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Steroid responsive regulation of IFNγ2 alternative splicing and its possible role in germ cell proliferation in medaka

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ABSTRACT

Interferon gamma (*IFNγ*) is an active player in estrogen dependent immuno-regulation of fish. The present work was aimed to characterize the alternatively spliced isoforms of *IFNγ2* in the gonadal sex development in medaka. Phylogenetic analysis demonstrated that IFNγ2a and 2b were clustered with fish specific interferon gamma. Our *in vitro* promoter and mini-genome analysis data confirmed that alternative splicing of *IFNγ2* is regulated by estrogens and androgens. Tissue distribution, quantitative PCR and *ISH* data demonstrated ubiquitous expression of *IFNγ2a*, while *IFNγ2b* was only expressed predominantly in female germ cells than males. This was further confirmed by germ cell specific GFP signals in the *IFNγ2b*-GFP over-expressed embryos and specific induction of *IFNγ2b* expression in the BrdU positive cells. All together our data suggest that steroid responsive alternatively spliced IFNγ2b isoforms might have some indirect roles in germ cell proliferation and thus can be an important candidate for immuno-reproductive interaction studies.

1. Introduction

Reproduction and self-maintenance are critical processes for animals, as both require substantial resource investment. The reproductive system is unique as its primary role is to assure the continuity of the species, while the immune system provides internal protection and thus facilitates continued health and survival (Lutton and Callard, 2006). In all vertebrates, the ovary has been long considered as an immunologically privileged site (Hunt and Johnson, 1999) and thus, reproduction related physiological changes (e.g., elevation in sex steroid concentrations) could directly regulate the immune functions (French and Moore, 2008). Hormones, like estrogen (E2), progesterone (P4) and testosterone (T), have demonstrated an overall inhibitory effect on leukocyte proliferation (Beagley and Gockel, 2003), and are also known to exert dose and time-dependent effects on apoptosis in mononuclear cells (Cutolo et al., 2003), T- and B-lymphocytes (Grimaldi et al., 2002), neutrophils (Molloy et al., 2003) macrophages (Yamaguchi et al., 2001) and cancer cells (Denmeade et al., 1996). The wide distribution of the estrogen receptors in the thymus (Tornwall et al., 1999), bone marrow (Smithson et al., 1998) and spleen (Samy et al., 2003) strongly suggests that estrogen plays a modulatory role in the immune system, such as differentiation, activation, proliferation, and antibody production from lymphoid cells.

Interferons (IFNs) are secreted proteins (cytokines) that induce an antiviral state in cells and play a major role in the defense against virus infection of vertebrates (Samuel, 2001). Structurally, both families of IFNs (type I and type II) belong to the class II alpha-helical cytokine family, but have different 3-dimensional structures and bind to different receptors. Virus infected cells recognize the viral dsRNA by Toll-like receptor 3 and activate several transcription factors (Interferon regulatory factor (IRF)-3 and NFkB), which further translocate to the nucleus and bind to type I IFN (IFNα/β) promoters. Then the secreted type I IFN protein binds to interferon receptors (IR) and activates the TYK2/JAK1 kinases, which phosphorylate the transcription factors, STAT1/STAT2. The activated STATs enter the cell nucleus, dimerize and associate with IRF-9, and attach to interferon-stimulated response elements (ISRE) in the promoters of interferon stimulated genes (ISGs). Finally, transcription of ISGs is induced which leads to the production of intracellular anti-viral proteins such as Mx protein, PKR and OAS (Schroder et al., 2004). On the other hand, although type II IFN (IFNγ) mediates autocrine signalling through the JAK–STAT pathway and stimulates the synthesis of MHC class I molecules and antiviral proteins (PKR, OAS etc.),

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the STAT1 attaches to only specific IFNγ activating sequences (GAS) in the promoters of IFNγ stimulated genes (Aggad et al., 2010). IFNγ is known to activate the macrophage proliferation by up-regulating the synthesis of NADPH-dependent phagocyte oxidase subunits $gp91^{phox}$ and $p67^{phox}$, nitric oxide synthase 2 (NOS2), p47 GTP-ase and guanylate-binding protein (MacMicking, 2004; Robertson, 2006; Zou and Secombes, 2011). Besides, IFNγ also up-regulates the MHC class II molecules in macrophages and other antigen-presenting cells (Robertson, 2006; Schroder et al., 2004).

IFNs and *IRFs* are also frequently implicated in ovarian growth, differentiation and apoptosis (Brannstrom and Norman, 1993). Among all *IFNs*, *IFN γ* is reported to be highly regulated by estradiol (Karpuzoglu-Sahin et al., 2001). The co-localized expression of ***IFNγ*** and aromatase in ovarian granulose cells of rat (Xiao and Findlay, 1992) and availability of GAS in the aromatase promoter region (Ramana et al., 2002) also suggest the steroidogenic influence of *IFN*. In the case of mice, males are more susceptible to many protozoan infections than females. Field and laboratory studies link the increasing susceptibility to infection with the circulating steroid hormones (Klein, 2004). Cernetich et al. (2006) used *IFN γ−/−* mice and found that the absence of ***IFNγ*** reduces the sex difference in post-infection mortality (Klein et al., 2008). Although the immune functionality of the ovary has been demonstrated in different teleost species, very little is known about the immune-reproductive interaction. Recently, several *IFNγs* have been isolated from teleost. A second form of *IFNγ* was found to have an unknown function in rainbow trout, which was predominant in the ovary (Purcell et al., 2009). Similar to other vertebrates, teleost *IFNγ* also possesses ERE (Estrogen Recognition Element), and ARE (Androgen Recognition Element), in the promoter region. However, their actual role is still unknown.

Alternative splicing variants of *IFNγ* possessing different functional properties have been reported in grass carp (Chen et al., 2010). Alternatively spliced gene variants are reported to be involved in sexual development and maintenance, e.g. *WT1* (Hammes et al., 2001), *P450c17* (Yu et al., 2003), and *Vasa* (Krovel and Olsen, 2004). The drosophila *Dsx* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs with different 3′ regions encoding related sex specific proteins i.e. DSXm in males and DSXf in females (Burtis and Baker, 1989). Combining all the facts, it is highly likely that alternative splicing of genes has a potential role in gonadal sex regulation, at least at the transcriptional level.

Medaka is an excellent model for sex determination and differentiation study because of numerous advantages, such as XX–XY sex determination system, well defined development process, availability of genome information, knowledge about the sex determining gene (dmy) (Matsuda et al., 2002), documentation of different inbreed strain, etc. Moreover, recently sex specific expression of alternatively spliced isoforms of vasa has been reported in medaka (Rao et al., 2011). *In silico* genome analysis has demonstrated the existence of alternative splicing in medaka *IFNγ*. Since in mammals *IFNγ* activities are sex biased, it would be interesting to know the probable correlation between the alternatively spliced isoforms of *IFNγ* and sexual development in medaka. Hence, this study was aimed to characterize the significance of these alternatively spliced isoforms in gonadal sex development in medaka.

2. Materials and methods

2.1. Plasmid construction

Expression plasmids were constructed using pcDNA3.1(+) vector backbone using complete ORFs of required genes, if not specifically mentioned. pGEM-T-easy plasmid of different genes were used for *in situ* hybridization (*ISH*) probe synthesis and realtime PCR standard preparation, whenever necessary. Promoter fragments (1.1–5.6 kb) of *IFNγ2* were isolated using specific primer set and cloned into pGL3 basic vector (Promega, USA) using Infusion-cloning kit (Clonetech, USA). IFNγ2–5′-utr, IFNγ2a ORF-GFP and IFNγ2a 3′utr, IFNγ2–5′utr, IFNγ2b ORF-GFP and IFNγ2b 3′utr were sequentially cloned into pCS2 vector, and named as pCS2-IFNγ2a-GFP-OE and pCS2-IFNγ2b-GFP-OE, respectively.

2.2. Experimental animals

The QurtE strain of medaka, *Oryzias latipes* (Wada et al., 1998), was used for this study. Fish were maintained at 26 ± 2 °C under a photoperiod comprising 14 h lightness and 10 h darkness. Eggs were collected within 30 min of fertilization and incubated in distilled water (Milli-Q) containing an antifungal solution (methylene blue, 2–3 ppm) at 26 ± 2 °C. Brooders and juveniles were fed with fresh artemia, while larvae were given artificial food.

2.3. Sample collection

Samplings were carried out at 3, 5, 6, 7, 8, 13 days after fertilization (daf) and 10, 20, 30, and 50 days after hatching (dah) following the reported description about medaka development (Iwamatsu, 2004). Samplings for RNA isolation were carried out in triplicate, each with a pool of 10 fish. Up to 13 daf, beheaded whole fish were used, while from 10 dah onwards, the gonads (ovary of 10 dah larvae, 350–400 μm long; testis of 10 dah larvae, 100–125 μm long) were sampled separately. Ten individual fish/gonads were pooled to make one sample for RNA isolation.

2.4. cDNA and protein analysis

cDNA and protein sequences were obtained from the NCBI, http://www.ncbi.nlm.nih.gov/, with accession numbers XP_003448178.1 (*Oreochromis niloticus*, IFNγ), BAG50577.1 (*Paralichthys olivaceus*, IFNγ), ADP55200.1 (*Hippoglossus hippoglossus*, IFNγ), NP_001117030.1 (*Salmo salar*, IFNγ1) NP_001118092.1 (*Oncorhynchus mykiss*, IFNγ1), CAR95729.1 (*Oncorhynchus mykiss*, IFNγ2), ACN41957.1(*Gadus morhua*, IFNγ), NP_001018629.1 (*Danio rario*, IFNγ1-1), NP_998029.1 (*Danio rario*, IFNγ1–2), BAM09180.1 (*Carassius auratus*, IFNγ), AEK80408.1 (*Labeo rohita*, IFNγ), CAJ51089.1 (*Cyprinus carpio*, IFNγ2b), NP_000610.2 (Homo sapiens, IFNγ1), (*Homo sapiens*, IFNγ2), ABM53145.1 (*Homo sapiens*, IFNγ3), ABN80441.1 (*Mus musculus*, IFNγ), NP_776511.1 (*Bos taurus*, IFNγ), NP_001028077.1 (*Macaca mulatta*, IFNγ), XP_004087027.1 (*Oryzias latipes*, IFNγ2a), (*Oryzias latipes*, IFNγ2b), XP_004085828.1 (*Oryzias latipes*, IFNγ1), XP_004085828.1 (*Oryzias latipes*, IFNα), NP_002161.2 (*Homo sapiens*, IFNA), and used for sequence analysis. The putative amino acid sequences were translated using expasy translation tools (http://web.expasy.org/translate). The putative signal peptides and domains were analysed using InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5). The secondary structural analysis, i.e. helix formation, helix–helix interactions, beta and gamma turns, were performed using ProFunc (http://www.ebi.ac.uk/thornton-srv/databases/profunc). Further, the nuclear localization signal and the *in silico* protein 3D structures were predicted using Predict protein (www.predictprotein.org) and SWISS MODELLING (http://swissmodel.expasy.org), respectively.

2.5. Tissue distribution experiments with RT-PCR

Total RNA was isolated from the ovary, testis, brain, heart, liver, kidney and intestine of 8-month-old adult fish with RNeasy kit (Qiagen, USA) following the manufacturer's directions. cDNA was synthesized with 1 μg of the total RNA using omniscript RT-PCR kit (Qiagen). Gene-specific primers (Appendix: Supplementary Table S1) were employed for the RT-PCR analysis. Positive and negative controls were set up to validate the distribution pattern with plasmid