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Expression analysis of *XPhyH-like* during development and tail regeneration in *Xenopus* tadpoles: Possible role of *XPhyH-like* expressing immune cells in impaired tail regenerative ability

Yuko Naora, Yuko Hishida, Taro Fukazawa, Takekazu Kunieda, Takeo Kubo *

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

Xenopus tadpoles have high regenerative ability of amputated tails except during the 'refractory period', when the ability is transiently lost. We previously demonstrated that distinct immune responses occur in tail stumps between the refractory and pre/post-refractory regeneration periods. Furthermore, treatment with an immunosuppressant, FK506, restores the tail regenerative ability during the refractory period. Based on these findings, we previously proposed that autoreactive immune cells infiltrate the tail stumps to attack blastema cells as 'non-self' during the refractory period, resulting in the impaired regenerative ability. The immune cells that attack the blastema cells, however, remained unclear. Here we screened for genes whose expression in the tail stumps was altered by FK506 treatment during the refractory period and identified a *Xenopus* homolog of phytanoyl-CoA dioxygenase (*PhyH*)-*like*. *XPhyH-like* expression transiently increased in tail stumps after amputation during the refractory period, and was reduced by FK506 treatment. *XPhyH-like* expression in the whole tadpole body specifically increased during the refractory period and was enriched in the blood cell fraction. These findings suggest that *XPhyH-like* is expressed in autoreactive immune cells that are distributed in the whole body during the refractory period and transiently infiltrate the tail stumps to attack the blastema cells as 'non-self'.

1. Introduction

Various animals possess the ability to regenerate lost organs or appendages, but the extent of the regenerative ability, and the organs/appendages and developmental stages with regenerative ability vary among animal species [1]. Although the molecular mechanisms underlying regenerative ability have been extensively investigated in some experimental animals, such as planarians, cockroaches, and newts, the reason for the variable regenerative ability remains largely unclear [1]. The African clawed frog *Xenopus laevis* possesses high tail and limb regenerative ability during the larval (tadpole) stages [2]. Tadpoles can regenerate complete tails with spinal cord, notochord, muscles, and fins within about 1 week after amputation. This regenerative ability is transiently lost, however, during certain developmental stages, called the 'refractory period (stage 45–47) [3,4]. Thus, the regenerative ability changes from positive (pre-refractory regeneration) to negative (refractory), and again to positive (post-refractory regeneration period) during the tadpole stages.

We previously used the differential display method to compare the responses against amputation show that distinct immune responses are activated in the tail stumps between the refractory and the post-refractory regeneration periods [5]. In addition, immunosuppressant (FK506 and Celastrol) treatments as well as immune cell depletion by knockdown of *PU.*1, a master transcription factor for monocyte/B cell development, [6], significantly restored the tail regenerative ability during the refractory period, indicating that immune responses activated during the refractory period impair the tail regenerative ability in *Xenopus* tadpoles [5]. We also showed that regulatory T-cells expressing *foxp*3 transiently infiltrate the amputated tail stumps during the post-refractory regeneration period [5,7]. This finding suggests that the tail regenerative ability could be restored because the regulatory T cells suppress the function of the immune cells that impair the tail regenerative ability during the refractory period. Based on these findings, we proposed that autoreactive immune cells recognize the blastema cells as 'non-self' during the refractory period, which results in impaired tail regenerative ability. The molecular and cellular mechanisms that impair tail regenerative ability during the refractory period, however, remain largely unclear.

In the present study, aiming to identify the molecular bases underlying the impaired tail regenerative ability during the refractory period, we again used the differential display method to

* Corresponding author.
E-mail addresses: naora@biol.s.u-tokyo.ac.jp (Y. Naora), Yuko_Hishida@nts.toray.co.jp (Y. Hishida), taro-fuka@brc.riken.jp (T. Fukazawa), kunieda@biol.s.u-tokyo.ac.jp (T. Kunieda), stkubo@biol.s.u-tokyo.ac.jp (T. Kubo).

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screen for genes whose expression in tail stumps is altered when the regenerative ability is restored by FK506-treatment during the refractory period. Among the three genes identified one that encodes *Xenopus* homolog of *phytanoyl-CoA dioxygenase-like* (*XPhyH-like*) was transiently induced in the tail stumps after tail amputation during the refractory period and reduced by FK506-treatment. Furthermore, *XPhyH-like* expression was enriched in the blood cell fraction, suggesting that the gene is expressed in immune cells that are related to the impaired tail regenerative ability during the refractory period.

2. Materials and methods

2.1. Tadpoles

Tadpoles in the pre-refractory regeneration and refractory periods were obtained by mating wild-type *Xenopus laevis* adults and keeping their offspring in the laboratory. Before mating, 500 U and 300 U of gonadotropin (Aska pharmaceutical) was injected to female and male adults, respectively. Tadpoles were kept in 0.2% salt water at 20 °C. Stage 39–41 tadpoles were used to study pre-refractory regeneration and stage 46–47 tadpoles were used for studying the refractory period, respectively. Tadpoles in the post-refractory regeneration period (stages 51–52) were purchased from a domestic company (Watanabe Zohshoku) and a private provider.

This study was carried out in accordance with the recommendations of in the Guidelines for Proper Conduct of Animal Experiments of Science Council of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Graduate School of Sciences, the University of Tokyo (Permit Number: 19–14 Z 07–08). All of the surgical manipulations, including the tail amputation and body parts collection, were performed after completely anesthetizing the tadpoles with 0.02% MS222 (Sigma–Aldrich).

2.2. FK506 treatment

Tadpoles were maintained in plastic dishes (90 mm in diameter) filled with 0.33×De Boer solution containing 3 μM FK506 (Calbiochem) dissolved in 0.1% dimethyl sulfoxide (DMSO) or 0.1% DMSO solution (vehicle control) after tail amputation at 8 days post fertilization (dpf). For determining FK506 treatment period needed to restore the tail regenerative ability, tadpoles were exposed to FK506 at the appropriate time points from 2 days before to 6 days after tail amputation. The solution was refreshed every other day and on the 6th or 7th day after tail amputation, tail regenerative ability was assessed. Tail regenerative ability was classified to four grades: perfect, whole tail structure was regenerated including fin, muscle, notochord and spinal cord; good, whole tail was regenerated, but length was shorter; partial, tail was regenerated, but lacked some tissues or had a curved axis; none, no regeneration was observed.

2.3. Differential display

Differential display screening was performed using the Differential Display Kit (TaKaRa) following the manufacturer's protocol, as described previously. Total RNAs were extracted from tail stumps dissected at 0 or 24 hours post-amputation (hpa) from tadpoles treated with FK506 or DMSO, during the refractory ($n = 80$) or the post-refractory regeneration period ($n = 12$).

2.4. cDNA cloning

To obtain the open reading frame sequences of the candidate gene fragments, Rapid Amplification of the cDNA Ends was performed using FirstChoice® RLM-RACE Kit (Invitrogen) following the manufacturer's protocol. The nucleotide sequences determined in this study have been submitted to DDBJ with accession numbers AB759708–AB759710.

The amino acid sequences of PhyH and PhyH-like of each organism were aligned by using multiple alignment program, MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and phylogenetic tree was constructed with the neighbor-joining method using Geneious software (Biomatters Ltd.). Accession numbers used for phylogenetic study are as follows shown in the (PhyH, PhyH-like) order; *Homo sapience* (NP_006205, NP_001094346), *Danio rerio* (NP_001017823, NP_001099060), *Xenopus tropicalis* (XP_002 935288, XP_002931851), and *Xenopus laevis* (NP_001086497, AB759708).

2.5. Quantitative (q) RT-PCR

Expression analysis by qRT-PCR of *XPhyH-like* was performed using total RNA collected from four sets of wound stumps from the refractory ($n = 20$) or the post-refractory regeneration period ($n = 5–6$) at 0, 5, 10, 15, 24 and 48 hpa. Developmental expression analysis of *XPhyH-like* was performed using total RNA collected from four sets of intact tadpoles ($n = 4–5$) at 4, 7, 14, 18, 21 and 25- dpf which were equivalent to Stages 39–40, 46, 47, 49, 50, and 50. Total RNA was extracted using an RNeasy mini Kit (QIAGEN) and reverse transcribed using SuperScript®III First Strand Synthesis System (life technologies). qRT-PCR of *XPhyH-like* was performed with gene-specific primers: 5′-TGATGTACTGTATTG GATGTTTACTGG-3′, and 5′-TTTAAAATAAAAATTGGCAAGGTAAGG-3′, using SYBR® *Premix Ex Taq* II (TaKaRa). The amounts of *XPhyH-like* transcripts were normalized with those of *EF1α*.

2.6. Blood collection and body parts sampling

Blood cells were collected from intact tadpoles during the refractory period. After tail amputation, tadpoles ($n = 48$) were collected into a mesh pack and immersed in saline (0.6% sodium chloride) with 2.5 mM EDTA in a centrifuge tube. Being left for bleeding for about 45 min, they were centrifuged at 100×*g* for 5 min for further bleeding. After centrifugation, tadpoles were removed and the resulting blood fraction was further centrifuged at 500×*g* for 5 min to precipitate blood cells. Pelleted blood cells were resuspended into 5 ml of saline including 2.5 mM EDTA, and the number of blood cells was counted using a hemocytometer. For body parts collection, tadpoles ($n = 5$) were dissected into three parts along the anterior-posterior axis; head: region from mouth to the anterior end of gut, trunk: region including gut, tail: region posterior than anus. Each sample was collected into chilled microtubes and rapidly frozen.

3. Results and discussion

3.1. Determination of FK506 treatment period required to restore tail regenerative ability during the refractory period

In our previous study, we demonstrated that immunosuppressant treatments drastically potentiate tadpole tail regenerative ability during the refractory period [5] It was not clear, however, when FK506 acts to restore tail regenerative ability because amputated tadpoles were treated with FK506 for entire period, 6 days, till regeneration completed. Therefore, to narrow down the time

point, we treated tadpoles with FK506 for various periods after tail amputation.

First, we examined whether earlier (0–2nd day) or later (3rd to 6th day) FK506 treatment after tail amputation was more effective in restoring the regenerative ability. Earlier FK506 treatment restored the regenerative ability to almost the same level as treatment during the entire period (0–6th day), whereas later treatment did not significantly restore regenerative ability (Fig. 1A). We then divided the earlier period (0–2nd day) into four 12-h periods (1st, 2nd, 3rd, and 4th 12-h periods), and examined the efficacy of FK506 treatment during each period to restore regeneration. FK506 treatment at the 1st 12-h period restored regenerative ability to almost the same level as treatment during the entire period, whereas treatments at the 2nd-, 3rd-, and 4th 12-h periods did not. These findings indicate that FK506 treatment immediately after tail amputation is important and sufficient to restore regenerative ability (Fig. 1B). Therefore, we next examined whether FK506 treatment prior to tail amputation also effectively restores tail regenerative ability. When tadpoles were exposed to FK506 for 24 h prior to tail amputation (−1 to 0 day), the regenerative ability was restored to almost the same level as treatment during the entire period after tail amputation (Fig. 1C). In contrast, 1 day-earlier FK506 treatment for 24-h (−2 to −1 day) failed to restore the regenerative ability (Fig. 1C), suggesting that the effect of FK506 diminished during 1 day prior to the amputation. Assuming that effect of FK506 sufficient to restore the tail regenerative ability remain in the tadpole for about 1 day, we estimated that FK506 is involved in restoring tail regenerative ability during the 0 to 1.5 days after tail amputation.

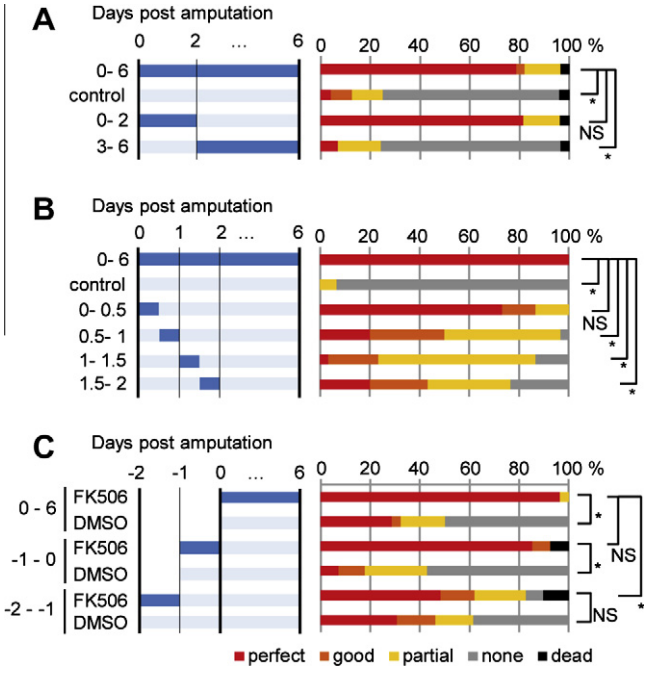

Fig. 1. Narrowing down the period of FK506-treatment to restore the regenerative ability during the refractory period. (A) FK506 treatment after the tail amputation for the first 2 days and the following 4 days. FK506 treatment at 0–2 days post amputation was sufficient for the identical tail regenerative ability with the treatment at 0–6 days post amputation, (B) Pulse treatment with FK506 for 12 h each during 0–2 days post amputation. FK506 treatment for 12 h just after the tail amputation enabled regeneration and (C) FK506 treatment prior to tail amputation. FK506 treatment 1 day prior to the tail amputation was needed for the regeneration. The blue and light blue bars on the left panels indicate the period of FK506 and DMSO (vehicle control) treatment. On the right panels, each color represents the extent of regeneration. $n = 24–30$ $^*P < 0.01$, Steel–Dwass method. NS: not significant.

We previously demonstrated that some chemokine genes, such as *CCLb*, *CCL5L1*, and *CCL5L2*, are induced approximately 15–24 h after tail amputation during the refractory period [5]. In addition, our previous observation showed that proliferation of blastema cells begins 18–24 h after tail amputation during the post-refractory regeneration period [5]. Importantly, however, FK506-treatments during the 2nd, 3rd, or 4th 12-h periods (i.e., 12–48 hpa) were not sufficient for the restoration of tail regenerative ability (Fig. 1B). It is thus plausible that immune responses that impair blastema cell proliferation, which can be repressed by FK506-treatment, occur till 1 day after the tail amputation during the refractory period.

3.2. Screening for genes whose expression in tail stumps changes depending on the regenerative ability

To identify the immune-related factors involved in the impaired tail regenerative ability during the refractory period, we used the differential display method to search for genes whose expression in the tail stumps changed depending on the tail regenerative ability. Because we previously demonstrated that FK506 treatment restores tail regenerative ability during the refractory period [5], we compared gene expression patterns in the tail stumps between FK506-treated and DMSO-treated (vehicle control) tadpoles (Fig. S1). Based on our critical period analyses, we selected 24 hpa as the comparison time point. In addition, to exclude the possibility of side effects of drug treatment on gene expression, we used tadpoles treated with FK506 or DMSO during the post-refractory regeneration period during which the regenerative ability is not sensitive to FK506 treatment (Fig. S1).

We first compared gene expression patterns between the FK506- and DMSO-treated tadpoles during the refractory period and identified 75 candidate gene fragments, 26 upregulated and 49 downregulated genes, on FK506-treatment. We then used qRT-PCR to confirm the differential expression of 22 of the 75 candidate genes. We used qRT-PCR again to examine whether the expression of these genes in the tail stumps differed between the FK506- and DMSO-treated tadpoles during the post-refractory regeneration period. As a result, we identified 3 candidate gene fragments (Clones 1–3). They were induced upon amputation in the tail stumps of tadpoles treated only with DMSO (non-regenerative condition; Clones 1 and 2), or FK506 (regenerative condition; Clone 3; Table 1).

Determination of whole coding sequences by RACE method revealed that Clone 1 was a gene with sequence similarity to phytanoyl-CoA dioxygenase (*PhyH*) and Clone 3 was a *Xenopus* homolog of *C-type lectin-like* (Table 1). Clone 2 contained no significant open reading frame (ORF), implying that it functions as a non-coding RNA (Table 1). We focused on Clone 1 because subsequent gene expression analysis revealed its clear induction upon amputation in the tail stumps of tadpoles treated with DMSO during the refractory period (Fig. 2C). The Clone 1 transcript contained the PhyH domain (Fig. 2A) whereas formed a distinct gene group from *PhyH* (Fig. 2B), indicating that Clone1 is a novel gene that is different from *PhyH*. Thus, we identified Clone1 as *Xenopus PhyH*-like (*XPhyH-like*).

In mammals, PhyH is an enzyme that contains PhyH domain and catalyzes the oxidation of phytanic acid derived from chlorophyll of plant origin. Although XPhyH-like resembles XPhyH in that it contains a PhyH domain, XPhyH-like differs from XPhyH in that XPhyH-like does not contain signal sequences. While XPhyH is suggested to localize in the peroxisome, XPhyH-like is predicted to be cytoplasmic. Some reports have demonstrated possible functions of PhyH in immunity in mice. In MRL/*lpr* mice, PhyH expression in the kidney is decreased in the early stage of lupus nephritis, suggesting its role to oppose the outbreak of autoimmune diseases [8].

Table 1
Candidate genes obtained with differential display screening.

#	Expression pattern in the refractory period	Length of fragment (bp)	Length of ORF (bp)	Similar gene	Subcellular localization
1	DMSO > FK506	194	789	Phytanoyl-CoA dioxygenase	Cytoplasm
2	DMSO > FK506	236	NA	NA	NA
3	DMSO < FK506	491	642	C-type lectin	Cytoplasm

Expression patterns were compared based on the band intensities between FK506 or DMSO- treated tadpoles during the refractory period. For the candidates that we could determine the open reading frame sequences, the sequence similarity and the subcellular localization were examined using Blastx (NCBI) and TargetP, respectively. NA: not applicable.

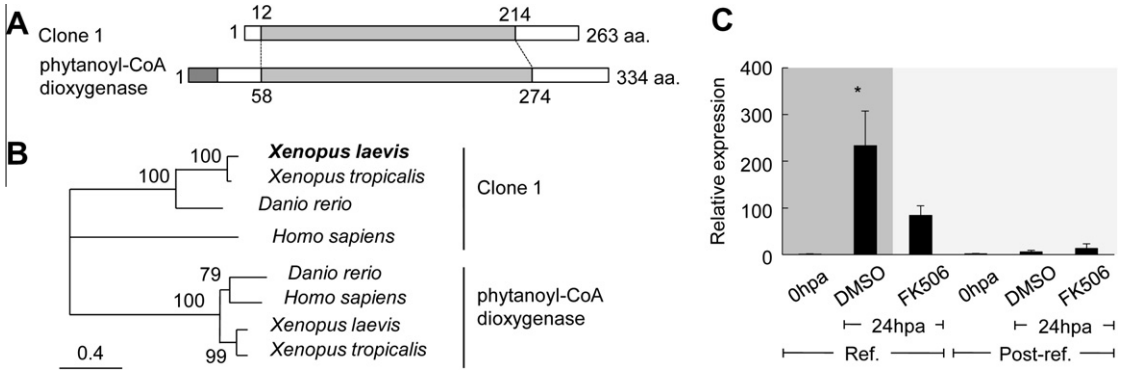

Fig. 2. Characterization of Clone 1, a novel gene that contained phytanoyl-CoA dioxygenase (PhyH) domain. (A) Comparison of primary structures of clone 1 and *Xenopus* phytanoyl-CoA dioxygenase revealed 22% sequence similarity in the PhyH domain. The dark-gray and light-gray boxes indicate the predicted mitochondrial targeting sequence and the PhyH domain, respectively. aa: amino acid residues, (B) Phylogenetic tree of clone 1 and phytanoyl-CoA dioxygenase. Clone 1 formed a distinct gene group from *PhyH* gene group. The numbers at the nodes represent bootstrap values and (C) Significantly higher expression of clone 1 in the DMSO-treated tadpoles during the refractory period was confirmed using qRT-PCR. The dark-gray and the light-gray color on the graph indicate non-regenerative and regenerative conditions, respectively. hpa: hours post amputation. (mean ± SEM, $n = 4$) Ref: refractory period, Post-ref: post-refractory regenerative period. $^{*}P < 0.01$, Tukey–Kramer method.

In addition, PhyH was isolated as a binding protein of FKBP52, an FK506-binding protein, from Jurkat cells [9]. PhyH binds to FKBP52 independently of the PTS2 signal, which is localized in the N-terminal part of PhyH [9]. Thus, even though XPhyH-like lacks the PTS2 signal, it is plausible that XPhyH-like also binds to FK506 via FKBP52 and is involved in immune responses in *Xenopus* tadpoles.

3.3. Gene expression analysis of XPhyH-like in tail stumps after amputation and during development

To examine how *XPhyH-like* is related to the impaired tail regenerative ability during the refractory period, we used qRT-PCR to analyze the expression profile of *XPhyH-like*. We first compared the time course of *XPhyH-like* expression in the tail stumps after amputation during the refractory period between FK506- and DMSO-treated tadpoles. Although *XPhyH-like* was transiently upregulated, with a peak at 10 hpa in both conditions, FK506 treatment significantly suppressed the upregulation (Fig. 3A), consistent with the differential display method results (Fig. 2C).

We next compared the time course of *XPhyH-like* expression in the tail stumps between the refractory and the post-refractory regeneration period. Although *XPhyH-like* was transiently upregulated, peaking at 5 hpa in both periods, the upregulation was more prominent during the refractory period than the post-refractory regeneration period (Fig. 3B). These findings suggest two possibilities that *XPhyH-like* is either expressed in a certain population of immune cells that transiently infiltrate the tail stump to impair tail regenerative ability during the refractory period, or that *XPhyH-like* is expressed transiently in some tail tissues that are related to immune responses (i.e., immune cells that were already present in the amputated tail stumps).

XPhyH-like expression levels differed twice even just after tail amputation (at 0 hpa) between in the post-refractory regeneration period and in the refractory period (Fig. 3B), which suggests that *XPhyH-like* expression changes depending on the developmental stages. To investigate this possibility, we analyzed *XPhyH-like* expression in the whole tadpole body at various developmental stages. We first confirmed the refractory period during development by examining the tail regenerative ability of tadpoles at 4–25 dpf (equivalent to Stage 39–50). We assessed the extent of the tail regeneration at 7 days after tail amputation and determined 4–7 dpf as the pre-refractory regeneration, 7–18 dpf as the refractory, and 18–25 dpf as the post-refractory regeneration periods (Fig. S2). *XPhyH-like* was expressed almost specifically during the refractory period and the expression was very low or almost not detectable during the pre- and post-refractory regeneration periods (Fig. 3C). These findings suggest that cells expressing *XPhyH-like* are distributed in the tadpole body only during the refractory period.

3.4. Tissue-preferential gene expression analysis of XPhyH-like

We previously identified *cathelicidin-like* and *RIN3* as genes whose expression was transiently induced in tail stumps, and found that these genes were expressed in leukocytes that transiently infiltrate the tail stumps [5]. We also demonstrated that regulatory T-cells expressing *foxp3* transiently infiltrate the tail stumps and thus *foxp3* expression was induced in the tail stumps [5]. Empirically, we assumed that *XPhyH-like* is expressed in immune cells that transiently infiltrate the tail stumps to impair the regenerative ability during the refractory period, and are also distributed throughout the tadpole body during the period.

Our attempts to detect *XPhyH-like* expression in the tail stumps using *in situ* hybridization failed, however, possibly due to its low expression levels and/or possible dynamic distribution of expressing cells as immune-cells (data not shown). We then assumed that

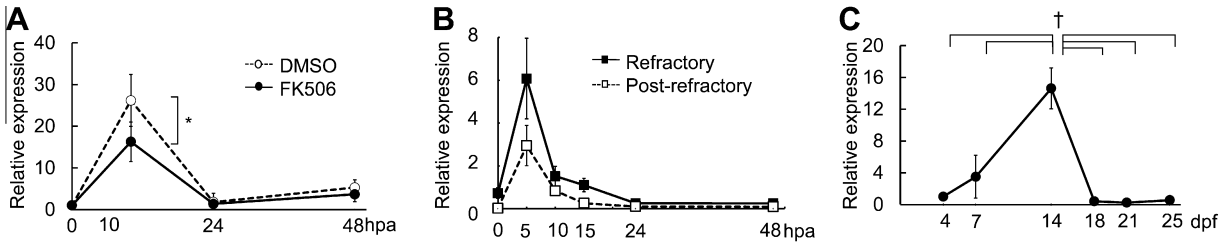

Fig. 3. Transient up-regulation of *XPhyH-like* after the tail amputation and during the refractory period. (A) Tail amputation during the refractory period evoked transient up-regulation of the transcript level (open circle), which was reduced by FK506-treatment (filled circle), (B) The up-regulation was more prominent during the refractory period (filled square) compared to the post-refractory regenerative period (open square) and (C) The transcript level in the whole tadpole body was specifically up-regulated at 14 dpf (the refractory period). The relative amounts of transcripts were obtained by taking the value of 0 hpa (A,B) or 0 dpf and (C) as 1, following the normalization using the transcript level of EF1α. (mean ± SEM, $n = 4$) hpa: hours post amputation, dpf: days post fertilization. $^{*}P < 0.05$, Student's t-test, $^{\dagger}P < 0.01$, Tukey–Kramer method.

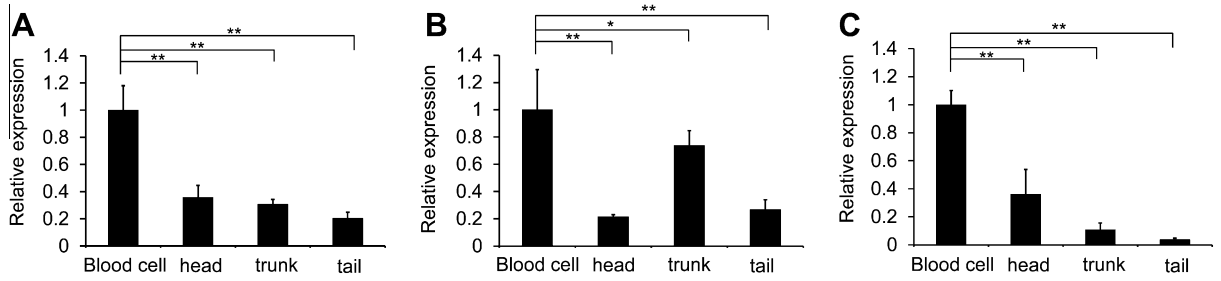

Fig. 4. Enriched expression of *XPhyH-like* in the blood cell fraction. (A) qRT-PCR analyses of *XPhyH-like* revealed significantly higher expression in the blood cell fraction, (B) *T cell receptor* (*TCR*) *alpha* showed higher expression in the blood cell fraction, resembling the pattern of *XPhyH-like* and (C) *CD45* showed higher expression in both the blood cell and the trunk fraction, indicating a broader distribution compared to the cells expressing *XPhyH-like*. (mean ± SEM, $n = 4$) $^{*}P < 0.05$, $^{**}P < 0.01$, Dunnet's test.

XPhyH-like expression could be enriched in the blood cell fraction. Therefore, we extracted total RNA from the blood cell fraction, head, trunk, and tail regions of tadpoles, and used qRT-PCR to compare the expression levels among these tissues. To validate whether immune cells are actually enriched in the blood cell fraction, we first compared the expression levels of *T cell receptor alpha* (*TCR*α, a T cell marker gene [10], and *CD45*, a pan-leukocyte marker gene [11], among these tissues. Expression of *TCR*α and *CD45* was 3- to 5-fold higher in the blood cell fraction than in the other fractions, except that expression of *CD45* in the blood cell fraction was only slightly (but significantly) higher than that in the trunk, indicating that immune cells are in fact enriched in the blood cell fraction (Fig. 4A and B). The expression of *XPhyH-like* was also 2.5- to 5-fold higher in the blood cell fraction than in the other tissues, strongly suggesting that *XPhyH-like* was expressed in immune cells enriched in the blood cell fraction (Fig. 4C). *XPhyH-like* expression in the blood cell fraction indicates the possibility that *XPhyH-like* expressing cells circulate in the whole body during the refractory period.

3.5. Possible role of PhyH-like-expressing immune cells in impaired tail regenerative ability

Taken together, our findings suggest that the immune cell population (s) expressing *XPhyH-like* is related to the impaired tail regenerative ability during the refractory period. In mammals, the 'central tolerance' eliminates auto-reactive (T or B) immune cells during their development [12]. However, in fact, there are several auto-reactive cells at periphery which escaped from the central tolerance; these cells are suppressed by the 'peripheral tolerance', which is controlled by regulatory T cells. In *Xenopus*, thymus begins to develop around the onset of the refractory period [12] and regulatory T-cells begin to transiently infiltrate the tail stumps in the post-refractory regeneration period [5]. It is noteworthy that *XPhyH-like* is expressed almost specifically during the refractory period in the whole tadpole body as this represents the inverse association between *XPhyH-like* expression level and the tail regenerative ability during the tadpole development. It is thus plausible that *XPhyH-like* expressing immune cells recognize the blastema cells as 'non-self' during the refractory period. This notion, however, needs to be verified by experiments showing that the *XPhyH-like* expressing cells actually attack the blastema cells and by identifying molecules of blastema cells that are recognized as 'auto-antigens'.

Our attempts to analyze the *in vivo* function of *XPhyH-like* by injecting morpholino antisense oligonucleotides (MOs) for *XPhyH-like* to the embryos, however, resulted in embryonic death, strongly suggesting that *XPhyH-like* is essential for the function of the immune cells expressing *XPhyH-like* and these cells are indispensable for tadpole survival and/or development (Fig. S3). Although the function of *XPhyH-like* is still not clear, we expect that the gene will be a useful marker for autoimmune cells that impair tissue regenerative ability in *X. laevis*. Further analysis of the presence or distribution of *XPhyH-like* expressing cells in the adult, another non-regenerative stage in *X. laevis*, or functional analyses of *PhyH-like* in mammals might provide further insight into the immune responses that impair tissue regenerative ability in vertebrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.005.

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