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Evolution of IFN subgroups in bony fish - 1:Group I-III IFN exist in early ray-finned fish, with group II IFN subgroups present in the Holostean spotted gar, *Lepisosteus oculatus*

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A B S T R A C T

The present study helps clarify when the fish type I IFN groups/subgroups evolved, by examination of the IFN genes present in the Holostean spotted gar, *Lepisosteus oculatus*, in relation to the IFN genes present in the Chondrostea (sturgeon). It confirms that all three IFN groups (I-III), and group II subgroups, existed prior to the appearance of teleost fish. Preliminary expression analysis in a gar cell line (GARL) suggests these IFN genes will have a role in antiviral defence in Holostean fish, in that they are induced by poly(I:C). A refined model of IFN evolution within the actinopterygian fish is proposed.

1. Introduction

Teleost fish possess a highly diversified type I interferon (IFN) repertoire, that can be classified into three groups (I-III), with subgroups known in some species for group I and II [1–3]. Group I IFNs exist in all teleost species whilst group II and III (IFNf) appear to be limited to certain species/lineages such as salmonids, cyprinids and some perciformes [4–8]. It is postulated that the group III IFN (to date found only in salmonids within the teleosts) is the closest sister group to the 4 cysteine containing type I IFN in cartilaginous fish, suggesting that IFNf is an ancient jawed vertebrate IFN [3,9]. A number of phylogenetic subgroups (IFNa-e and h) have also been identified in the group I and II IFNs [9,10]. Salmonids have the most subgroups (IFNa-e) and also the largest numbers of genes [9,11]. The group I and II subgroups have some functional differences. For example, in addition to the secreted isoforms, two intracellular isoforms that result from alternative splicing of an IFNa gene (a group I IFN) are able to trigger expression of antiviral genes in host cells [12]. Moreover, the phylogenetic groups/subgroups exhibit distinct expression patterns in cell types and in response to immune stimuli and viral/bacterial infection [9,10,13,14]. These findings demonstrate that teleost fish IFNs are phylogenetically and functionally diverse in immune defence against pathogen infection.

Fish group II and III IFNs have 4 conserved cysteines, which is believed to be the primordial form of vertebrate type I IFNs [1,15]. They are hypothesised to have arisen from a 4 cysteine containing IFN ancestral gene that itself was derived from the IL-10 cytokine family. The loss of two cysteines in teleost group I IFN is thought to have been an independent event giving rise to the 2 cysteine IFN forms known in cartilaginous fish [15] and mammals (ie IFN-β and IFN-ε). It is unclear when fish type I IFN appeared and when group I and II diverged into the different subgroups. Boudinot et al. (2016) proposed a bony fish 4 cysteine containing type I IFN ancestor may have duplicated into the group I and II IFN genes in the actinopterygian lineage prior to the teleost specific whole genome duplication (TSWGD) event [16], with their model explaining the subgroups found at the two loci present in teleosts, with subsequent gene expansion and loss. A recent paper searching for IFN genes in two species of sturgeon, *Acipenser dabryanus* and *Acipenser sinensis* (Chondrostea), intriguingly found several members of the IFNe subgroup [17], confirming that the appearance of group I IFN indeed preceded the teleosts but that these fish have either lost the group II genes or they have yet to be discovered. With the aim to help verify IFN subgroup evolution in fish, the present study analysed the type I IFN genes in the genome of spotted gar (*Lepisosteus oculatus*), another primitive actinopterygian fish group (Holostea) which has not undergone the TSWGD [18]. Using an *in silico* data mining approach (https://www.ncbi.nlm.nih.gov/, http://www.ensembl.org/index.html), 8 IFN genes were found in 2 different scaffolds. Prediction of the putative transcripts revealed that these genes belong to group I, II and III, confirming the presence of all three IFN groups in spotted gar and revealing for the first time that fish group II IFN genes had diversified into subgroups prior to the appearance of teleost fish. In addition, further analysis of Chondrostean (sturgeon) IFN genes revealed

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that all three IFN groups are also present at this level of phylogeny, with IFNc and IFNf now identified in these fish for the first time.

2. Materials and methods

2.1. Longnose gar liver cell line and stimulation

The cell line, GARL, was derived in 1998 from the liver of the longnose gar (*Lepisosteus osseus*) in the laboratory of Dr Niels Bols, University of Waterloo, Canada. The invitromatics of GARL, which is the discipline of describing the history, development, characterization, and storage of cell lines [19], will be described in a separate manuscript at a later date. For the current experiments, GARL was used for expression analysis of IFN genes from spotted gar since it is from the same Genus and no spotted gar cell lines exist as far as the authors are aware. It was maintained at 20 °C in Leibovitz L15 (L-15) medium supplemented with 10% new born bovine calf serum (NCBS, New Zealand origin, ThermoFisher), 100 U/ml penicillin and 100 μg/mL streptomycin (Invitrogen). The GARL cells were passaged into 25 cm^2 flasks and cultured for 2 days at 20 °C prior to use. The cells were stimulated for 6 h or 24 h with poly(I:C) (100 μg/mL, Sigma), pokeweed mitogen (100 μg/mL, Sigma), lipopolysaccharide (LPS, 50 μg/mL, Sigma) or equal volume of PBS (control). At the end of the stimulation period the cells were lysed in TRIzol (Sigma), according to the manufacturer's instructions.

2.2. In silico identification sequence confirmation

By *in silico* data mining of the NCBI and Ensembl databases (https://www.ncbi.nlm.nih.gov/, http://www.ensembl.org/index.html), spotted gar linkage group (LG) 15 (Acc. No., NC_023193) and a scaffold (Acc. No., NW_006270535) were obtained and analysed for putative transcripts using the GenScan program [20]. The transcripts were then subject to Blast search in the NCBI database to confirm their identity. Primers (Supplementary Table S1) were subsequently designed for PCR cloning of the complete coding region of individual IFN genes. To obtain the PCR template, the GARL cells were stimulated for 6 h with LPS or poly(I:C) as described above and mixed for RNA extraction. Total RNA was extracted using TRIzol and reversed transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (ThermoFisher). PCR was performed using the MyTaq™ HS DNA Polymerase (Bioline) and the resultant products were ligated into the pGEM-T Easy vector (Promega) at 4 °C overnight. The ligation reaction was transformed into *E. coli* TAM competent cells (ActivMotif) and plated onto the LB petri dishes containing 100 μg/mL ampicillin. After overnight culture at 37 °C, colony PCR was performed to screen positive clones. Plasmid DNA of positive clones was prepared using a Qiagen plasmid miniprep kit and sequenced by Eurofin Genomics.

In view of the multiple IFN subgroups discovered in gar, a similar analysis was undertaken for a Chondrostean species, the sterlet (*Acipenser ruthenus*), where the genome was interrogated for IFN genes and the predicted sequences compared to those from gar.

2.3. Sequence analysis

The Basic Local Alignment Search Tool (BLAST) program was used to confirm the identity of obtained sequences. The gene organization was predicted using the Splign program listed on the NCBI server. Protein translation was performed using the Virtual Ribosome programme (version 2.0) and signal peptides predicted using the SignalIP 4.1 program. The net charge and molecular weight were predicted using tools listed on the ExPASy server (https://web.expasy.org/compute_pi/). Sequence identity and similarity were analysed using the MATGAT program [21]. Multiple sequence alignment was performed using the Cluster Omega program and the conserved amino acids were shaded by the BoxShade Server. The alpha helices were predicted using Jpred 4 [22]. Synteny analysis of type I IFN loci was conducted using Genomicus (database version 96.01) and the latest version of genomes for spotted gar and zebrafish. A phylogenetic tree was constructed by the Neighbour-Joining method using the MEGA7 program and bootstrapped 1,000 times. A multiple sequence alignment was generated by the Clustal Omega program and used for the phylogenetic tree analysis using the MEGA7 software.

2.4. Gene expression analysis by real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the TRIzol reagent (Sigma) and reverse-transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (ThermoFisher). In order to ensure that no genomic DNA could be amplified under the RT-qPCR conditions, at least one primer was designed to cross an intron. Gene primers are listed in Table 1. To obtain the sequence for elongation factor-1 alpha (EF-1α) of longnose gar, a partial fragment was cloned by a homology-cloning approach using rainbow trout EF-1α primers and sequenced. The EF-1α primers are also given in Table 1. The expression levels of IFN paralogues were calculated as arbitrary units after normalization against the expression

Table 1
Information of type I IFN paralogues in spotted gar.

Gene	Genomic DNA [a]	Location	Orientation	cDNA [b]	Signal/mature peptide (aa)	Cysteine number	pI [c]	MW (kDa) [c]
IFNe1	LG15, NC_023193.1	7,519,119–7,522,607	F	MN267410	22/161	2	10.15	19.00
IFNe2	LG15, NC_023193.1	7,523,280–7,526,024	F	MN267411	22/157	2	9.30	18.38
IFNb[d]	LG15, NC_023193.1	7,547,000–7,550,000	F	ENSLOCT00000016253.1	21/166	5	7.79	19.06
IFNc1	LG15, NC_023193.1	7,535,000–7,537,000	F	MN267412	20/169	4	8.93	19.63
IFNc2[d]	LG15, NC_023193.1	7,555,905–7,558,369	F	XM_015362125.1	20/170	4	9.21	20.01
IFNc3	LG15, NC_023193.1	7,565,000–7,566,000	F	MN267413	20/175	4	6.75	20.38
IFNc4[d]	LG15, NC_023193.1	7,566,500–7,575,000	F	ENSLOCT00000016258.1	20/170	4	4.64	19.46
IFNf	Scaffold, NW_006270535.1	2,359–5,356	R	XM_015339977.1	23/155	4	9.63	17.92

[a] accession number of genomic DNA and chromosome location.
[b] accession number of cDNA.
[c] pI and molecular weight of mature peptide.
[d] predicted ORF and protein sequence.

level of EF-1α. The RT-PCR reactions were set up in triplicate in a volume of 10 μL, containing 4 μL of cDNA template and 6 μL of SyBr green master mix, and run on the Roche LightCycler 480 II system using the following programme: an initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 25 s, with the fluorescence recorded after every cycle. Melting curves were obtained at the end of each PCR reaction to confirm that only one product was amplified. The PCR products were verified by sequencing.

2.5. Statistical analysis

The data were analysed using the SPSS Statistics package 24 (SPSS Inc., Chicago, Illinois). Briefly, real-time quantitative PCR measurements were scaled, with the lowest expression level as 1, and log2 transformed [23]. One-way analysis of variance (ANOVA) and the LSD post hoc test were used to analyse the expression data, with $P \leq 0.05$ between treatment and control groups considered significantly different.

3. Results

3.1. Sequence analysis of type I IFN paralogues

Eight type I IFN genes were obtained by sequence analysis in the NCBI and Ensembl database (Supplementary Figs. S1–S8), and designated here as IFNb, IFNc1-4, IFNe1-2, and IFNf. The IFNb, IFNc1-4 and IFNe1-2 were clustered in chromosome LG 15 (Acc. No., NC_023193) with the same transcription orientation, whilst the IFNf was found on a separate scaffold (Acc. No, NW_006270535). The IFNf could not be identified in the spotted gar genome in the Ensembl database.

To verify if the obtained sequences were expressed and could be modulated, a cell line was developed from the longnose gar liver and named GARL. The GARL cells appeared to be epithelial like and were relatively larger in size than most cells derived from teleost fish (Supplementary Fig. S9). The GARL cells were stimulated with LPS or poly(I:C) to obtain cDNA templates for cloning of gar type I IFN genes. Using primers designed from the predicted sequences (Supplementary Table S1), the coding regions of IFNe1, IFNe2, IFNc1, IFNc3 and IFNf were amplified by PCR and confirmed by sequencing. However, the IFNb, IFNc2 and IFNc4 could not be amplified. It may be that these IFNs are not be expressed in the GARL cells or they exist as pseudogenes, or a few of the IFN genes did not get amplified (ie b1, c2 and c4) which could be due to small differences in the longnose gar sequence from the spotted gar sequence. The NCBI accession numbers of the IFN genes are listed in Table 1. That the sequences obtained by cloning from GARL cells were the same as those in the gar genome also verified the cell line was gar-derived, as expected.

All the spotted gar type I IFN genes have complete open reading frames (ORFs) that encode proteins of 178–195 aa, with a signal peptide of 20–23 aa and a mature peptide of 155–175 aa. The mature IFN peptides are similar in size, with molecular weight ranging from 17.92 kDa to 20.38 kDa, but differ considerably in pI values (Table 1). The IFNe1 and e2 molecules contain two cysteine residues in the mature peptide, whilst IFNc1-4 and IFNf have four, and IFNb has five. Multiple sequence alignment revealed that the two cysteines of IFNe1 and e2 aligned well with Cys1 and Cys3 in zebrafish IFNa (IFNphi1) and IFNd (IFNphi4), indicating that they belong to the fish group I IFN subgroup (Fig. 1). Similarly, the positions of the four cysteines of IFNc1-4 and IFNf were well conserved with those of zebrafish IFNc1 (IFNphi2) and IFNc2 (IFNphi3), typical of fish group II and group III IFNs. All the gar type I IFNs consist of five conserved alpha helices (helix A, C, D, E and F), with IFNe1, e2, c3 and c4 containing an additional short helix B between helix A and C. IFNe1 and e2 also share a relatively high sequence identity of 76.0% at the protein level (Table 2), whilst the sequence identities among the IFNc molecules are moderate (41.7–55.5%), but those between gar IFN groups/subgroups are low (21.7–32.3%).

Table 2
Protein sequence homology of spotted gar type IFN paralogues.

	IFNe1	IFNe2	IFNb	IFNc1	IFNc2	IFNc3	IFNc4	IFNf
IFNe1		**76.0**	25.4	25.6	21.7	24.4	22.5	25.8
IFNe2	82.5		22.3	26.9	22.2	24.0	23.0	25.0
IFNb	43.9	41.2		32.3	31.2	27.5	30.0	26.1
IFNc1	44.4	43.4	52.4		**53.4**	**41.7**	**55.5**	25.5
IFNc2	41.1	40.5	57.4	71.1		**45.8**	**46.5**	23.8
IFNc3	42.6	41.5	52.8	63.6	62.1		**47.1**	26.8
IFNc4	43.9	40.8	53.1	70.4	67.9	67.3		26.6
IFNf	45.4	43.6	47.6	48.1	46.3	46.2	46.4	

Note: Upper and lower panel indicate percentage of identity and similarity, respectively.

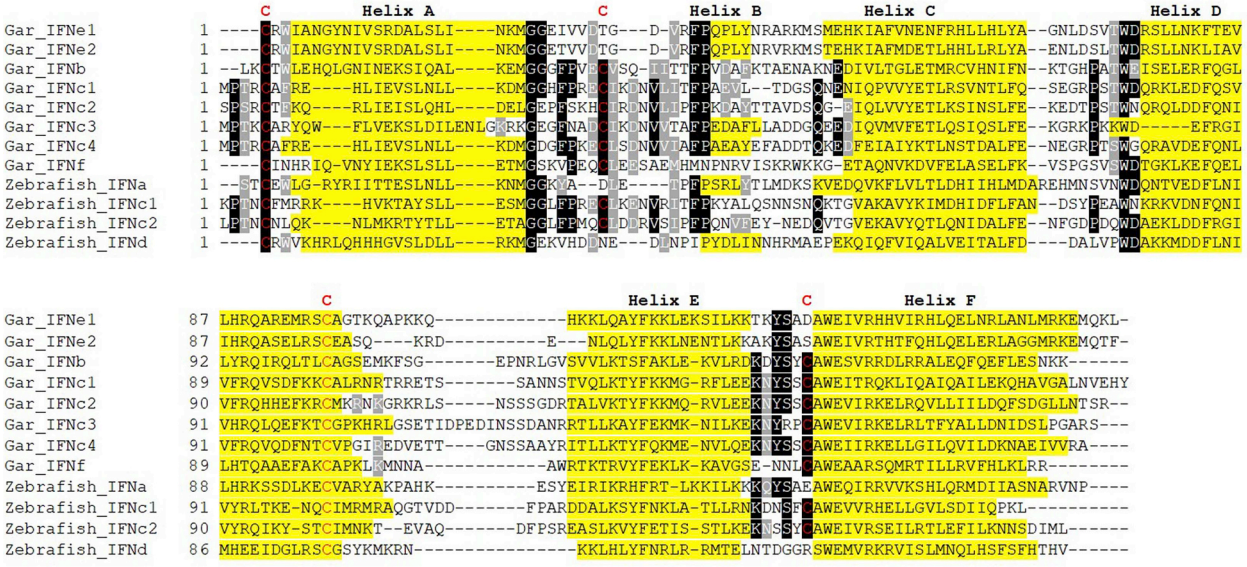

Fig. 1. Multiple sequence alignment of putative mature peptides of spotted gar and zebrafish type I IFNs. The multiple alignment was generated using the Clustal Omega program, and conserved amino acid residues were shaded using BOXSHADE (version 3.2). The conserved cysteine residues are in red and the predicted alpha helices are highlighted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

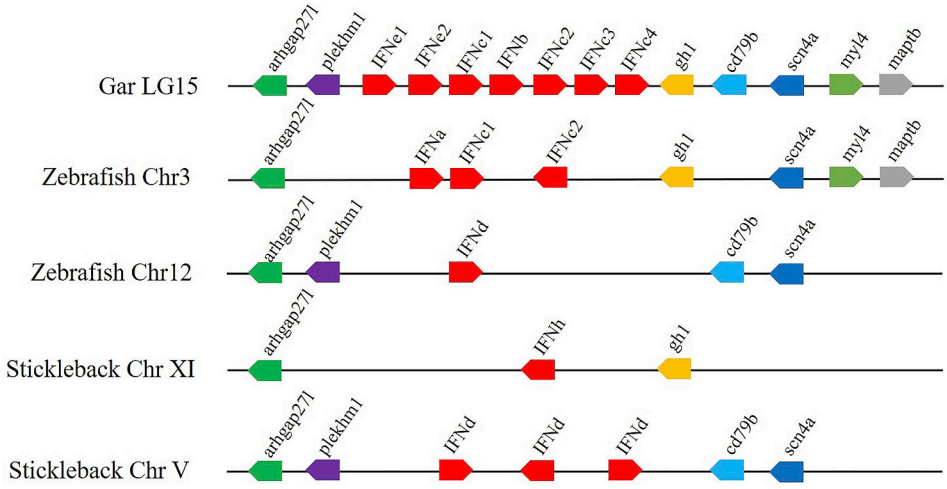

Fig. 2. Comparative synteny analysis of type I IFN genes in spotted gar and zebrafish. The synteny was predicted using the Genomicus program (database version 96.01) or extracted from recently released reference genomes at the NCBI. The accession numbers are NC_023193 (spotted gar), NC_007114 and NC_007123 (zebrafish).

3.2. Synteny analysis

The IFN gene synteny was comparatively analysed in the spotted gar, zebrafish and stickleback. Spotted gar is an Holostean, an early actinopterygian (ray-finned) fish that did not undergo the TSWGD (Fig. 2). Seven of the gar type I IFN genes (IFNb, IFNc1-4 and IFNe1-2) were found at a single locus in gar chromosome LG 15 (Acc. No., NC_023193), which also contained arhgap27, plekhm1, gh1, cd79b, scn4a and my14. The homologous IFN loci could be found in zebrafish, however in two different chromosomes (Chr 3 and 12); one linked with gh and my14, and the other linked with plekhm1 and cd79b, with arhgap27 and scn4a at both, as reported previously (Boudinot et al., 2016). Two homologous IFN loci are also be found on two chromosomes (Chr XI and V) in stickleback, one linked with gh, and the other one linked with plekhm1 and cd79b, with arhgap27 at both. The IFNf gene was located in a small scaffold (Acc. No., NW_006270535; 12,147 bp) where no other genes could be identified. It is not clear whether the IFNf scaffold is linked with the other IFN locus in the gar genome.

3.3. Gene organisation analysis of spotted gar type I IFN genes

The spotted gar type I IFN genes (excluding 5′ and 3′ untranslated regions) possess a five exon/four intron structure, as seen in teleost IFN genes (Fig. 3). All four introns are in phase 0. Exons 1, 2, 4 and 5 of IFNe1 and e2 have the same length whilst exon 3 of IFNe2 is 12 bp

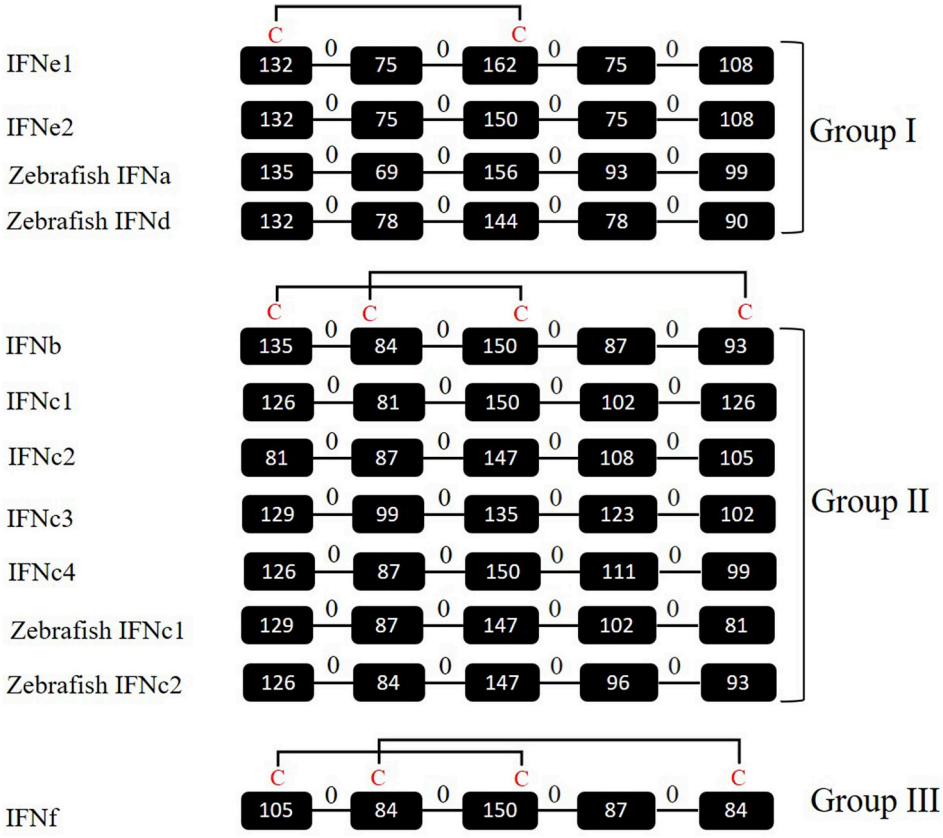

Fig. 3. Genomic organisation of IFN genes in spotted gar and zebrafish. The black boxes represent coding regions of exons and lines between boxes represent introns. The exon size (bp) is numbered in the box, and the intron phase is indicated above the line. Conserved cysteine residues and putative disulphide bonds are marked.

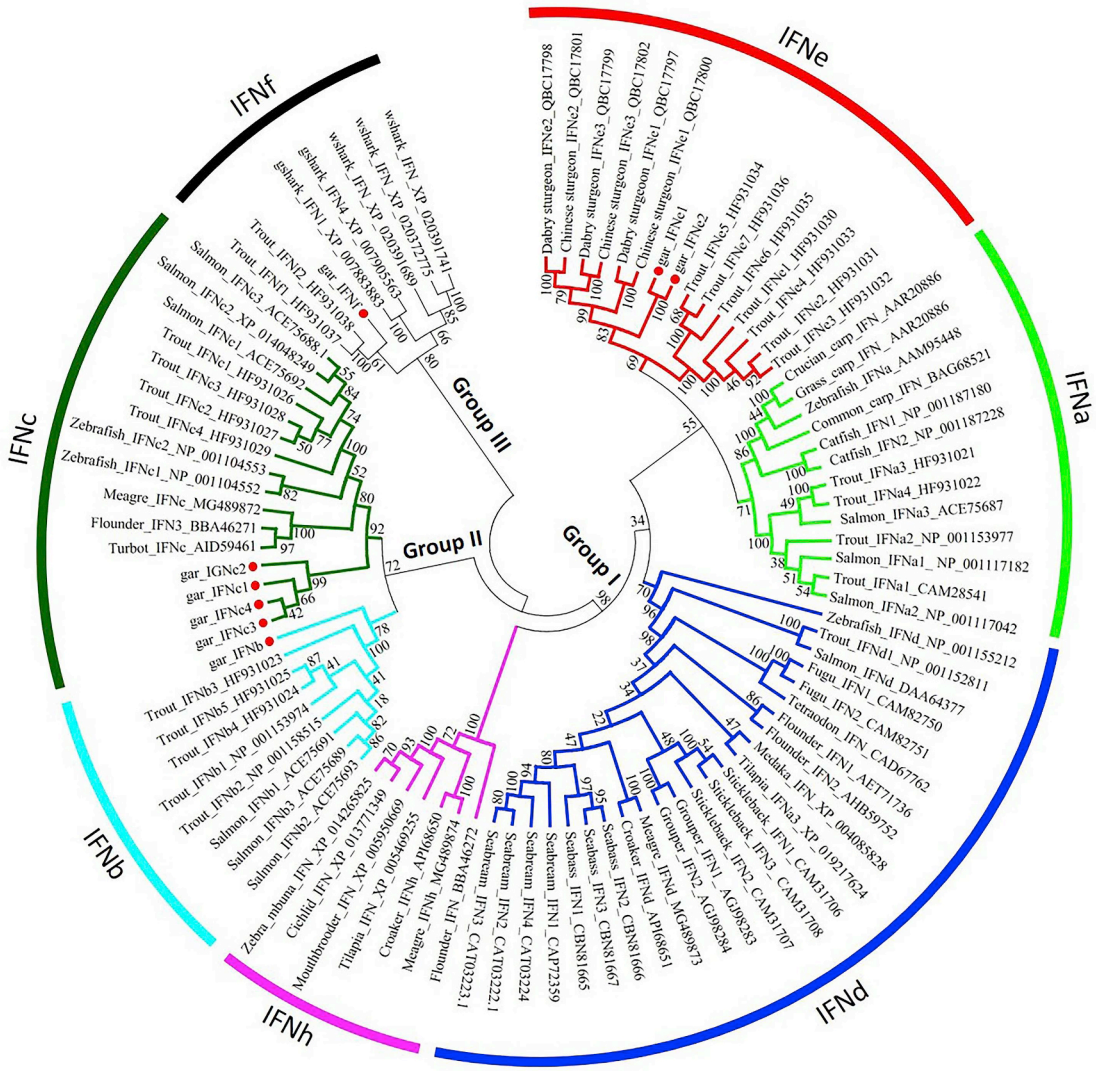

Fig. 4. Phylogenetic tree analysis of fish type I IFNs. The phylogenetic tree was constructed by the Neighbour-Joining (NJ) method within the MEGA 7.0 program, using a multiple alignment of full length protein sequences. The JTT matrix-based method with pairwise deletion option was chosen to compute the evolutionary distance. The percentage bootstrap value is shown as a percentage based on 1,000 replicates. The spotted gar IFNs are indicated by red dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

shorter than the corresponding exon of IFNe1. The two conserved cysteine residues predicted to form an intracellular disulfide bond in the mature peptide of IFNe1 and e2 are located in exon 1 and exon 3 respectively. In IFNb, IFNc1-4 and IFNf, the four conserved cysteine residues predicted to form two intracellular disulfide bonds (Cys1-Cys3 and Cys2-Cys4) are located in four different exons, Cys1 in exon 1, Cys2 in exon 2, Cys3 in exon 3 and Cys4 in exon 5. The exon 4 of IFNe1 and e2 (group I IFNs) is relatively small vs that of IFNb and IFNe1-4 (group II), contrasting with the comparable size of the other four exons.

3.4. Phylogenetic tree analysis of fish type I IFN molecules

Fish type I IFNs are classified into three groups, I-III. According to the phylogenetical relationships, group I and II can also be divided into 6 distinct subgroups (IFNa-e and IFNh[1]), with IFNf being hypothesised to be an ancient isoform in Gnathostome vertebrates [3,9]. In the phylogenetic tree (Fig. 4), the gar IFNf together with trout IFNfs were clustered with cartilaginous fish IFNs to form a branch with a high bootstrap value (80%), in agreement with its ancient origin. In addition to IFNf, the spotted gar also has 5 additional members of the group II IFNs. The IFNb and IFNc molecules were sister groups, forming an extended branch with a bootstrap value of 72%. The tree also confirmed the phylogenetic grouping of the two gar group I IFNs, with gar IFNe genes orthologous with those in sturgeon and rainbow trout [9,17]. Taken together, the results indicate that the three known fish IFN groups are present in the spotted gar, with expansion of group II genes into two subgroups (b, c).

Since the presence of group II and III IFN genes has been clearly demonstrated in gar, but were apparently absent in a first report on Chondrostean IFN genes, where only IFNe were found [17], we re-examined this issue. Our search for sterlet IFN genes discovered 5 molecules (Supplementary Figs. S10–S14), that when analysed for group/subgroup revealed the presence of two IFNf, two IFNb-like and one IFNe (Supplementary Fig. S15). Thus, it is clear that all three type I IFN groups were present at an even earlier stage of actinopterygian evolution.

[1] Use of IFNg is omitted from this terminology to avoid confusion with IFN-γ, a type II IFN.

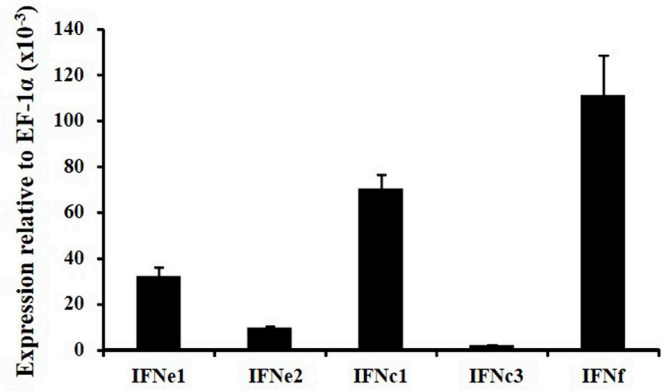

Fig. 5. The constitutive expression of longnose gar type I IFN genes in GARL cells. The transcript levels of IFN genes were normalized against the expression of EF-1α. The data are presented as means +SEM (n = 3).

3.5. The expression of gar type I IFN genes in GARL cells

The expression of the IFN genes was examined in unstimulated GARL cells. A partial fragment of EF-1α (Acc. No., MN267409) was obtained by a homology-cloning approach and used as an internal reference gene for the RT-qPCR analysis. In the unstimulated cells, the expression level of IFNf was the highest among the IFN genes analysed, moderate for the IFNc1, e1 and e2, and lowest for IFNc3 (Fig. 5). No expression of IFNb, IFNc2 and IFNc4 was detected. Next, the GARL cells were stimulated with LPS (50 μg/mL), PWM (100 μg/mL) or poly(I:C) (μg/mL) for 6 h and 24 h to examine their effects on the IFN expression. IFNe1, e2, c1, c3 and f were all significantly induced at 6 h and 24 h after stimulation with poly(I:C) but not with LPS or PMW (Fig. 6). The highest increase of expression (14- fold) was detected for IFNf at 6 h after stimulation. Again, no expression of IFNb, IFNc2 and IFNc4 was detected.

4. Discussion

Teleost fish possess three groups of type I IFNs [3]. Whilst group III IFN (IFNf) is considered to be ancient, with homologous genes in extant cartilaginous fish, the origin of the group I and II IFN genes and how early they evolved in bony fish evolution is less clear. A recent study of two Chondrostean (sturgeon) species discovered three IFNe in both species, and raised a number of possibilities regarding IFN evolution in the actinopterygians. Firstly, could particular IFN groups have been lost in certain lineages, since IFNf was not discovered. When did the group I

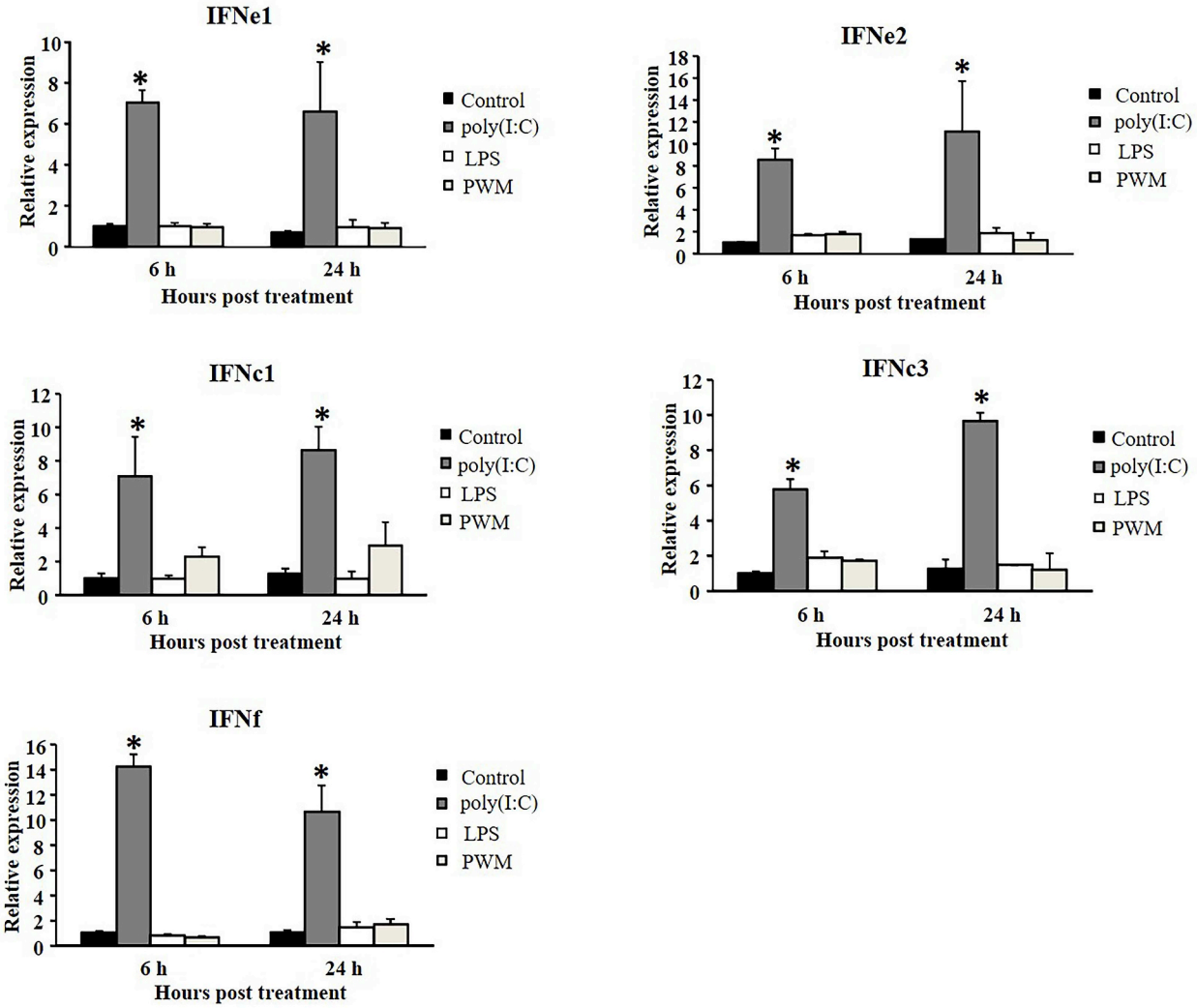

Fig. 6. Modulation of the expression of gar type I IFN genes in GARL cells by PAMPs. The GARL cells were stimulated with poly(I:C) (100 μg/mL), LPS (50 μg/mL) or pokeweed mitogen (PWM) (100 μg/mL) for 6 h or 24 h. The transcript levels of IFN genes were normalized against that of EF-1α and compared between the treatment groups and control groups. The data are presented as means +SEM (n = 3). *, $p < 0.05$ between treated groups and their respective control group.

and group II IFNs diverge, since perhaps group II genes also have been lost in Chondrosteans. Can IFNe be considered the first group I genes to have appeared, from which the other group I subgroups are derived (ie IFNa, IFNd, IFNh). To help shed light on these issues, we analysed the IFN repertoire in another early actinopterygian fish group, the Holostea, and in view of the results obtained also revisited the Chondrostea where further genes were discovered.

Our analysis of the spotted gar genome discovered eight type I IFN genes, including isoforms from group I, II and III. In addition, it was apparent that the group II IFNs had expanded into two phylogenetic subgroups (b, c) prior to the TSWGD. That group I and III IFN were present was not surprising in view of results in sharks and sturgeon, but these data apparently verified that Chondrostean fish may have lost IFNf, that IFNe was the first group I IFN subgroup to have evolved (in line with the sturgeon findings) and that group II IFN possibly evolved after the Chondrostei diverged from the Neopterygii. Since the sturgeon study had used RNA –sequencing analysis of spleen from healthy fish, it was possible that some IFN genes may have been missed, hence we revisited this issue by analysis of another sturgeon species, the sterlet (*Acipenser ruthenus*). In our analysis of sterlet we found five IFN genes, that were grouped with the three known type I IFN groups in fish, and appeared to be two IFNb-like, one IFNe and two IFNf genes. Thus IFNf genes are also present in Chondrostea, IFNe indeed seems to be the first group I genes to have appeared but that group II genes were also present at this level of phylogeny. From these data we propose a revised model of IFN evolution in actinopterygian fish (Fig. 7), similar to that of Boudinot et al. (2016) but where the timings of group/subgroup appearance is more refined.

Type I IFNs belong to the IL-10 cytokine family consisting of multiple α-helices and are structurally conserved in teleost fish. Crystal structural analyses of zebrafish homologues has shown that both group I (IFNa/IFNphi1) and group II (IFNc1/IFNphi2) IFNs contain 5 well-defined α-helices, a typical feature of mammalian type I IFNs [2]. In relation to Holostean type I IFNs, in the present study all the gar IFNs have five predicted α-helices, supporting the notion that the overall protein structure of type I IFNs remains similar in vertebrates despite low sequence homology (Fig. 1, Table 2). Of note, multiple sequence alignment showed that the regions between helix D and F were most variable and IFNc3, c4, e1 and e2 had an identifiable short helix B (Fig. 1). Interestingly, all the gar group II IFNs but not the group I IFNs (ie IFNe1 and e2) contain predicted glycosylation sites (NxS or NxT). However, a recent study of group I IFNs in black carp demonstrated that glycosylation is not essential for antiviral activity [24].

Multiple isoforms of IFNs are common and the genes encoding them are usually clustered in the genome. In the spotted gar, seven of the eight IFN genes are arranged in the sequence IFNe1-e2-c1-b-c2-c3-c4, with the same transcriptional orientation and flanked by the arhgap27 and plakhm1 genes at the 5′ upstream region and gh1 and cd79 genes at the 3' downstream region (Fig. 2). This syntenic clustering of IFNs with gh1 and cd79 has been shown to be conserved in fish and can be found in all the jawed fish, as seen in the elephant shark genome [25], although fish group I/II IFN genes are not found in sharks. Unfortunately we were unable to find a linkage of the IFNf gene to this locus in gar, but in view of data from other fish groups it seems likely it will be shown to be linked when a better draft of the genome is available. The data in gar support our previous hypothesis that the IFN loci have been unstable throughout the vertebrate evolution, where insertions (birth) and deletions (death) of IFN genes have taken place to generate diversity [9]. In salmonids, it has been shown that more than 23 copies of rainbow trout IFN genes have been found in three scaffolds where the IFN genes are spaced by numerous transposon genes [9,11]. In contrast to the expansion of group II IFNs in the spotted gar, only a single subgroup of group I IFNs (IFNe) was found, from which the teleost group I IFNs appear to have expanded into a further three subgroups (IFNa, d and h) after the TSWGD, in one case (IFNd) possibly as a direct consequence of the TSWGD event which generated two type I IFN loci.

Fish IFNs are differentially expressed in tissues and cells. In teleost fish, group I IFNs are inducible in a wide range of cell types in response to viral infection or PAMPs, whilst group II IFNs appear limited to leucocytes [1,11,14]. Svingeru et al. (2012) report that certain

Fig. 7. Proposed evolutionary model of type I IFNs in bony fish.

peripheral blood leucocytes of Atlantic salmon are able to express high levels of IFNb and IFNc following stimulation with R848, a ligand for TLR3/7. In brown trout, IFN-d, -e, and -f have been shown to be induced in kidney and spleen at an early stage of infection with viral hemorrhagic septicemia virus relative to other subgroups [9]. Recently, the expression of IFNe was examined in sturgeon for the first time. The IFNe genes are constitutively expressed in lymphoid tissues such as head kidney and blood, and can be up-regulated in primary splenocytes by poly(I:C) and to some degree by LPS [17]. In the gar epithelial-like GARL cell line, constitutive expression was detected for IFNc1, e1, e2 and f, with very low levels of c3 (Fig. 5). This demonstrates that a single cell type can express all three groups of IFNs in this species. In addition, all five genes were up-regulated in GARL cells stimulated with poly(I:C) but not when stimulated with LPS or PWM. These results imply that the expression of IFNs may be regulated differently in chondrosteans and holosteans. Future bioactivity studies of gar and sturgeon IFNs will confirm whether functional diversification of the IFN molecules occurs at the protein level.

In conclusion, the present study helps clarify when the fish type I IFN groups/subgroups evolved. It confirms that all three IFN groups, and group II subgroups, existed prior to the appearance of teleosts. Preliminary expression analysis suggests these genes will have a role in antiviral defence in Holostean fish, in that they are induced by poly (I:C). A refined model of IFN evolution within the actinopterygian fish is proposed.

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Appendix A. Supplementary data

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