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Short communication

Molecular characterization and transcriptional expression of a B cell transcription factor Pax5 in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Pax5 (Paired Box 5), a nuclear transcription factor expressed in B cell specifically, is a key regulator for B cell activation. In this study, we cloned and identified a Pax5 gene (OnPax5) from Nile tilapia (*Oreochromis niloticus*), which has an open reading frame of 1278 bp, encoding deduced amino acid sequence of 425 residues. OnPax5 contains a conserved DNA-binding domain encoding the paired box, an octapeptide, a homeobox homology region, a transactivation and a repressor domain. *OnPax5* is constitutively expressed in various analyzed tissues of tilapia, with a relatively high expression in lymphoid organs, including spleen (SPL), anterior kidney (AK), and thymus. What's more, *OnPax5* is highly expressed in leukocytes especially in IgM^+ lymphocytes sorted from peripheral blood (PBL), SPL and AK. When stimulated with lipopolysaccharide (LPS) *in vivo*, *OnPax5* expression was significantly up-regulated in PBL, SPL and AK. Upon stimulation with LPS, pokeweed mitogen and mouse anti-OnIgM monoclonal antibody *in vitro*, the expression of *OnPax5* was also significantly up-regulated in leukocytes from SPL and AK. Taken together, Pax5, the B cell lineage specific activator factor, might get involved in B cell activation in Nile tilapia.

1. Introduction

During the B cell development in vertebrate, transcription factors play important roles by interacting with specific DNA sequences within the enhancers or promoters of target genes [1,2]. According to the structural and functional similarities of their DNA-binding domains, transcription factors are divided into groups including leucine zipper, zinc-finger, helix-loop-helix, homeodomain and winged-helix families [3]. The paired box (Pax) gene is one family of homeodomain class transcription factors involved in development, which contains at least nine members Pax1 through Pax9 in mammals [4–6]. All members are highly conserved during evolution containing a conserved DNA-binding domain encoding the Pax, consisting of a 128 amino acid sequence at the amino-terminal side of the protein [7,8]. Pax5 is one of the best studied members of the Pax family, named B cell-specific activator protein (BSAP) as well [9]. Along with Pax2 and Pax8, Pax5 is classified in subclass III of these genes [5], which contains a bipartite “paired domain” responsible for DNA binding, a sequence encoding a characteristic octapeptide, a homeobox homology region, a transactivation domain, and a repressor domain [10]. Like other transcription factors, the alternative splicing of RNA transcripts on Pax5 results in different isoforms of Pax5 mRNAs expressed in different B cell lines [11].

Pax5 expressed at almost all B cell developmental stages, from the pro-B cells to the mature B cells but not in the terminally differentiated B cells [7,9,12,13]. It has been implicated in the regulation of several B cell specific genes, such as CD19 [14] and CD20 [15,16], and controlling B cell development, proliferation [17,18] and antibody secretion [19–21] in mammals. In addition, it had been taken as the B cell differentiation stage specific expression factor (excepted in terminally differentiated plasma cells) [7,22].

Teleost fish does not possess bone marrow or lymph nodes as mammals, but it possesses the anterior kidney (AK) as a main site for B cell lymphogenesis instead [23–25]. Compared to mammals, the study of Pax5 in teleost is limited in few species, including rainbow trout [26,27], fugu [28] and zebrafish [29,30]. In zebrafish, Pax5 was demonstrated to regulate the development of the utricular macula and vestibular function [29]. Besides, both in fugu and rainbow trout, Pax5 was regarded as a critical transcription factor in regulating B cell differentiation [26–28], providing a new biomarker for teleost B cell development [27]. However, the role of its involvement in teleost B cell activation is still not clear, especially in Nile tilapia. Nile tilapia (*Oreochromis niloticus*) is an important economically cultured fish in the

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world and China is the largest tilapia farming country [31]. The studies on Nile tilapia immune system are popular in recent years [32–38] and leukocytes are playing important roles in both innate immune and adaptive immune responses [39–42]. Even though the importance of teleost transcription factors as in mammals, the study of their roles on B cell activation in Nile tilapia is deficient. Pax5, as an important transcription factor with high conservation between species, would provide a comparative approach to study the B cell activation in Nile tilapia.

In this study, the Pax5 (OnPax5) gene of Nile tilapia (*Oreochromis niloticus*) was cloned and identified, and analyzed the expression at tissues, leukocytes, IgM^+ and IgM^- lymphocytes in various organs. Then, the LPS-challenged tilapia *in vivo* was going to investigate the transcriptional expression of OnPax5 in peripheral blood (PBL), spleen (SPL) and AK. The OnPax5 expressions of leukocytes from AK and SPL were also investigated after stimulation with LPS, pokeweed mitogen (PWM) and mouse anti-OnIgM monoclonal antibody (mAb). These results would provide clues for the better understanding of transcription factor Pax5 in teleost fish.

2. Materials and methods

2.1. Experimental animals and immune challenge experiment

Healthy Nile tilapias (*Oreochromis niloticus*) were obtained from Guangdong Tilapia Breeding Farm (Guangdong, China) and acclimatized to laboratory conditions at 28 ± 2 °C (automatic filtering aquaculture system with a stocking rate of 10 g/L) for three weeks in 300 L tanks [32,33,35,37]. Fish used in this study (100 ± 10 g mean weight) were maintained with a density of 10 animals per tank and experiment was conducted by the University Animal Care and Use Committee of the South China Normal University.

With the aim of performing the tissue distribution profiling of OnPax5, tissue samples including peripheral blood, liver, thymus, gills, anterior kidney, posterior kidney, spleen, intestine, muscle and skin were collected (n = 3). To determine the role of OnPax5 in B cell stimulation response, LPS (055:B5 from *E. coli*; Sigma, USA) was used in stimulation experiment by intraperitoneal injection with 100 μL (1 mg/mL in sterile PBS) [32,37]. The control group was stimulated with PBS. At 0 h, day 1 (D1), D3, D6, D10, D14, D21 and D28 post-stimulation, tissue samples (including PBL, SPL and AK) were individually collected. All the samples got above were frozen by liquid nitrogen immediately and stored at −80 °C until RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA of each tissue collected in *2.1* from three animals was extracted by using Trizol Reagent (Invitrogen, USA) as the guidelines. RNA was quantified using electrophoresis on a 1.0% agarose gel (BIOWEST, Spain) and Nanodrop 2000 (Thermo Scientific, USA). An aliquot of 1 μg of total RNA was used to synthesize cDNA from each tissue with the Prime-Script ™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. Finally, the synthesized cDNA was diluted ten folds and used in quantitative real-time PCR (RT-qPCR).

2.3. Identification of the open reading frame sequences (ORF) of OnPax5

A cDNA library was constructed from the spleen tissue of healthy Nile tilapia. The ORF cDNA of OnPax5 was identified according to the NCBI reference sequence (GenBank Graphics: XM_005456847.3). The PCR primers used here were designed by Primer Premier 5.0, with upstream primer (Pax5-F: 5′-ATGGACCAGGGGCCCAAGAGTCCTG-3′) and downstream primer (Pax5-R: 5′-TCAGTGGCGGTCGTAGGTGGAG GCT-3′). To verify authenticity of the OnPax5 sequence, the PCR products were electrophoresed on a 1.0% agarose gel, then ligated into the pMD-18T vector (TaKaRa, Japan) and transformed into competent *E. coli* cells TOP10 (Tiangen, China).

2.4. Molecular characterization and structural analysis of OnPax5

The obtained OnPax5 nucleotide sequence was translated with ExPASy tools (https://www.expasy.org/tools/). Signal peptide was analyzed by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignment of amino acid sequences was completed using the DNAMAN software. Similarity analyses of the amino acid sequences were performed by NCBI protein blast programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein domains were defined by simple modular architecture research tool (SMART) (http://smart.emblheidelberg.de/) and NCBI Conserved Domains (https://www.ncbi.nlm.nih.gov/cdd/?term=). Phylogenic trees were constructed based on the putative amino acid sequences using the neighbor-joining method, with 1000 bootstrap replications and displayed in MEGA 7.0 software [35,37].

2.5. Determination of OnPax5 transcript levels by qPCR

Spatial transcript levels of *OnPax5* in isolated ten tissues of healthy fish, and the temporal expression levels of *OnPax5* in three tissues of LPS-stimulated animals (section *2.1* and *2.2*) were individually evaluated by qPCR using a 7500 Real Time PCR System (Life Technologies, USA) following the essential guidelines. Reaction was performed in a 20 μL system containing 3 μL of diluted cDNA, 4 μL of each primer (2 μM), 10 μL of 2 × ExTaq™ SYBR premix, 0.4 μL Rox Reference Dye II and 2.6 μL DEPC H_2O (TaKaRa, Japan). The cycling parameters were performed as reference [32,33,35–37]. The gene-specific primers were employed to amplify *OnPax5* (qPax5-F, 5′-CCGGAAGTCCATATTCCC ATC-3'and qPax5-R, 5′-GACCCATAATCCTGCTGAAACACT-3′) and Nile tilapia *β*-actin (q*β*-actin-F, 5′-GAGAGGGAAATCGTGCGTGACA-3'; q*β*-actin-R, 5′-AGGAAGGAAGGCTGGAAGAGGGC-3′). The baseline was set automatically by the 7500 Real Time System software.

In spatial mRNA distribution, the relative *OnPax5* transcript level in each tissue was normalized against *β*-actin using the $2^{-\Delta CT}$ method [43] and showed as the ratio of the comparation with *OnPax*5 expression in spleen. In temporal transcriptional profiling, the relative-fold change in mRNA levels after the stimulation was computed with respect to the expression in corresponding PBS-injected controls using the $2^{-\Delta\Delta CT}$ method [44], and then compared with that of 0 h, which was considered as the baseline.

2.6. Leukocyte separation and stimulation in vitro

Leukocytes isolated from anterior kidney and spleen were according to the methods described in the publications [32,35,38] with some modification. The leukocytes at the interface of the Histopaque®1077 (Sigma, USA) were collected and washed three times in RPMI-1640 medium (Gibco, USA). The cell mount was counted in trypan blue, and the viability of the separated cells was in good condition. Cells were re-suspended to a concentration of 1×10^7 cells/mL in RPMI-1640 with 10% fetal bovine serum (FBS; Gibco, USA) and cultured *in vitro* for 5 h at 25 °C [35]. Then removed the adherent cells (mainly monocytes/macrophages) and the non-adherent leucocytes were carefully collected and re-cultured in complete medium as the concentration of 1×10^7 cells/mL [45]. Followed by treatment with a final concentration either 40 μg/mL of LPS, 10 μg/mL PWM or 10 μg/mL mouse anti-OnIgM mAb [35,37] with the control of PBS. All groups were incubated at 25 °C and sampled at the time of 0 h, 12 h, D1, D2, D4 and D6 post-stimulation for qPCR analysis of *OnPax5* expression as described above. The relative expression of OnPax5 in experimental group was compared with PBS group. Fold change of *OnPax5* was calculated by using the $2^{-\Delta\Delta CT}$ method [44]. Moreover, the protein levels after stimulation were detected by flow cytometer (BD, USA) as described in reference [26]. The relative expression of OnPax5 was shown as the ratio of the

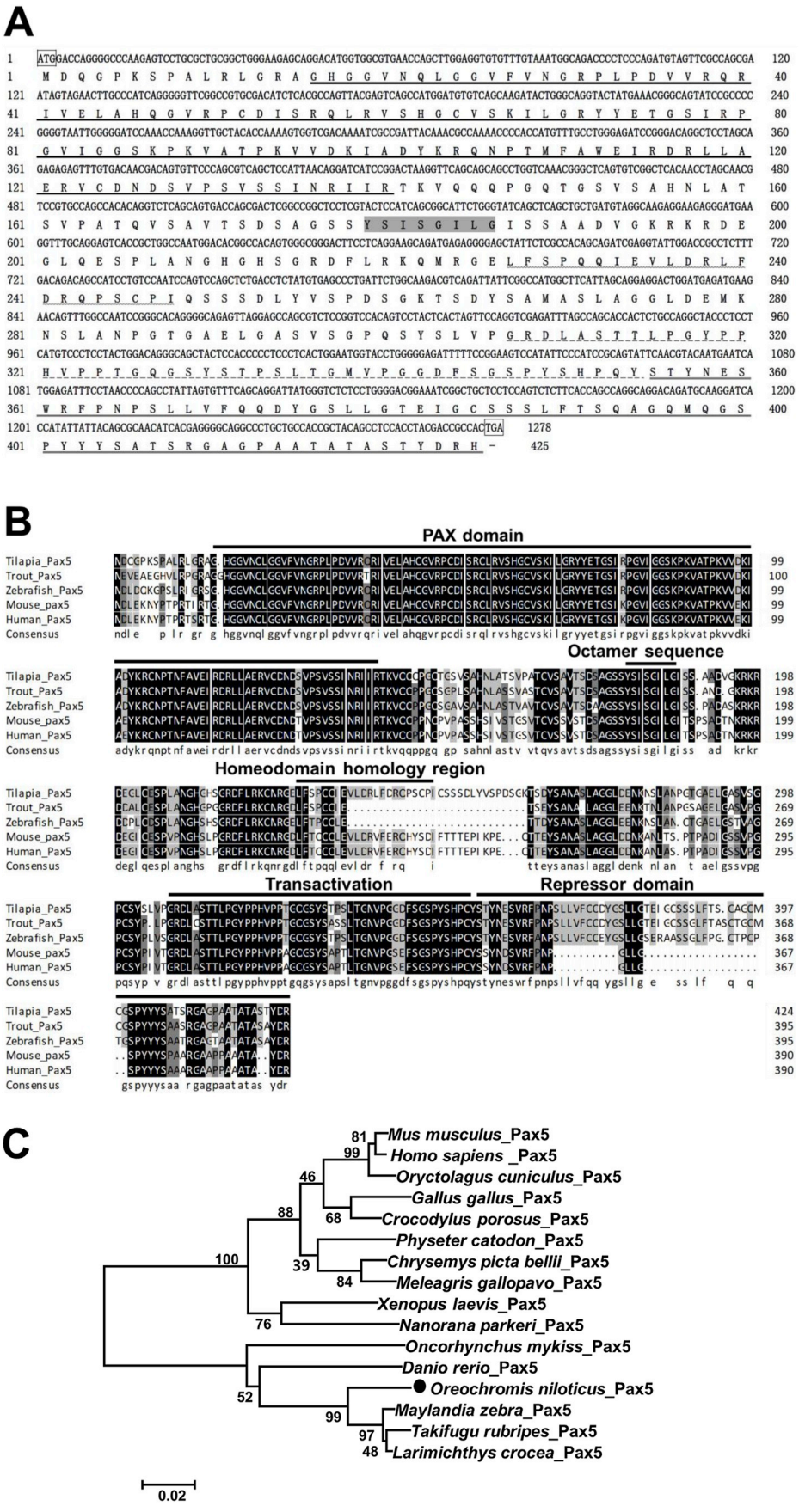

Fig. 1. Sequence (A), multiple sequence alignment (B) and phylogenetic tree analysis (C) of Nile tilapia Pax5 with known Pax5 sequences. (A) cDNA and deduced amino acid sequence of OnPax5. The first ATG (initial codon) and the last TGA (termination codon) is in a box; PAX domain is underlined with a single line; octamer sequence is in gray shadow; homeodomain homology region is in wave line; transactivation is dashed underlined and repressor domain is double lined. (B) Multiple sequence alignment of the OnPax5 amino acid sequence with other known species. The conserved motif and domains are overlined. The accession numbers of the sequences are as follows, rainbow trout (*Oncorhynchus mykiss*, NP_001118154.1), zebrafish (*Danio rerio*, NP_571713.1), mouse (*Mus musculus*, Q02650.1) and human (*Homo sapiens*, Q02548.1). (C) Phylogenetic tree analysis of the amino acid sequences of Pax5 protein were constructed by the neighbor-joining method and bootstrapped 1000 times using the MEGA 7.0 software. The other accession numbers of the sequences are as follows, *Oryctolagus cuniculus* (XP_008263358.1), *Gallus gallus* (NP_989755.1), *Meleagris gallopavo* (XP_010724574), *Chrysemys picta bellii* (XP_005308893.1), *Crocodylus porosus* (XP_019410974.1), *Xenopus laevis* (NP_001079237.1), *Nanorana parkeri* (XP_018407927.1), *Physeter catodon* (XP_007127593.1), *Maylandia zebra* (XP_004564341.1), *Larimichthys crocea* (XP_019130671.1), *Takifugu rubripes* (NP_001032960.1).

percentage in positive group compared to the control group.

2.7. Sorting of leukocyte populations from PBL, SPL and AK and qPCR analysis

Whole leukocytes (including lymphocytes, monocytes/macrophages and so on) separated from PBL, SPL and AK by Histopaque® 1077 (Sigma, USA) as described in *2.6*, was suspended by Trizol regent (1×10^7 cells/sample) and frozen by liquid nitrogen immediately then stored at −80 °C until RNA extraction. To further explore the transcriptions in lymphocytes, IgM^+ (positive) and IgM^- (negative) lymphocytes were sorted by a flow cytometer (BD, USA) as we previous did [46]. The separated leukocytes from PBL, SPL and AK were re-suspended in RPMI-1640 (5% FBS) and incubated for 1 h on ice with anti-OnIgM mAb (1 mg/mL, 1:2000 dilution) labeled with Alexa Fluor 647 (Thermo, USA) [35,37,46], respectively. After washed three times, BD FACS Arial III was used to detect their forward scatter (FSC) and side scatter (SSC) to exclude the granulocyte gate. Then, on the basis of the fluorescence emitted by the sample, IgM^+ and IgM^- cells were sorted. The purity of the sorted cells was confirmed with more than 95% purity by sorted back. The RNA isolation of collected cells was performed by Trizol regent. Total RNA isolation and cDNA synthesis were executed as described in *2.2*. The relative *OnPax5* transcript level in each sample was normalized against *β*-actin using the $2^{-\Delta CT}$ method [43] and showed as the value of $2^{-\Delta CT}$.

2.8. Western blotting analysis of sorted cells

The sorted IgM^+ and IgM^- lymphocytes from PBL described in *2.7* were collected in aliquots of 2×10^6 cells. Whole-cell protein lysates were prepared by re-suspending cells in 80 μL of a sample buffer containing 5% 2-ME, and proteins were separated by size using denaturing 12% SDS-PAGE gels [35,37] and proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Membranes were incubated in blocking solution (0.5% BSA in PBS) for 1 h, followed by a 1 h incubation in dilution (0.1% BSA in PBS) with primary antibody (Ab), rabbit anti-Pax5 monoclonal Ab (1:5000, 1 mg/mL; Abcam, USA). Three 10 min washes in PBS were then followed after 1 h incubation and secondary Ab, goat anti-rabbit IgG-HRP conjugated (1 mg/mL; Southern Biotech, USA), 1:2000, was added in dilution, and membranes were washed three more times in PBS and developed using a chemiluminescence kit (ECL; Beyotime, China) according to the instructions of the manufacture and acquired by Tanon5500 automatic chemiluminescence imaging analysis (Tanon, China). The densitometric data of OnPax5 and *β*-actin protein expression were analyzed with ImageJ software. The expression level of OnPax5 was corrected by the endogenous control *β*-actin expression (Yeasen, China), which was shown as the mean intensity ratio of OnPax5 to *β*-actin.

2.9. Statistical analysis

Data were presented as mean ± standard deviation (SD) of three individual samples (n = 3) in the *in vivo* experiment or six fish (n = 6) *in vitro* study. In qPCR results, the value of each sample was the mean of the individual replications. The relative protein levels of OnPax5 by densitometric analysis were expressed as mean ± SD with three individual replications. Differences were considered as significant at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). All plots were made by Sigma Plot 10.0 software in this study.

3. Results and discussion

3.1. Molecular characterization of OnPax5

Both in mammals and teleost, domain structure of Pax5 comprises a paired domain responsible for DNA binding, an octapeptide, a homeobox homology region, a transactivation and a repressor domain [10–12,26,29]. The Pax5 homology (OnPax5) from Nile tilapia was characterized and the result showed that the OnPax5 is similar to the reported sequences in other species. The ORF of OnPax5 contains 1278 bp, encoding a polypeptide of 425 amino acids (aa) (Fig. 1A). OnPax5 does not have signal peptides, but contains a PAX domain (16–143 aa), an octamer sequence (179–186 aa), a homeodomain homology region (228–250 aa), a transactivation (306–354 aa) and a repressor domain (355–425 aa) (Fig. 1A and B).

Sequence alignment showed that the amino acid sequence of OnPax5 shared high similarity with their mammalian counterparts (70% with mouse and human) and the known sequences of Pax5 in teleost, sharing 82% with zebrafish and 84% with rainbow trout (Fig. 1B). The homeobox homology region of OnPax5 is similar to mouse and human; however, it is different from the known sequences in trout, fugu and zebrafish [26,28–30], with the absence of a 25 aa sequence in trout, fugu and zebrafish Pax5 (Fig. 1B). The transactivation domain of OnPax5 likes other teleost's Pax5, with a 30 aa sequence which is deficient in mouse and human Pax5 [26]. Different from trout but the same as zebrafish, the cysteine residue presented in OnPax5 transactivation domain does not exist, which residue (aa 281) is unique to trout. In addition, the cysteine residue in repressor domain of OnPax5 is the same as trout (aa 355), but it is absent from zebrafish, mouse and human [14,26,28–30]. Until now, the functional significance of these species-specific differences has not been reported. To further analyze the evolutionary relationship of OnPax5 with other vertebrates, the phylogenetic tree was built and shown in Fig. 1C, indicating that fish and non-fish vertebrates were classified into two branches.

Pax5 is an alternatively spliced transcription factor that regulates B cell development and it has been demonstrated that there exist different isoforms of Pax5 mRNAs in mouse and trout [11,27]. According the above analysis, the sequence we obtained in Nile tilapia would be one of the existing isoforms. This isoform has been reported in previous studies, which expressed during the early stages of B cell differentiation in mammals and teleost [7,11,12,26,29]. Right now, the other isoforms of Pax5 in Nile tilapia are not discovered in our study; however, it is considered in our later work.

3.2. Expression of OnPax5 in tissues and cells

The tissue level gene expression of *OnPax5* was analyzed by using qPCR and gene expression level was normalized with the housekeeping gene *β*-actin. *OnPax5* was highly expressed in the anterior kidney, spleen and thymus (Fig. 2A). Among mammals' hematopoietic cells, Pax5 gene expression is restricted to the B cell lineage, which initiated in pro-B cells and is abundant at the pre-B and mature B cell differentiation stages, but is absent in terminally differentiated plasma cells [7,22]. In teleost, B cells reside in various tissues but are abundant in anterior kidney and spleen [47]. Although thymus is the site of thymocyte maturation, previous studies were demonstrated that a few IgM-producing B cells existed in teleost in thymus [48,49]. In addition, the *OnPax5* expression in the muscle was the lowest (Fig. 2A), due to the possibility of the low B cells in this tissue.

In order to analyze the *OnPax5* expression in leukocytes from different immune tissues, we separated the leukocytes from PBL, SPL and AK. The results showed that *OnPax5* expressed the highest level in leukocytes from spleen, and the lowest was expressed in AK leukocytes (Fig. 2B). The possibility of tissue-dependent expression is, as the report in rainbow trout, that spleen and peripheral blood contain mostly resting mature B cells, while anterior kidney contains both developing B and Ig-secreting B cell populations and few resting, mature B cells [26]. The similar findings of the *OnPax5* expression in leukocytes in both fishes indicated that the B cell differentiation level at PBL, SPL and AK in Nile tilapia might be consistent with that in rainbow trout, because Pax5 gene is abundant at the pre-B and mature B cell differentiation

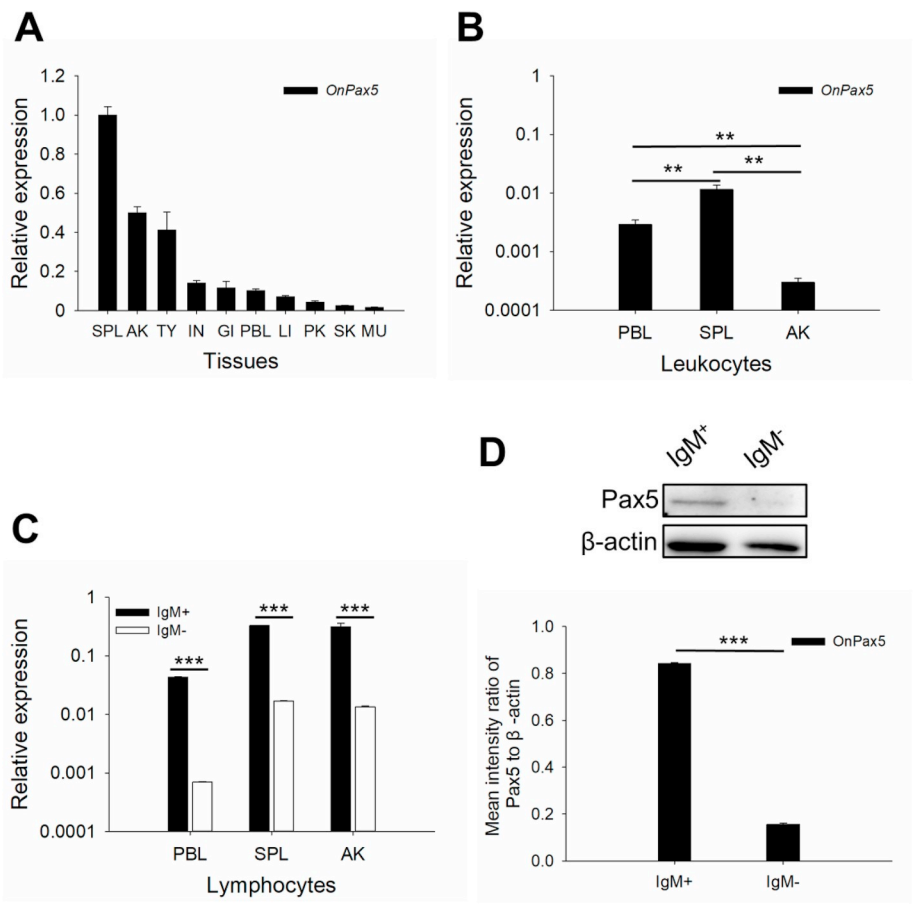

Fig. 2. The expression levels of OnPax5 in tissues (A), leukocytes separated from PBL, SPL and AK (B), mRNA (C) and protein levels (D) in sorted lymphocyte populations from PBL, SPL and AK. SPL, spleen; AK, anterior kidney; TY, thymus; IN, intestine; GI, gills; PBL, peripheral blood; LI, liver; PK: posterior kidney; SK, skin; MU, muscle. OnPax5 mRNA transcription was evaluated by qPCR. Protein level was determined in IgM^+ and IgM^- lymphocytes from PBL and showed as the mean intensity ratio to *β*-actin. Vertical bars represent the mean ± SD (n = 3). $^{**}p < 0.01$, *** means $p < 0.001$.

stages, but is absent in terminally differentiated plasma cells [7,22].

To further investigate the *OnPax5* expression in lymphocytes, we used the mouse anti-OnIgM mAb [35,37] to sort out IgM^+ and IgM^- lymphocytes by flow cytometer [47,50]. The results were shown in Fig. 2C that *OnPax5* expressed highly in IgM^+ lymphocytes compared to IgM^- cells. The finding was consistent with the report in rainbow trout IgM^+ and IgM^- cells [47,51]. Western-blot analysis of IgM^+ and IgM^- cells from PBL indicated that the differential expression was confirmed at the protein level (Fig. 2D). Although the anti-Pax5 antibody used in western-blot analysis is a commercial antibody anti-human Pax5, the specificity of antibody to the 250–350 aa sequence of Pax5 is identical to the amino acid fragments in Nile tilapia (Fig. 1B). Therefore, the strategy could be an alternative solid way for fish immunologists to study B cells by using commercial antibodies anti-conservative sites of transcriptional factors in teleost, especially during the conditions of the absence of serological reagents as previous studies in rainbow trout [26,52–55].

3.3. Response to LPS stimulation in vivo

Lipopolysaccharide is the principal component of the outer membrane from Gram-negative bacteria [56], which can be recognized by monocytes and macrophages of the innate immune system and induce systemic inflammation [57]. What's more, LPS is the potent polyclonal activators of leucocytes in mammals via interaction with toll-like receptor 4 [58]. LPS is a stimulation mitogen for teleost leucocytes which has been demonstrated in many fish species, including trout [26], snapper [45] and tilapia [37]. Upon LPS stimulation by intraperitoneal injection, *OnPax5* expression was significantly up-regulated in all three examined tissues of PBL, SPL and AK, but with different expression patterns (Fig. 3). After LPS stimulation, *OnPax5* expression was up-regulated faster (D1 vs. D3 post-stimulation) and attained the maximum more quickly (D3 vs. D21 post-stimulation) in PBL and AK than SPL (Fig. 3). In addition, the maximal increase of *OnPax5* expression in SPL (4.73-fold) was higher than those in AK (3.34-fold) and PBL (2.14-fold) (Fig. 3). The quick up-regulation occurring in the three immune tissues indicated that *OnPax5* may be involved in innate immunity by the B cell activation, since B cells take parts in the innate immune system [59,60]. The different dynamics of *OnPax5* up-regulation in different tissues may reveal that the B cell populations are non-uniform distribution in Nile tilapia, as reported in rainbow trout [26,52,53].

3.4. Expression of OnPax5 in AK and SPL leucocytes upon stimulation in vitro

In order to examine the *OnPax5* expression upon stimulation *in vitro*, leukocytes were separated from anterior kidney and spleen, and were stimulated with three B cells stimuli, LPS, PWM and mouse anti-IgM mAb, respectively. Interestingly, upon challenges of these stimuli, the *OnPax5* expression was all significantly up-regulated in both spleen and anterior kidney (Fig. 4). After LPS stimulation, the peak of *OnPax5* expression was observed at D4 (13.7-fold) in AK and at D2 (4.2-fold) in SPL (Fig. 4 A, D). When activated with PWM, the maximum of *OnPax5* transcription appeared earlier than that of LPS, at D1 (120.7-fold) in both AK and SPL (7.5-fold) (Fig. 4B, E). Upon the mouse anti-OnIgM Ab challenge, the highest expression of *OnPax5* reached at D1 (23.1-fold) in both AK and SPL (2.7-fold) (Fig. 4C, F). At protein level, the OnPax5 protein expression in AK was significantly elevated at D4 (1.37-fold), D2 (1.98-fold) and D4 (1.37-fold) after stimulated by LPS, PWM and mouse anti-OnIgM Ab, respectively (Fig. 5A–C). In spleen, OnPax5 protein expression was significantly up-regulated at D4 (1.21-fold), D4 (1.71-fold) and D2 (1.22-fold) after stimulated by LPS, PWM and mouse anti-OnIgM Ab, respectively (Fig. 5D–F). The up-regulated protein level of OnPax5 was a little later than that at the molecular level, which may

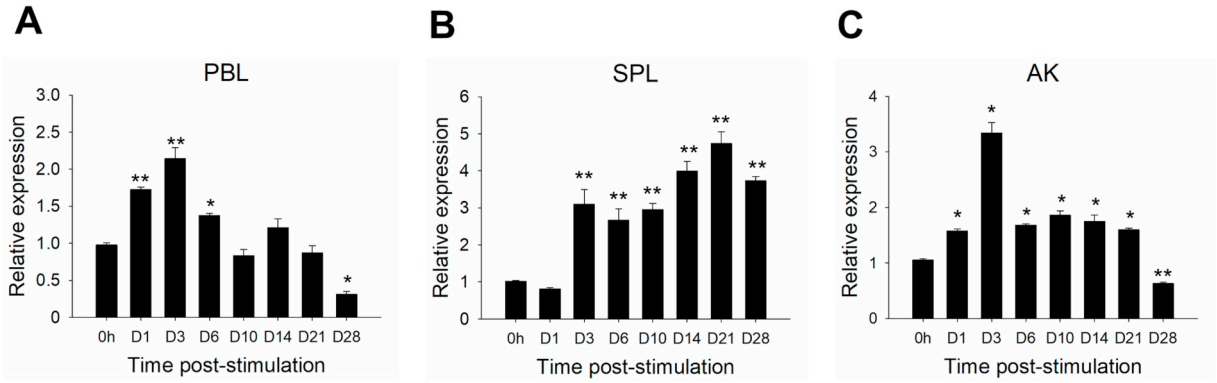

Fig. 3. Temporal expression profile of the OnPax5 mRNA in PBL (A), SPL (B), and AK (C) after LPS stimulation *in vivo*. Expression values were normalized to those of *β*-actin. Data are expressed as the mean fold change (means ± SD, n = 3) from the control group. Significant differences among the samples at different time in the same tissue are presented with the asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

be because of the posttranslational modification. The LPS is a polyclonal activator of B lymphocytes, while PWM is a potent T cell-dependent mitogen to induce B cell proliferation and differentiation to the plasma cell phenotype [61]. Further, anti-IgM mAb is able to activate B cells by cross-linking the B cell receptor on the IgM^+ B lymphocytes specially [35,37,62]. Both in AK and SPL, the up-regulation of *OnPax5* by LPS was slower than that by PWM and anti-IgM mAb may be due to the different stimulatory pathway in recruited different cells during the stimulation. Further studies are required to elucidate the precise role and mechanism of Pax5 during the stimulation in fish. Since Pax5 is believed to be a key protein for B cell activation [18,26,27], the transcription of *OnPax5* was significantly up-regulated after stimulation, indicating that *OnPax5*, as in mammals, was likely to get involved in B cell activation in tilapia.

4. Conclusion

In conclusion, we cloned and identified the transcription factor OnPax5 from Nile tilapia and explored the transcriptional expression upon stimulation with LPS *in vivo*, which indicated OnPax5 might get involved in B cell activation. By *in vitro* stimulation of the leukocytes with different B cells stimuli, the data revealed that *OnPax5* would take part in B cell activation. Based on its conserved structure and function as other Pax5, it is likely that OnPax5 is a useful marker to distinguish the B cell subpopulations in Nile tilapia as in mammals and rainbow trout. The functional analyses of Pax5 factor will provide a sight for better understanding its role in B cell activation in teleost.

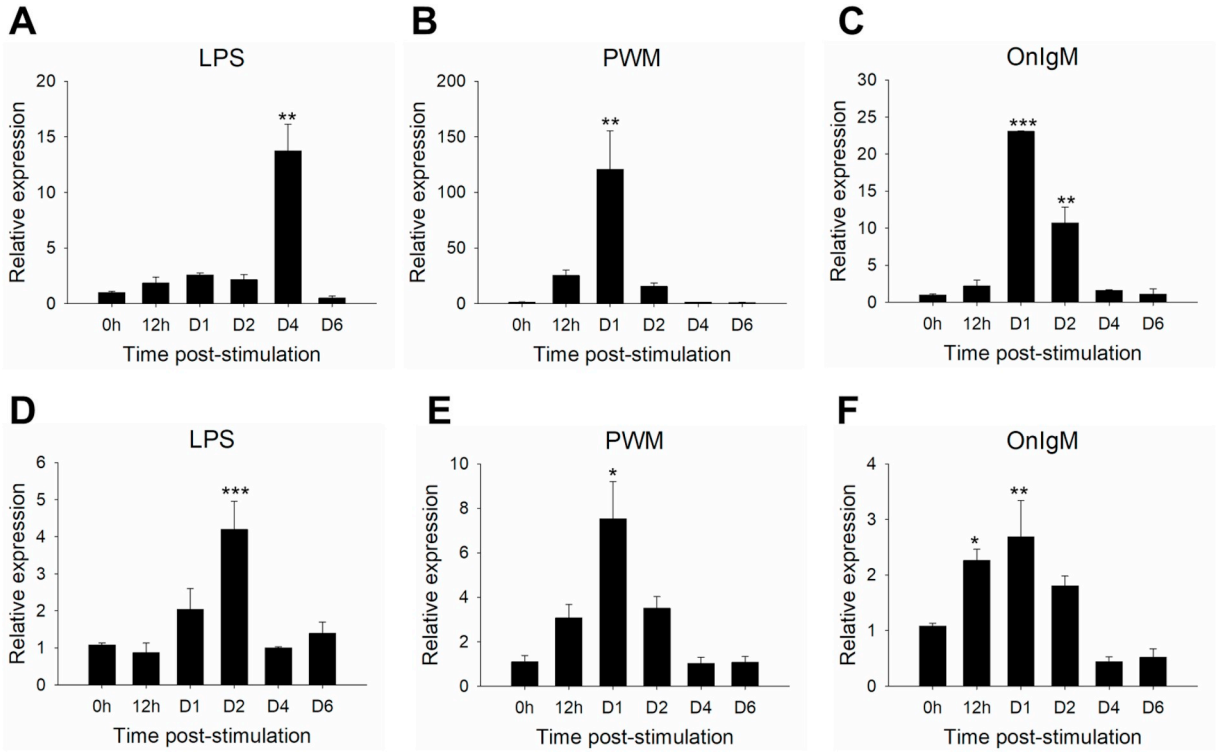

Fig. 4. *OnPax5* expression in stimulated anterior kidney (A–C) and spleen leukocytes (D–F) *in vitro* as measured by qPCR. The stimulus are as follows: (A, D) LPS, (B, E) PWM and (C, F) mouse anti-OnIgM mAb. All data were normalized to the housekeeping gene *β*-actin and are presented as fold-change (means ± SD, n = 6) relative to the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

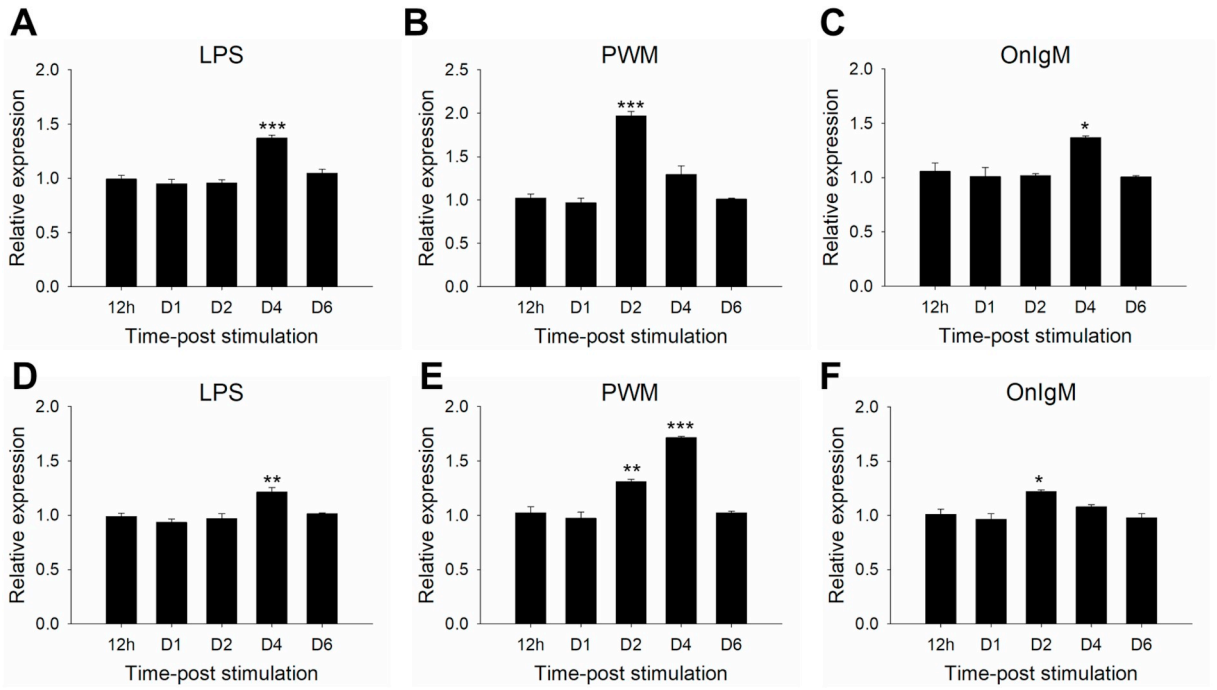

Fig. 5. The relative expression of OnPax5 at protein level in stimulated anterior kidney (A–C) and spleen leukocytes (D–F) *in vitro* as measured by flow cytometer. The stimulus are as follows: (A, D) LPS, (B, E) PWM and (C, F) mouse anti-OnIgM mAb. All data were presented as fold-change (means ± SD, n = 6) relative to the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.059.

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