Molecular cloning and expression analysis of Foxp3 from Nile tilapia

Jing Wei, Lintian Yu, Lina Sun, Xiaoping Zhang, Minghui Li, Wenchuang Qi, Linyin Zhou, Deshou Wang*

Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Science, Southwest University, Chongqing 400715, China

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ABSTRACT

Foxp3 is a crucial transcription factor for the development and function of CD4+CD25+ regulatory T cells (Tr) which play a key role in preventing autoimmune diseases and maintaining maternal-fetal tolerance in mammals. However, the knowledge of Foxp3 and its regulation in teleosts is limited. In the present study, the Foxp3 cDNA was cloned from Nile tilapia and characterized. The full length cDNA of Nile tilapia (nt)Foxp3 contained a 5′UTR of 104 bp, a 3′UTR of 239 bp and an open reading frame of 1134 bp. The putative ntFoxp3 contained a C2H2 zinc finger domain, a winged-helix/fork head domain and a leucine zipper-like domain with a consensus of V-x(6)-L-x(5)-L-x(6)-L, which are typical motifs of a Foxp3. The highest mRNA expression of ntFoxp3 was observed in the spleen, with moderate expression in the head kidney, intestine, kidney, liver and brain. Stimulation of peripheral blood mononuclear cells with PHA and LPS led to a significant increase of ntFoxp3 mRNA expression at 6 and 24 h, respectively. In contrast, stimulation with PMA had no effect during the sampling period. After injecting 1 × 10⁶ cells of live Aeromonas hydrophila into adult female Nile tilapia, the mRNA expression of ntFoxp3 significantly increased in the gill and intestine at 6 h, while unchanged in the spleen. In all the tissues examined, a clearly up-regulation of ntFoxp3 expression was detected at 24 h after the second challenge. These results suggest that ntFoxp3 might be involved into lymphocyte activation and immune responses as reported in mammals. Meanwhile, the correlation between the expression profile of ntFoxp3 and serum estrogen (17-beta-estradiol, E2) concentration was investigated by real-time PCR and enzyme immunoassay. The results showed that E2 could regulate the expression of ntFoxp3 in the intestine and kidney but not in the spleen. This work will help future investigations into Tr cells and extend our understanding of interactions between sex hormones and immune related molecules in teleosts.

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1. Introduction

In mammals, it has been well documented that Foxp3 plays an essential role in naturally occurring CD4+CD25+ regulatory T cells (Tr) development and function which are crucial in preventing autoimmune diseases and maintaining maternal-fetal tolerance through being immunosuppressive in both innate and adaptive immune responses (Afshan et al., 2012; Bennett et al., 2001; Ng et al., 2001; Schubert et al., 2001; Stephens and Mason, 2000; Valencia and Lipsky, 2007). In Scurfy mice, a frame shift mutation of Foxp3 results in early lethality due to a CD4+ T cell-mediated lymphoproliferative disease (Schubert et al., 2001). In humans, mutation in FOXP3 results in a fatal autoimmune disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome associated...
with autoimmune disease in multiple endocrine organs) (Bennett et al., 2001; Kobayashi et al., 2001). Extensive studies have further established a critical role for Foxp3 in CD4+CD25+ Tr development and function (Chen et al., 2003; Fontenot et al., 2003). Accordingly, the knowledge about signals that govern the transcription of Foxp3 is greatly required. Meanwhile, it has been reported that Estrogens (17-beta-estradiol, E2) treatment and pregnancy in mice and human can drive CD4+CD25- T cells to CD4+CD25+ T cells associated with enhanced Foxp3 expression in vitro and in vivo which possess similar regulatory function as naturally occurring CD4+CD25+ Tr (Offern and Vandenberg, 2005; Polanczyk et al., 2004, 2005).

It is well established that teleosts possess T cells and T cell-mediated immune response like mammals. Many of teleost T cell specific development-related genes have been cloned, which have similar expression profiles and functions to their mammalian counterparts (Castro et al., 2011). What’s more, the way in which T cells undergo antigen receptor rearrangements in teleosts is similar to what they do in mammals, which would often generate receptors that recognize autoantigens (Haire et al., 2000; Meeker et al., 2010; Meeker and Trede, 2008). Thus, active regulatory elements such as those which control tolerance and functional equivalents of mammalian Tr might accompany the development of the molecular potential for adaptive autoimmune in teleosts. Recently, the Foxp3 cDNAs from zebrafish (Danio rerio), rainbow trout (Oncorhynchus mykiss), pufferfish (Tetraodon nigroviridis), Atlantic salmon (Salmo salar) and grass carp (Ctenopharyngodon idellus) have been cloned, their functional characteristics and involvement in the immune response have also been primarily investigated (Mitra et al., 2010; Quintana et al., 2010; Wang et al., 2010; Wen et al., 2011; Yang et al., 2012; Zhang et al., 2011). However, the factors which regulate the transcription of Foxp3 in teleosts remain to be explored. In teleosts, E2 plays a pivotal role in reproductive physiology, such as sex determination, differentiation and maintenance. Meanwhile, it has been reported that E2 has the potential to compromise immunocompetence of fish, such as a reduced phagocytic activity, lower respiratory burst activity, decreases of plasma lysozyme and immunoglobulin levels in seabass (Lateolabrax japonicas) and common carp after estrogen exposure (Cuesta et al., 2007; Thilagam et al., 2009). Nevertheless, the currently available information on an immunomodulatory role of E2 is scant and by no way conclusive in teleosts. Therefore, it would be interesting to investigate the relationship between sex hormones such as E2 and the transcription of Foxp3 in teleosts.

As a worldwide farmed fish, the Nile tilapia (Oreochromis niloticus) is an excellent model for the study of the interaction between sex hormones and immune molecules due to the availability of monosex offspring and short spawning cycle (14 days). However, the knowledge of the immune characteristics from tilapia is very limited till now. In the present study, the full length cDNA of Nile tilapia (O. niloticus) Foxp3 (ntFoxp3) was cloned and characterized, and the expression profiles of ntFoxp3 in immune responses and in 6-month-old Nile tilapias (XX:XY) with different serum E2 concentrations were investigated by real-time PCR and enzyme immunoassay.

2. Materials and methods

2.1. Animals

Nile tilapias were kept in recirculating aerated water tanks at 26°C. All genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from control females (XX) with sperm from either sex reversed males (XX) or super males (YY), respectively. All animal experiments were carried out in accordance with the Guidelines for Care and Use of Laboratory Animals prescribed by the Regulation of Animal Experimentation of Chongqing, China.

2.2. Cloning of ntFoxp3 cDNA

Total RNAs were extracted from spleen and head kidney of adult Nile tilapias using RNAiso PLUS (Takara, Japan) according to the manufacturer’s instructions. The quality and concentration of total RNAs were analyzed by agarose gel electrophoresis and optical density reading at 260 and 280 nm. One microgram of total RNA was reverse-transcribed into cDNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan).

By searching the GenBank and comprehensive bioinformatics analysis, degenerate PCR primers for ntFoxp3 were designed (Table 1). To obtain the full length of ntFoxp3, 3′- and 5′-RACE-ready cDNA were reverse transcribed from Nile tilapia spleen and head kidney mRNA mix using the SMART RACE Kit (Clontech, USA) according to the manufacturer’s instructions, and three pairs of primers (Table 1) were designed, respectively. PCR products were resolved by agarose gel electrophoresis and target DNA fragments were purified, subcloned and sequenced. After sequencing the RACE product, one pair of gene-specific primers (Table 1) were designed based on the end sequences of the cDNA and used to amplify the full-length cDNA. Then, the product was sequenced again to confirm the nucleotide sequences.

2.3. Sequence analysis of ntFoxp3

After full length cDNA sequences of ntFoxp3 were obtained, the similarity with other known sequences were analyzed by BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the multiple sequence alignment was generated using CLUSTALW (Chenna et al., 2003) and GENEDOC program (http://genedoc.software.informer.com/). Similarity and identity at amino acid level were calculated with the program Matrix Global Alignment Tool (MatGAT) V2.0.3 (Campanella et al., 2003). The protein structure was identified by the PredictProtein Program (http://www.expasy.org/tools). SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to detect the presence of signal peptides (Bendtsen et al., 2004). The phylogenetic tree was constructed based on the full amino acid sequences of Foxp3 using neighbor-joining algorithm.
within MEGA version 5.0 and was bootstrapped 2000 times (Tamura et al., 2011).

2.4. Real-time PCR

The mRNA level of ntFoxp3 and β-actin (a stable housekeeping gene) was quantified by real-time PCR using delta–delta Ct (threshold crossing value) calculations. Primer pairs used in this study were gene-specific and intron-spanning (Table 1). Prior to use in real-time PCR, each primer set was validated for use by gel analysis of RT-PCR products. Only reactions generating single products of the correct size were used for real-time analysis. The efficiencies of the PCR reaction for each primer pair of ntFoxp3 and β-actin were 97.89% and 99.52%, respectively, which were determined through standard curves. Real-time PCR was performed on the StepOnePlus™ Real-Time PCR System (Life Technologies, USA) in a final volume of 20 μl with 10 μl SYBR® Premix Ex Taq™ (TAKARA, Japan), 2 μl diluted (1:10) RT samples, and 0.2 μM of each primer. The amplification protocol was an initial denaturation and activation step at 94 °C for 30 s followed by 40 cycles of 94 °C for 5 s, 62 °C for 30 s. Melting curve program was carried out routinely to confirm the presence of a single PCR product (60–90 °C with a heating rate of 1 °C/s and a continuous fluorescence measurement). The expression level of each gene was calculated as arbitrary units that had been normalized to the expression level of β-actin.

2.5. Tissue expression patterns of ntFoxp3

Eleven tissues (head kidney, kidney, spleen, gill, intestine, heart, liver, muscle, ovary, testis and brain) were collected from 9 healthy adult Nile tilapias. Total RNA was prepared individually from each tissue and reverse transcribed to cDNA. The real-time PCR was performed as described in Section 2.4.

2.6. Expression of ntFoxp3 in peripheral blood mononuclear cells (PBMC) with or without stimulation

Heparin anticoagulant was collected from 3 healthy adult Nile tilapias, then diluted with equal volume of D-Hank's solution (Gibco BRL, NY, USA) and layered on the fish lymphocyte preparation medium (Histopaque 1.080 kg/l) (TBD, Tianjin, China). Following centrifugation at 800 × g for 30 min, a layer of PBMC was collected and washed twice with PBS. The cell pellet was resuspended in RPMI-1640 (Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL, NY, USA) and seeded in 24-well plate (Becton Dickinson, NJ, USA) with the density of 1 × 10⁶ cells/well. PBMC were exposed to 50 μg/mL LPS or 20 μg/mL PMA (Sigma Aldrich, MO, USA) for 6, 12 and 24 h at 28 °C under 5% CO₂ and saturated humidity. RNA preparation and real-time PCR analysis were performed as described above.

2.7. Expression of ntFoxp3 after live Aeromonas hydrophila infection

Adult healthy female Nile tilapias (100–250 g) were injected intraperitoneally (i.p.) with 1.0 × 10⁷ cells live A. hydrophila and boosted after 20 d. The control group was injected i.p. as the infection group with PBS. The gill, intestine and spleen were collected from three treated Nile tilapias each time at 6, 24, 48, 96 h, 7 d post challenge and 24 h after the second challenge, respectively. As a time-matched control, the gill, intestine and spleen were also collected from the control group. The expression levels of ntFoxp3 in each sample were quantified as described above.

![Table 1](https://example.com/table1.png)

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Fig. 1. Multiple alignment of the deduced amino acid sequences of ntFoxP3 with the other known vertebrate FoxP3. The multiple alignment was produced using ClustalW and conserved amino acids were shaded using GENEDOC (V3.21). The ZnF-C2H2, leucine zipper-like and Fork head domains were indicated by boxes. The GenBank accession numbers of sequences used in this analysis were as follows: human, NP_054728; pig, NP_001121910; horse, NP_001156744; mouse, NP_001121199; African clawed frog, NP_001121199; zebrafish, AAAS485; grass carp, AEV45614; Atlantic salmon, NP_001185776; spotted green pufferfish, ADD91631; rainbow trout a, NP_001233262; rainbow trout b, NP_001233261.

2.8. The correlation between the serum E2 concentrations and the expression levels of ntFoxp3

The 6-month-old male Nile tilapias (XY) were injected i.p. with 50 μl sesame oil or 100 ng E2 (Sigma, St. Louis, MO) dissolved in 50 μl sesame oil per fish. Forty-eight hours after injection, the peripheral blood, intestine, kidney and spleen from the control and E2-treated males were collected. Meanwhile, the same tissues were also collected from the 6-month-old control females and
Fadrolose-treated females which were obtained by rearing 3-month-old genetic female Nile tilapias (XX) with 400 μg Fadrolose (LookChem limited company, Chengdu, China) g feed for 90 d. Fadrolose is the inhibitor of E2 synthase (aromatase), which could significantly down-regulate the E2 concentration of females (Wang et al., 2004).

After the blood samples agglutinated, the supernatants were collected and frozen at −20 °C until assayed. The E2 concentrations of the supernatants were determined in triplicate by enzyme immunoassay using commercially available kits (Cayman Chemical Company, USA). The expression levels of ntFoxp3 in the aforementioned tissues were investigated by real-time PCR.

2.9. Statistical analysis

Statistical analysis was performed with Student’s t-test for the comparison between two groups. Multiple group comparison was conducted by one-way ANOVA. Differences were considered significant at p < 0.05.

3. Results

3.1. Sequence analysis of ntFoxp3

The full length of ntFoxp3 cDNA was 1477 bp, which included a 5’UTR of 104 bp, an open reading frame of 1134 bp and a 3’UTR of 239 bp with the poly A stretch. The open reading frame encoded 377 amino acids with a theoretical molecular weight 43,813 Da and a theoretical pI 7.9. The putative primary structure of ntFoxp3 deduced from the cDNA sequences shared similar protein architecture with other known Foxp3, which contained a C2H2 zinc finger domain and a winged-helix/fork head domain. In addition, the ntFoxp3 contained a leucine zipper-like domain with a consensus of V-x-(6)-L-x-(6)-L-x-(6)-L, which could facilitate dimerization in many gene regulatory proteins (Fig. 1). Comparison of amino acid sequences of ntFoxp3 with other vertebrate Foxp3 revealed an identity higher than 50% to its homologs from pufferfish, Atlantic salmon and rainbow trout, while relatively low identity to grass carp (37.7%) and zebrafish (38.7%), and the lowest identity (30.3%) to frog (Xenopus laevis). Nonetheless, ntFoxp3 possessed more than 50% amino acid similarity to those from the other vertebrates except for frog (46.2%). Phylogenetic analysis indicated that ntFoxp3 together with the other known fish members formed a fish-specific Foxp3 clade that also grouped with the other vertebrate Foxp3, which suggested that ntFoxp3 was the ortholog of mammalian Foxp3 (Fig. 2).

3.2. Tissue distribution of ntFoxp3

The message levels of ntFoxp3 transcripts were readily detectable in all of the tissues analyzed. The highest expression level of ntFoxp3 was observed in the spleen, while the lowest level in the testis. Moderate expression levels were detected in the head kidney, intestine, kidney, liver and brain, relative less in the ovary, muscle, gill and heart (Fig. 3).

![Phylogenetic tree of ntFoxp3 with the other known vertebrate Foxp3.](image)

**Fig. 2.** Phylogenetic tree of ntFoxp3 with the other known vertebrate Foxp3. Zebrafish Foxp4 was used as an outer group. The neighbor-joining tree was constructed using the MEGA 5.0 program based on the amino acid sequences of Foxp3 available in the GenBank database. Bootstrap values were shown as % of 20,000 repetitions. The accession numbers were given in Fig. 1 except for zebrafish Foxp4 (NP 001196420).

3.3. Modulation of ntFoxp3 expression in PBMC

As shown in Fig. 4, stimulation of PBMC with 50 μg/mL PHA led to a significant increase of ntFoxp3 mRNA level at 6, 12 and 24 h (p < 0.05) respectively, while the prominent increase was at 24 h, and the fold change was approximately 8.0 compared with that in the control at the matched time. In the LPS-stimulated (20 μg/mL) group, the up-regulation of ntFoxp3 mRNA level was observed only at 24 h (p < 0.05), not at 6 or 12 h. In contrast, PMA (50 ng/mL)
had no effect on the expression of ntFoxp3 during the sampling period.

3.4. Expression of ntFoxp3 following live Aeromonas hydrophila infection

As shown in Fig. 5, a significant increase of ntFoxp3 expression was observed in the gill and intestine at 6 h (Fig. 5A and B) \( (p < 0.05) \), which formed an inverted “U” shaped expression curve from 0 h to 7 d. In contrast, the expression of ntFoxp3 unchanged in the spleen at 6 h (Fig. 5C) \( (p > 0.05) \). In all the examined tissues, a prominent up-regulation of ntFoxp3 expression was detected at 24 h after the second challenge (Fig. 5) \( (p < 0.05) \). Meanwhile, there was no significant change of the expression levels of ntFoxp3 from the different tissues in the time-matched controls (data not shown).

3.5. The correlation between the serum E2 concentrations and the expression levels of ntFoxp3

Among the 6-month-old control females, Fadrozole-treated females, control males and E2-treated males, the highest expression levels of ntFoxp3 in the intestine and kidney were detected in the control females with the highest concentration of E2, while the lowest expression levels of ntFoxp3 in the control males with the lowest concentrations of E2. After the 3-month-old control females were fed with 400 \( \mu \)g Fadrozole/g feed for three months, the serum E2 concentrations were dramatically down-regulated corresponding with the decreasing mRNA expression levels of ntFoxp3 in the intestine and kidney \( (p < 0.05) \), while the serum E2 concentrations were dramatically up-regulated after administrating 6-month-old males with 100 ng E2 at 48 h corresponding with the increasing mRNA expression levels of ntFoxp3 \( (p < 0.05) \) (Fig. 6A and B). However, there was no significant difference of the expression levels of ntFoxp3 in the spleen among the 6-month-old control females, Fadrozole-treated females, control males and E2-treated males \( (p > 0.05) \) (Fig. 6C).

4. Discussion

The ntFoxp3 cDNA encoding 377 amino acids was cloned and characterized. The ntFoxp3 had no predicted signal peptide but had well conserved domains which are special features of a Foxp3, including a \( C_2 \)H\(_2\) zinc finger domain, a winged-helix/Fork head domain and a leucine zipper-like domain. These domains have been implicated in DNA binding, nuclear translocation or dimerization in mammals (Koh et al., 2009). Phylogenetic analysis and
identity/similarity analysis further suggested that ntFoxp3 was a true ortholog of mammalian Foxp3. The high levels of ntFoxp3 expression in the immune related organs and regulation by mitogens (such as PHA and LPS) and live bacterial infection suggested that ntFoxp3 might be involved into lymphocyte activation and immune responses as reported in mammals.

In mammals, it is well documented that PHA activates T cells mainly by binding to the T cell receptor/CD3 complex, while PMA uses a different pathway directly by activation of protein kinase C which leads to the activation of T cells; and LPS activates different cell subpopulations mediated by Toll-like receptor 4, including B cells (DeGuisé et al., 1996; Jeevanandam et al., 1999). In this study, PHA led to a significant increase of ntFoxp3 mRNA level at 6, 12 and 24 h, while LPS only at 24 h when compared with the time-matched control (Fig. 4). The differential expression patterns of ntFoxp3 between PHA-stimulated and LPS-stimulated PBMCs may be related to the differences in cell populations that compose PBMCs and provide evidence for the involvement of ntFoxp3 in the activation of different subpopulations of PBMCs. By the way, no significant change of the expression levels of ntFoxp3 was detected in PMA-stimulated PBMCs, the potential reasons are still unknown and need to be further investigated.

A. hydrophila is mainly found in aquatic environments and causes fish illness such as ulcers, fin rot, tail rot, and hemorrhagic septicemia through open wounds or by ingestion of an adequate number of the organisms (Swain et al., 2007). In the present study, after live A. hydrophila infection in vivo, the expression profiles of ntFoxp3 were up-regulated in the gill and intestine which are known as the important parts of the mucosal associated lymphoid system in teleosts. The results provided the evidence of the involvement of ntFoxp3 in the immune response. Nevertheless, the expression profile of ntFoxp3 was unchanged in the spleen after the first challenge. What are the underlying mechanisms explaining the discrepancy is not clear and remains to be further studied owing to the limited knowledge of the immune characteristics in teleosts (Rauta et al., 2012). For example, how lymphoid cell populations are geographically compartmentalized, what are the definition of distinct phenotypical and functional subsets of lymphocytes, what are the key regulatory loops controlled by T cells in the immune response. All of which remain to be further studied and validated to understand the fish immunedefenses.

In mammals, it has been reported that Foxp3 is transcribed not only in lymphocytes, including CD4+CD25+ Tr, γδT cells, a minority of naive CD4+CD25− T cells, CD8+CD25− T cells, but also outside of the lymphoid system, including epithelial cells of multiple organs and tumor cells of various types, which implies that Foxp3 might have potential novel biological functions outside of Tr cells (Aarts-Riemens et al., 2008; Du et al., 2008). Consistent with this, the message levels of ntFoxp3 transcripts were readily detectable outside of the lymphoid tissues such as liver, brain, ovary, muscle, etc. Moreover, the N-terminal before the C2H2 zinc finger domain was highly divergent among fish, amphibians and mammals. Therefore, it will be very interesting to investigate the potential biological roles

Fig. 6. Impact of E2 on the mRNA expression levels of ntFoxp3. (A) Serum concentrations of E2 in the 6-month-old control females (XX, Ctrl), Fadrozole-treated females which were obtained by feeding 3-month-old genetic females with 400 μg Fadrozole/g feed for 3 months (XX, Fadrozole), 6-month-old control males (XY, Ctrl) and E2-treated males which were obtained by administrating 6-month-old males with 100 ng E2 at 48 h (XY, E2). (B) The expression levels of ntFoxp3 were examined by real-time PCR. The values were given as the average ± SEM (n = 3). Data presented were representative results from four individual experiments. Groups denoted by different letters represented significant difference at p < 0.05. Ctrl, control.
of Foxp3 in lower vertebrates compared with the counter-parts in mammals (Koh et al., 2009; Lopes et al., 2006).

E2 are produced primarily by the ovaries, and in smaller amounts by other tissues such as the liver, brain, adrenal glands and breasts (Oldle and Leeb-Lundberg, 2009). Previous studies suggest that E2 has multiple effects on the development and regulation of the immune system, and has the potential to compromise immunocompetence of fish (Cuesta et al., 2007; Thilagam et al., 2009). A correlation between the expression levels of ntFoxp3 in the tissues and the serum E2 concentrations was determined in this study, which suggested that the serum E2 concentrations and the expression levels of ntFoxp3 in the kidney and intestine but not spleen were positively correlated. Whether the up-regulation of Foxp3 by E2 might cause an increase of Tr-like cells in the immune tissues needs to be further explored. Nonetheless, there was no difference in expression levels of ntFoxp3 in the kidney and intestine between the Fadrozole-treated females and E2-treated males (Fig. 6A–C), which might be attributed to the differential regulation by endogenous and exogenous E2. Meanwhile, stimulation of PBMC with E2 could not enhance the expression of ntFoxp3 in vitro in our study (data not shown), which was in agreement with the result in mice that indicates E2 alone could not enhance the Foxp3 expression in vitro (Tai et al., 2008).

In conclusion, our study not only supports the involvement of ntFoxp3 in the immune response, but also provide insight into the regulation of Foxp3 by sex hormones such as E2 for the first time in a fish species. This work will aid further investigation into Tr-like cells and regulation mechanisms in teleosts.

Acknowledgments

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