneration. We injected high dose of MS-275 and Trichostatin A (TSA), another well-known pan-HDAC inhibitor (Yoshida et al., 1990), in the stump ends of juvenile axolotls in an attempt to minimize the indirect systematic effect from high dosage of HDAC inhibitor treatments. The experimental protocol is shown in Fig. 4A. The injections started right after amputation and repeated every other day until 34 dpa. Two microliters of 25 mM MS-275 or 10 mM TSA, and equal volume of 2% DMSO (as vehicle control), were injected into the stump ends. Progression of the regeneration stages were classified as early bud (EB), mid bud (MB), late bud (LB), early differentiation (ED), late differentiation (LD), and near complete regeneration (NCR). The definition of regeneration stages is presented in Supplementary Fig. 1 and Supplementary Table1. In the control group, 3 of the regenerating limbs reached to ED and 7 reached to LD stage of regeneration at 34 dpa (Fig. 4B and E). In contrast, injection of MS-275 or TSA completely inhibited blastema formation and limb regeneration (Fig. 4C, D and 4E). The juveniles receiving injection of these two kinds of HDAC inhibitors were well and did not show any abnormality in activities and feeding. HDAC inhibition by this local injection model in juveniles was more profound with 58.5% and 67.2% reduction of HDAC activities by MS-275 and TSA treatment, respectively (Fig. 4F), in contrast to the only milder reduction (14.7–34.8%, Fig. 2A) for the larvae incubated in 25 μM MS-275-containing solution. The results that both MS-275 and TSA profoundly reduced HDAC activities and completely inhibited limb regeneration indicated that their effects were through specific HDAC inhibition instead of other off-target functions.

Local injection of a 25 mM of MS-275 into juveniles did not impair wound healing, but profoundly inhibited blastema formation leading to failure of regeneration, as observed at 13 dpa (Fig. 5A and B). Revealed by 5-ethynyl-2′-deoxyuridine (EdU) labeling, percentage of cell proliferation in the mesenchyme was significantly reduced in the stumps of MS-275-treated axolotls (Fig. 5C, D, 5C′, 5D′, and 5E). Taken together, these data indicated that the local injection of HDAC inhibitor in juvenile axolotls resulted in significant reduction of cell proliferation and profound inhibition of limb regeneration.

2.4. HDAC1 expression was preferentially in WE and was nerved-mediated during limb regeneration in juvenile axolotls

Since MS-275 is a specific inhibitor of class I HDACs, we focused on the expression of HDAC1 in axolotl limb regeneration. Wound epidermis and underlying mesenchyme could be easily separated in juvenile axolotl limbs to examine the expression of HDAC1 in respective tissues. In control juveniles, HDAC1 expression was significantly up-regulated between 3 and 4 dpa in the WE (Fig. 6A, red solid line). The expression was also slightly increased in the underlying mesenchyme but not statistically significant (Fig. 6A, red dotted line). In contrast, neither WE (Fig. 6A, blue solid line) nor underlying mesenchyme (Fig. 6A, blue dotted line) showed up-regulation of HDAC1 in the denervated limbs. Consistently, *in situ* hybridization (ISH) of HDAC1 revealed more abundant HDAC1 expression in WE and neighboring skin and to a lesser extent in the underlying mesenchyme (Fig. 6B). ISH using sense probe served as a control (Fig. 6C). Taken together, these data indicated that the up-regulation of HDAC1 was in both WE and underlying mesenchyme, and this up-

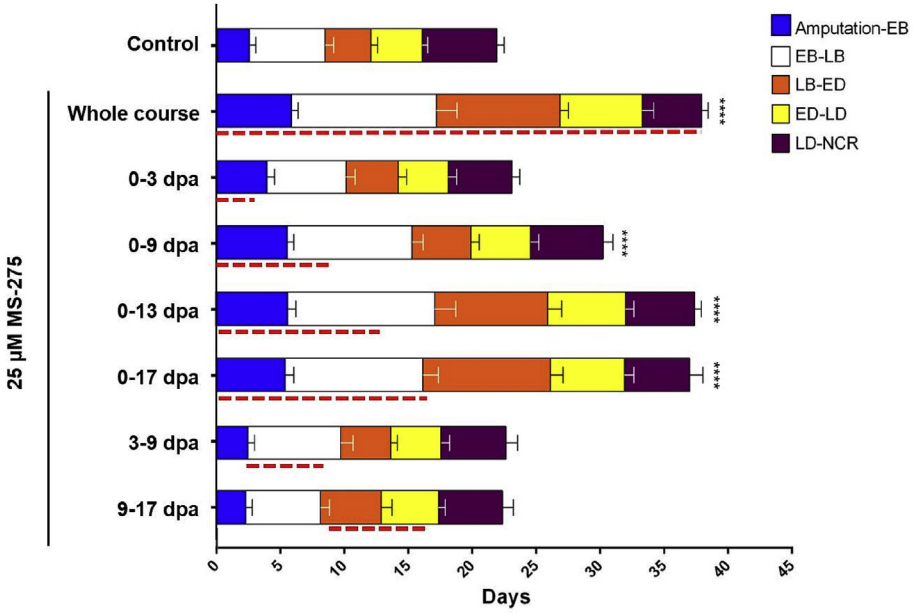
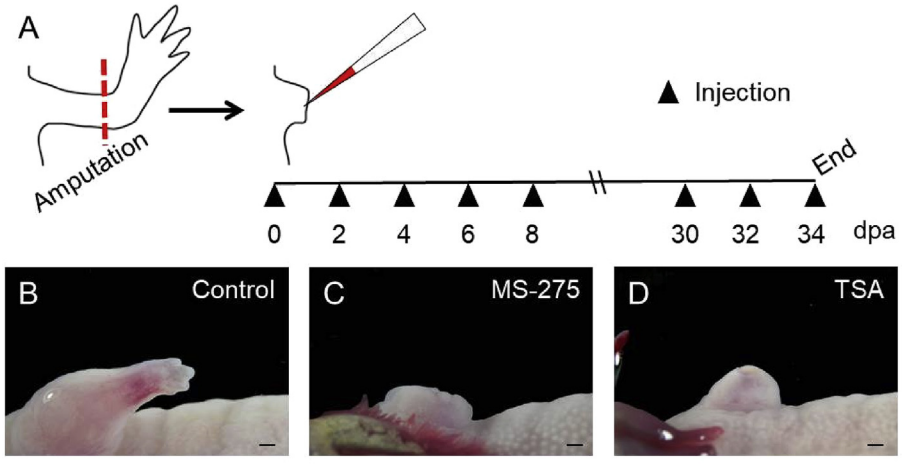


Fig. 3. Stage progression of limb regeneration in larvae axolotls incubated in 25 μM MS-275. Red dash line under each bar represents respective period of MS-275 treatment. Treatment for the whole course (0–37 dpa) significantly delayed regeneration stage progression. Treatment since day 0 showed the longer treatment the more delay in the progression (0–3 to 0–17 dpa). Note the delay reached maximum when the treatment was extended up to 13 dpa. Both the progression of 0–13 and 0–17 dpa groups were similar to the whole course group. Of particular interest, 3–9 dpa group and 9–17 dpa groups were similar to vehicle control, indicating treatment in the initial 3 days was critical for the delay in regeneration progression. EB = early bud; LB = late bud; ED = early differentiation; LD = late differentiation; NCR = near complete differentiation. ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



E

Injection	Total number	Regenerative stage					
		NR	EB	MB	LB	ED	LD
Control	10	0	0	0	0	3	7
25 mM MS-275	10	10	0	0	0	0	0
10 mM TSA	10	10	0	0	0	0	0

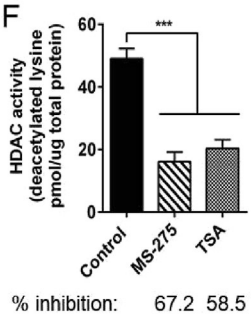


Fig. 4. MS-275- and TSA-induced regeneration inhibition by decreasing HDAC activities in juvenile axolotls. (A) Summary of experimental protocol indicating injection of MS-275 or TSA into the stump every other day since amputation till 34 dpa. The injection was divided into 3 groups: 2% DMSO (control), 25 mM MS-275, and 10 mM TSA. Representative photos at 34 dpa show a limb regenerating into LD stage in control group (B), and no regeneration in MS-275 group (C) and TSA group (D). (E) A table showing the total outcome of these 3 groups at 34 dpa. (F) HDAC activities were significantly reduced after MS-275 or TSA treatment in the stump ends. % inhibition= (activity in control − activity in treatment)/activity in control × 100%. NR = no regeneration; EB = early bud; MB = mid bud; LB = late bud; ED = early differentiation; LD = late differentiation. Scale bars = 1 mm ***$p < 0.001$.

regulation was nerve-dependent.

2.5. Partially rescuing HDAC1 expression and limb regeneration in denervated limbs by BMP7, FGF2, and FGF8

Nerve factors released from the stump nerve ends play an important role in blastema formation and limb regeneration (Tsonis, 1996; Suzuki et al., 2005; Stocum, 2011). ALM studies also demonstrated that nerves could induce successful ectopic limb formation (Endo et al., 2004). In ALM studies, grafting of gelatin beads soaked in a mixture of BMP7, FGF2, and FGF8 (BFF) could act as a substitute for nerve ends to induce accessory limb formation (Makanae et al., 2014). The substitution effects indicated that these 3 nerve factors may be, at least in part, the main factors inducing blastema formation and limb regeneration.

Thus, we hypothesized that these 3 molecules might rescue limb regeneration through reactivation of HDAC1 expression in the denervated limbs. The experimental protocol is shown in Fig. 7A. At 35 dpa, representative photos show limb regeneration to LD stage (Fig. 7B) in a control non-denervated axolotl, to ED stage in a denervated limb treated with BFF (Fig. 7C), and no regeneration at all in a denervated limb treated with vehicle control aPBS (Fig. 7D). Fig. 7E shows the numbers of limbs in each group that reached various regeneration stages at 35 dpa. All the non-denervated control limbs developed into LD stage. In the denervation + BFF group, 2 limbs failed to regenerate, 1 regenerated into MB, 4 into LB, 1 into ED, and 2 into LD stage. In contrast, all the denervation + aPBS limbs failed to regenerate.

Furthermore, we analyzed the expression of HDAC1 in WE from 1 to 6 dpa. The data showed that the expression of HDAC1 was significantly increased at 4 dpa in denervation + BFF group but not in denervation + aPBS group (Fig. 7F). These results were consistent with our hypothesis that BFF can, at least in part, substitute for nerve factors to induce HDAC1 expression and thereafter regeneration.

3. Discussion

Axolotl limb regeneration is initiated by amputation that leads to wound healing. In response to pro-regenerative signals, the cells around the wound edge are recruited to form a blastema (Gardiner et al., 1986; Endo et al., 2004). Blastema formation is mediated by interactions between nerves and WE (Stocum, 2011). Denervation prior to amputation does not affect wound healing but blocks blastema formation and limb regeneration (Todd, 1823). ALM studies also demonstrated that nerves could trigger successful ectopic limb formation (Endo et al., 2004; Satoh et al., 2007).

HDACs are essential to many biological processes. Numerous studies have shown that HDACs can affect cell growth and proliferation (Glozak and Seto, 2007). Thus, HDAC inhibitors have been used in clinical cancer therapy (Liu et al., 2006). However, the roles of HDACs in tissue regeneration remain poorly understood and are still under debate. Some reports suggest that HDACs might inhibit regeneration. For example, mice treated with HDAC inhibitors demonstrated increased proliferation, collagen deposition, and stem cell numbers at the amputation site, resulting in enhanced digital regeneration (Wang et al., 2010). HDAC inhibitors have also been shown to reduce cell apoptosis and improve functional recovery in regeneration after spinal cord injuries in mice (Lv et al., 2011). On the other hand, HDACs have also been frequently reported to improve regeneration. For instance, pharmacological blockade of HDACs increased histone acetylation levels and led to failure of tail regeneration in tadpoles (Tseng et al., 2011; Taylor and Beck, 2012). Other studies showed inhibition of HDAC activities resulted in delay of liver regeneration and hepatocyte proliferation (Chou et al., 2011), and also impeded keratinocyte proliferation and promotion of terminal differentiation through suppression of cell cycle regulators and up-regulation of differentiation genes (Elder and Zhao, 2002; Markova et al., 2007). In addition, HDAC1-null mice showed disruption of hair follicle regeneration (Hughes et al., 2014). Moreover, blocking class I HDACs activities with MS-275 resulted in more tubular injury and

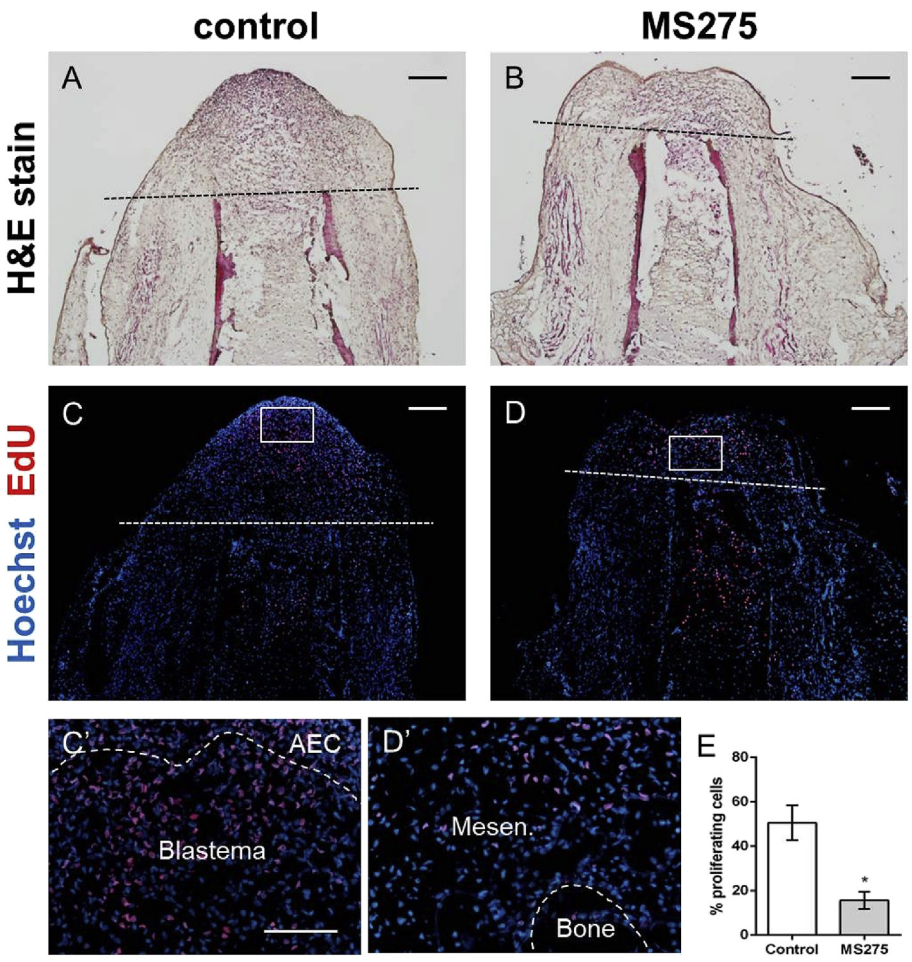


Fig. 5. MS-275-induced inhibition of limb regeneration by decreasing cell proliferation in juvenile axolotls. (A–B) Hematoxylin and Eosin staining of control and 25 mM MS-275-treatment showed inhibition of blastema formation at 13 dpa by local injection in the stump every other day. (C–D) MS-275 application blocked cell proliferation. Black dash lines indicate the plane of amputation (A, B, C, D). Nuclei that incorporated EdU are red, and all the nuclei are counterstained by Hoechst (blue). (C′) Higher magnification view of the boxed region in (C). Dotted line indicates the boundary between the basal keratinocytes of the AEC (upper) and the blastema cells (lower). (D′) Higher magnification view of the boxed region in (D). Dotted line indicates the bone. Mesen. = mesenchyme. (E) Percentage of proliferating cells in the mesenchyme was significantly reduced in the MS-275-treated (n = 3) than control (n = 3). Scale bars = 500 μm (A, B, C, D) and 100 μm (C′, D′). *p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

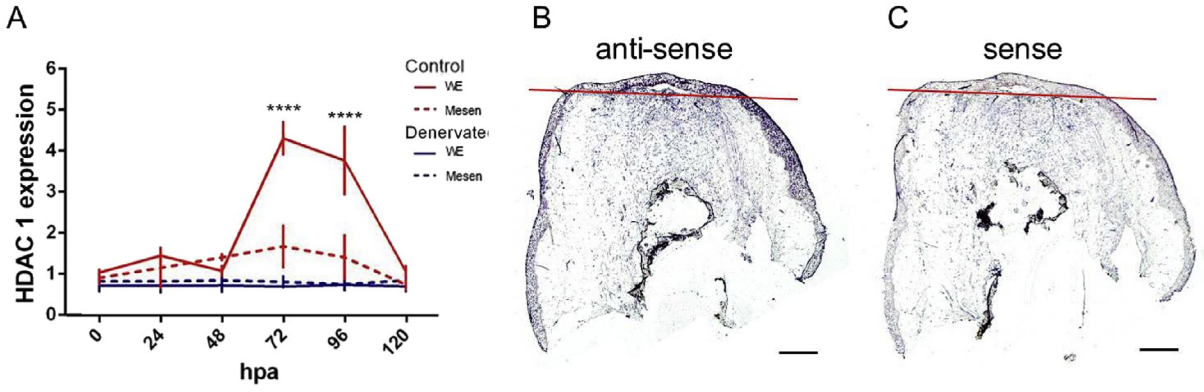
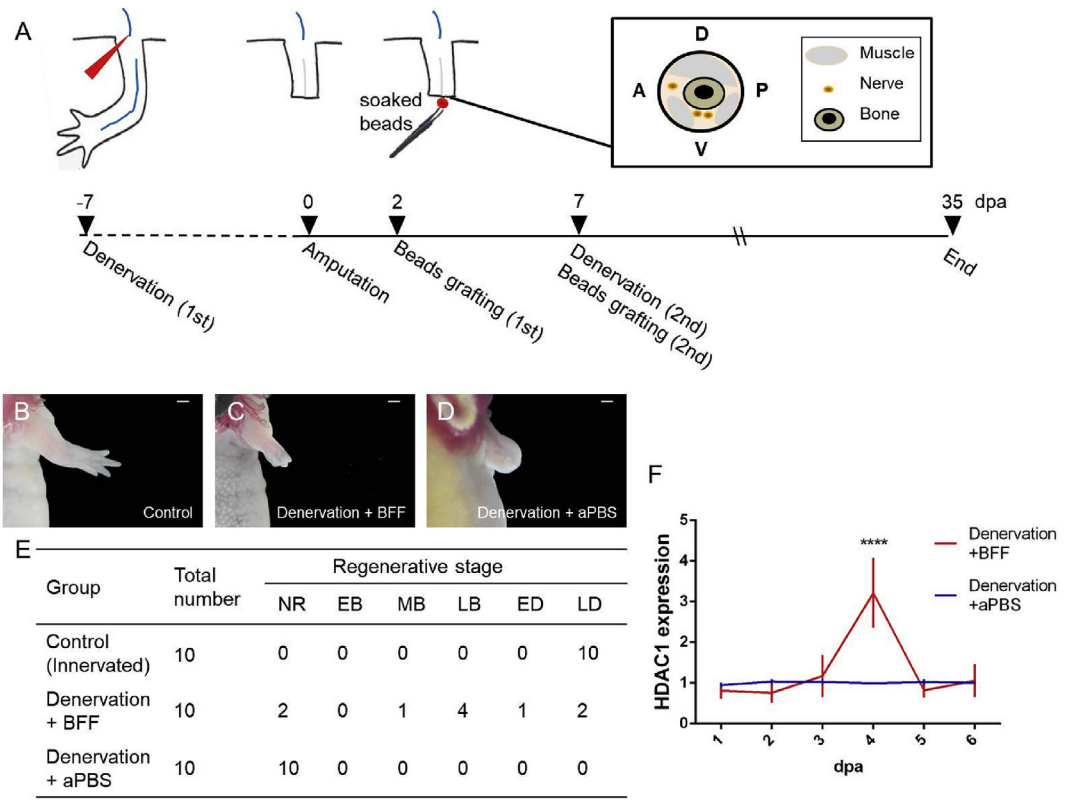


Fig. 6. Nerve-mediated HDAC1 expression preferentially in WE of juvenile axolotls. (A) Q-PCR analyses show the expression level of HDAC1. In control (innervated) juvenile axolotls, expression of HDAC1 was significantly elevated between 3 and 4 dpa in WE. This elevation in the underlying mesenchyme was not statistically significant. Preceding denervation before amputation completely abolished the elevation of HDAC1 expression in both WE and mesenchyme. (B) ISH showing that HDAC1 was expressed in the most of the WE and neighboring skin at 3 dpa. (C) Sense probe control. Even sense probe gave a faint signal, but one much weaker than that using antisense probe. Red lines in B and C indicate amputation line. Scale bars = 500 μm (B, C). ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

apoptosis, impairing renal regeneration (Tang et al., 2014).

Our study showed that HDACs played positive roles in axolotl limb regeneration. Interestingly, a bi-phasic up-regulation of HDAC1 was noted in both larvae and juvenile axolotls (Fig. 1). First phase of up-regulation was in the pre-blastema stage. Then, it returned to the basal level. A second phase started since blastema formation and returned to the basal level again approximately before start of early differentiation stage. This bi-phasic expression pattern of HDAC1 may explain why MS-275 incubation of larvae during day 0–3, day 3–9 and day 9–17 had no inhibition effect on regeneration and suggest ether wave of HDCA1 up-regulation is indispensable for the regeneration (Fig. 3). However, the biological significance of this bi-phasic up-regulation of HDAC1 instead of persistent up-regulation is currently unknown. Then we performed loss-of-function studies using an HDAC inhibitor MS-275. By incubating



E

Group	Total number	Regenerative stage					
		NR	EB	MB	LB	ED	LD
Control (Innervated)	10	0	0	0	0	0	10
Denervation + BFF	10	2	0	1	4	1	2
Denervation + aPBS	10	10	0	0	0	0	0

Fig. 7. Dependence of HDAC1 expression on nerve factors during limb regeneration in juvenile axolotls. (A) Experimental protocol shows amputation was performed 7 days following 1st denervation. Beads soaked either in aPBS containing BMP7, FGF2, and FGF8 (BFF) or aPBS alone were for the first time grafted under WE at 2 dpa. Second denervation to avoid nerve reconnection and 2nd time bead grafting were performed at 7 dpa. The regeneration was observed till 35 dpa. (B, C, D) At 35 dpa, representative photos show a limb regenerating into late differentiation stage in control group in B, a limb regenerating into early regeneration stage in denervation + BBF group in C, and a limb without regeneration in denervation + aPBS in D. (E) A table showing the outcome of the 3 groups at 35 dpa. (F) Q-PCR of HDAC1 expression in WE shows a brief elevation at 4 dpa in Denervation + BFF group (2 days after BFF bead grafting) but not in Denervation + aPBS group. NR = no regeneration; EB = early bud; MB = mid bud; LB = late bud; ED = early differentiation; LD = late differentiation. In the inset of A, A = anterior; P = posterior; D = dorsal; V = ventral. Scale bars = 1 mm (B, C, D). ****$p < 0.0001$.

larvae in Holtfreter's solution containing MS-275, inhibition of HDAC activities was mild and limb regeneration was only delayed but able to reach NCR stage after longer periods (Figs. 2 and 3). However, local injection of either MS-275 or TSA at a high dose resulted in higher % inhibition of HDAC activities and hence complete inhibition of limb regeneration (Fig. 4). Similar effects of these two different HDAC inhibitors, MS-275 and TSA, indicated that their regeneration-inhibition effects are indeed through HDAC inhibition instead of other non-specific functions.

This local injection method is better than incubation method to deliver HDAC inhibitors. Local injection of higher dose of inhibitors will keep higher concentration of the inhibitors at the local region, while avoiding possible systemic detrimental effects caused by whole larvae incubation. This approach kept these juveniles alive with normal feeding activities, and resulting in much stronger HDAC1 inhibition and more severe impairment of limb regeneration. Cell proliferation in the mesenchyme was strongly inhibited under the treatment with MS-275 (Fig. 5E), suggesting HDACs might stimulate cell proliferation and promote blastema formation. In line with our observations, recent studies have indicated that class I HDACs play an important role in cell proliferation (Rosato et al., 2003; Spallotta et al., 2013; Chen et al., 2015).

To investigate whether HDAC up-regulation is linked to nerve stimulation, we compared time-course HDAC1 expression in WE and underlying mesenchyme on normal innervated versus post-denervation limbs. For this experiment, juvenile axolotls were used instead of larvae because denervation on the brachial plexus and separation between WE and mesenchyme tissues could be more easily performed on juveniles. HDAC1 up-regulation was shown preferentially in WE and less in mesenchyme. In contrast, the expression of HDAC1 was found mainly in the mesenchyme of Xenopus tail regenerating bud (Tseng et al., 2011). The reason for this discrepancy is unknown. More strikingly, HDAC1 expression in both WE and mesenchyme was not elevated in the denervated limbs. This strongly suggested that HDAC1 up-regulation is nerve-mediated.

Since it has been noted that salamander limb regeneration is nerve-dependent nearly 2 centuries ago (Todd, 1823), identification of nerve-secreted factors that induce blastema formation and limb regeneration became a hot issue (Carlson, 2007; Singer, 1952; Stocum, 2011). Numerous candidate factors have been found, including transferrin (Kiffmeyer et al., 1991; Mescher et al., 1997), anterior gradient protein (Kumar et al., 2007), FGFs (Satoh et al., 2011) and neuregulin-1 (Farkas et al., 2016). In ALM studies, grafting of gelatin beads soaked in a mixture of BFF could act as substitutes for nerves to induce accessory limb formation (Makanae et al., 2014). Following this approach, supplementation of these 3 factors was used in our following experiments.

It's therefore interesting to clarify whether application of corresponding nerve factors in the stumps of denervated limbs would rescue HDAC1 up-regulation and hence regeneration. Our current data showed that application of nerve-derived factors, BFF, soaked in beads could briefly induce HDAC1 expression and led to partial improvement in regeneration. There are some potential reasons for why the effects on HDAC1 up-regulation and induction of regeneration were not complete in this experiment. First, there may be other nerve factors in addition to BFF that are also needed. Second, naturally occurring spatiotemporal concentrations of these factors in the tissues could not be accurately mimicked by bead grafting.

How HDACs regulate tissue regeneration is largely unknown at the molecular level. HDACs may epigenetically modify chromatin structure to regulate gene expression and thus biological functions. Apart from their epigenetic regulations, HDACs may also physically bind to a number of regulatory partners. Despite lack of DNA binding activity, HDACs can interact with transcriptional activators and repressors and become incorporated into large transcriptional complexes (Grunstein, 1997; Shahbazian and Grunstein, 2007). Thus further investigations of the molecular and cellular regulatory mechanisms of HDACs in axolotl limb regeneration are warranted. Comprehensive understanding of this process will enable exciting new biomedical therapies for promoting regenerative repair of complex structures.

In conclusion, our present data indicate that elevation of HDAC expression in WE and mesenchyme after amputation is nerve-mediated through nerve factors such as BMP7, FGF2, and FGF8. Furthermore, HDACs may serve as a relay between nerve factors and cell proliferation in the pre-blastema and blastema stage to initiate an orchestrated scenarios of blastema formation and limb regeneration (Fig. 8).

4. Materials and methods

4.1. Axolotl husbandry, limb amputation, and denervation

Axolotls, *Ambystoma mexicanum*, were reared to larvae and juveniles (3–4 cm and 8–16 cm snout to tail tip length, respectively) for experiments. Juvenile axolotls were fed fish pellets three times a week and larvae fed brine shrimp every day. All the axolotls were kept in modified Holtfreter's solution (118.4 mM NaCl, 1.3 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgSO_4.7H_2O$). The axolotls for experiment were anesthetized in a 0.1% MS-222 (Sigma-Aldrich, St. Louis, MO, USA) solution prior to any manipulation or imaging. Limb amputation was performed on the middle upper arms in both larvae and juveniles, and denervation over brachial plexus was conducted in juveniles as previously described (Wu et al., 2013). One round denervation was performed for short term follow-up experiments (less than 6 days). For follow-up observations longer than 20 days, a second round denervation was performed 7 days apart to maintain fully denervation state (Farkas et al., 2016; Farkas and Monaghan, 2015). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine and were conducted in accordance with the approved guidelines.

4.2. Q-PCR for determining HDAC expression

In larvae, stump ends were harvested at 0, 12, 24, 36, 48, 72, 120, and 168 hpa. In 8–12 cm juveniles, stump ends were harvested at 0, 1, 2, 3, 4, 5, 6, 10, 20, 28, and 33 dpa. To more easily conduct denervation and separation of WE from underlying mesenchyme for analyses, juvenile axolotls were used instead of larvae. Stumps at 0, 24, 48, 72, 96, and 120 hpa were harvested and their WE and underlying mesenchyme were separated for Q-PCR analysis. At 0-time point, proximal 2 mm of the amputated parts were harvested immediately after amputation and the epidermis and underlying soft tissue were separately collected. Total RNAs of the collected tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription using Superscript III reverse transcriptase (Invitrogen) was done at 50 °C. The first strand cDNAs were diluted 1:9 and used as templates to perform Q-PCR. Reactions were performed in a total volume of 10 μl using the SYBR Green kit (Stratagene, La Jolla, CA, USA) with addition of each primer at 0.8 μM following the manufacturer's instructions. Sequences of gene-specific primers (Supplementary Table 2) were designed based on the sequences of our next generation transcriptome sequencing (Wu et al., 2013) and axolotl ESTs from Sal-Site (Smith et al., 2005). Q-PCR was performed using ABI StepOne™ Real-Time PCR System and data were analyzed using StepOne™ software version 2.1 (Applied Biosystems, Foster City, CA, USA).

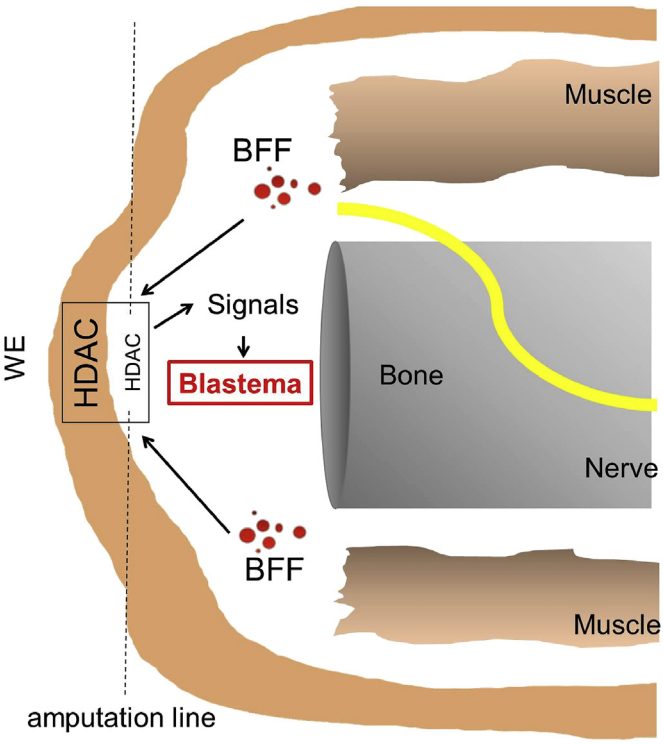


Fig. 8. Schematic diagrams illustrating summary of this study. After amputation, nerve factors including BMP7, FGF2 and FGF8 (BFF) are secreted from nerve ends to induce expression of HDACs including HDAC1 more in WE and less in mesenchyme. HDACs may induce cell proliferation and other signals to promote regeneration. Summary: axolotl limb regeneration is HDAC-dependent and HDAC up-regulation after amputation is nerve-dependent.

4.3. In situ hybridization (ISH)

ISH was performed as previously described (Wu et al., 2013) on 12 cm juveniles. HDAC1 cDNA fragment (230 bp) was amplified by RT-PCR using sense primers linked to SP6 promoter sequence and antisense primers linked to T7 promoter sequence. Sense and antisense RNA probes were synthesized using DIG RNA-labeling kit (Roche, Indianapolis, IN, USA). RNA probes were prepared using primers: HDAC1 (anti-sense), 5′-CGTAATACGACTCACTATAGGGAGAGTGAGAGGCCTTCCAAAAT G-3'; and HDAC1 (sense), 5′-CGATTTAGGTGACACTATAGAA-GAGTCTTAATGCAGGGAGGGTTG-3'. Juveniles with post-amputation limb stumps were fixed overnight at 4 °C in 4% paraformaldehyde in 1 × phosphate buffered saline (PBS) and subsequently embedded in Cryomatrix (Thermo Scientific, Miami, FL, USA) and frozen at −80 °C until sectioning at 10 μm. Following prehybridization, the stumps were hybridized overnight in a hybridization solution (50% formamide, 5 × SSC, 50 μg/mL yeast RNA, 50 μg/mL heparin, and 1% tween-20) containing the DIG-labeled RNA probe (5–10 μg/mL) at 60 °C. After hybridization, the DIG-labeled probes were detected using anti-DIG-alkaline phosphatase antibody (Roche, Indianapolis, IN, USA) and developed using a mixture of the 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride (BCIP/NBT) solution (Sigma-Aldrich) dissolved in 1 mM levamisol solution (Sigma-Aldrich).

4.4. Nuclear protein extraction and determination of HDAC activity

Nuclear protein was extracted by Nuclear Extract kit (Active motif, Carlsbad, CA, USA). In brief, the regenerating samples at indicated time points were cut and immediately placed in ice-cold PBS containing phosphatase inhibitors. The tissues were transferred to a hypotonic solution for 30 min at 4 °C and followed by homogenization using dounce homogenizer to disrupt tissues and form single-cell slurry. The

cytoplasmic fraction was lysed in a lysis buffer with pipetting. After incubation and centrifugation, the nuclei were isolated and nuclear proteins were extracted and stored at −80 °C until used.

HDAC activities in the nuclear extracts were measured by HDAC Activity Colorimetric Assay kit (BioVision, Mountain View, CA, USA) following manufacturer's instructions. This assay system uses a short peptide substrate that contains an acetylated lysine residue that can be deacetylated by certain HDAC enzymes. Once deacetylated, the substrate would be sensitized to react with a lysine developer to produce a chromophore. The chromophore was measured in an ELISA plate reader at 405 nm and their concentration was determined by a series of standard deacetylated lysine concentrations.

4.5. Incubation of larvae axolotls in MS-275-containing solution

Stock solution of MS-275 was made up using dimethyl sulfoxide (DMSO). Solutions were then diluted to desired concentrations using modified Holtfreter's solution. Limb amputation was performed on the middle upper arms. After amputation, the 3–4 cm larvae were individually separated in 6-well plates filled with 6 mL Holtfreter's solution containing various concentrations of MS-275 (Selleckchem, Houston, TX, USA) for indicated periods of treatment or indicated concentrations of MS-275 for various periods of treatment. 0.1% DMSO was used as a control. Each group included 5 animals with MS-275 treatment at various concentrations and for various durations as shown in Fig. 2A–D and Fig. 3, respectively. The solution containing MS-275 was changed daily to maintain the stability of the inhibitor. The axolotls were observed microscopically every day to record stage progression.

4.6. HDAC inhibitor injection into amputated limbs of juvenile axolotls

Stock solution of MS-275 and TSA (ApexBio Technology, Houston, TX, USA) were made up using DMSO. The stock solutions were then diluted to the final treatment concentration with filtered 0.8 × amphibian PBS (aPBS; dilute 1× PBS into 0.8× PBS with dH_2O). The glass Pasteur pipettes (Kimble Chase, Vineland, NJ, USA) for the injection was sharpened manually (external tip diameter: 40 μm). Two microliters of 25 mM of MS-275, 10 mM of TSA or 2% DMSO diluted in aPBS as a control were injected using the glass Pasteur pipettes into the stumps right beneath WE of 15–16 cm juveniles every other day since amputation.

4.7. Morphological criteria of limb regeneration stages

To record regeneration stage progression, morphological criteria for limb regeneration stages of EB, MB, LB, ED, LD and NCR were determined with modification from previous studies (Iten and Bryant, 1973; Tank et al., 1976) as shown in supplementary table 1 and supplementary Fig. 1.

4.8. EdU labeling

To detect proliferating cells in the regenerating bud, the 15–16 cm juvenile received intraperitoneal injection of 100 μg/g body weight of EdU (Invitrogen) 20 h prior to tissue collection. Collected tissues were fixed in 4% PFA in 1 × PBS, embedded in Cryomatrix (Thermo Scientific). On the sections, EdU labeling was demonstrated by using the reagents provided in the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) following manufacturer's instructions. The slides were mounted with fluorescence mounting medium (DakoCytomation, Carpinteria, CA, USA) and EdU-positive nuclei were detected under a fluorescence microscope.

4.9. Bead grafting

Dried gelatin beads with 200–400 μm diameter were allowed to swell in aPBS (control) or aPBS containing mouse BMP7, FGF2, and FGF8 (BFF, for 0.1 μg/μl each, R&D systems, Minneapolis, MN, USA) (Makanae et al., 2014). Denervation was performed on 8–12 cm juvenile axolotls 7 days prior to amputation. Two days after amputation, 3 beads soaked in aPBS with or without BFF were grafted under WE at locations approximately corresponding to the 3 major nerve ends (Fig. 7A, indicated as "nerve" in the cross section cartoon of an upper arm). These juvenile axolotls received a second round of denervation at 7 dpa and followed by a second round of placement of experimental group-specific beads. Limb regeneration stages were recorded at 35 dpa.

4.10. Microscopy and image processing

Bright-field and fluorescence images were obtained using Olympus BX51 and Olympus SZX7 microscopes (Olympus, Tokyo, Japan). The images were processed using Photoshop (Adobe Systems, San Jose, CA, USA) and only brightness/contrast functions were performed simultaneously on experimental and control images together to increase the signal/noise ratio.

4.11. Statistics

All the experiments were performed on triplicate animals. Data are presented as mean ± SEM. Two-way ANOVA and Bonferroni's post hoc test or one-way ANOVA and Dunnett's multiple comparison test were used to compare multiple means between groups. All the statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). P values less than 0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.02.011.

References

Beck, C.W., 2009. Beyond early development: Xenopus as an emerging model for the study of regenerative mechanisms. Dev. Dynam. 238, 1226–1248.
Boilly, B., Albert, P., 1990. In vitro control of blastema cell proliferation by extracts from epidermal cap and mesenchyme of regenerating limbs of axolotls. Roux Arch. Dev. Biol. 198, 443–447.
Bolden, J.E., et al., 2006. Anticancer activities of histone deacetylase inhibitors. Nat. Rev. Drug Discov. 5, 769–784.
Brockes, J.P., Kumar, A., 2008. A Comparative aspects of animal regeneration. Annu. Rev. Cell Dev. Biol. 24, 525–549.
Carlson, B.M., 2007. Principles of Regenerative Biology. Academic Press, Cambridge.
Chen, S., et al., 2015. Histone deacetylase 1 and 2 regulate Wnt and p53 pathways in the ureteric bud epithelium. Development 142, 1180–1192.
Chou, C.W., et al., 2011. HDAC inhibition decreases the expression of EGFR in colorectal cancer cells. PLoS One 6, e18087.
Delcuve, G.P., et al., 2012. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin. Epigenet. 4, 5.
de Ruijter, A.J., et al., 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370, 737–749.
Elder, J.T., Zhao, X., 2002. Evidence for local control of gene expression in the epidermal differentiation complex. Exp. Dermatol. 11, 406–412.
Endo, T., et al., 2004. A stepwise model system for limb regeneration. Dev. Biol. 270, 135–145.
Farkas, J.E., Monaghan, J.R., 2015. Housing and maintenance of Ambystoma mexicanum, the Mexican axolotl. In: Kumar, A., Simon, A. (Eds.), Salamanders in Regeneration Research: Methods and Protocols. Humana Press, New York, pp. 27–46.
Farkas, J.E., et al., 2016. Neuregulin-1 signaling is essential for nerve-dependent axolotl limb regeneration. Development 143, 2724–2731.

Gardiner, D.M., et al., 1986. The migration of dermal cells during blastema formation in axolotls. Dev. Biol. 118, 488–493.
Globus, M., Vethamany–Globus, S., 1985. In vitro studies of controlling factors in newt limb regeneration. In: Sicard, R.E. (Ed.), Regulation of Vertebrate Limb Regeneration. Oxford University Press, New York, pp. 106–127.
Glozak, M.A., Seto, E., 2007. Histone deacetylases and cancer. Oncogene 26, 5420–5432.
Goss, R.J., 1956. Regenerative inhibition following limb amputation and immediate insertion into the body cavity. Anat. Rec. 126, 15–27.
Grunstein, M., 1997. Histone acetylation in chromatin structure and transcription. Nature 389, 349–352.
Haberland, M., et al., 2009. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. 10, 32–42.
Hess-Stumpp, H., et al., 2007. MS-275, a potent orally available inhibitor of histone deacetylases–the development of an anticancer agent. Int. J. Biochem. Cell Biol. 39, 1388–1405.
Hoxha, E., et al., 2011. HDAC1 plays an important role in the differentiation of embryonic stem cells and induced pluripotent stem cells into cardiovascular lineages. Dev. Biol. 356, 221.
Hu, E., et al., 2003. Identification of novel isoform selective inhibitors within class I histone deacetylases. J. Pharmacol. Exp. Therapeut. 307, 720–728.
Hughes, M.W., et al., 2014. Disrupted ectodermal organ morphogenesis in mice with a conditional histone deacetylase 1, 2 deletion in the epidermis. J. Invest. Dermatol. 134, 24–32.
Iten, J.E., Bryant, S.V., 1973. Forelimb regeneration from different levels of amputation in the newt, Notophthalmus uzrzdescens: length, rate and stages. Wilhelm Roux Arch. Entwickl. Mech. Org. 173, 263–282.
Kiffmeyer, W.R., et al., 1991. Axonal transport and release of transferrin in nerves of regenerating amphibian limbs. Dev. Biol. 147, 392–402.
Kumar, A., et al., 2007. Molecular basis for nerve dependence of limb regeneration in an adult vertebrate. Science 318, 772–777.
Liu, T., et al., 2006. Histone deacetylase inhibitors: multifunctional anticancer agents. Cancer Treat Rev. 32, 157–165.
Loyd, R.M., Tassava, R.A., 1980. DNA synthesis and mitosis in adult newt limbs following amputation and insertion into the body cavity. J. Exp. Zool. 214, 61–69.
Lv, L., et al., 2011. Valproic acid improves outcome after rodent spinal cord injury: potential roles of histone deacetylase inhibition. Brain Res. 1396, 60–68.
Makanae, A., et al., 2014. Co-operative Bmp- and Fgf-signaling inputs convert skin wound healing to limb formation in urodele amphibians. Dev. Biol. 396, 57–66.
Markova, N.G., et al., 2007. Inhibition of histone deacetylation promotes abnormal epidermal differentiation and specifically suppresses the expression of the late differentiation marker pro-filaggrin. J. Invest. Dermatol. 127, 1126–1139.
McCusker, C., et al., 2015. The axolotl limb blastema: cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods. Regeneration 2, 54–71.
Mescher, A.L., 1976. Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. J. Exp. Zool. 195, 117–128.
Mescher, A.L., et al., 1997. Transferrin is necessary and sufficient for the neural effect on growth in amphibian limb regeneration blastemas. Dev. Growth Differ. 39, 677–684.
Rosato, R.R., et al., 2003. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1. Cancer Res. 63, 3637–3645.
Satoh, A., et al., 2007. Nerve-induced ectopic limb blastemas in the Axolotl are equivalent to amputation-induced blastemas. Dev. Biol. 312, 231–244.
Satoh, A., et al., 2010. Cummings GM, Bryant SV, Gardiner DM. Neurotrophic regulation of fibroblast dedifferentiation during limb skeletal regeneration in the axolotl (Ambystoma mexicanum). Dev. Biol. 337, 444–457.
Satoh, A., et al., 2011. Blastema induction in aneurogenic state and Prrx-1 regulation by MMPs and FGFs in Ambystoma mexicanum limb regeneration. Dev. Biol. 355, 263–274.
Shahbazian, M.D., Grunstein, M., 2007. Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. 76, 75–100.
Shaw, T., Martin, P., 2009. Epigenetic reprogramming during wound healing: loss of polycomb-mediated silencing may enable upregulation of repair genes. EMBO Rep. 10, 881–886.
Singer, M., 1952. The influence of the nerve in regeneration of the amphibian extremity. Q. Rev. Biol. 27, 169–200.
Singer, M., Salpeter, M., 1961. Regeneration in vertebrates: the role of the wound epithelium. In: Zarrow, M.X. (Ed.), Growth in Living System. Basic Books, New York, pp. 277–311.
Singer, M., 1974. Trophic functions of the neuron. VI. Other trophic systems. Neurotrophic control of limb regeneration in the newt. Ann. NY Acad. Sci. 228, 308–322.
Smith, J.J., et al., 2005. Sal-Site: integrating new and existing ambystomatid salamander research and informational resources. BMC Genomics 6, 181.
Spallotta, F., et al., 2013. Detrimental effect of class-selective histone deacetylase inhibitors during tissue regeneration following hindlimb ischemia. J. Biol. Chem. 288, 22915–22929.
Stocum, D.L., 2011. The role of peripheral nerves in urodele limb regeneration. Eur. J. Neurosci. 34, 908–916.
Suzuki, M., et al., 2005. Nerve-dependent and -independent events in blastema formation during Xenopus froglet limb regeneration. Dev. Biol. 286, 361–375.
Tang, J., et al., 2014. Class I HDAC activity is required for renal protection and regeneration after acute kidney injury. Am. J. Physiol. Renal. Physiol. 307, 303–316.
Tank, P.W., et al., 1976. A staging system for forelimb regeneration in the axolotl, Ambystoma mexicanum. J. Morphol. 150, 117–128.
Tassava, R.A., Mescher, A.L., 1975. Roles of injury, nerves, and wound epidermis during initiation of amphibian limb regeneration. Differentiation 4, 23–24.
Tassava, R.A., Garling, D.J., 1979. Regenerative responses in larval axolotl limbs with skin grafts over the amputation surface. J. Exp. Zool. 208, 97–110.
Taylor, A.J., Beck, C.W., 2012. Histone deacetylases are required for amphibian tail and limb regeneration but not development. Mech. Dev. 129, 208–218.
Todd, T.J., 1823. On the process of reproduction of the members of the aquatic salamander. Q. J. Sci. Lit. Arts 16, 84–96.
Tseng, A.S., et al., 2011. HDAC activity is required during Xenopus tail regeneration. PLoS One 6, e26382.
Tsonis, P.A., 1996. Limb Regeneration. Cambridge University Press, New York.
Wallace, H.H., 1981. Vertebrate Limb Regeneration. Wiley, Chichester.
Wang, G., et al., 2010. The effects of DNA methyltransferase inhibitors and histone deacetylase inhibitors on digit regeneration in mice. Regen. Med. 5, 201–220.
Wu, C.H., et al., 2013. De novo transcriptome sequencing of axolotl blastema for identification of differentially expressed genes during limb regeneration. BMC Genomics 14, 434.
Yoshida, M., et al., 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. 265, 17174–17179.