Marine Genomics 31 (2017) 25–31

Contents lists available at ScienceDirect

Marine Genomics

journal homepage: www.elsevier.com/locate/margen

NFAT5 genes are part of the osmotic regulatory system in Atlantic salmon (*Salmo salar*)

Marlene Lorgen [a], Even H. Jorgensen [b], William C. Jordan [c,1], Samuel A.M. Martin [a,*,2], David G. Hazlerigg [b,2]

[a] Institute of Biological and Environmental Sciences, University of Aberdeen, AB24 2TZ, UK
[b] Department of Arctic and Marine Biology, Faculty of BioSciences Fisheries & Economy, University of Tromsø, Norway
[c] Zoological Society London, Institute of Zoology, London NW1 4RY, UK

ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form 13 June 2016
Accepted 13 June 2016
Available online 18 June 2016

Keywords:
Nuclear activated factor of T-cells
Salinity
Salmonid
Thyroid hormone
Whole genome duplication

ABSTRACT

The anadromous Atlantic salmon utilizes both fresh and salt water (FW and SW) habitats during its life cycle. The parr-smolt transformation (PST) is an important developmental transition from a FW adapted juvenile parr to a SW adapted smolt. Physiological changes in osmoregulatory tissues, particularly the gill, are key in maintaining effective ion regulation during PST. Changes are initiated prior to SW exposure (preparative phase), and are completed when smolts enter the sea (activational phase) where osmotic stress may directly stimulate changes in gene expression. In this paper we identify 4 nuclear factor of activated T cells (NFAT5, an osmotic stress transcription factor) paralogues in Atlantic salmon, which showed strong homology in characterized functional domains with those identified in other vertebrates. Two of the identified paralogues (NFAT5b1 and NFAT5b2) showed increased expression following transfer from FW to SW. This effect was largest in parr that were maintained under short day photoperiod, and showed the highest increases in chloride ion levels in response to SW exposure. The results of this study suggest that NFAT5 is involved in the osmotic stress response of Atlantic salmon.

1. Introduction

The Atlantic salmon is an anadromous species, spending the first one to three years of life in a freshwater (FW) environment before migrating downstream and out to sea for one or multiple winters before returning to its natal stream to spawn. A process termed the parr-smolt transformation (PST) facilitates this exploitation of FW and salt water (SW) environments. During PST, FW juveniles called parr undergo numerous physiological changes to become saltwater adapted 'smolts' (McCormick, 2013). The success of PST is vital to survival during FW to SW transfer, which occurs in a synchronized fashion in response to increasing photoperiod, with the aim of entering the sea during the narrow 'smolt window' in the spring when SW survival rates are highest.

The pathways governing detection of environmental salinity via molecular osmotic sensors in teleosts is not clear, however, a number of candidate genes that may serve this function have been identified, including adenyl cyclase (Saran and Schaap, 2004) and calcium sensing receptor (CaSR) (Nearing et al., 2002). The expression of downstream target genes is assumed to be modulated by osmotically-regulated transcription factors (Fiol and Kültz, 2007). One such transcription factor is the osmotic response element binding protein (OREBP), also known as tonicity response element binding protein (TonEBP) or nuclear factor of activated T-cells 5 (NFAT5). NFAT5 is the most ancestral of the NFAT gene family, showing high similarity to the single NFAT genes identified in *Drosophila melanogaster* and in pearl oyster (Graef et al., 2001; Huang et al., 2015), with high homology in the DNA-binding domain and the rel-homology domain (RHD). The NFAT5 DNA binding domain within the RHD regulates osmotic responses by binding to osmotic response elements (OREs) (Cheung and Ko, 2013).

During osmotic stress, changes in extracellular tonicity cause rapid changes in nuclear abundance of NFAT5 via nucleocytoplasmic trafficking mechanisms, with hyper-tonicity inducing nuclear accumulation of NFAT5 and hypo-tonicity resulting in nuclear export in mammals (Ko et al., 2000; Woo et al., 2000). In addition, increased NFAT5 mRNA levels have been observed during hyper-osmotic stress in mammals (Ko et al., 2000). Although hyper-tonically induced nuclear transport acts to generate a prompt response in downstream gene transcription, increased NFAT5 synthesis is also important for sustaining osmo-adaptation in the presence of chronic hyper-tonic stress (Cheung and Ko, 2013).

NFAT5 is involved in osmo-sensory signal transduction in killifish (*Fundulus heteroclitus*) gill, binding to OREs in the promoter of iodothyronine deiodinase 2 and initiating transcription in response to hypo-osmotic stress (Lopez-Bójórquez et al., 2007). Many of the physiological changes occurring during PST in the Atlantic salmon are directly

* Corresponding author at: Institute of Biological and Environmental Sciences, University of Aberdeen, AB24 2TZ, UK.
E-mail address: sam.martin@abdn.ac.uk (S.A.M. Martin).
[1] Deceased 25th May 2011.
[2] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.margen.2016.06.004

or indirectly regulated by thyroid hormones (THs) (Dickhoff et al., 1978; Hoar, 1988, Lorgen et al., 2015), which require conversion from pro-hormone thyroxine (T4) to active thyroid hormone triiodothyronine (T3) to become functional (Darras and Herck, 2012). The iodothyronine deiodinase (dio) gene family acts to locally regulate the availability of T3 and the action of dio2 results in increased T3 availability in vertebrates (Darras and Herck, 2012).

To date, no NFAT5 genes have been characterized in the salmonid family. In this paper we have identified a repertoire of 4 NFAT5 paralogues in the Atlantic salmon, which show high similarity in structure to those characterized in other vertebrates. We show an increase in mRNA expression of two of the paralogues following 24 hour SW challenge in vivo and hypothesize that these genes may act to mitigate osmotic stress during FW to SW transition in salmonid smolts.

2. Methods

2.1. Characterisation of NFAT5 repertoire in Atlantic salmon

2.1.1. NFAT5 paralogue identification in the Atlantic salmon genome

Searches for teleost NFAT5 nucleotide sequences on NCBI and Ensembl revealed the presence of 2 NFAT5 paralogues in teleosts; NFAT5a and 5b. Blast searches were carried out against the Atlantic salmon genome (*Salmo salar* Linnaeus, 1758; Taxid: 8030, version AGKD00000000.4, Lien et al., 2016) on NCBI using BlastN default parameters and available NFAT5a and NFAT5b nucleotide sequences from *Takifugu rubripes* (ENSTRUG00000011018.1 and ENSTRUT00000045105, respectively) to identify homologous genes in *S. salar*. BlastN was also used to search the Rainbow Trout (*Oncorhynchus mykiss*) (CCAF00000000.1, Berthelot et al., 2014) and Northern Pike (*Esox lucius*) (AZJR00000000.2, Rondeau et al., 2014) WGS databases for NFAT5 paralogues using the full length Atlantic salmon sequences obtained from searching the *S. salar* WGS database.

For each paralogue identified, the intron/exon structure was determined using GENSCAN (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin, 1997) combined with manual alignment and the amino acid sequences were generated by translation with ExPASY (Gasteiger et al., 2003). Multiple sequence alignment of predicted amino acid sequences was performed using CLUSTALW2 (http://align.genome.jp) (Larkin et al., 2007).

Synteny analyses were carried out using the Generic Genome Browser (version 2.55) on SalmoBase (http://salmobase.org/cgi-bin/gb2/gbrowse/salmon_GBrowse_Chr_NCBI/). The data source for the browser was Ssal ICSASG_v2. 100 kbp up- and downstream of each *S. salar* NFAT5 paralogue were analysed along with the same region in *Esox Lucius* (NCBI Genome Data Viewer, data source ASM72191v2), *Takifugu rubripes*, *Lepisosteus oculatus*, *Xenopus* and *Mus musculus* (Ensembl genome browser) NFAT5a and NFAT5b.

2.1.2. Identification of conserved NFAT5 protein domains

Conserved domains (as described in Cheung and Ko, 2013) were identified by amino acid alignments with *Homo sapiens* NFAT5 protein isoform c. NFAT5 consists of a rel-like homology domain (RHD), a canonical nuclear export signal (NES), an auxiliary export domain (AED), a dimerization domain (DD) within the RHD, and 3 transactivation domains (AD1, AD2, AD3), AD1 at the N-terminal and AD2 and AD3 at the C-terminal (Tong et al., 2006; Lopez-Rodriguez et al., 2001). Phylogenetic trees predicting evolutionary relationships were generated with MEGA6 software (Tamura et al., 2013) using the amino acid sequence alignment of the RHDs with the neighbour-joining method and 10,000× iteration of bootstrapping (Fig. S1B).

2.2. SW challenge experiments

Fertilized Atlantic salmon eggs from a commercial hatchery (Aquagen, Kyrksæterøra, Norway) were raised at the University of Tromsø Aquaculture research station. Fish were held at 10 °C under constant light (LL) from the free feeding stage and fed continuously with pelleted salmon food (Skretting, Stavanger, Norway) using automatic feeders. Photoperiod manipulation was carried out to generate fish that were either prime smolt condition and able to osmoregulate well, or those that were maintained under photoperiod conditions that meant they were poor at osmoregulating. At the start of the experiment, fish were either maintained under LL or transferred to short-day photoperiod (SP, 8L:16D). After 8 weeks under SP a subset of fish from the SP group were switched back to LL (to stimulate PST). Fish were transferred to SW for 24-hr ($n = 6$) at the time points indicated in before sampling, and FW individuals were also sampled ($n = 6$) as time matched controls. Euthanization was by overdose with 0.05% v/v aqueous 2-phenoxyethanol (Sigma Aldrich, UK). Gills were collected in RNA-Later for subsequent RNA extraction. Blood was taken from the caudal vein into heparinised tubes and centrifuged at 500 ×*g* for 15 min to collect plasma, and sodium, potassium and chloride were analysed with ion selective electrodes using standard solution on the COBAS c111 auto analyser (Roche Diagnostics, Norway). Length and weight measurements were also taken throughout the duration of the study as an indication of successful PST by way of a reduction in condition factor which was calculated using the equation, CF = body weight in grams × 100 × fork length (in cm)^−3.

2.3. NFAT5 gene expression analysis by qPCR

2.3.1. RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of gill tissue, which was homogenized in TRI-reagent (Invitrogen) using tungsten carbide beads (3 mm, Qiagen) in a mixer mill MM30 (Retsch) following the manufacturer's instructions. The resulting RNA pellet was washed twice with cold 80% ethanol and dissolved in nuclease-free water (Sigma). RNA concentration was determined by a nanodrop ND1000 spectrophotometer (LabTech) and RNA integrity by the Agilent Bioanalyser 2100. RNA was stored at −80 °C until required for cDNA synthesis.

Synthesis of cDNA was performed using the Quantitect cDNA Synthesis kit (Qiagen) starting with 2 μg of total RNA, according to the manufacturer's protocols, briefly described here. 4 μl of gDNA wipeout was added to 2 μg total RNA in a total volume of 24 μl with water and incubated at 42 °C for 2 min to remove any genomic DNA contamination. A mastermix consisting of 8 μl buffer, 2 μl primer and 2 μl reverse transcriptase per reaction was then added to the treated RNA and incubation at 42 °C continued for another 25 min before a final 5 minute incubation at 95 °C. cDNA was diluted to a final volume of 100 μl representing an original concentration of 500 ng μl^{-1} RNA before use in subsequent PCR and qPCR assays.

2.3.2. Measurement of RNA expression by qPCR assay

The mRNA expression of 4 NFAT5 paralogues was assayed by real time PCR. Confirmation of primer (Table S1) specificity was by sequence analysis. A volume of 3 μl of cDNA was used as template in a final volume of 20 μl with 10 μl of 2× GoTaq® SYBR-green qPCR master mix (Promega), 5 μl nuclease-free water (Promega) and 2 μl of primers. qPCR was carried out in 96 well plates on a DNA Engine Opticon™ 107 System (MJ Research Inc.). The PCR cycles were 95 °C for 5 min then 40 cycles of 95 °C for 30 s, 60–65 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. Primer specificity was further confirmed by the presence of a single peak in a melting curve with reads every 0.5 °C from 70 and 92 °C.

RNA expression was calculated from a standard curve generated by plotting log dilution against threshold cycle number (C(t)) obtained from a dilution series ran in the same plate as the plate of interest. Efficiency was calculated as $E = 10^{\wedge(-1/slope)}$ using serial dilutions, where slope was obtained from a plot of C(t) against log input cDNA