Identification of two FoxP3 genes in rainbow trout (*Oncorhynchus mykiss*) with differential induction patterns

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1. Introduction

FoxP3 is a member of the forkhead box (Fox) gene family that comprises a large and diverse group of transcription factors that share a ‘winged helix’ DNA binding domain consisting of three alpha helices flanked by two ‘wings’ of beta strands and loops (Jonsson and Peng, 2005). This gene family is found exclusively in animals and fungi and plays important roles in development, metabolism, cancer and ageing, as well as in immune regulation (Carlsson and Mahlapuu, 2002; Jonsson and Peng, 2005). Genetic defects in FoxP3 result in mice with a scurfy phenotype (scaly skin, running, squinted eyes, hepatosplenomegaly, enlarged lymph
nodes, and variable thickening of the ears) and in humans with IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy and X-linked syndrome), a severe perinatal autoimmune syndrome resulting from defects in regulatory T cell (Treg) development and consequently activation of conventional T cells with specificity for self antigens (Lal and Bromberg, 2009). Thus FoxP3 is currently the most intensively studied forkhead family member in immunological research and such studies show that FoxP3 is a “master” regulator of the development and suppressive function of Tregs (Jonsson and Peng, 2005). Sustained expression of FoxP3 is required for Treg differentiation and to prevent deviation of Treg cells into effector T cell lineages. It confers dependence of Treg cell survival and expansion on growth factors such as interleukin (IL)-2, provided by activated effector T cells (Josefowicz and Rudensky, 2009). Foxp3 mediates its function by acting as a transcriptional repressor as well as a transcriptional activator. Consistent with its dual role FoxP3 represses NF-AT-mediated transcription, e.g. ectopic expression of FoxP3 results in a marked reduction of IL-2 production following stimulation (Schubert et al., 2001) but induces the transcription of CD25, CTLA4, and GITR genes (Chen et al., 2006).
Fig. 2. Trout FoxP3b cDNA sequence and its translation. Three potential polyadenylation signals are boxed. The ZnF_C2H2 and FH domains predicted using the SMART program (Letunic et al., 2009), as well as a leucine zipper-like domain are underlined. The proline residues in a proline-rich region upstream of the ZnF_C2H2 domain are boxed. The forward and reverse primer binding sites for primers used for 3′- and 5′-RACE, respectively, are shaded with names above.

Mammalian FoxP3 contains a large amino-terminal region required for transcriptional activation and repression, a central C2H2 zinc-finger domain, a leucine zipper domain implicated in multimer formation and suppressor function, and a C-terminal Forkhead (FH) domain (Lopes et al., 2006; Chae et al., 2006). Activation of Tregs by TCR engagement results in up-regulation of FoxP3 expression, followed by rapid nuclear transport and binding to chromatin to regulate the expression of target genes. FoxP3 was found to bind regions for 700 genes and for an intergenically encoded microRNA (Zheng et al., 2007). Both the leucine zipper and FH domains are required for sequence specific DNA binding (Koh et al., 2009). Interestingly, the basic sequence (RKKR in humans and mice) in the C-terminal of the FH domain, that can function as a nuclear translocation signal (NLS) in other proteins, does not function as a NLS. Instead, nuclear transportation of murine FoxP3 is brought about by three distinct domains: the N-terminal 51 amino acids (aa), the C-terminal 12 aa and the domain immediately N-terminal to the FH domain (Hancock and Ozkaynak, 2009). Although not involved in DNA binding, the N-terminal of FoxP3 associates with other transcription factors and other intracellular proteins. Human FoxP3 is expressed as two isoforms, a full-length form and a smaller form lacking exon 2. The full-length FoxP3 can interact with and inhibit transcriptional activation mediated by retinoic acid receptor-related orphan receptor...
(ROR)x. This interaction and repression by FoxP3 is mediated by a LxxLL motif encoded by exon 2 as mutation of this LxxLL motif abolished the interaction (Du et al., 2008). FoxP3 was also found to directly interact with RORyt, the master transcription factor of Th17, and to suppress RORyt-mediated IL-17A promoter activation via the exon 2 region of FoxP3 (Ichiyama et al., 2008). Lastly, the N-terminal 106–190 aa of FoxP3 is physically associated with histone acetyltransferase and deacetylase that is involved in epigenetic regulation of gene expression (Li et al., 2007).

Whilst FoxP3 regulates the transcription of genes critical for development and function of Tregs, the signals that govern the transcription of FoxP3 itself are still unclear. Signaling pathways activated by TCR engagement up-regulate the expression of FoxP3. Multiple NF-AT and AP-1 binding sites in the FoxP3 promoter positively regulate the trans activation of the FoxP3 promoter after triggering of the TCR (Mantel et al., 2006). TGF-β signaling has a critical role on FoxP3 transcription. The transcription factor Smad3 is induced by TGF-β and cooperates with NF-AT to induce FoxP3 transcription (Tone et al., 2008). Retinoic acid can enhance TGF-β signaling by increasing the expression and phosphorylation of Smad3, thus increasing FoxP3 transcription even in the presence of IL-6 and IL-21 (Schambach et al., 2007). The γ-chain cytokines, including IL-2, 4, 7, 9, 15 and 21, that signal through JAK/STAT pathways, have all been implicated in the regulation of FoxP3 expression. For example, IL-2, -7 and -15 all up-regulate FoxP3 expression in Tregs (Wuest et al., 2008), whilst IL-4 if present at the time of T cell priming inhibits FoxP3 expression mediated by direct binding of GATA3 to the FoxP3 promoter (Mantel et al., 2007). Other pathways, including the NOTCH1, Interferon/IRF and PI3K/AKT/MTOR pathways, as well as epigenetic changes such as histone acetylation and CpG dinucleotide methylation, are all implicated in the regulation of FoxP3 transcription (Shen et al., 2009; Lal and Bromberg, 2009).

Although the role of FoxP3 in Treg development and suppressive function has been extensively studied in mammals, little is known about this molecule in other vertebrates except for a recent report on the identification of a zebrafish FoxP3 (Mitra et al., 2010). In the present study, two cDNA sequences encoding two paralogues of mammalian FoxP3 have been identified in rainbow trout (Oncorhyncus mykiss). We found the two paralogues are differentially expressed in early development stages, and in some adult tissues (e.g. muscle). We also show that FoxP3 gene expression can be modulated in vitro in splenocytes and thymocytes.

2. Materials and methods

2.1. Cloning of rainbow trout FoxP3

TBLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul et al., 1997) of rainbow trout ESTs with mammalian or a zebrafish FoxP3 (Acc No. A8WF7) protein sequences returned two sets of ESTs that when translated had homology to the C-terminal of FoxP3. The translation of ESTs, BX298952 and BX298953, that come from the same clone was tentatively designated as rainbow trout FoxP3a, whilst of the ESTs, BX295262 and BX298355, was designated as FoxP3b according to the unified nomenclature for the winged/helix/forkhead transcription factors (Kaestner et al., 2000). To obtain the full-length cDNA sequences of the FoxP3 molecules, 3′- and 5′-RACE was carried out using spleen SMART cDNA as described previously (Wang and Secombes, 2003). The primer binding sites for RACE are shown in Figs. 1 and 2 for FoxP3a and FoxP3b, respectively, and the primer sequences are in Table 1. The reverse primers are located within the 3′-untranslated region (UTR) to ensure a successful 5′-RACE product that contained the 5′UTR and complete coding sequence (CDS). The PCR products were cloned and sequenced as described previously (Wang and Secombes, 2003; Wang et al., 2008).

2.2. Sequence analysis

The nucleotide sequences generated were assembled and analyzed with the AlignIR programme (LI-COR, Inc.). The protein identification was carried out on http://www.expasy.org/tools/(Gasteiger et al., 2005). The domain structure was predicted using the SMART6 program (http://smart.embl-heidelberg.de/smart/) (Letunic et al., 2009), and presence of a signal peptide was determined by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) according to Emanuelsson et al. (2007). Global sequence comparison was performed using the Megalign program (Campanella et al., 2003). Multiple sequence alignments were generated using ClustalW (Chenna et al., 2003) and shaded using BOXSHADE (version 3.21; www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were created by the neighbour-joining method using the MEGA program (V4.1) (Tamura et al., 2007) based on a CLUSTAL multiple alignment and were bootstrapped 50,000 times.

2.3. Real-time PCR quantification of gene expression

The expression of tFoxP3a and tFoxP3b as well as a common reference gene, elongation factor-1α (EF-1α), was quantified by real-time PCR using SYBR green (Invitrogen) and a LightCycler 480 real-time PCR system (Roche) as described previously (Wang et al., 2008, 2009). The primers used for real-time PCR are given in Table 1 and were pre-tested to ensure that each primer pair could not amplify genomic DNA using the real-time PCR protocols. For comparison of the relative expression level of two paralogues, a standard was constructed with a mixture of equal molar amounts of PCR products of the tFoxP3a and tFoxP3b genes. A serial dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as a reference for quantification. The expression level of each gene was calculated as arbitrary units that had been normalized to the expression level of EF-1α.

2.4. Expression of tFoxP3a and tFoxP3b in vivo in healthy fish

The selection of tissues, RNA preparation, cDNA synthesis and real-time PCR analysis of gene expression was described previously (Wang et al., 2007, 2009). Briefly, six healthy rainbow trout (average weight ±SEM = 106 ± 5 g) were killed and fourteen tissues (tail fins, thymus, gills, skin, scales, muscle, liver, spleen, head kidney, caudal kidney, intestine, ovary, heart, and brain) were collected. Total RNA was prepared individually from each tissue and converted to cDNA, thus 84 (6 × 14) cDNA samples were used in the gene expression analysis. The real-time quantification of gene expression was as described above. The comparative expression level of each gene was expressed as arbitrary units where one unit is equal to the average expression level of tFoxP3a in liver.

2.5. Ontogeny of the expression of tFoxP3a and tFoxP3b

To investigate if the expression of FoxP3 is correlated to immune capacity in early life, the ontogeny of the expression of tFoxP3a and tFoxP3b was examined. Juvenile stages of rainbow trout were raised in the Institut National de la Recherche Agronomique experimental fish facility at Jouy-en-Josas. Eyed eggs (egg, 17/12/09 ~ 280 degree days, DD), immediate post-hatch fry (hatch, 16/01/09, ~370 DD), pre-first feeding fry (FF, 4/2/09, 560 DD), at the stage of full disappearance of the yolk sac, and fry 3 weeks following first feeding (Fry, 25/2/09, 770 DD) were sampled. The fish were maintained at 10 °C in recirculated water. Egg, hatch and FF samples were
stored directly in RNA Later (Ambion), whilst (first feeding) fry were killed by anaesthetic overdose in 2-phenoxycethanol, and then stored in RNAlater according to the manufacturer’s instructions. Total RNA was prepared by dissolving the samples in TRI reagent (Ambion) assisted by Tungsten Carbide Beads (3 mm, QIAGEN), and a shaker (Retsch MM300) for 4 min (30 times/s). The RNA samples prepared were quantified using a Nanodrop ND-1000 (Agilent) and converted to cDNAs as described previously (Wang et al., 2009). Six samples for each developmental stage were prepared. To obtain enough RNA, each sample contained two eyed eggs or two larvae at hatching, but a single PFF or Fry was sufficient. The real-time quantification of gene expression was as described above. The comparative expression level of each gene was expressed as arbitrary units where one unit is equal to the average expression level of FoxP3b in eyed eggs.

### 2.6. Modulation of gene expression of tFoxP3a and tFoxP3b in splenocytes

As a high level of expression of both tFoxP3a and tFoxP3b was detected in the spleen in the tissue distribution experiment above, the initial investigation of potential modulation of tFoxP3 gene expression was carried out using splenocytes. The preparation of splenocytes was as described previously (Wang et al., 2007). A quantity of 3 x 10^6 freshly prepared splenocytes was seeded into 25-cm² cell culture flasks (Nunc) in 5 ml of complete medium (L-15 plus 10% FCS). The freshly prepared splenocytes were stimulated with Escherichia coli (strain O55:B5) lipopolysaccharide (LPS, 25 μg/ml, Sigma–Aldrich), polyinosinic:polycytidylic acid (poly I:C, 50 μg/ml, Sigma–Aldrich), and phytohaemagglutinin (PHA, a lectin from Phaseolus vulgaris, 10 μg/ml, Sigma–Aldrich) for 4, 8, 20, and 24 h at 22°C. The treatment was terminated by dissolving the cells in TRIzol. The RNA preparation and real-time PCR analysis of expression of tFoxP3a and tFoxP3b was as described above. The expression level of each sample was normalized to that of EF-1α. The expression level of FoxP3a and FoxP3b was expressed as arbitrary units where one unit was equal to the average expression level of tFoxP3a in the control at 4 h. A fold change was also calculated by comparing the average expression level of treated samples to that of the controls at the same time points.

### 2.7. Dose–response of the expression of tFoxP3a and tFoxP3b modulated by PHA

As PHA was the only factor among the factors tested above that highly increased the expression of tFoxP3a and tFoxP3b in splenocytes from 4 to 24 h after stimulation, we further examined the effect of different modulation of PHA on this induction in splenocytes, as well as in thymocytes, as thymus had the highest expression level of tFoxP3a and tFoxP3b in vivo. Different amounts of PHA (0, 1, 5 and 25 μg/ml) were added to freshly prepared splenocytes and thymocytes from individual fish for 24 h and the expression of tFoxP3a and tFoxP3b was examined as above. The comparative expression level of tFoxP3a and tFoxP3b was expressed as arbitrary units where one unit equals the average expression level of tFoxP3b in the controls. A fold change was also calculated by comparing the average expression level of treated samples to that of the controls.

### 2.8. Statistical analysis

Real-time quantitative PCR measurements were analyzed using the nonparametric Mann-Whitney test within the SPSS package 17.0 (SPSS Inc., Chicago, IL), or student T tests, with p < 0.05 between treatment groups and control groups considered significant.

### 3. Results

#### 3.1. Sequence analysis and characterisation of tFoxP3a and tFoxP3b

The full-length cDNAs of two trout FoxP3 (tFoxP3) paralogues have been cloned. The tFoxP3a cDNA (FM883710) is 2085 bp long encoding for 438 aa with a theoretical molecular weight of 49,962 Da and a theoretical pl of 8.5. The tFoxP3b cDNA (FM883711) is 1838 bp encoding for 442 aa with a theoretical molecular weight of 50,439 Da and a theoretical pl of 9.1. The tFoxP3a has a single ATTAC motif in the 3′-UTR and a polyadenylation signal (AATAAA) in vivo. Different amounts of PHA were added to the cells, and the expression between different samples was compared with the nonparametric Mann–Whitney test. The expression level of tFoxP3a and tFoxP3b were expressed as arbitary units.

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Fig. 3. Multiple alignment of the deduced amino acid sequences of \( \text{FoxP3a} \) and \( \text{FoxP3b} \) with known vertebrate \( \text{FoxP3} \) molecules. The multiple alignment was produced using ClustalW and conserved amino acids shaded using BOXSHADE (V3.21). Dashes (–) indicate gaps, stars (*) indicate complete conservation and dots (.) indicate similarity. The ZnF \( \text{C2H2} \), the leucine zipper-like and the FH domains are indicated by solid lines above the alignment. A LxxLL motif conserved only in mammalian \( \text{FoxP3} \) is highlighted under the alignment. The aa identities (%) at the N-terminals and C-terminals, defined by the N-terminal of the ZnF \( \text{C2H2} \) domain, of trout \( \text{FoxP3a} \), and \( \text{FoxP3s} \) from platypus and humans to other \( \text{FoxP3} \) molecules are indicated at the end of the alignment. The final 34 aa of the platypus \( \text{FoxP3} \) was removed from the alignment. The accession numbers for sequences used in this analysis are given in Fig. 4.
The tFoxP3a and tFoxP3b translation share 84% identity with each other, but only share 40-41% with zebrafish FoxP3 and even lower (31-36%) identities with FoxP3 molecules from other higher vertebrates. This is in contrast to the other FoxP subfamily members where fish members share higher sequence identities to members from higher vertebrates, e.g. fish FoxP2’s share 71-78% identities to mammalian FoxP2s (Table 2). Nevertheless, the FoxP3 molecules from fish do show higher identities/similarities with FoxP3 members from higher vertebrates compared with other FoxP subfamily members (Table 2). Multiple alignment of amino acid sequences of all known fish and selected higher vertebrate FoxP3 molecules showed greatest conservation in the ZnF of all known fish and selected higher vertebrate FoxP3 molecules (Fig. 3). Nevertheless, all the FoxP3 sequences upstream of the ZnF, leucine zipper-like and FH domains that are important for the known function of mammalian FoxP3 (Fig. 3). However, the N-terminal sequence upstream of the ZnF2 domain that is responsible for transcriptional repression is less conserved between fish, amphibians, monotreme mammals (Platypus) and eutherian mammals (Fig. 3). For example, the identities of the N-terminal of tFoxP3a (with similar results for tFoxP3b) to FoxP3 from other species is 22–33% lower than an equivalent analysis of the C-terminal region. Although FoxP3 from platypus and human share similar identities to all the fish and frog FoxP3 molecules both at the N-terminal and C-terminal regions, the platypus FoxP3 has 17–20% lower identities to FoxP3 from eutherian mammals (pig, cow, mouse and humans) at the N-terminal compared to the C-terminus. A LxxLL motif in the N-terminal, encoded by exon 2 of human FoxP3, is involved in the interaction with RORα to inhibit transcriptional activation mediated by RORα (Du et al., 2008). This LxxLL motif is conserved in all mammalian FoxP3 molecules, but is missing in fish and frog FoxP3 molecules (Fig. 3). Nevertheless, all the FoxP3 sequences upstream of the C2H2 domain are proline rich.

To further reveal the identities of tFoxP3a and tFoxP3b, a phylogenetic tree analysis was carried out using known fish FoxP subfamily members and selected FoxP3 members from higher vertebrates. Fig. 4 shows a neighbour-joining tree where tFoxP3a and tFoxP3b group together and with zebrafish FoxP3 to form a fish-specific FoxP3 clade that also grouped with other vertebrate FoxP3 with a high bootstrap value (100%). Other FoxP subfamilies all form independent clades, suggesting that this tree is reliable and the FoxP3 paralogues are co-orthologs of mammalian FoxP3.

3.2. Comparative expression study of tFoxP3a and tFoxP3b transcripts in vivo

The expression of tFoxP3a and tFoxP3b was comparatively studied using equal mole references in the same real-time PCR plate. Thus the arbitrary units of their relative expression is on an equal molar basis. Whilst the expression of both tFoxP3a and tFoxP3b was detectable in all the 14 tissues examined, the liver expresses the lowest level of both tFoxP3a and tFoxPb and the thymus expresses the highest level of both tFoxP3a and tFoxPb (Fig. 5). A high level of expression of both tFoxP3a and tFoxP3b was also detected in the spleen, head and caudal kidney, the central immune organ in fish, as well as gills and intestine, important sites where many pathogens enter the host. The expression level of the two tFoxP3 genes was not significantly different in any of the tissues examined with the exception of muscle where tFoxP3a expression was higher than that of tFoxP3b in the six fish tested (Fig. 5).

3.3. Ontogeny of the expression of tFoxP3a and tFoxP3b

The high expression of tFoxP3a and tFoxP3b in thymus and other immune related tissues suggests that the two paralogues of mammalian FoxP3 may have a role in T cell development and host immune regulation as seen in mammals. We examined the expression of these two paralogues at different stages of trout development, from eyed eggs to feeding fry, which represent a critical period when the fish encounter potential pathogens from the environment and food. Interestingly, the expression of tFoxP3a was maintained high and almost constant from eye development to 3 weeks post-fertilization (Fig. 6). However, the expression of tFoxP3b at the eyed egg stage was more than a hundred fold lower relative to that of tFoxP3a (p < 0.05), but increased steadily over the developmental
Fig. 4. A phylogenetic tree of the trout FoxP3 paralogues with known vertebrate members of the FoxP subfamily of the forkhead protein family. The tree was constructed using the neighbour-joining method within the MEGA4 program (Tamura et al., 2007). Node values represent percent bootstrap confidence derived from 50,000 replicates. The accession number is followed by the common species name and the molecule.

stages and reached a comparable level to that of tFoxP3a in fry 3 weeks post-feeding (Fig. 6).

3.4. Modulation of tFoxP3a and tFoxP3b expression in splenocytes

As the spleen expressed a high level of both tFoxP3a and tFoxP3b, splenocytes were used to examine possible factors that might modulate their expression. The expression of both tFoxP3a and tFoxP3b decreased over time, e.g. the expression levels are lower at 8 h than at 4 h (Fig. 7, p < 0.05) presumably due to a decrease in the numbers of cells that express these two molecules. The treatments with different stimulants were effective in that up-regulation of a set of known reference genes responsive to these stimulations was found (data not shown). There were no significant differences in the expression of both tFoxP3a and tFoxP3b between the time-matched controls and the groups stimulated with poly I:C and various cytokines (rIL-1β, rIFNγ and rIL-15). However, the expression of both genes was increased by the T cell stimulant PHA from 4 to 24 h (Fig. 7, p < 0.05). Interestingly, the fold increase of tFoxP3b expression was more prominent than for tFoxP3a. In addition, the expression of tFoxP3a was down-regulated by LPS at 4 h and the expression of tFoxP3b was up-regulated by PMA at 4 h (p < 0.05).

3.5. PHA dose-dependent induction of expression of tFoxP3a and tFoxP3b

The differential responses of the two FoxP3 paralogues to PHA stimulation (Fig. 7) may relate to different sensitivities to PHA. Thus a dose–response (1–25 μg/ml of PHA) experiment was performed with splenocytes and thymocytes, as the latter had the highest level of expression of both tFoxP3a and tFoxP3b. The expression level in control cells was 4.4 (tFoxP3a) and 3.2 (tFoxP3b) fold higher in thymocytes relative to splenocytes (Fig. 8, p < 0.05), in agreement with the tissue expression study (Fig. 5). 1–25 μg/ml PHA was effective at increasing the expression of both tFoxP3a and tFoxP3b (p < 0.05). A PHA concentration as low as 1 μg/ml was sufficient to induce the expression of tFoxP3a, with higher concentrations hav-
The expression of tFoxP3a and tFoxP3b transcripts was determined by real-time RT-PCR in 14 tissues from six fish. The transcript level was first calculated using a serial dilution of references that contained equal molar amounts of the probes for each gene. The relative expression level for each gene was expressed as arbitrary units, normalized against the expression level of EF-1α/H9251. For comparison, the expression level of tFoxP3a in liver, which had the lowest level, was defined as 1. The results represent the average ± SEM.

In contrast, the expression of tFoxP3b induced by PHA was clearly concentration dependent, with peaks in expression seen at 5 μg/ml PHA in thymocytes and at 25 μg/ml PHA in splenocytes. Although the expression of tFoxP3a and tFoxP3b was generally higher in thymocytes than in splenocytes, expression was more inducible in splenocytes (Fig. 8).

4. Discussion

Two paralogues of the mammalian FoxP3 gene that share 83.9% identity at the amino acid sequence level, have been cloned in rainbow trout. Both tFoxP3a and tFoxP3b have no predicted signal peptides but all have well conserved ZnF_C2H2, FH and leucine zipper-like domains (Figs. 1–3), typical of a FoxP3 (Koh et al., 2009). These two paralogues share higher sequence identities to members of the FoxP3 subfamily than to any other FoxP subfamilies (Table 2). Phylogenetic analysis revealed that tFoxP3a and tFoxP3b grouped with zebrafish FoxP3 to form a fish-specific FoxP3 clade, that also grouped with higher vertebrate FoxP3s with a high bootstrap value (100%) and separate from the other FoxP subfamilies. In addition, the zebrafish FoxP3 has been shown to be syntenically conserved compared to mammals (Mitra et al., 2010). Such data suggest that tFoxP3a and tFoxP3b are true co-orthologues of mammalian FoxP3.

The well conserved ZnF_C2H2, leucine zipper-like and FH domains in fish and amphibian FoxP3 molecules suggest that the lower vertebrate FoxP3 molecules possess the structural features needed to mediate nuclear translocation and DNA binding as a transcription factor. However, the basic sequence (RRKR in platypus and RKKR in eutherian mammals) in the C-terminal of the FH domain, that can function as a nuclear translocation signal (NLS) in other proteins, was not conserved in amphibians and fish. Mutations in this NLS region in mouse FoxP3 do not affect its nuclear translocation (Hancock and Ozkaynak, 2009). This suggests that this basic sequence may not be necessary for the nuclear translocation of FoxP3.

Whilst the C-terminal from the ZnF_C2H2 domain is well conserved among all the FoxP3 molecules, the N-terminal before the ZnF_C2H2 domain is highly divergent between fish, amphibians, monotremes and eutherian mammals (Fig. 3). Interestingly the platypus FoxP3 has 17–20% lower identities to FoxP3 from eutherian mammals at the N-terminus compared to the C-terminus, even though there is a less than 6% difference in identities between the N- and C-termini amongst the eutherian FoxP3s. The N-terminal region of human and mouse FoxP3 is required for FoxP3-mediated repression of transcription (Lopes et al., 2006). This region was found to be physically associated with RORα, RORγt, acetyltransferase and deacetylase. The LxxLL motif in the N-terminal, encoded by exon 2 of human FoxP3, is involved in the interaction with RORα to inhibit transcriptional activation mediated by RORα (Du et al., 2008). This LxxLL motif is conserved in all mammalian FoxP3 s but
is the fish and amphibian molecules (Fig. 3). The N-terminal of mouse FoxP3 was also found to directly interact with RORγt and to suppress RORγt-mediated IL-17A promoter activation (Ichiyama et al., 2008). The N-terminal 106–190 aa of human FoxP3 was found to be associated with histone acetyltransferase and deacetylase that is involved in epigenetic regulation of gene expression (Li et al., 2007). The N-terminal 51 aa of mouse FoxP3 can also contribute to nuclear transportation (Hancock and Ozkaynak, 2009). The poor conservation within the N-terminal of FoxP3 in fish, amphibians and platypus suggests these molecules may have different roles compared to human and mouse FoxP3 that defines the Treg cell lineage and mediates immune regulatory function. The functional roles of fish FoxP3 and their potential role in fish regulatory T cell development and repressive function remains to be determined.

It is worth noting that the zebrafish FoxP3 gene has four ATTTA motifs in the 3′-UTR suggesting that this gene is transiently transcribed (Mitra et al., 2010). The ATTTA motif is a highly conserved sequence and confers mRNA instability. This motif is repeated three or more times in the 3′-UTR of RNAs encoding many of the short-lived cytokines and oncogenes (Akashi et al., 1994). However, only a single ATTTA motif was found in the 3′-UTR of tFoxP3a and none in tFoxP3b. The human and mouse FoxP3s (human full-length reference sequence NM_014009, and mouse AF277994) also only have one ATTTA motif in the 3′-UTR. This suggests that there is no/limited role of ATTTA motif-mediated mRNA instability or degradation of FoxP3 genes in trout, human and mouse.

It was not surprising to isolate two FoxP3 genes in this study, as many immune important genes, e.g. IL-1β, TNFα, interferon-γ, MCSF, IL-17 family members, have been found to have multiple copies in rainbow trout and other fish species (Wang et al., 2010). These multiple copies might be the result of tandem gene duplication events, but are more likely from a teleost-wide genome duplication or in salmonids a more recent salmonid genome duplication event (Koop et al., 2008). The retention of multiple copies of duplicated genes in an organism after a genome (gene) duplication event might confer various advantages as the duplicated genes may evolve different regulatory mechanisms and/or different functions. Indeed, the two tFoxP3 paralogues were differentially expressed, as seen in the different developmental stages (Fig. 6) and following PHA stimulation (Figs. 7 and 6). Whether the two tFoxP3 paralogues may have evolved different functions remains to be determined.

The expression of mammalian FoxP3 is restricted to a specialized Treg subset with dedicated suppressor function, and controls Treg lineage development (Zheng et al., 2007). Mammalian FoxP3+ T cells can be generated from thymus or converted in the periphery to maintain immune homeostasis (Feuerer et al., 2009). Consistent with this, the expression of both tFoxP3a and tFoxP3b was detectable in all the fourteen tissues examined albeit with highest expression levels in the thymus (Fig. 5). Relatively high levels of expression of both tFoxP3a and tFoxP3b were also detected in immune related organs including the spleen, kidney, gills and intestine. Although the cells expressing tFoxP3 genes are unknown at present, the high level of expression in thymus and immune related organs, and up-regulation by the T cell stimulant PHA, suggests that the tFoxP3 genes may have a role in T cell development and host immune regulation as seen in mammals. Interestingly, in the absence of stimulation FoxP3 expression decreased in control cultures over time (e.g. 8 h cultures expressed only half the level of FoxP3 relative to 4 h cultures), presumably due to a decreasing number of cells expressing FoxP3 when the cells are cultured in vitro without supplementation of cytokines and growth factors.

Fish, including salmonids, have critical early stages of development, particularly from hatching to feeding when the protection provided by the egg shell is lost and water and food borne antigens are met directly for the first time in life whilst the adaptive
immune defence has not fully developed (Zapata et al., 2006). Thus fish need to develop immunocompetence early and in parallel there is a requirement for immunoregulatory mechanisms to prevent host damage and autoimmunity. In our study, the expression of tFoxP3a was at a high and almost constant level from eyed eggs to fry 3 weeks after feeding (Fig. 6). However, the expression of tFoxP3b at the eyed egg stage was more than a 100-fold lower relative to that of tFoxP3a, but increased steadily over the developmental stages and reached a comparable level to that of tFoxP3a in fry 3 weeks after feeding (Fig. 6). Whilst the presence of cells equivalent to mammalian Tregs in fish is still to be proven, the high and constant expression of FoxP3a and increased expression of FoxP3b genes over the early developmental stages in rainbow trout might suggest that an immunoregulatory mechanism mediated by the expression of FoxP3 may exist in fish. However, the constitutive expression of tFoxP3a was higher than that of FoxP3b in the muscle in the six healthy fish tested (Fig. 5). Considering the huge mass of muscle in the fish body, the high level of FoxP3a over the developmental stages from eyed egg to feeding fry might be mainly contributed by the expression in the muscle, suggesting a potential role of FoxP3a in muscle development or function. Determination of the cells expressing FoxP3 paralogues in the muscle may help to clarify if there is a role of t FoxP3a in muscle.

In conclusion, we have identified two FoxP3 paralogues in rainbow trout. In mammals, FoxP3 expressing Treg (CD4+CD25+) cells play a key role in homeostasis of the immune system (Feuerer et al., 2009). Since CD4 has been cloned from a few fish species and IL-2, the cytokine binding to CD25, has also been found to exist in fish (Bird et al., 2005; Diaz-Rosales et al., 2009) it is possible that fish Treg cells similar to mammalian CD4+CD25+ FoxP3+ cells may also exist. The cloning of the two trout FoxP3 paralogues will help to clarify the existence of potential Treg cells and their development in fish. However, the structural and expression analysis of the two trout isoforms suggests that tFoxP3a and tFoxP3b may be subject to differential regulation and could have evolved novel functions.

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