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Endogenous retinoic acid mediates the early events in salamander limb regeneration

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Urodeles including newt and salamander have remarkable regenerative capacity during the postembryonic life span. Some of the unique features are the formation of the well-developed wound epidermis and the active dedifferentiation process in the early phase of regeneration. These are regarded as key events for the successful regeneration since no further regenerative activity is possible without them. In this study, we investigated the role of retinoic acid (RA) in salamander limb regeneration by blocking RA synthesis using disulfiram, an inhibitor of aldehyde dehydrogenase that oxidizes retinal to RA. Disulfiram treatment resulted in delaying the limb regeneration processes via inhibition of wound epidermis formation and dedifferentiation process. When RA was administered after disulfiram treatment, the inhibitory effect of disulfiram was rescued. In addition, disulfiram treatment after the dedifferentiation stage resulted in the mild retardation of limb regeneration, suggesting that RA might also be involved in the blastema outgrowth. Furthermore, salamander *MMP-9* gene expression was also inhibited by disulfiram treatment. Collectively, our findings indicate that endogenous RA may play an important role(s) in the early phase of limb regeneration by regulating the expression of molecules responsible for the modification of intracellular and extracellular environment during salamander limb regeneration.

Keywords: dedifferentiation; salamander limb regeneration; retinoic acid; *MMP-9*

DEVELOPMENTAL BIOLOGY

Introduction

Regeneration is the recovery of body parts that have been lost or destroyed during the postembryonic life span. Although mammals have a limited regenerative power, urodeles including salamander and newt possess an outstanding regenerative ability even in the adult state. The body parts to be regenerated in urodele include limbs, tail, lens, jaw and spinal cord (Carlson 2007). Especially, limbs of urodele have been used extensively as a typical model system of regeneration. Limb regeneration is composed of four steps: wound healing, dedifferentiation, blastema formation and redifferentiation (Stocum 1979). At dedifferentiation stage, the mature cells lose their differentiated characteristics and re-enter cell cycle to produce blastema cells. Therefore, dedifferentiation is a critical event between the regenerating and the non-regenerating systems (Tanaka 2003; Stocum 2004). During dedifferentiation, extensive tissue remodeling such as modification of extracellular matrix (ECM) is undergoing in the stump by the activities of various enzymes such as matrix metalloproteinases (MMPs), lysosomal enzymes and peptidases (Ju and Kim 1998, 2000, 2010; Park and Kim 1999; Yang et al. 1999; Carlson 2007; Santosh et al. 2011). Since ECM is involved in the stabilization of the differentiation state of cells, the degradation of ECM in the mature stump tissue is likely to cause the disorganization of the differentiated state and triggers the early event of regeneration (Hay 1993; Daley et al. 2008).

Retinoic acid (RA) is considered as one of the key molecules in regeneration since it has a noticeable effect on the dedifferentiation state in the regenerating salamander limb (Ju and Kim 1994, 1998, 2010; Shimizu-Nishikawa et al. 2001). RA is an endogenous derivative of vitamin A, which is involved in many developmental processes including embryogenesis, spermatogenesis and epithelial tissue differentiation (Marill et al. 2003; Clagett-Dame and Knutson 2011; Rhinn and Dollé 2012). Alcohol dehydrogenases metabolize vitamin A (retinol) to retinal, which is, in turn, converted to all-trans RA or 9-cis RA by the retinal dehydrogenases (Duester 2000). RA modulates the gene expression through RA response element, which is mediated by RA receptors (RARs) and retinoid X receptors (Duong and Rochette-Egly 2011).

In the salamander limb regeneration, exogenous supply of RA at the dose of pharmacological level results in the extensive dedifferentiation in the stump tissue and pattern duplication. This was demonstrated by the increased expression and activity of marker enzymes of dedifferentiation such as MMP-9, cathepsin D and lysosomal acid phosphatase after RA treatment both in gene expression and enzyme activity (Ju and Kim 1998, 2000, 2010; Park and Kim 1999; Yang et al. 1999; Santosh et al. 2011). The pattern duplication in the regenerating amphibian limbs by

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exogenous RA suggests that RA resets positional memory of blastema cells (Kim and Stocum 1986; Crawford and Stocum 1988; Ludolph et al. 1990). In the developing chick limb bud, RA differentially concentrates on the posterior region compared to the anterior region (Thaller and Eichele 1987). Interestingly, local application of RA to the anterior margin disrupts the established gradient of RA on chick wing bud and induces mirror-imaged pattern duplication of wing skeletons in the anteroposterior axis (Eichele et al. 1985). Furthermore, inhibition of RA synthesis suppresses chick wing bud development, implying that RA also might be required for the initiation of the chick wing bud outgrowth (Stratford et al. 1996). In addition, RA gradient is actively established in development and regeneration processes of amphibian limbs (Scadding and Maden 1994; McEwan et al. 2011).

In this study, we examined the role of endogenous RA in salamander limb regeneration by inhibition of RA synthesis using disulfiram, an inhibitor of aldehyde dehydrogenase. Our findings indicate that RA may be involved in the wound epidermis formation and initiation of dedifferentiation via up-regulation of *MMP-9* gene expression during salamander limb regeneration.

Materials and Methods

Animal maintenance

Salamander (*Hynobius leechii*) larvae were maintained as described previously (Ju and Kim 1994). Briefly, the larvae were individually reared in latticed cages to prevent cannibalism and fed finely chopped beef liver. At the time of amputation, the larvae were 20 to 30 mm in length from snout to tail.

Experimental manipulation

For surgical manipulations, animals were anesthetized in 0.02% benzocaine solution. The forelimbs of each animal were amputated bilaterally through the distal stylopodium (upper arm) level. Any protruding limb cartilage due to the contraction of soft tissue was trimmed to make a flat cut surface immediately after amputation. Disulfiram (Tetraethylthiuram disulfide, Sigma, USA) dissolved in dimethyl sulfoxide (DMSO) was injected to each salamander larvae intraperitoneally at the dose of 100 μg/g body weight using microliter syringe (Hamilton, USA). RA (all trans, type XX, Sigma) dissolved in DMSO was injected intraperitoneally at the dose of 40 μg/g body weight. To compare the effect of the administration stage in the course of regeneration, animals were injected at 0 (immediately), 1, 4 and 8 day(s) after amputation with disulfiram. Same volume of DMSO was injected intraperitoneally as a control.

Histology

Limbs were collected at each time course, and they were fixed in Bouin solution. Histological examination was performed as described previously (Ju and Kim 1994). The specimens were processed for paraffin embedding, serially sectioned at 10 μm and stained with Ehrlich hematoxylin and eosin.

Whole mount in situ *hybridisation*

Limb regenerates were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for overnight. The limb regenerates injected with disulfiram were analyzed for *Hynobius MMP-9* gene expression by whole mount *in situ* hybridization. The riboprobe of MMP-9 was transcribed by T7 RNA polymerase from Kpn I linearized MMP-9 cDNA using DIG-labeling kit (Boehringer Mannheim, Germany). The whole mount *in situ* hybridization was performed according to the Wilkinson's protocol with some modifications (Wilkinson 1992; Ju and Kim 1998). After hybridization, color reaction was carried out using BM purple alkaline phosphatase substrate (Boehringer Mannheim) in a dark box with gentle rocking for several hours or overnight. The specimens were washed 3 times with stop solution (1mM EDTA, 0.1% Tween 20 in PBS).

Results

Retardation of regeneration by disulfiram treatment

We investigated the role of RA in the regenerating salamander limbs using disulfiram, an inhibitor of aldehyde dehydrogenase that mediates RA synthesis. In order to find the most effective stage of the regeneration by the disulfiram injection, *Hynobius* larvae were intraperitoneally injected with disulfiram at 0 day (DS 0), 1 day (DS 1), 4 days (DS 4) and 8 days (DS 8) after amputation. We first examined the days required to reach the medium bud stage from amputation. As shown in Figure 1, the rate of regeneration in disulfiram-injected group was evidently retarded compared to that in non-injected control group. The retardation was especially pronounced when the disulfiram was injected in the early stage of limb regeneration. While control limb regenerates took for 7 days to reach the medium bud stage, disulfiram-treated regenerates took for 20 days, 17 days, 12 days and 9 days in the groups of DS 0, DS 1, DS 4 and DS 8, respectively.

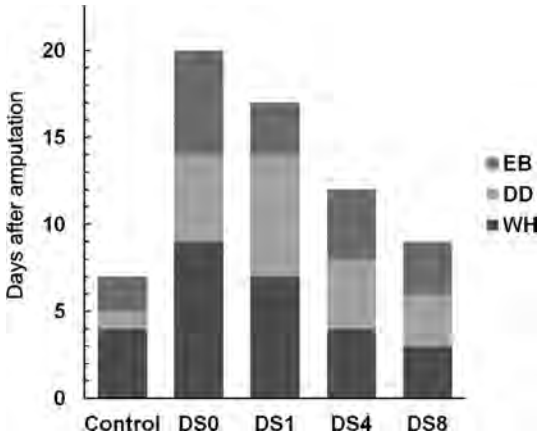

Figure 1. Regeneration rate in the disulfiram-treated salamander limbs. Amputation was performed through the level of elbow in the forelimbs of *Hynobius leechii* in these series of experiments. The regeneration rates of limbs in all disulfiram-treated groups are decreased compared to that of the control. Animals were injected with disulfiram at 0 (immediately; DS 0), 1 (DS 1), 4 (DS 4) and 8 days (DS 8) after limb amputation. The various boxes denote days corresponding to the specified regeneration stages up to the medium bud stage after amputation in the limb regenerates (Tank et al. 1976; Ju and Kim 1994). Non-injected animals are used as a control. WH, wound healing stage; DD, dedifferentiation stage; EB, early bud stage.

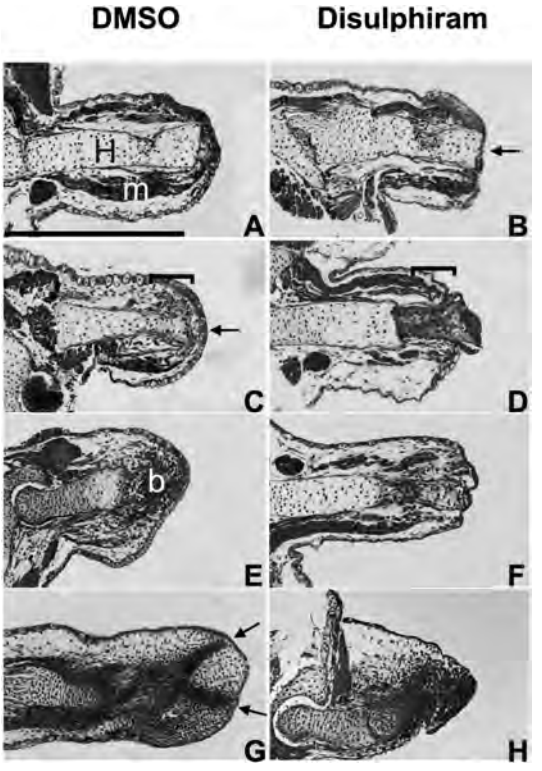

Figure 2. Histological analysis of limb regenerates treated with disulfiram at day 0. Salamanders were injected with DMSO (A,C,E,G) or with disulfiram (B,D,F,H) immediately after amputation (DS 0). Scale bar; 1mm. (A,B) Limb regenerates at 1 day after amputation (1 day after injection). Note the thinner wound epithelium (arrow) in disulfiram-treated regenerate compared to the DMSO-treated control; H, humerus; m, muscle. (C,D) Limb regenerates at 4 days after amputation (4 days after injection). In DMSO-treated control regenerate, dedifferentiation is extensive (bracket) under the thickened wound epithelium (arrow). However, the level of dedifferentiation is low, (bracket) and the wound epithelium is not well-developed in disulfiram-treated regenerate. (E,F) Limb regenerates at 7 days after amputation (7 days after injection). While blastema develops well in control regenerate, blastema cannot be discernible in disulfiram-treated regenerate; b, blastema. (G,H) Limb regenerates at 17 days after amputation (17 days after injection). Note the condensation of mesenchymal cells for digit formation (arrows) in DMSO-treated regenerates. An abnormal cellular condensation is formed at the tip of the disulfiram-treated limb.

Histological analysis of disulfiram-treated limb regenerates

We further performed histological analysis of limb regenerates throughout regeneration period. In DS 0 group, wound epithelium of limb regenerates was thinner than that of control group at 1 day after amputation (1 day after injection) (Figure 2A and B). While control wound epithelium thickened for a few days after wound healed, it remained thin in disulfiram-treated limb regenerates (Figure 2C and D). In addition, disulfiram treatment resulted in inhibition of dedifferentiation process and blastema formation (Figure 2E and F). At 17 days after amputation (17 days after injection), control limb regenerates reached to palette stage (Figure 2G). However, limb regenerate was still remained at the blastema formation stage in disulfiram-injected group (Figure 2H). The histological aspects of DS 1 group were similar to that of the DS 0 group (data not shown).

To examine the effect of disulfiram on the process of dedifferentiation, one group of animals was subjected to disulfiram injection at 4 days after amputation (DS 4). At 4 days after amputation, the dedifferentiation is at its peak level in the normally regenerating limbs (Ju and Kim 1994). As shown in Figure 3A and B, wound epithelium and blastema were not well developed in disulfiram-treated regenerate at 5 days after amputation (1 day after injection). At 12 days after amputation (8 days after injection), outgrowth of disulfiram-treated blastema appears to be more retarded compared to that of control regenerate (Figure 3C and D).

We further tested whether disulfiram affects the blastema outgrowth even after it has been initiated. Disulfiram was given to one group of animals at 8 days after amputation (DS 8) when the control limb

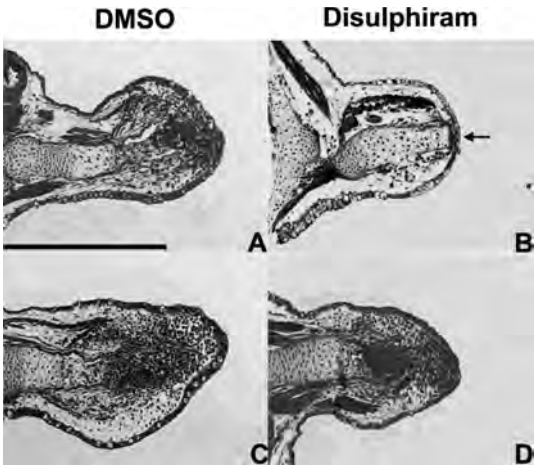

Figure 3. Histological analysis of limb regenerates treated with disulfiram at day 4. Salamanders are injected with DMSO (A,C) or with disulfiram (B,D) at 4 days after amputation. Scale bar; 1mm. (A,B) Limb regenerates at 5 day after amputation (1 day after injection). While extensively dedifferentiated internal tissues and distal accumulation of blastema cells are noted in DMSO-treated regenerate, wound epithelium (arrow) is thinner and less dedifferentiated tissues are observed in disulfiram-treated regenerate. (C,D) Limb regenerates at 12 days after amputation (8 days after injection). The outgrowth of blastema appears to be retarded in disulfiram-treated regenerate compared to control.

regenerate was at medium bud stage (Figure 4). The regenerate at 9 days after amputation (1 day after injection) showed a little shrunken blastema compared

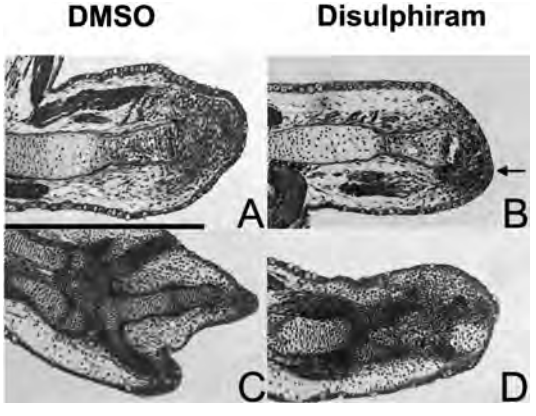

Figure 4. Histological analysis of limb regenerates treated with disulfiram at day 8. Salamanders are injected with DMSO (A,C) or with disulfiram (B,D) at 8 days after amputation. Scale bar; 1mm. (A,B) Limb regenerates at 9 days after amputation (1 day after injection). The disulfiram-treated blastema (arrow) is somewhat smaller than that in the control. (C,D) Limb regenerates at 20 days after amputation (12 days after injection). The digits are almost formed in the control regenerate. Disulfiram treatment resulted in slow regeneration rate, but limb regenerate reached the palette stage.

to the control (Figure 4A and B). Although the rate of regeneration in disulfiram-injected group was somewhat slower than that of control group, disulfiram-treated limb regenerate reached the palette stage at 20 days after amputation (12 days after injection) (Figure 4C and D). These findings indicate that disulfiram treatment might inhibit the process of wound epithelium formation and dedifferentiation during salamander limb regeneration.

RA rescues the phenotype caused by disulfiram treatment

To see whether the inhibitory effect of disulfiram on limb regeneration is due to the depletion of endogenous RA, disulfiram injection was done immediately after amputation and RA was subsequently injected at 4 days after the disulfiram injection. Although wound epithelium formation was somewhat inhibited, dedifferentiation was evident (Figure 5A and B). At 12 days after amputation and disulfiram injection (8 days after RA injection), a spike-like blastema under the well-developed wound epithelium was observed (Figure 5C). The digit formation was completed almost by 20 days after amputation and disulfiram injection (16 days after RA injection) at which time point the control regenerate showed similar morphology (Figure 5D).

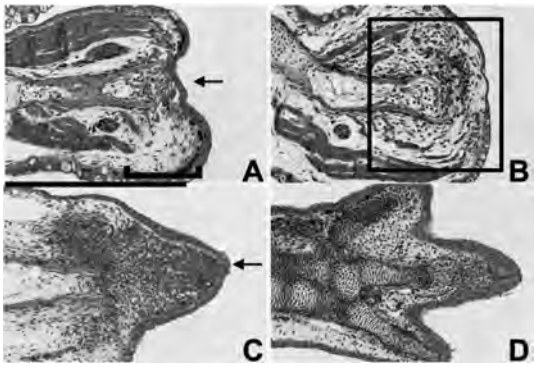

Figure 5. Histological analysis of limb regenerates treated with disulfiram and RA. Disulfiram injection was done immediately after amputation. RA was injected 4 days after disulfiram injection. (A) Limb regenerate at 5 days after amputation and disulfiram injection (1 day after RA injection). The wound epithelium (arrow) is still hypomorphic, but the extensive dedifferentiation process (bracket) can be seen in the distal portion of the regenerate. (B) Limb regenerate at 8 days after amputation and disulfiram injection (4 days after RA injection). Note the active dedifferentiation in progress (box). (C) Limb regenerate at 12 days after amputation and disulfiram injection (8 days after RA injection). Note the well-formed blastema (arrow). (D) Limb regenerate at 20 days after amputation and disulfiram injection (16 days after RA injection). The digits are completely formed, and regenerate showed similar morphology to the control regenerate of equivalent days.

These results indicate that the low level of dedifferentiation and the retarded growth of blastema in disulfiram-treated limb regenerates can be rescued by exogenous supply of RA.

The effect of disulfiram on MMP-9 gene expression

Since *MMP-9* gene expression is up-regulated in the dedifferentiation stage during salamander limb regeneration, we investigated the effects of disulfiram treatment on its expression by whole mount *in situ* hybridization. In control DMSO-injected group, *MMP-9* gene expression started to be detected in the wound epithelium at 2 days after amputation (1 day after injection) (Figure 6A). The expression level of *MMP-9* gene was significantly up-regulated and reached the peak level in the distal region of the mesenchymal tissue at 8 days after amputation (7 days after injection) (Figure 6C). As the regenerate entered the notch stage, the *MMP-9* gene was expressed only in the edge of the hand plate (data not shown). Consistent with our findings, axolotl *MMP-9* gene expression showed the bimodal activation during limb regeneration (Yang et al. 1999). In the disulfiram-injected limb regenerates, the expression region of *MMP-9* was not changed but the expression level was very low and expression region was restricted to the tip of the regenerates (Figure 6B and D). These results suggest that endogenous RA may up-regulate *MMP-9* expression in salamander limb regeneration.

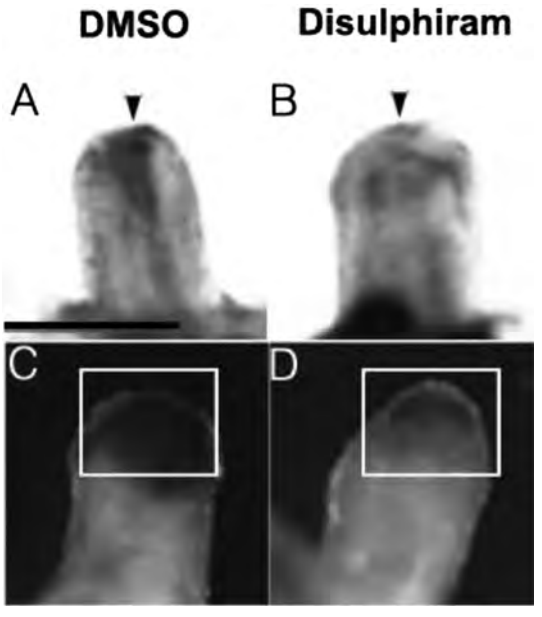

Figure 6. The gene expression pattern of *MMP-9* in the disulfiram-treated regenerating salamander limbs. Salamanders were injected either with DMSO (A,C) or disulfiram (B,D) 1 day after amputation. (A,B) Limb regenerate at 2 days after amputation (1 day after injection). Low level of *MMP-9* gene expression was detected in wound epithelium of both DMSO- or disulfiram-treated regenerates (arrowhead), but the expression level is somewhat higher in the control regenerate. (C,D) Limb regenerates at 8 days after amputation (7 days after injection). *MMP-9* gene appears to be expressed remarkably in the whole blastema (box). However, in disulfiram-treated regenerate, the expression is low and its signal is restricted to the distal region of blastema (box).

Discussion

A number of studies suggest that the RA is essential in various developmental processes including limb regeneration. Exogenous RA is well known to induce the duplication in three cardinal axes, i.e., proximodistal, dorsoventral and anteroposterior axes and probably to respecify the positional identity of the blastema cells in the limb regeneration (Kim and Stocum 1986; Crawford and Stocum 1988; Ludolph et al. 1990). In addition, administration of RA both intensifies the level of dedifferentiation and extends the duration of dedifferentiation (Ju and Kim 1994, 1998, 2000, 2010; Shimizu-Nishikawa et al. 2001). Although it has been reported that the inhibition of RA's action blocks limb regeneration of urodele (Del Rincón and Scadding 2002), how the depletion of endogenous RA by disulfiram affects the process of regeneration has not been studied thoroughly at the histological and molecular levels. In this study, we found that disulfiram treatment results in retardation of limb regeneration processes. Especially, limb regeneration was severely inhibited when disulfiram was injected before dedifferentiation stage, suggesting that RA is more critically involved in the wound epithelium formation and initiation of dedifferentiation process than the maintenance of dedifferentiation state. Interestingly, it has been reported that disulfiram treatment at the initiation of limb bud development completely blocks the chick limb bud formation. However, the administration of disulfiram after the formation of limb bud shows little effect on the chick limb development (Stratford et al. 1996).

Our findings also indicate that development of wound epithelium plays important role(s) in the process of dedifferentiation. In fact, the denervation of newt limbs resulted in the formation of thin wound epithelium due to the lack of mitogenic effect of nerve (Bryant et al. 1971; Satoh et al. 2008; Kumar et al. 2010). Moreover, wound epithelium is presumed to inhibit the redifferentiation of neighboring mesenchymal cells and promotes them to re-enter the cell cycle by production of signaling factors such as fibroblast growth factors (Stocum 1996). Therefore, the lack or sub-threshold level of RA induced by disulfiram treatment might be involved in underdevelopment of

wound epithelium, which in turn causes low activity of dedifferentiation in salamander limb regeneration. Although disulfiram delayed limb regeneration, we did not observe abnormal regeneration or complete inhibition of regeneration. This could be possible due to the resumed supply of RA by the diminishing disulfiram level with time. Alternatively, it is possible that low amount of RA was synthesized even under disulfiram treatment so that this treatment alone might not be enough to completely block RA synthesis in the regenerating salamander limbs.

The reduced expression of *MMP-9* gene by disulfiram treatment indicates that RA might be involved in the dedifferentiation process at the molecular level. In fact, RAR α (RA receptor α) binds to the promoter of *MMP-9* gene when it was up-regulated by RA treatment (Zaragozá et al. 2007; Lackey and Hoag 2010). Considering that the MMPs are involved in the ECM degradation and tissue remodeling, it is possible that the MMP-9 regulates epidermal-mesenchymal tissue interaction by being involved in the modification of ECM like basement membrane.

Collectively, our findings suggest that endogenous RA may play crucial role in the early events such as wound epithelium formation and initiation of dedifferentiation via up-regulation of *MMP-9* gene expression during salamander limb regeneration. To better understand the precise mechanism of endogenous RA-mediated limb regeneration, it is necessary to investigate exact expression pattern of RA receptors and genome-wide analysis of target genes regulated by RA.

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References

Bryant SV, Fyfe D, Singer M. 1971. The effects of denervation on the ultrastructure of young limb regenerates in the newt, Triturus. Dev Biol. 24:577–595.

Carlson BM. 2007. Principles of regenerative biology. Burlington, MA: Academic press. p. 85–87.

Clagett-Dame M, Knutson D. 2011. Vitamin A in reproduction and development. Nutrients. 3:385–428.

Crawford K, Stocum DL. 1988. Retinoic acid coordinately proximalizes regenerate pattern and blastema differential affinity in axolotl limbs. Development. 102:687–698.

Daley WP, Peters SB, Larsen M. 2008. Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci. 121:255–264.

Del Rincón SV, Scadding SR. 2002. Retinoid antagonists inhibit normal patterning during limb regeneration in the axolotl, *Ambystoma mexicanum*. J Exp Zool. 292: 435–443.

Duester G. 2000. Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. Eur J Biochem. 267:4315–4324.

Duong V, Rochette-Egly C. 2011. The molecular physiology of nuclear retinoic acid receptors. From health to disease. Biochim Biophys Acta. 1812:1023–1031.

Eichele G, Tickle C, Alberts BM. 1985. Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: temporal and spatial aspects. J Cell Biol. 101:1913–1920.

Hay ED. 1993. Extracellular matrix alters epithelial differentiation. Curr Opin Cell Biol. 5:1029–1035.

Ju BG, Kim WS. 1994. Pattern duplication by retinoic acid treatment in the regenerating limbs of Korean salamander larvae, *Hynobius leechii*, correlates well with the extent of dedifferentiation. Dev Dyn. 199:253–267.

Ju BG, Kim WS. 1998. Upregulation of cathepsin D expression in the dedifferentiating salamander limb regenerates and enhancement of its expression by retinoic acid. Wound Repair Regen. 6:349–357.

Ju BG, Kim WS. 2000. Cloning of a cDNA encoding cathepsin D from salamander, *Hynobius leechii*, and its expression in the limb regenerates. DNA Seq. 11:21–28.

Ju BG, Kim WS. 2010. Lysosomal acid phosphatase mediates dedifferentiation in the regenerating salamander limb. Anim Cells Syst. 14:73–81.

Kim WS, Stocum DL. 1986. Retinoic acid modifies positional memory in the anteroposterior axis of regenerating axolotl limbs. Dev Biol. 114:170–179.

Kumar A, Nevill G, Brockes JP, Forge A. 2010. A comparative study of gland cells implicated in the nerve dependence of salamander limb regeneration. J Anat. 217: 16–25.

Lackey DE, Hoag KA. 2010. Vitamin A upregulates matrix metalloproteinase-9 activity by murine myeloid dendritic cells through a nonclassical transcriptional mechanism. J Nutr. 140:1502–1508.

Ludolph DC, Cameron JA, Stocum DL. 1990. The effect of retinoic acid on positional memory in the dorsoventral axis of regenerating axolotl limbs. Dev Biol. 140:41–52.

McEwan J, Lynch J, Beck CW. 2011. Expression of key retinoic acid modulating genes suggests active regulation during development and regeneration of the amphibian limb. Dev Dyn. 240:1259–1270.

Marill J, Idres N, Capron CC, Nguyen E, Chabot GG. 2003. Retinoic acid metabolism and mechanism of action: a review. Curr Drug Metab. 4:1–10.

Park IS, Kim WS. 1999. Modulation of gelatinase activity correlates with the dedifferentiation profile of regenerating salamander limbs. Mol Cells. 9:119–126.

Rhinn M, Dollé P. 2012. Retinoic acid signalling during development. Development. 139:843–858.

Santosh N, Windsor LJ, Mahmoudi BS, Li B, Zhang W, Chernoff EA, Rao N, Stocum DL, Song F. 2011. Matrix metalloproteinase expression during blastema formation in regeneration-competent versus regeneration-deficient amphibian limbs. Dev Dyn. 240:1127–1141.

Satoh A, Bryant SV, Gardiner DM. 2008. Regulation of dermal fibroblast dedifferentiation and redifferentiation during wound healing and limb regeneration in the Axolotl. Dev Growth Differ. 50:743–754.

Scadding SR, Maden M. 1994. Retinoic acid gradients during limb regeneration. Dev Biol. 162:608–617.

Shimizu-Nishikawa K, Tsuji S, Yoshizato K. 2001. Identification and characterization of newt rad (ras associated with diabetes), a gene specifically expressed in regenerating limb muscle. Dev Dyn. 220:74–86.

Stocum DL. 1979. Stages of forelimb regeneration in *Ambystoma maculatum*. J Exp Zool. 209:395–416.

Stocum DL. 1996. A conceptual framework for analyzing axial patterning in regenerating urodele limbs. Int J Dev Biol. 40:773–783.

Stocum DL. 2004. Amphibian regeneration and stem cells. Curr Top Microbiol Immunol. 280:1–70.

Stratford T, Horton C, Maden M. 1996. Retinoic acid is required for the initiation of outgrowth in the chick limb bud. Curr Biol. 6:1124–1133.

Tanaka EM. 2003. Cell differentiation and cell fate during urodele tail and limb regeneration. Curr Opin Genet Dev. 13:497–501.

Tank PW, Carlson BM, Connelly TG. 1976. A staging system for forelimb regeneration in the axolotl, *Ambystoma mexicanum*. J Morphol. 150:117–128.

Thaller C, Eichele G. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature. 327:625–628.

Wilkinson DG. 1992. Whole mount in situ hybridization of vertebrate embryos. In: Willkinson DG, editor. In situ hybridization, a practical approach. London: Oxford University Press. p. 75–83.

Yang EV, Gardiner DM, Carlson MR, Nugas CA, Bryant SV. 1999. Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. Dev Dyn. 216:2–9.

Zaragozá R, Gimeno A, Miralles VJ, García-Trevijano ER, Carmena R, García C, Mata M, Puertes IR, Torres L, Viña JR. 2007. Retinoids induce MMP-9 expression through RARalpha during mammary gland remodeling. Am J Physiol Endocrinol Metab. 292:E1140–E1148.