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Correlation between *Shh* expression and DNA methylation status of the limb-specific *Shh* enhancer region during limb regeneration in amphibians

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Abstract

The *Xenopus* adult limb has very limited regeneration ability, and only a simple cartilaginous spike structure without digits is formed after limb amputation. We found that expression of *Shh* and its downstream genes is absent from the regenerating blastema of the *Xenopus* froglet limb. Moreover, we found that a limb enhancer region of the *Shh* gene is highly methylated in the froglet, although the sequence is hypomethylated in the *Xenopus* tadpole, which has complete limb regeneration ability. These findings, together with the fact that the promoter region of *Shh* is hardly methylated in *Xenopus*, suggest that regenerative failure (deficiency in repatterning) in the *Xenopus* adult limb is associated with methylation status of the enhancer region of *Shh* and that a target-specific epigenetic regulation is involved in gene re-activation for repatterning during the *Xenopus* limb regeneration process. Because the methylation level of the enhancer region was low in other amphibians that have *Shh* expression in the blastemas, a low methylation status may be the basic condition under which transcriptional regulation of *Shh* expression can progress during the limb regeneration process. These findings provide the first evidence for a relationship between epigenetic regulation and pattern formation during organ regeneration in vertebrates.
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Keywords: DNA methylation; MFCS1; *Shh*; Regeneration; Limb

Introduction

Axial pattern formation in regenerating limbs is evident in *Xenopus* tadpoles and urodeles. Expression of genes involved in axial pattern formation during limb development has been examined in their regenerating blastemas, and it has been proposed that molecular cascades mediated by these genes are involved in repatterning their lost limbs also during limb regeneration (Endo et al., 1997, 2000; Gardiner et al., 1995; Imokawa and Yoshizato, 1997; Matsuda et al., 2001; Torok et al., 1999, 1998). The fact that *Hoxa9*/*Hoxa13* genes and *Hoxd11*/other 5′ *HoxD* genes (Gardiner et al., 1995; Torok et al., 1998) are reactivated in the urodele limb blastema raises the possibility that patterns both along the proximal–distal (PD) and anterior–posterior (AP) axes are reconstructed. *Sonic hedgehog* (*Shh*) has also been detected in the posterior mesenchyme of the blastemas (Imokawa and Yoshizato, 1997; Torok et al., 1999), suggesting that an SHH signaling-dependent mechanism along the AP axis is re-established, although the expression and function of other genes related to the SHH signaling, regulators of *Shh* expression such as *Gli3* and *dHAND*, during limb regeneration have not been investigated.

Although the *Xenopus laevis* tadpole (stages 52–53; Nieuwkoop and Faber, 1956) exhibits complete limb regeneration ability as urodele amphibians do, the *Xenopus* froglet (a young adult after metamorphosis) forms a simple cartilaginous spike structure after the limb is amputated (Dent, 1962; Endo et

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al., 2000; Satoh et al., 2005). Expression analyses of patterning-related genes in the *Xenopus* froglet blastema, including some of the above genes, have revealed that the froglet blastema has some defects in gene reactivation (Endo et al., 2000; Matsuda et al., 2001; Satoh et al., 2006). Taken together with the fact that the early step for limb regeneration, including formation of proliferative blastema cells, occurs normally in the amputated froglet limb as in urodele amphibian limbs (Gardiner et al., 2002; Suzuki et al., 2005, 2007, 2006), these findings of a lack of gene re-expression suggest that insufficient patterning ability in the *Xenopus* froglet limb is closely related to its low regenerative capacity. *Shh* has been suggested not to be expressed in the froglet blastema (Endo et al., 2000), implying that the SHH signaling pathway is not active in the blastema. This possibility is supported by results of the following experimental studies. Roy and Gardiner (2002) showed that an amputated axolotl limb treated with cyclopamine, a Hedgehog pathway inhibitor, forms a spike-like regenerate. A similar spike-like limb regenerate has been observed in a cyclopamine-treated *Xenopus* tadpole (Satoh et al., 2006). $Shh^{-/-}$ mutant mice exhibit a severely defected phenotype in limbs, which contains a shaft-like truncated bone (Chiang et al., 2001; Kraus et al., 2001). Furthermore, mice that lack a *cis*-acting region regulating limb-specific *Shh* expression exhibit a limb phenotype similar to that in the $Shh^{-/-}$ mutant mice (Sagai et al., 2005). These studies demonstrate that defects in AP patterning due to deficient *Shh* function cause limb malformation, suggesting that the absence of *Shh* is closely related to formation of the malformed structure, a spike, in *Xenopus* froglet limbs. *Xenopus* has a complete set of genes for regulation of *Shh* expression in the limb bud and its blastema, at least in the tadpole stage, and thus, the absence of *Shh* expression in the adult blastema is probably not due to a lack or insufficiency of the gene regulatory element for *Shh* expression in the *Xenopus* genome. The fact that *Shh* expression can be controlled in the larval stage but not in the adult stage raises the possibility that epigenetic regulation is involved in the failure of *Shh* reactivation in the adult limb. Epigenetic regulation of gene expression is generally involved in both the developmental process and stem cell differentiation (Bird, 2002; Hsieh and Gage, 2004; Palacios and Puri, 2006). Artificial controls of DNA methylation level sometimes give rise to changes in gene expression level and timing, resulting in abnormality in those processes (Li et al., 1992; Martin et al., 1999; Stancheva and Meehan, 2000). Moreover, recent studies have shown that epigenetic regulation is important for gene expression also during tissue regeneration. During repair of damaged muscles in mammals, for example, DNA demethylation in muscle progenitor cells plays a key role in the induction of re-expression of *myoD* and *myf5*, early myogenic marker genes (Palacios and Puri, 2006), suggesting relationships between incomplete expression of genes, epigenetic regulation of gene expression, and ability of regeneration.

To elucidate relationships between AP patterning and decline of repatterning ability in the froglet limb blastema, we focused on the SHH signaling pathway, particularly on epigenetic regulation of *Shh* expression as a possible cause of failure of *Shh* reactivation in the blastema. In the present study, we compared expression of SHH signaling-related genes in the *Xenopus* tadpole and froglet blastemas and found that expression levels of *Shh* and its downstream target genes are diminished, although upstream components of *Shh*, *dHAND* and *Gli3*, are expressed in the froglet blastema. We also found that MFCS1, which contains a limb-specific *Shh* enhancer, is highly methylated in the froglet limb blastema. Our epigenetic analyses of the *Shh* enhancer and promoter sequences in the *Xenopus*, newt, and axolotl further revealed a clear relationship between methylation level of the sequences and regeneration ability of their limbs.

Materials and methods

Animals and limb amputation

Xenopus laevis tadpoles were raised in our laboratory from induced breeding of adult frogs and allowed to develop until they reached stages 50–56 (Nieuwkoop and Faber, 1956). *X. laevis* froglets (15–20 mm in length from snout to vent) were obtained from animal suppliers. The animals were kept at 24 °C in dechlorinated water.

For limb amputation, tadpoles at stage 52–53 and froglets were anesthetized in 0.05% ethyl-3-aminobenzoate (Sigma) dissolved in Holtfreter's solution. Hindlimbs of tadpoles and forelimbs of froglets were amputated at the prospective ankle (Tschumi, 1957) and at wrist level, respectively. The amputation surface of froglets was trimmed to be flat.

Adult newts (*Cynops pyrrhogaster*) and axolotls (*Ambystoma mexicanum*) were purchased from a local animal supplier. The surgery method for their limbs was the same as that of froglets.

In situ hybridization

X. laevis Gli1 and *Gli3* clones for probes were kindly provided by Dr. Kazuhito Takeshima (Takabatake et al., 2000). Partial sequences for probes of *X. laevis dHAND* (NCBI number AF228335, from 526 to 1115) (Angelo et al., 2000), *Shh* (NCBI number U26314, from 334 to 859) (Paganelli et al., 2001), *Ptc1* (NCBI number AB037686, from 3404 to 3706) and *Ptc2* (NCBI number AB037688, from 579 to 1029) (Takabatake et al., 2000) were cloned by RT-PCR using total RNA from *Xenopus* regenerating hindlimb blastemas. A digoxygenin-labeled RNA probe for each gene was prepared according to the manufacturer's protocol (Roche). For making sections, specimens were fixed in MEMFA, cryoprotected with 30% sucrose, and embedded in O.C.T compound (Sakura). Sections were cut at a thickness of 10 μm with a cryostat. The procedure used for section *in situ* hybridization was essentially the same as that described by Yoshida et al. (1996).

Genomic DNA preparation and cloning of MFCS1 and Shh promoter sequence

Tissues were placed in DNA lysis buffer (50 mM Tris–HCl [pH 7.5], 125 mM NaCl, 10 mM EDTA, 1% SDS, and 8 M urea) containing 1 mg/ml Proteinase K (Invitrogen) and incubated overnight at 37 °C. Samples were then extracted with phenol and phenol/chloroform, ethanol precipitated, and suspended in TE. Purified DNA was used for genomic PCR to clone MFCS1 and *Shh* promoter. The primer sets for genomic PCR are shown in Supplementary Fig. 1. The PCR product was cloned into the pCRII-TOPO vector (Invitrogen) and sequenced.

Bisulfite sequencing analysis

Tissue samples for extraction of genomic DNA for bisulfite sequencing analysis were collected as follows. Samples of stage 53 hindlimb buds and blastemas were collected from 10 tadpoles of *X. laevis*, and samples of eyes and hearts were obtained from three tadpoles at stage 53. Five and two *Xenopus*

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