Steroid responsive regulation of IFN γ 2 alternative splicing and its possible role in germ cell proliferation in medaka.

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ABSTRACT

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

Keywords: *IFN γ 2*, medaka, alternative splicing,

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, 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 suggest 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 the 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 recognizes the viral dsRNA by Toll-like receptor 3 and activates several transcription factors (Interferon regulatory factor (IRF)-3 and NFkB), which further translocates to the nucleus and binds to type I IFN (IFN α/β) promoters. Then the secreted type I IFN protein binds to interferon receptor (IR) and activates the TYK2/ JAK1 kinases, which phosphorylates the transcription factors, STAT1/STAT2. The activated STATs enter the cell nucleus, dimerizes and associates with IRF-9, and attaches 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 ($IFN\gamma$) mediates autocrine signaling through the JAK-STAT pathway and stimulates the synthesis of MHC class I molecules and antiviral proteins (PKR, OAS etc), the STAT1 attaches to only specific $IFN\gamma$ activating sequences (GAS) in the promoters of $IFN\gamma$ stimulated genes (Aggad et al., 2010). $IFN\gamma$ 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 & Secombes 2011). Besides, $IFN\gamma$ also up-regulates the MHC class II molecules in macrophages and other antigen-presenting cells (Schroder et al., 2004; Robertson, 2006).

IFNs and *IRFs* are also frequently implicated in ovarian growth, differentiation and apoptosis (Brannstrom and Norman, 1993). Among all *IFNs*, $IFN\gamma$, is reported to be highly regulated by estradiol (Karpuzoglu-Sahin et al., 2001). The co-localized expression of $IFN\gamma$ and aromatase in ovarian granulosa cells of rat (Xiao and Findley, 1992) and availability of GAS in aromatase promoter region (Ramana et al., 2002) also suggest the steroidogenic influence of $IFN\gamma$. In 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 $IFN\gamma$ -/- mice and found that the absence of $IFN\gamma$ 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 $IFN\gamma$ has been isolated from teleost. A second form of $IFN\gamma$ was found to have unknown function in rainbow trout, which was predominant in the ovary (Purcell et al., 2009). Similar to other vertebrates, teleost $IFN\gamma$ 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 $IFN\gamma$ possessing different functional properties has been reported in grass carp (Chen et al, 2010). Alternatively spliced gene variants are reported to be involved in sexual development and maintenance, eg. *WT1* (Hammes et al., 2001) *P450c17* (Yu et al., 2003), *Vasa* (Krovel et al., 2004). The drosophila *Dsx* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs with different 3' regions encoding related sex specific protein i.e. DSXm in

males and DSXf in females (Burtis et al., 1989). Combining all the facts, it is highly likely that alternative splicing of genes has potential role in gonadal sex regulation, at least at 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 inbreeds 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 *IFN γ* . Since in mammals, *IFN γ* activities are sex biased, it would be interesting to know the probable correlation between the alternatively spliced isoforms of *IFN γ* 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 *IFN γ 2* were isolated using specific primer set and cloned into pGL3 basic vector (Promega, USA) using Infusion-cloning kit (Clontech, USA). *IFN γ 2-5'*-UTR, *IFN γ 2a* ORF-GFP and *IFN γ 2a 3'utr*, *IFN γ 2-5'utr*, *IFN γ 2b* ORF-GFP and *IFN γ 2b 3'utr* were sequentially cloned into pCS2 vector, and named as pCS2-*IFN γ 2a-GFP-OE* and pCS2-*IFN γ 2b-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 $26 \pm 2^\circ\text{C}$ under a photoperiod comprising of 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 $26 \pm 2^{\circ}\text{C}$. 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 13daf, beheaded whole fish were used, while from 10 dah onwards, the gonads (ovary of 10dah larvae, 350-400 μm long; testis of 10dah 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*, IFN γ), BAG50577.1 (*Paralichthys olivaceus*, IFN γ), ADP55200.1 (*Hippoglossus hippoglossus*, IFN γ), NP_001117030.1 (*Salmo salar*, IFN γ 1) NP_001118092.1 (*Oncorhynchus mykiss*, IFN γ 1), CAR95729.1 (*Oncorhynchus mykiss*, IFN γ 2), ACN41957.1 (*Gadus morhua*, IFN γ), NP_001018629.1 (*Danio rario*, IFN γ 1-1), NP_998029.1 (*Danio rario*, IFN γ 1-2), BAM09180.1 (*Carassius auratus*, IFN γ), AEK80408.1 (*Labeo rohita*, IFN γ), CAJ51089.1 (*Cyprinus carpio*, IFN γ 2b), NP_000610.2 (*Homo sapiens*, IFN γ 1), (*Homo sapiens*, IFN γ 2), ABM53145.1 (*Homo sapiens*, IFN γ 3), ABN80441.1 (*Mus musculus*, IFN γ), NP_776511.1 (*Bos taurus*, IFN γ), NP_001028077.1 (*Macaca mulatta*, IFN γ), XP_004087027.1 (*Oryzias latipes*, IFN γ 2a), (*Oryzias latipes*, IFN γ 2b), XP_004085828.1 (*Oryzias latipes*, IFN γ 1), XP_004085828.1 (*Oryzias latipes*, IFN α), NP_002161.2 (*Homo sapiens*, IFN α), 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 analyzed 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 (SI table 1) were employed for the RT-PCR analysis. Positive and negative controls were set up to validate the distribution pattern with plasmid DNA and water as templates, respectively. A 320-bp fragment of *EF1a* was amplified (as an internal control) from medaka to test the quality of the cDNAs used in the PCR (Chakraborty et al., 2011, Su et al., 2011). The PCR conditions consisted of initial denaturation at 94°C (3 min), followed by 30 cycles of 94°C (30 s), 58°C (30 s), and 72°C (2 min and 30 s). The reaction was ended by a further incubation for 10 min at 72°C. PCR was performed on a GeneAmp PCR system (9700 thermal cycler, Applied Biosystems, USA) and products were separated on 1.2% agarose gels.

2.6. Quantification of *IFN γ 2* by RT-PCR

Changes in *IFN γ* gene expression during development were quantified using realtime PCR (ABI prism 7000 sequence detection system, Applied Biosystems, USA). cDNA synthesis was carried out using a QuantiTect RT-PCR kit (Qiagen) and 100 ng of total RNA. This was done in triplicate for each developmental stage as described above. First strand cDNA was diluted to 100 µl for subsequent use. Gene-specific RT-PCR was performed using SYBR green master mix (Applied Biosystem) and cDNA from 5 ng RNA, according to the manufacturer's instructions. The PCR conditions included initial denaturation at 94°C (2 min) followed by 40 cycles of 94°C (30s) and 60°C (1 min). Relative expression was calculated using *RPS18* expression (internal control) (Rienzo et al., 2013; Zhao et al 2014). Average relative expression derived from triplicates was used for further analysis. The specificity of primer sets, throughout this range of detection, was confirmed by the observation of a single amplification product of the expected size, T_m (melting temperature) and sequenced. All assays were quantitative, with standard curves (mean Ct vs. log cDNA

dilution) having slopes between -2.99 and -3.34, a linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution of >0.985 in each case. All test cDNAs were run in duplicates. Sequences of gene-specific primers are listed in SI table 1.

2.7. Histology and in situ hybridization (ISH)

Bouin fixed, paraffin embedded samples (at least 10 fish per group) were used for standard HE staining, while, 4% paraformaldehyde fixed samples were used for ISH. All the histological analyses were performed using 5 μ m sections. For ISH, probes for sense and anti-sense digoxigenin-labeled RNA strands were transcribed *in vitro* with a RNA labelling kit (Roche Diagnostics GmbH, Mannheim, Germany) from plasmid DNA containing the ORFs of respective genes. Sections were deparaffinized, hydrated, treated with proteinase K at 10 μ g/ml (Roche), and hybridized with the sense or anti-sense DIG-labeled RNA probe at 60°C for 18-24 h. The hybridization signals were then detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and NBT, as described previously (Chakraborty et al., 2011). Double ISH protocols were obtained from Nakamoto et al. (2010). Whole mount ISH (WISH) was performed using previously published protocols (Inoue and Wittbrodt, 2011).

2.8. In vitro analysis

The genomic sequence of *IFN γ 2* was taken from Medaka Ensemble database (http://www.ensembl.org/Oryzias_latipes/Info/Index) and analysed using Promoter 2 (<http://www.cbs.dtu.dk/services/Promoter/>). The ERE, ARE, Glucocorticoid responsive element (GRE), interferon regulatory elements (IRE) were manually marked according to their respective conserved sequences (Fox et al., 1991; Purcell et al., 2009). A five kb *IFN γ 2* promoter was isolated from medaka genomic DNA and directionally cloned in pGL3 promoter-less luciferase vector. The promoter analysis was performed using a previously described protocol (Chakraborty et al., 2011). Briefly, HEK293 cells were seeded in 24-well plates at 5 X 10⁵ cells/well in Dulbecco's modified Eagle's Medium (Sigma). After 24 h, cells were transfected with pGL3-promoter-luciferase plasmid in triplicates. Four hour after transfection, 1 μ l of E2/ MT was added to the well. Luciferase assay was performed after 48 h of transfection. The experiment was repeated thrice for reproducibility.

For splicing variant analysis, the mini genome was constructed using -2.205kb to polyadenylation signal sequence (ultra contig 279) fused with mCherry, which was expected to express only as IFN γ 2a fused protein. The HEK-293 cells were similarly transfected with mini genome construct and exposed to steroid. The cells were either used for live microscopy or collected for RNA isolation and realtime PCR analysis.

2.9. Over expression of IFN γ

Briefly, the mRNAs were synthesized from pCS2-IFN γ 2a-GFP-OE and pCS2-IFN γ 2b-GFP-OE plasmids with mMESSAGE mMACHINE SP6 kit (Ambion, USA) following polyA addition with poly-A tailing kit (Ambion). The purified RNA(s) was injected in one/two cell stage medaka embryos at 1 ng/ μ l. Pictures were taken at stages 15, 22, 27 and 32 (Iwamatsu, 2004) using Nikon confocal microscope for each embryo, separately. The Z-stage confocal sections of each live embryo were further analysed to ascertain the PGC numbers at each stage. The embryos showing reporter gene expression were then grown separately until 10 dah on a 24 well dish (1 embryo/well) and fixed/preserved for histological and realtime PCR analysis.

2.10. Chemical treatment

The chemicals, 17 β -estradiol (E2, Sigma, USA), aromatase inhibitor (AI, LKT labs, USA), dihydroxyprogesterone (DHP, Sigma, USA), methyltestosterone (MT, Sigma, USA), and Bromodeoxyuridine (BrdU, Sigma, USA), were dissolved in DMSO (Nacalai tesque, Japan) and used for treatment. For embryonic treatment, one/two cell stages medaka embryos (100 embryos/50 ml of water) were treated till 18 daf (if not otherwise specifically mentioned). The embryos were reared as mentioned above, with daily water exchange. The adults were treated for 72 hours with daily water exchange, if not otherwise mentioned. For BrdU treatment experiment, 20 adult XY fish were treated with 10mg/L BrdU for 24 hours. After that, half of the fishes were treated with E2 and other half were exposed to vehicle DMSO.

2.11. Data analysis

Statistical differences in relative mRNA expression between experimental groups were assessed by One Way ANOVA of log-transformed data, followed by

Tukey's test, or Student's t-test. All statistical analyses were performed using graph pad prism software. All experimental data are shown as the mean $\pm$ S.E.M. Differences were considered statistically significant at $P < 0.05$, if not otherwise mentioned. The correlations were calculated using Pearson correlation coefficient method.

3. Results

3.1. Sequence and phylogenetic analysis

RACE PCR identified two different *IFN γ* alternatively spliced isoform from the gonadal cDNA of medaka. *In silico* genome comparison showed that *IFN γ 2a* possessed 8 exons (ORF-) while *IFN γ 2b* had 6 exons (ORF-) (SI Fig. 1 and Fig. 1b). Alignment analysis depicted that medaka *IFN γ 2a* and *2b* displayed the highest similarity with *IFN γ* of *Oreochromis niloticus* (56.4 and 54.8% respectively), followed by *Paralichthys olivaceus* (53.7, 53%, respectively), *Takifugu rubripes* (45.9 and 44.8%, respectively) and *IFN γ 2* of *Onchorhynchus mykiss* (39 and 32.8% respectively) (Fig1b). The two alternatively spliced isoforms showed 98.1% similarity with each other, at mRNA level. However, both *IFN γ 2a* and *2b* showed very low similarity with *IFN γ 1* of medaka (17% and 15.25%, respectively) (Fig.1).

A phylogenetic tree was constructed based on the amino acid sequences of vertebrate interferon gamma. It showed that even though fish *IFN γ 1* and *IFN γ 2* formed different cluster, they were derived from a common ancestor, *IFN γ* , of higher vertebrate (Fig. 1A).

3.2. Putative protein analysis

Putative domain analysis showed that both *IFN γ* proteins possess a signal peptide (21 amino acid) at the N terminal region. *IFN γ 2a* also possesses one of each four-helical-cytokine-like core, four-helical-cytokine core and a nuclear localization signal (NLS), while *IFN γ 2b* is only predicted to have the first one (SI Fig. 2A, SI Fig. 3A). Secondary structure prediction showed that both *IFN γ* proteins significantly differed from each other, especially in number of helixes, beta and gamma turns (SI Fig. 2B, SI Fig. 3B). Protein 3D monomeric models also verified the differences among these two proteins (SI Fig. 2C, SI Fig. 3C).

3.3. Tissue distribution and ontogeny

Tissue distribution analysis revealed a ubiquitous distribution of *IFN γ 2a* with stronger expression in the ovary, testis, heart, liver and kidney. However, *IFN γ 2b*

expression was restricted to the ovary and testis (Fig. 1C). Ontogeny analysis showed that even though *IFN γ 2a* did not depict any sexual dimorphism during development (Fig. 2A-B), *IFN γ 2b* remained female biased throughout life (except at 20dah) (Fig. 2C-D). *ISH* analysis using adult ovaries and testes demonstrated that *IFN γ 2b* expressed in phase II and III oocytes, and spermatogonia (Fig 2E-F). Similar to realtime PCR data, strong expression of *IFN γ 2b* was found in the ovarian germ cells (Fig. 2G-H). *WISH* analysis of medaka embryos also demonstrated a gonad specific expression of *IFN γ 2b* in XX fish (Fig. 3B-C) and ubiquitous expression of *IFN γ 2a* in both XX and XY fish (data not shown).

3.4. Steroid dependent activation of *IFN γ 2* promoter

In order to ascertain whether the steroid responsive regulation of *IFN γ* is direct/indirect, we performed *in silico* analysis of *IFN γ 2* promoter. The analysis depicted several potential ERE, ARE, GRE and IRE sites in the promoter region (SI Fig. 4A). To find out the roles of these elements in steroidogenic activation of *IFN γ 2*, we analysed 4 different lengths of promoter (5598bp, 3248bp, 2205bp, 1100bp) using luciferase assay. We found that both E2 and MT could differentially activate the luciferase activity (SI Fig. 4B) of all the tested fragments. Highest E2 dependent activation was obtained from -2205bp fragment, while maximum MT responsive luciferase activity was found in -1100bp. In general, our data points out the possible steroidogenic involvement in *IFN γ 2* regulation.

3.5. Effect of steroid on *IFN γ* expression

In order to find out the role of *IFN γ 2* on germ cell development and maintenance, we treated medaka embryo (0daf) with E2, and adults with E2, MT, DHP and AI. We found that E2 treatment induced embryonic *IFN γ 2b* expression in XY fish in a stage dependent manner (Fig 3A). The changes were subtler in 6daf than 8daf, while, no significant increase in *IFN γ 2a* was observed till 8daf (data not shown). Although, *WISH* expression of *IFN γ 2b* was only restricted to the XX gonad, and E2 treated embryonic XY gonad (Fig. 3D), no changes in *IFN γ 2a* and *IFN γ 2b* expression were observed in the E2 treated XX embryos (data not shown).

During adulthood, E2 treatment followed similar activation pattern, i.e. higher activation of *IFN γ 2b* (3.5 fold) than *IFN γ 2a* (0.025 fold) in XY gonad (Fig. 4A-B). However, in XX gonad, no significant differences were observed in both cases.

Interestingly, both MT and AI treatment preferentially induced *IFN γ 2a* expression in XX while reduced the same in XY fish. But, *IFN γ 2b* expression was found to be suppressed in the MT treated XX fish. However, no differences in *IFN γ 2a* and *2b* expression were found in the DHP treated group. The data collected from steroid treatment of adults were plotted as ratio to their corresponding control to get better idea about the changes in sex related expression (Fig. 4A-B). This highlights the possible involvement of steroids in the regulation of preferential alternative splicing of *IFN γ 2*.

3.6. Alternative splicing of *IFN γ* is steroid dependent

To confirm the above results, we transfected the HEK293 with mini genome (-2205bp – polyA) fused with mCherry (Fig. 5A) and treated the cells with either E2 or MT. As expected, mCherry expression was only observed in MT treated cells, which suggested the production of *IFN γ 2a* after MT treatment (Fig. 5B-C). Realtime analysis of the same samples also suggested similar patterns of *IFN γ 2a* expression. In contrast, we observed an elevated expression of *IFN γ 2b* in E2 treated samples, but not in the MT treated ones (Fig. 5D). These data confirm that E2 accelerates the production of *IFN γ 2b* while MT prefers *IFN γ 2a*.

3.7. *IFN γ* and germ cell mitosis

Our data suggested that *IFN γ 2b* is more important for E2 responsive gonadal development. For better understanding of *IFN γ 2b* responsive gonad development, we injected the pCS2-*IFN γ 2b*-GFP-OE mRNA into one/two cell stage medaka embryos. We found a strong GFP expression from blastula stage, which further localized in germ cells by 4daf and continued till 3dah (Fig. 6B-C). pCS2-*IFN γ 2a*-GFP-OE mRNA was used as a control, which showed ubiquitous expression as expected (Fig 6A). Parallel to that, our realtime ontogeny data also depicted a female specific surge during 6daf (Fig. 2B), just before the initiation of germ cell proliferation. This points towards the role of *IFN γ 2b* in gonad development, especially, germ cell proliferation. In order to further confirm the role of *IFN γ 2b* in germ cell proliferation, we treated the adult XY medaka with BrdU for 24 hour, followed by E2 for a period of another 48 hours. The gonads were analysed for both BrdU proliferation (by immunohistochemistry) and *IFN γ 2b* expression (by realtime PCR). We found that, E2 induced the gonadal BrdU-index and *IFN γ 2b*-expression by 3-5 fold and 6-10 fold,

respectively. The BrdU index and *IFN γ 2b* both showed a positive correlation with each other (Fig. 7).

4. Discussion

Alternative splicing is known to increase the mRNA expression diversity and gonad development to some extent. However, the role played by the alternatively spliced variants of the interferon related genes in gonadal sex maintenance is relatively unknown. In the present study, for the first time, we isolated 2 different alternatively spliced variants of *IFN γ 2*, i.e. *IFN γ 2a* and *IFN γ 2b*, and analysed their responsiveness in gonadal development and maintenance. Tissue distribution, ontogeny, steroid treatment and *ISH* data point towards a possible involvement of *IFN γ 2b* in germ cell proliferation. The promoter analysis suggested a steroid responsive regulation of *IFN γ 2*, and the *in vitro* mini- genome analysis also confirmed that the abundance of specific alternatively spliced isoforms are dependent on E2 or MT concentration. Further, over-expression of *IFN γ 2b* and BrdU experiment analysis also emphasized the importance of *IFN γ 2b* in germ cell proliferation.

It is well accepted that, during evolution, the genomes of most vertebrates had two rounds of whole genome duplications (Ohno, 1970), whereas the genomes of most bony fishes had another extra round (3 round duplication) and thus evolved as the modern fish (Amores et al., 1998; Hoegg et al., 2004; Meyer and Van de Peer, 2005). *In silico* genome analysis revealed that the two different medaka *IFN γ s* (1 and 2) are located in separate contigs. However, no *IFN γ 2* like sequences were found in the *Xenopus*, chicken, mouse, rat and human genome database. Moreover, the phylogenetic analysis suggested that medaka *IFN γ 1* and *IFN γ 2* diverged from *IFN γ* of mammals (Purcell et al., 2009). Taken together, occurrence of *IFN γ 2* is a specific phenomenon to fish group, which suggests that *IFN γ 2* duplication might play unique roles in teleost. In this context, Purcell et al. (2009) reported that *IFN γ 2* expresses in the ovary, while *IFN γ 1* does not. Since, our main focus of this study was to explore the possibility of existing correlation between immunity and reproduction, we focused on *IFN γ 2* in this investigation.

Short and long transcript forms of *IFN* have been reported in channel catfish, Atlantic salmon and zebrafish, which were derived by differential promoter use and alternative splicing (Levraud et al., 2007; Milev-Milovanovic et al., 2006) in the exon 1. We also identified two alternatively spliced isoforms of *IFN γ 2* in medaka.

Moreover, we found several potential EREs, AREs in the promoter region and observed an E2/MT dependent alternative splicing. This phenomenon could be attributed to the differential promoter use. However, in case of medaka *IFN γ 2*, the modification in exon5 and 6, gave rise to a shorter protein. Both forms differ in 3'utr length, which might be responsible for differential cellular localization signal. Our putative protein analysis shows that only *IFN γ 2a*, but not *IFN γ 2b*, possess immune enhancement responsive “four helical cytokine core” and NLS, which might point towards the neo-functionalization of the later gene (Aggad et al., 2010; Schroder et al., 2004). Our RNA-overexpression data suggests that *IFN γ 2b* is localized in the germ cell while *2a* is more ubiquitous (data not shown). Moreover, unlike other IFN proteins (Robertsen et al., 2003), because of autocrine activity (characteristic of IFN γ) and the non-availability of certain domain and NLS, medaka IFN γ 2b is predicted to be embedded in extra cellular matrixes only (data not shown), which suggests its localised role(s) at expression site (Schroder et al., 2004).

IFN γ 2 has been reported to have a wide expression range in several teleostian tissues, i.e. rainbow trout (Purcell et al., 2009), grass carp (Chen et al., 2010), and is suggested to have an independent immune related activity (Chen et al., 2010). Similarly, we observed a ubiquitous expression of *IFN γ 2a* in adult fish, suggesting more conserved immune related roles in medaka. Further, we observed a positive age-dependent increment in *IFN γ 2a* expression, without any sex differences, which supported the fact that IFN γ 2a retained the immune related functions of IFN γ 2 in medaka. Several reports suggest that expression of *IFN γ* is sexually biased; females having more expression than males, owing to their steroidogenic regulation (Cernetich et al., 2006). Our present data suggests that major sex steroids play significant role in *IFN γ 2* alternative splicing and regulation. Moreover, several available reports also suggest that alternative spliced isoforms are essential for sexual development (Hammes et al., 2001; Yu et al., 2003; Krovel et al., 2004). The female dominated *IFN γ 2b* expression in all stages of ovary highlights its possible reproductive roles in medaka. Critical observation of ontogeny data suggests that *IFN γ 2b* expression sequentially increases with age of female fish and germ cell population. It is noteworthy to mention that, in medaka, proliferation of germ cell starts at 3 daf and takes a progressive pace from 7 daf (Saito et al., 2007). Our *ISH* data further showed that *IFN γ 2b* expressions were localized in the early stage germ cells (oogonia) to pre-vitellogenic oocytes. This indicates the potential involvement of

IFN γ 2b in early stage germ cell proliferation and maintenance. Moreover, E2 is known to induce germ cell proliferation in medaka. Our data confirmed that E2 was able to accelerate the *IFN γ 2b* expression in both E2 treated XY embryos and adults, which, along with BrdU treatment data (discussed later), suggested a steroid responsive gonad related function of *IFN γ 2b*. This was further supported by the fact that females have more estrogen than males, which might have led to higher production of *IFN γ 2b* in females.

Estrogen and its receptors are well known regulators of *IFN γ* (Gourdy et al., 2005, Panchanathan et al., 2010) in mice. Apart from E2, several other steroid recognition sites are found in promoter regions of mice, suggesting a probable multi-steroidogenic regulation (Panchanathan et al., 2010). Our promoter analysis also predicted several EREs and AREs, whose activities were confirmed *in vitro*, indicating a potential steroid based regulation (Panchanathan et al., 2010). The promoter analysis also suggested that although half-ERE was able to activate the *IFN γ* transcription but full-ERE takes the edge. And E2, owing to its advantageous role over MT in transcription initiation, probably has more regulatory importance *in vitro*.

A clear link between steroid regulated transcription and alternative splicing has been established in mouse, human and zebrafish. Several alternative splicing-regulating proteins interact with E2, which in turn controls the transcription-coupled alternative splicing of the first intron in a gene specific manner, thus impacting the rate of co-transcriptional RNA processing (Bhat-Nakshatri et al., 2013). Since, estrogen is converted from testosterone in the presence of aromatase, the aromatase inhibitor treated XX fish displayed gene profile similar to XY males. This could be due to the fact that AI tips the balance towards MT from E2 (Paul- Prashant et al., 2013) by restricting the conversion of testosterone to E2, which further leads to higher testosterone accumulation. In the present study, the reduction of *IFN γ 2b* expression in AI treated XX fish and simultaneous increase of *IFN γ 2a*, further supports the steroid dependent alternative splicing of *IFN γ 2*. Both MT dependent upregulation of *IFN γ 2a* in XX and E2 responsive *IFN γ 2b* expression in XY, support the correlation among steroid concentration and preferential alternative splicing. Additionally, mini genome analysis depicted *IFN γ 2a-mCherry* (fused) expression in MT elevated situation while, E2 addition induced *IFN γ 2b* expression, thereby confirming the above fact.

Interestingly, E2 treated females did not show any further increase in *IFN γ 2b* expression compared to normal females. Even though the estrogen production

increased along with age of medaka, we found no significant difference in *IFN γ 2b* expression between 50dah and 240dah samples. This might be due to the restricted expression in specific stages of oocyte or the saturated levels of *IFN γ 2b* in females, which in any case, needs further investigation. On the other hand, apart from E2 production, androgen is also known to directly affect the granulosa cell differentiation and follicular maturation in many vertebrates (Gleicher et al., 2011). Similarly in medaka, it is possible that during oocyte maturation, the testosterone concentration increases locally which further leads to higher amount of *IFN γ 2a* production, as a part of immune enhancement mechanism of the germ cells/ other gonadal cells. Sertoli cell specific mutation of androgen receptor was reported to cause degradation of early spermatids by producing auto-antibodies (Meng et al., 2011). Androgen specific upregulation of immune enhancement property containing *IFN γ 2a* might be associated with maintaining the gonadal cell population, which can be further supported by the equivalent *IFN γ 2a* expression in the male and female gonads.

In medaka, circulating sex steroid concentration determines the gonadal sex and regulates the germ cell proliferation. Germ cell mitosis is essential for the gonadal development and maintenance. First round of germ cell mitosis initiates around 3-4 daf, while proliferative mitosis starts from 7daf (Saito et al., 2007). Corresponding to the germ cell mitosis initiation, we observed an increase in *IFN γ 2b* expression from the same period (3-4 daf), which peaked at 7daf. Taken together, our ontogeny data suggests that *IFN γ 2b* corroborates a high similarity with the progression of gonadal mitosis in medaka. Our *WISH* data also depicts that *IFN γ 2b* is predominantly expressed in ovarian mitotic cells (early germ cell population). Similarly, GFP-fused *IFN γ 2b* mRNA expressions were confined to a very small population of germ cells, which along with the fact that *IFN γ 2b* works only in extra cellular matrix, suggests their localized role in germ cell maintenance. On the other hand, E2 is potentially known to regulate mitosis and meiosis in medaka. In the present study, we also confirmed the short-term E2 treatment effects by BrdU cell proliferation index. As *IFN γ 2b* is highly regulated by estrogen, extremely positive correlation ($r=0.98$) was seen between the BrdU index and *IFN γ 2b* expression. Moreover, their expression in BrdU positive cells made us to think that *IFN γ 2b* might also possess some inexplicit role(s) in germ cell proliferation. However, more functional analysis using cutting edge knockout techniques might give more explanation about their detailed role in germ cell mitosis in medaka.

5. Conclusion

Our observations have corroborated that alternative splicing of medaka $IFN\gamma 2$ is regulated by the circulating steroids. Although $IFN\gamma$ is long known as a cytokine related gene, our data suggests that it might also have potential effect on gonadal development. This is the first demonstration that fish $IFN\gamma 2$ probably plays an important role in gonadal sex differentiation and germ cell proliferation in vertebrates. However, further in depth analysis are necessary to elucidate its exact role in reproduction.

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Figure legends:

Fig. 1. Phylogenetic analysis, exon characteristics and tissue distribution of medaka *IFN γ 2* (*IFN γ 2a*, *IFN γ 2b*) alternatively spliced isoforms. Phylogenetic analysis (A) was carried out using ClustalW. The tree was generated with PHYLIP and viewed with PhyloDendron online software. Values on the tree represent bootstrap scores of 1000 trials. Medaka *IFN α 1* was used as an out-group. Graphical representation showing the putative exon-intron distribution of medaka *IFN γ 2* alternatively spliced isoforms (B), where the splice detector primer positions are marked with dotted arrow (refer SI Table 1 for primer sequence). RT-PCR analysis of the two alternative spliced forms of *IFN γ* in various tissues of adult medaka (C), where B, brain; P, Pituitary; G, Gill; H, heart; L, liver; S, Spleen; I, Intestine; T, Testis; O, ovary; K, kidney; M, Muscle; F, Fin; m, DNA ladder; -, negative control; EF1a in lowest panel as internal control.

Fig. 2. mRNA expression analysis of *IFN γ 2a* and *IFN γ 2b* in medaka by realtime PCR (A-D) and *in situ* hybridization (*ISH*) (E-H) using different age group samples. Changes in mRNA levels of *IFN γ 2a* (A) and *IFN γ 2b* (B) in beheaded XX and XY embryos of medaka collected at 3, 5, 6, 7, 8 and 13 daf. The ontogeny of *IFN γ 2a* (C) and *IFN γ 2b* (D) mRNA in gonads, collected at 10, 20, 50 and 240 dah. Data are shown as the mean $\pm$ S.E., and expressed as relative abundance corrected for *RPS18*. Letters above the bars indicate that these groups differ significantly from each other at $p < 0.05$. The cellular localization of *IFN γ 2a* (E, adult ovary; F, adult testis) and *IFN γ 2b* (G, adult ovary; H, adult testis) were determined by *ISH*. Note the ubiquitous gonadal expression of *IFN γ 2a* (E, F) and early germ cell specific expression (marked by blue arrow) of *IFN γ 2b* (G, H). Bar length -200 μ m.

Fig. 3. Effects of E2 (estradiol-17 β) on embryonic gonadal expression of *IFN γ 2b* in XX and XY embryos of medaka collected at 6 and 8 daf. Realtime PCR showed an estrogen dependent increase in *IFN γ 2b* expression in XY embryos (A). Fertilized eggs were maintained in water containing E2 or ethanol at a concentration of 10 ng/ml for 6 and 8 days. Data are shown as the mean $\pm$ S.E., and expressed as relative

abundance corrected for *RPS18*. Letters above the bars indicate that these groups differ significantly from each other at $p < 0.05$. *IFN γ 2b* expression in gonads of 8daf XX (B), XY (C) and E2 treated XY (D) medaka were further confirmed by whole mount *ISH*. Representative expression is indicated with red arrow heads. Note that expression was only detected in XX and E2 treated XY gonadal regions but not in XY gonads.

Fig. 4. Effects of steroids on *IFN γ 2a* and *2b* expression during adulthood. Realtime PCR showed a steroid/chemical dependent increase in *IFN γ 2a* (A) and *IFN γ 2b* (B) expression in XY embryos Note: 1, E2; 2, MT; 3, DHP; and 4, AI. Data are shown as the mean $\pm$ S.E., and expressed as relative abundance corrected for *RPS18* which were further normalized against vehicle control data. Letters above the bars indicate that these groups differ significantly from each other at $p < 0.05$. Double *in situ* hybridization showed the E2 responsive changes in cellular localization patterns of *IFN γ 2a* (green) *IFN γ 2b* (red) in adult XY testis (C). Note: Representative *IFN γ 2b* expressions were marked with white arrowhead while the blue arrowhead marks the overlapping expression (yellow) of both isoforms in early germ cell population.

Fig. 5. Assessment of steroid dependent alternative splicing of *IFN γ 2* *in vitro*. The schematics of construct used for analyzing the splicing event consists of *IFN γ 2* genomic fragment, starting from -2205b to just before stop codon of *IFN γ 2a*, aligned with mCherry (A). MT treatment induced production of *IFN γ 2a* fused mCherry expression (B) while E2 failed to produce the same (C). Realtime PCR showing the amount of *IFN γ 2a* and *2b* in different treatment groups (D). Data are shown as the mean $\pm$ S.E., and expressed as relative abundance corrected for *RPS18*. Letters above the bars indicate that these groups differ significantly from each other at least at $p < 0.05$.

Fig. 6. Over-expression of *IFN γ 2a* and *2b*. *IFN γ 2a* over-expression led to ubiquitous GFP expression in all over the body (A) while *IFN γ 2b* started expression in few cells at early blastula stages (B) and later the expression congregates in germ cells only (C). Inset showing the bright field images of whole embryos.

Fig. 7. Role of *IFN γ 2b* on mitotic cell proliferation. The graph represents the correlations between *IFN γ 2b* expression and BrdU positive germ cell population (Correlation coefficient (r)=0.86, p<0.01). Note, E2 treatment (r= 0.98, p<0.01) up-regulated 2-3 times more germ cell proliferation compared to DMSO treatment (r= 0.12, p>0.01), and the same induced 6-10 fold in *IFN γ 2b* expression. The dotted line represents the trend line (R^2 value, 0.96) of E2 dependent time responsive effect on mitotic germ cell proliferation (BrdU positive cells) and *IFN γ 2b* expression. Different symbols represent different time period of sampling, and black and white symbols were used for DMSO and E2, respectively.

Figure-1

Figure-2

Figure-3

Steroid treatment and *ifny2b* modulation in embryonic medaka

Figure-4

Figure-5

Figure-6

Figure-7