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Transcriptomic responses of S100 family to bacterial and viral infection in zebrafish

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ABSTRACT

The S100 family proteins are a group of small acidic polypeptides and have diverse functions in regulating many aspects of physiological processes. They are structurally conserved and possess two EF-hands which are central for calcium-mediated functions. In this study, 14 S100 cDNA sequences were determined in zebrafish and their genomic organizations confirmed. Re-analyzing the gene synteny of the S100 loci identified two major S100 loci in Chr16 and Chr19 which share remarkable conservation with the S100 locus in human Chr1, suggesting they may have evolved from a single locus during the teleost specific whole genome duplication event. It appears that the homologues of human S100G and S100P have been lost in zebrafish. Expression analysis reveals that S100W, ICN1 and ICN2 are markedly expressed in embryos. Further, the transcripts of S100 genes are relatively abundant in mucosal tissues such as gills and gut. Intraperitoneal injection of poly(I:C) resulted in up-regulation of most S100 genes in the gut and spleen, with highest induction of S100V2 and S100Z detected. In fish challenged with spring viremia of carp virus (SVCV), expression of most S100 family genes was increased in the spleen between day 1 and 7 post infection, with consistent induction seen for the S100A1, S100A10b, S100B, S100ICN1, S100T, S100U, S100V1 and S100Z. Interestingly, intraperitoneal injection of *Edwardsiella tarda* down-regulated S100 expression in the gut but resulted in induction in the spleen. The results demonstrate that the S100 family genes are differentially modulated by bacterial and viral pathogens in zebrafish.

1. Introduction

The S100 protein family consists of a group of small acidic peptides of 10–12 kDa and has a wide range of biological functions in regulating cell growth and motility, maintaining Ca^{2+} homeostasis, and mediating cell migration and anti-microbial defences [1–4]. Initially identified as soluble proteins in 100% saturated ammonium sulfate solution in 1965 [5], S100 peptides are known to be one of the largest protein families with the EF-hand and Ca^{2+}-binding activities. In humans, approx. 25 members have been identified, with 16 S100 genes clusters in chromosome 1q21 and 4 in chromosome 21q22, Xp22, 4p16 and 5q12, respectively [6,7].

The S100 proteins are structurally similar and comprise two similar helix-loop- helix EF-hands for calcium-binding which are separated by a hydrophobic hinge region [8]. The C-terminal EF-hand contains a canonical EF signature motif whilst the N-terminal EF-hand possesses a modified Ca^{2+} binding motif which is unique to the S100 proteins. This canonical C terminal EF motif has a signature sequence of D1xN3xD5xxxxF10xE12 and is dispensable for high affinity binding with Ca^{2+}. Modifications of this motif lead to loss of binding activity with Ca^{2+} [6]. The hinge region is less conserved and is postulated to be responsible for the specific biological functions of individual S100 proteins. In addition to binding Ca^{2+}, S100 proteins can bind other metal ions including Zn^{2+}, Ca^{2+}, and Mn^{2+} [9].

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The S100 proteins form homo- or hetrodimers to exert functions within cells or as secreted proteins [9]. Having no enzymatic activities, they need to interact with other cellular proteins to be active. Once released to the extracellular space by non-conventional secretion pathways, the S100 proteins bind to the cell surface receptor for advanced glycation end products (RAGE) to activate NFkB and MAPK signaling, and to promote apoptosis, inflammation, chemotaxis and cell proliferation [3,4]. It has been shown that calprotectin, a S100A8 and A9 heterodimer, can elevate expression of pro-inflammatory factors and is associated with pathogenesis of several inflammatory diseases in humans [10,11]. Some members of S100 family proteins such as S100A8, S100A9, and S100A12, also termed calgranulins, possess antimicrobial activity [12,13].

The S100 genes are expressed in a wide spectrum of cell types. Immune cells such as neutrophils store a considerable amount of S100A8 and S100A9 protein [14]. Human S100A8, S100A9 and S100A12 are shown to be highly expressed in neutrophils, monocytes, macrophages, fibroblasts, endothelial cells and keratinocytes and can be induced by stress and cytokine stimulation. In cancer cells, S100A5 and S100A7 exhibit marked expression [15]. These data demonstrate that S100 proteins are important players in regulating cell growth and immune responses.

The S100 genes have been reported in fish. *In silico* analyses of fish genomes identified multiple S100 genes in jawed and jawless fish including jawless sea lamprey (*Petromyzon marinus*), cartilaginous fish (*Callorhinchus milii* and *Squalus acanthias*) and several teleost species [16]. Interestingly, the S100 genes are not found in any invertebrate species. To date, 14 S100 genes have been predicted in teleosts, 6 in cartilaginous fish and 10 in sea lamprey [16]. Among the teleost, eight genes could not be assigned as orthologues to mammalian counterparts and are considered to be teleost specific genes. As expected, the predicted fish S100 proteins are structurally conserved and possess two characteristic EF hands. Recent protein crystallography shows that the homodimeric structure of zebrafish S100Z has a tetramerisation interface similar to that of human S100A4 [17]. Zebrafish S100 genes have been shown to be expressed in keratinocytes, hair cells, ionocytes and olfactory receptor neurons in larva [16]. However, apart from the abovementioned studies, there is scarce information about the S100 family proteins in fish. Whether the S100 family proteins are involved in immune defence in fish has not been studied. In the present study, the expression of the S100 family genes was examined in zebrafish after infection of pathogenic bacteria and virus.

2. Materials and methods

2.1. Fish

Zebrafish (*Danio rerio*) (approx. 5 g) were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. Fish were maintained at 25 °C ± 2 °C in aerated tanks and fed with brine shrimp twice daily. Fish were anaesthetized with 0.4% ethyl 3-aminobenzoate methanesulfonate (MS222, Sigma-Aldrich, USA) prior to experimental procedures including injection and killing for tissue sampling. All the experimental procedures were conducted under the local guidelines of use of animals for research and approved by the ethics committee of laboratory animals of Shanghai Ocean University (SHOU-DW-2019-003).

2.2. Sequence validation and analysis

The zebrafish S100 family genes were previously predicted from the genome [16]. To verify the cDNA sequences, primers were designed based on the predicted sequences to amplify the full length open reading frame (ORF). The primers are listed in Supplement Table 1. RT-PCR was performed using the cDNA template mix generated from different tissues including gills, gut, head kidney and spleen. After checking the size by electrophoresis, the PCR amplicons of the predicted size were ligated into the pMD-19T vector (Promega, USA) and positive clones with inserts sent to Sangon Biotech (Shanghai, China) for sequencing.

Table 1
The physicochemical properties of S100 family.

Name	Nucleotide (bp)	Amino acid	Theoretical MW	pI
A1	288	96	10787.35	4.61
A10a	306	102	11636.30	4.86
A10b	300	100	11156.79	5.41
A11	255	85	9228.30	4.89
B	285	95	11109.42	4.41
ICN1	285	95	10407.00	5.04
ICN2	291	97	10715.32	5.04
S	306	102	11353.70	4.56
T	288	96	10960.21	4.49
U	720	240	26755.69	5.02
V1	315	105	11687.27	4.59
V2	300	100	11066.73	6.82
W	309	103	11975.46	5.79
Z	297	99	11238.79	4.69

Sequence homology was obtained using the MatGat programme (Version 2.0). The theoretical molecular weight and pI values were predicted using the PeptideMass programme listed on the ExPASy website (https://www.expasy.org/). Multiple alignment of protein sequences was generated using the DNAMAN programme (Version 6.0). The size of exons and introns was obtained by comparing the genomic sequences retrieved from the Ensembl database (http://www.ensembl.org/index.html) with the corresponding cDNA sequences obtained in the present study. Gene synteny analysis was performed using the genomic data deposited in the Ensembl database.

2.3. Gene expression in embryos and tissues

Zebrafish embryos were sampled for examining S100 gene expression during embryogenesis. Embryos were collected and pooled in an Eppendorf tube at 10 h (50 embryos), 24h (30 embryos), 48 h (30 embryos) and 72 h (30 embryos) after fertilisation. In total, five tubes of embryos were taken at each time point. RNA extraction, cDNA synthesis and qPCRs were performed described below.

Tissues including gills, head kidney, gut, and spleen were sampled from 5 healthy fish for assessing gene expression. Three tissue replicate samples were collected, each containing tissues pooled from 6 individual fish. The tissues were homogenized in TRIzol reagent (Thermo Fisher, USA) using the QIAGEN® TissueLyser II system (Qiagen, Germany) and stored at −80 °C before RNA extraction.

2.4. In vivo injection of fish with poly(I:C)

Two hundred forty fish were randomly divided into 2 groups, each containing 120 fish. The experimental and control group were intraperitoneally (i.p.) injected with 10 μL of poly(I:C) (100 μg/μL in PBS) and 10 μL of PBS, respectively. After 12, 24, 48 and 72 h, tissues including gut and spleen were sampled from 10 fish and pooled into an Eppendorf tube for RNA extraction. For each time point, 3 tubes of tissues from control and experimental group were taken.

2.5. Infection of Edwardsiella tarda (E. tarda)

E. tarda was provided by Dr Haixie Xie, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and prepared according to the protocols described previously [18]. Briefly, *E. tarda* was taken from −80 °C freezer and cultured on tryptic soy agar (TSA) at 28 °C overnight. A single colony was transferred to tryptic soy broth (TSB) medium and shaken at 170 rpm at 28 °C overnight. The culture was

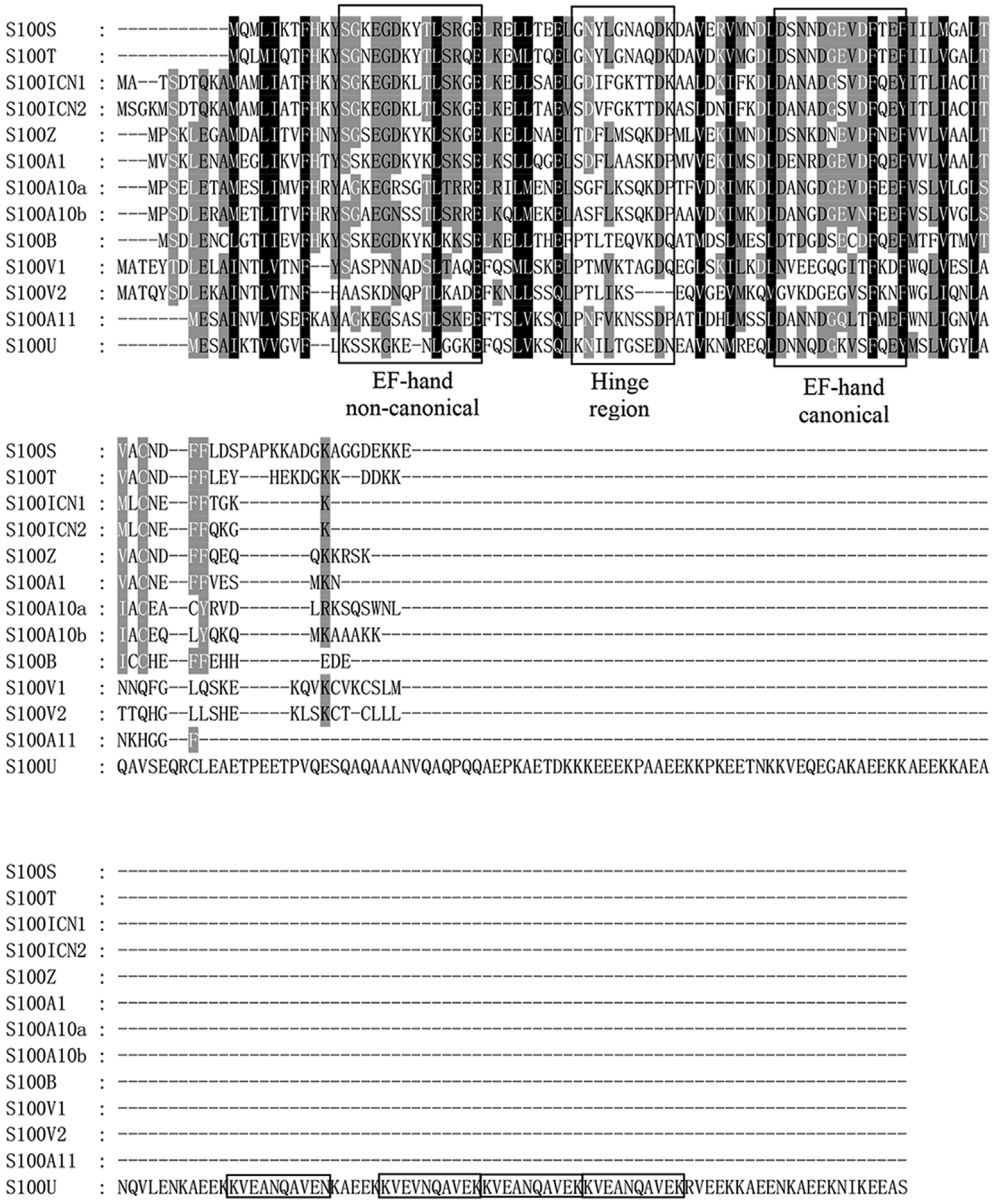

Fig. 1. Sequence alignment of S100 proteins. Multiple alignment was generated the DNAMAN programme. Identical and similar amino acids are highlighted in black and grey respectively. The EF-hand, hinge region and repeats in S100U are boxed.

then diluted 1:10 (v/v) with fresh medium and further cultured for 3 h to reach logarithmic phase. The bacteria were counted using the spectrophotometry and plating dilutions [19].

For challenge experiment, control (120 fish) and experimental group (120 fish) were i.p. injected with 10 μL of PBS or 10 μL of bacterial suspension containing 1×10^5 CFU (colony forming unit) of *E. tarda*, respectively. At 3, 6, 9, 24 and 72 h, tissues including gut and spleen were sampled from 8 fish and pooled into an Eppendorf tube for RNA extraction. For each time point, 3 tubes of tissues from control and experimental group were collected and homogenized in TRIzol reagent using the QIAGEN® TissueLyser II system. The tissue lysates were stored at −80 °C before use.

2.6. Infection of spring viremia of carp virus (SVCV)

Spring viremia of carp virus (SVCV) was propagated in EPC cells at 25 °C in a CO_2 incubator [20]. The EPC cells were infected with SVCV and when the cytopathic effect (CPE) was clear, the supernatant was collected and centrifuged at 4000 g for 30 min. Virus aliquots were stored at −80 °C before use.

Similar to the bacterial challenge experiment, two groups of fish, each containing 120 fish, were i.p. injected with 10 μL of MEM medium (control) or 10 μL of 1×10^5 CFU SVCV solution (challenge group). At days 1, 3, 5, 7 and 14 post injection, tissues including gut and spleen were sampled from 8 fish and pooled for RNA extraction as described below.

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIzol Reagent (Thermo Fisher, USA) according to the manufacturer's protocol. For 1 mL tissue lysate, 200 μL of chloroform was added and vortexed vigorously for 15 s. The lysate solution was then placed on ice for 5 min and centrifuged at 13000 rpm for 15 min at 4 °C. The upper phase was carefully transferred into a fresh 1.5 mL tube and an equal volume of isopropanol added. The solution was then mixed by inverting the tube for several times, placed at room temperature for 15 min, and centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant was discarded and the RNA pellet

Table 2
Nucleotide (lower left panel) and amino acid (upper right panel) sequences were analysed for homology (percentage identity) between members of the S100 family. Highlighted in bold are the highest and lowest conservation between the different members.

	A1	A10a	A10b	A11	B	ICN1	ICN2	S	T	U	V1	V2	W	Z
A1		51.0	54.2	36.5	47.9	51.1	50.0	58.0	55.2	32.6	24.5	24.4	33.7	71.9
A10a	42.5		69.0	36.5	33.7	38.3	38.3	43.6	40.2	26.3	21.0	23.2	34.7	50.5
A10b	45.4	55.9		37.6	36.8	44.7	43.6	45.7	46.2	30.1	30.6	31.2	35.4	57.6
A11	39.7	39.7	40.0		30.6	34.1	32.9	35.8	33.3	37.8	31.3	34.2	36.5	31.8
B	41.6	36.8	40.2	38.4		40.9	43.0	38.6	39.8	24.7	19.4	24.7	28.4	42.1
ICN1	51.5	45.5	48.5	41.6	50.1		**87.4**	58.1	54.7	27.6	22.6	24.7	30.1	51.1
ICN2	50.5	42.3	45.7	39.9	49.0	**80.1**		55.8	51.2	25.3	21.1	23.1	29.0	50.0
S	42.8	35.9	40.1	37.4	39.8	49.5	48.4		80.2	25.3	**18.1**	21.3	29.5	59.3
T	43.2	40.1	39.5	40.3	37.4	48.5	46.8	54.2		28.0	21.1	20.0	28.0	56.7
U	37.0	30.3	35.5	33.8	**29.8**	37.6	38.2	36.3	37.7		22.7	27.2	28.1	26.1
V1	36.6	34.8	36.3	40.7	33.5	42.9	38.5	35.1	36.1	31.7		51.0	19.4	23.7
V2	33.0	36.7	37.2	41.2	34.5	35.7	37.4	34.4	34.2	30.7	38.5		24.7	25.0
W	42.6	37.4	42.4	42.5	37.9	43.3	41.1	38.1	41.8	36.1	34.5	40.5		41.6
Z	56.0	38.1	45.9	42.0	36.5	48.3	47.7	41.8	41.0	31.6	35.5	32.5	32.7	

washed twice with 1 mL of 75% ethanol. The RNA pellet was dried briefly at room temperature and dissolved in RNase-free water. The RNA concentration was quantitated using a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA) and the first-strand cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) which contained the gDNA eraser enzyme for removal of contaminated genomic DNA. The resultant cDNA samples were stored at −20 °C until use.

2.8. Quantitative real time PCR (qPCR) analysis

The information of primers for qPCR analysis is given in Supplement Table 1. The primers were tested using RT-PCR and the resultant PCR amplicons ligated into pMD-19T vector (Promega, USA). The ligation reaction was transformed into competent *E. coli* DH5α cells and positive clones were screened for plasmid preparation using the plasmid Mini Kit I (OMEGA, USA). Three clones were sequenced to confirm primer specificity. After sequence verification, plasmid DNA was diluted in a series of 10 fold. Gene copy numbers were calculated and used for establishing the standard curve for qPCR quantitation of gene expression.

The qPCR reaction was set up as follows: 5 μL SYBR® Green PreMix Ex Taq™ II (YEASEN, China), 1 μL cDNA template, 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), and 3.6 μL H_2O. The qPCR reactions were performed using the Roche LightCycler® 96 system (Roche) using the following conditions: 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 62 °C for 30 s, 72 °C for 10 s, followed by 1 cycle of 95 °C for 10 s, 65 °C for 60 s, 97 °C for 1 s. The elongation factor 1 alpha (EF1α) was used as an internal reference gene to normalize gene expression. Fold change of gene expression was calculated by comparing the expression level of experimental group with that of respective control group (defined as 1).

2.9. Statistical analysis

The SPSS 17.0 software package was used for statistical analysis of data generated by quantitative real-time PCR. For the challenge experiments, the expression data were analysed by One-way analysis of variance, followed by the LSD post hoc test, with $P < 0.05$, $P < 0.01$ or $P < 0.001$ considered significantly different between experimental group and control group.

3. Results

3.1. Sequence analysis

A previous study performed *in silico* analysis on the S100 family genes in telelosts including zebrafish [16]. Surprisingly, the predicted sequences were not verified and few follow-on studies were undertaken in lower vertebrates. In the present study, the ORF sequences of 14 S100 genes were confirmed by RT-PCR cloning and their genomic organizations determined (GenBank accession numbers: MK622798-810). Akin S100 peptides in higher vertebrates, the zebrafish S100 peptides translated from the ORFs were small in size, ranging from 85 aa to 105 aa, and lacked a canonical signal peptide (Table 1) with the exception for the zebrafish S100U which consisted of 240 aa and contained several KE rich repeats at the C-terminal region (Fig. 1). All the zebrafish S100 peptides possessed two conserved EF-hand motifs which were separated by a hinge region. The predicted pI values of zebrafish S100 peptides were relatively low, between 4.41 and 5.41, except for the S100V2 which had a pI value of 6.82 (Table 2), suggesting that they were negatively charged. Despite the conserved common features, zebrafish S100 molecules shared low sequence homology at both the nucleotide and protein level (Table 2).

By comparing the genomic sequences and corresponding cDNA sequences, the genomic structure of zebrafish S100 genes was determined (Fig. 2). Interestingly, all the S100 genes had 3 exons and 2 introns, a conserved organization also shared by majority of the human S100 genes. Although the putative S100 peptides were small, the genes encoding some of the peptides including S100S (13,403 bp), S100U (15,722 bp) and S100Z (19,663 bp) contained large introns (Figs. 1 and

Fig. 2. Genomic structure of the zebrafish S100 genes. The size of exons (black box) and introns (line) is indicated.

2). In a previous study, four chromosomes were identified to harbor the S100 genes in the zebrafish genome [16]. However, re-analysis of these loci in the present study revealed that gene synteny between zebrafish and humans was remarkably conserved (Fig. 3). Six genes including S100A1, S100A10b, S100ICN1, S100ICN2, S100T and S100V1 were clustered in zebrafish Chr16 and linked with the cct3 gene. The other major S100 locus was located in the Chr19 where 5 genes including S100A10a, S100A11, S100S, S100U and S100V2 were placed in a region containing the rit1 gene. The rit1 and cct3 gene were also seen to be situated in the human S100 locus where 16 S100 genes were tandemly arranged. These synteny data suggest that the two S100 loci in zebrafish Chr16 and Chr19 could have split from an ancestral locus that was possibly linked with the rit1 and cct3 gene. It is likely that this event could have occurred in the ancestor of ray-finned fish since the rit1 and cct3 gene were present in the same S100 locus in the spotted gar (unpublished data). The data also imply that the S100 locus in zebrafish Chr21 and in human Chr4/Chr5 shared a common origin and so did that in zebrafish Chr22 and human Chr21/ChrX (Fig. 3).

3.2. Constitutive expression in embryos and tissues

The expression of S100 family genes were examined in embryos (Fig. 4). At 10 h post fertilization (hpf), the S100A1 was expressed at a relatively high level whilst the S100ICN1, ICN2, S, T, U, V1, V2, and W at moderate levels. Negligible expression was seen for S100A10a, A11, B, and Z. At 24 pfh, the highest expression was detected for S100W, followed by S100ICN1, V1, ICN2, A10b, A11 and V2, whilst that others was low. Notably, the transcripts of S100A1 decreased significantly. At 48 pfh and 72 pfh, the expression levels of S100W, ICN1 and ICN2 remained high whilst the rest of S100 genes were weakly expressed.

To determine the tissue expression of S100 family genes, 4 tissues including systematic (head kidney and spleen) and mucosal (gills and gut) immune tissues were analysed by qPCR analysis (Fig. 5). In general, the S100 genes were constitutively expressed at a higher level in mucosal tissues (gills and gut) than systemic tissues (head kidney and spleen). For example, the transcript levels of S100V1 and S100V2 were much higher in gills and gut than that in head kidney and spleen. At the gene level, S100A1 (in all tissues), S100A10a (in gut), S100A11 (in gills) and S100ICN1 (in gills) were relatively highly expressed. In the head kidney, the S100A1 transcript level was shown to be the highest, followed by S100ICN1 and S100T.

3.3. Modulation of gene expression by poly(I:C)

Poly(I:C) is a synthetic double stranded RNA analog of viral nucleotide pathogen associated molecular patterns and is able to activate antiviral response in fish. To assess its effects on S100 gene expression, zebrafish were i.p. injected with poly(I:C) (10 μg per 5 g fish) and gut and spleen collected for assessing gene expression by qPCR. At 12 h post injection, 8 genes including S100A10b, S100A11, S100S, S100T,

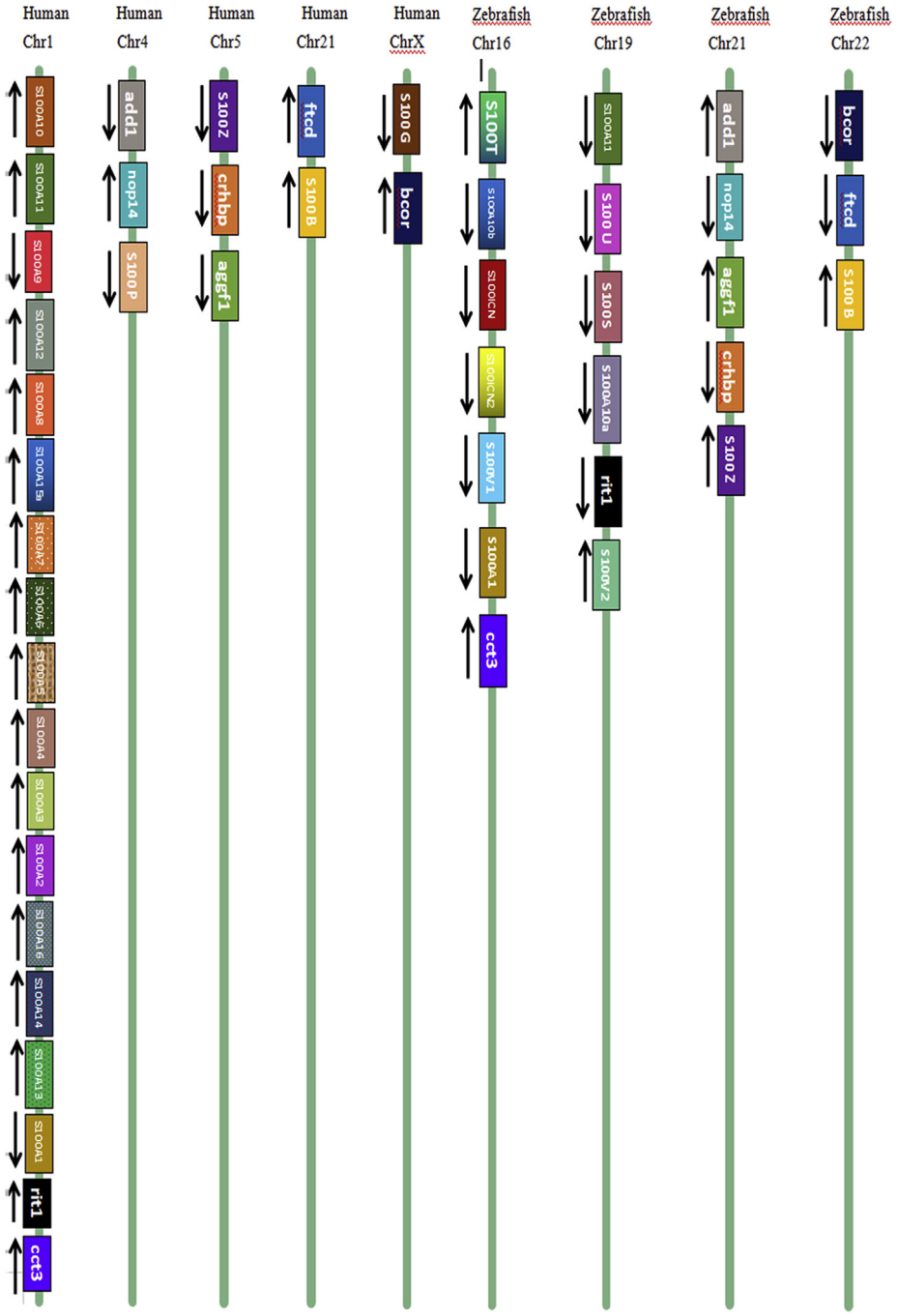

Fig. 3. Gene synteny analysis of zebrafish S100 genes. Gene synteny data were obtained from the Ensembl database. The orthologues between humans and zebrafish are shown in the same colour. Arrows indicate transcription orientation (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

S100U, S100V1, S100V2 and S100Z were significantly up-regulated in the gut whilst S100A1 and S100ICN2 were down-regulated (Fig. 6). Strikingly, the expression of S100Z increased 24 fold compared to control. Conversely, in the spleen, none of the S100 genes was up-regulated whilst S100A1, S100T, S100V1, S100V2 and S100ICN2 were down-regulated. At 24 h, only S100V2 and S100Z were induced in the gut, with the expression of other genes either inhibited or unaltered.

3.4. Gene expression after challenge with SVCV

To further understand the roles of S100 genes in antiviral response, zebrafish were infected with SVCV, a double stranded negative RNA virus causing high mortalities in cyprinids. Fish were i.p. injected with SVCV 1×10^5 CFU [21]. Gut and spleen were examined for gene expression by qPCR during the 14 days infection period. Fig. 7 showed that at day 1 post injection, alteration of S100 gene expression was apparent. Up-regulation of expression was detected for S100A1, S100A10b, S100B, S100ICN1, S100T, S100U, S100V1 and S100Z in the spleen but not in the gut. The S100A11 was decreased in the gut. The most increases (> 5 fold) were seen for S100T, S100V1 and S100Z. However, gut displayed decreased expression for most genes including S100A10a, S100A10b, S100B, S100ICN1, S100ICN2, S100S, S100V1 and S100V2. At day 3, the numbers of genes modulated by SVCV reduced significantly in both tissues, with a moderate increase of

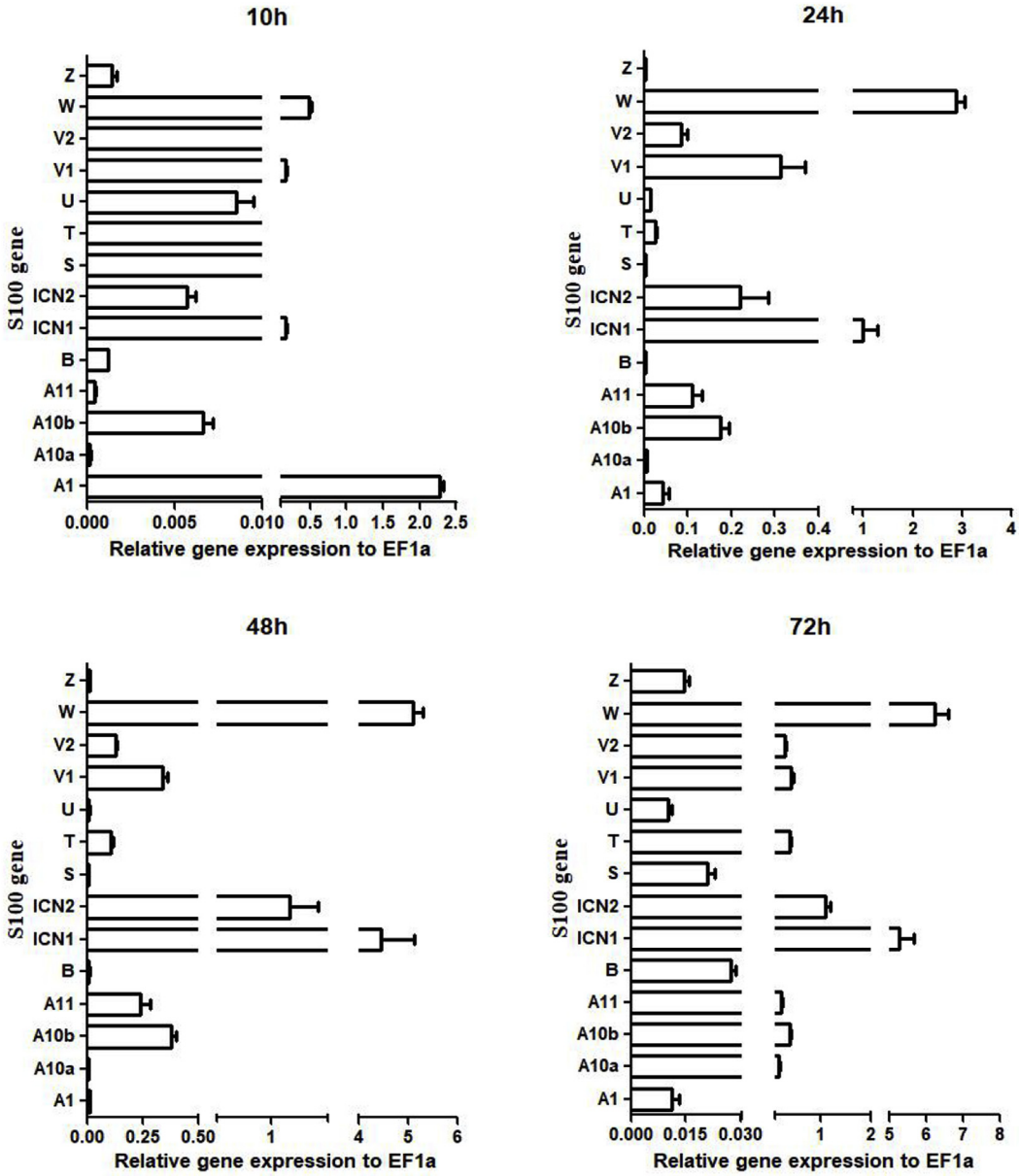

Fig. 4. Expression analysis of S100 family genes during embryogenesis. Embryos were collected and pooled in an Eppendorf tube at 10 h (50 embryos), 24 h (30 embryos), 48 h (30 embryos) and 72 h (30 embryos) after fertilization and 5 sets of samples for each time point were used for qPCR analysis. The expression of S100 genes was normalized to that of EF1a. Data are presented as the mean + SD (n = 5).

expression detected for S100B, S100S and S100U in the spleen. There was no change of gene expression in the gut compared to the control group (PBS). At day 5 and 7, the S100 expression was largely unaffected by SVCV infection and showed no clear patterns. At day 14, similar to the expression patterns at day 1, induced S100 gene expression of S100A10a, S100A10b, S100B and S100ICN2 was detected only in the spleen. Down-regulated genes included S100A1, S100S, S100T, S100V1 and S100V2 in the spleen and S100A1, S100T and S100U in the gut.

3.5. Gene expression after challenge with E. tarda

E. tarda is an intracellular bacterial pathogen infecting a wide range of fish species. To examine the kinetics of S100 expression, fish were challenged with 1×10^5 CFU *E. tarda* by i.p. injection and gut and spleen were sampled at 3, 6, 9, 24 and 72 h for qPCR analysis. Overall, among the genes responsive to infection, there were more genes down-regulated than that up-regulated in the spleen before 24 h (Fig. 8). For example, there were 10 genes down-regulated in the spleen at 9 h. Highly constitutive expressed genes such as 100A10a, S100T and S100Z exhibited a significant decrease of transcripts in the gut at the early stage of infection (at 3, 6 and 9 h) (Figs. 5 and 8). However, at 72 h, most S100 genes showed increases of expression. Up-regulated genes were detected mostly in the gut, especially during the early stage of infection (at 3 and 6 h) (Fig. 8). For example, transcripts of S100A, S100A10a and S100ICN2 significantly elevated in the gut at 3 h after injection and so did that of S100A10b, S100B, S100ICN1, S100ICN2 and S100V1 at 6 h. In the spleen, the S100ICN1 and S100ICN2 gene were shown to be consistently induced.

4. Discussion

The S100 proteins are key intracellular calcium sensors that play an important role in mediating calcium-associated cellular responses that affect calcium homeostasis, cell mobility, cell differentiation and cell

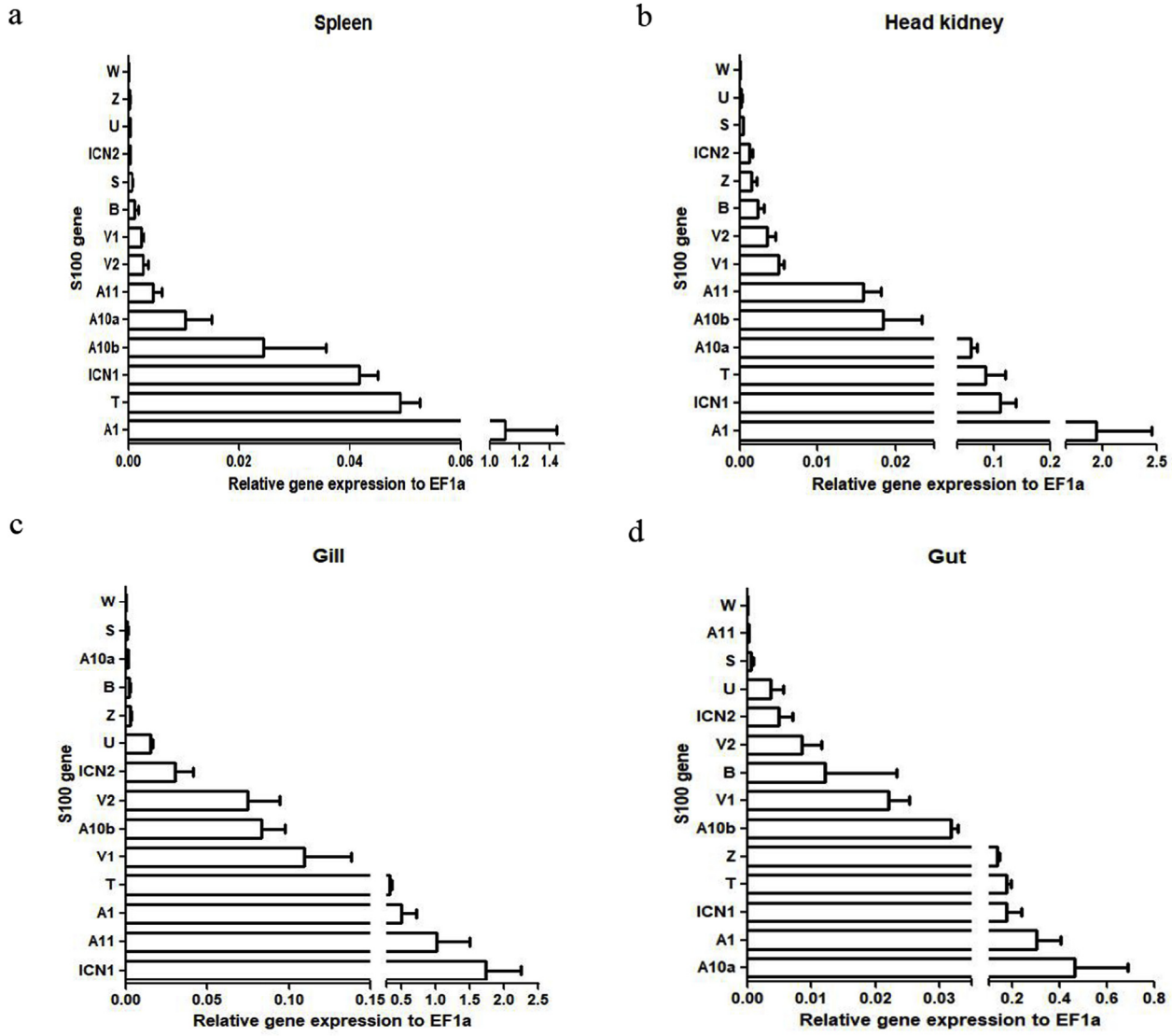

Fig. 5. Tissue expression of zebrafish S100 genes. Tissues from 6 fish were pooled for RNA extraction and qPCR analysis. The expression of S100 genes was normalized to that of EF1a. Data are presented as the mean + SD (n = 3).

survival [2,11]. In the present study, 14 S100 genes were sequenced in zebrafish and their transcriptomic modulation after infection of bacterial and viral pathogens was investigated. It has been shown that the expression of S100 family could be modulated differentially in response to bacterial and viral infection. The results provide insights into the evolution and functions of S100 proteins in immune defence in teleost fish.

Sequence data indicate that the zebrafish S100 proteins possess several conserved features. They are acidic, small in size (except for zebrafish S100U), and contain a typical S100 family structure of 2 EF-hands separated by a hinge region. The EF-hand signature of D-x-[DNS]-[ILVFYW]-[DENSTG]-[DNQGHRK]-[GP]-[LIVMC]-[DENQST-AGC]-xx-[DE] is present in all the zebrafish S100 proteins. A recent study suggests that the S100 proteins underwent strong selection [11]. It should be noted that the zebrafish S100U has an extended acidic C-terminal tail containing 4 consensus repeats of K-V-E − [AV]-Q-A-V-E − [KN], whose biological functions are unclear.

Four S100 gene loci have been reported in the zebrafish genome [16]. However, re-analysis of these loci in the present study unveiled novel findings about the evolution and phylogeny of S100 family in fish. It is evident that the two major loci accommodating most of the S100 genes in zebrafish are located in two different chromosomes and linked with the rit1 and cct3 gene respectively. In contrast, in spotted gar and humans, the corresponding chromosomal locus harbouring the majority of S100 genes is also clustered with the rit1 and cct3 gene, suggesting that this S100 locus could have split into two loci as seen in zebrafish (Fig. 3). It has been proposed that teleosts have undergone a third whole genome duplication event after split from the ancestor of tetrapods 350 million years ago [22]. Since the S100 proteins are small in size, it is not reliable to determine the definite orthologous relationships of S100 genes seen in zebrafish Chr16 and Chr19 with that located in human Chr1 by phylogenetic tree analysis. In addition to the aforementioned 2 S100 loci, zebrafish have 2 additional loci located in Chr21 and Chr22, each containing a single S100 gene. This is in contrast to that in humans where 4 S100 loci are found in Chr4, Chr5, Chr21 and ChrX, all containing a single S100 gene. Conserved gene synteny of zebrafish S100B locus (clustered with the bcor and ftcd gene) with the two human loci comprising S100B/ftcd and S100G/bcor suggests that they share a common ancestral origin. Similarly, the S100Z locus in zebrafish Chr21 is homologous to the two human loci containing S100Z and S100P. It seems probable that the S100G and S100P seen in humans could have been duplicated from the S100B and S100Z respectively.

The expression patterns of S100 genes differ significantly in

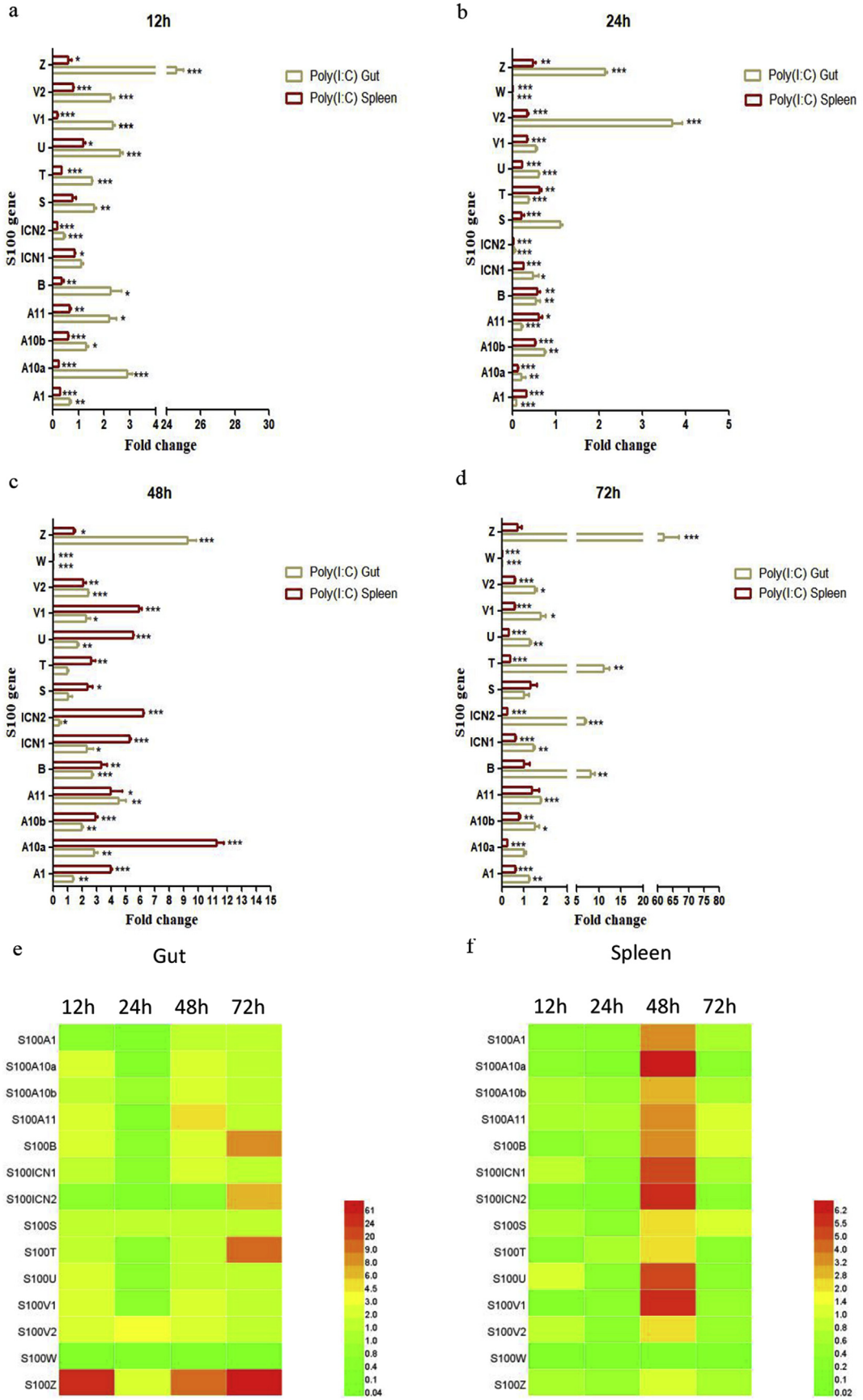

Fig. 6. Expression patterns of S100 genes in response to poly(I:C). Gut and spleen were sampled and pooled from 10 fish for RNA extraction and qPCR analysis. The expression of S100 genes was normalized to that of EF1a. Fold change of gene expression was calculated by comparing the expression level of poly(I:C) treated group with that of control group. Data are presented as mean + SD (n = 3). $P < 0.05$, $P < 0.01$ and $P < 0.001$ are considered significantly different and indicated by *, ** and ***, respectively. The heatmap of gene expression was generated using the Heatmap Illustrator 1.0 programme.

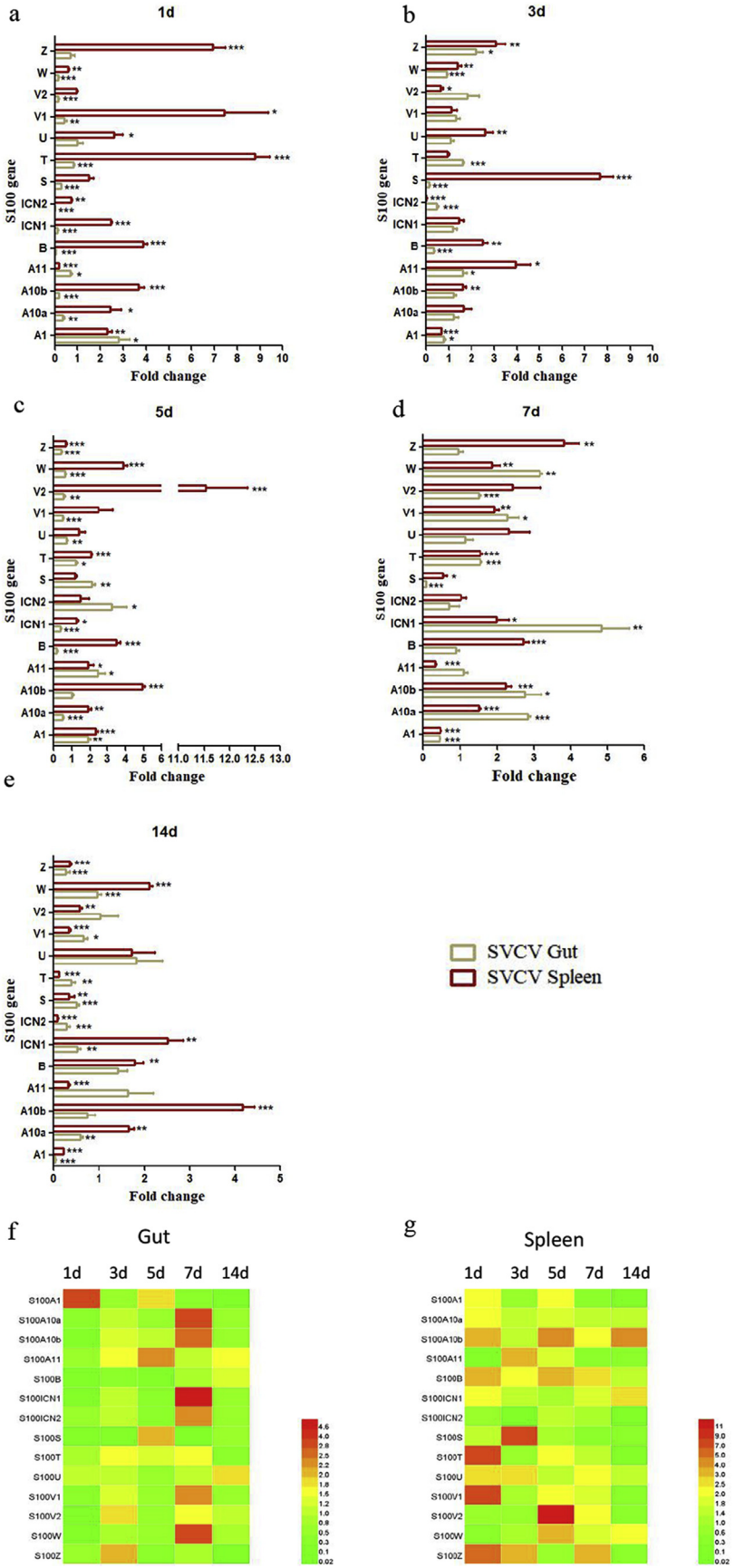

Fig. 7. Expression patterns of S100 genes after infection of SVCV. Gut and spleen were sampled and pooled from 10 fish for RNA extraction and qPCR analysis. The expression of S100 genes was normalized to that of EF1a. Fold change of gene expression was calculated by comparing the expression level of SVCV infected group and control. Data are presented as mean + SD (n = 3). $P < 0.05$, $P < 0.01$ and $P < 0.001$ are considered significantly different and indicated by *, ** and ***, respectively. The heatmap of gene expression was generated using the Heatmap Illustrator 1.0 programme.

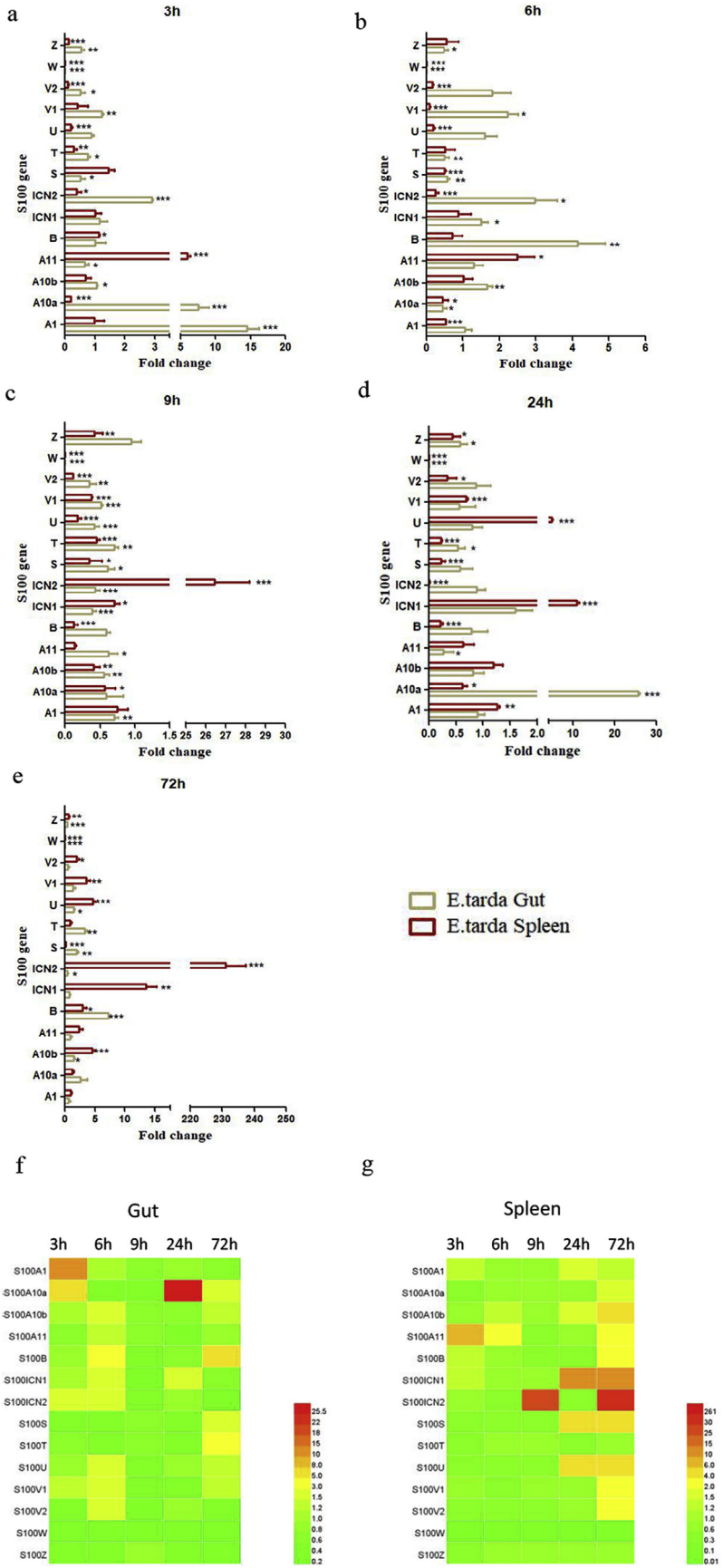

Fig. 8. Expression patterns of S100 genes after infection of *E. tarda*. Gut and spleen were sampled and pooled from 10 fish for RNA extraction and qPCR analysis. The expression of S100 genes was normalized to that of EF1a. Fold change of gene expression was calculated by comparing the expression level of *E. tarda* infected group and that of control. Data are presented as mean + SD (n = 3). $P < 0.05$, $P < 0.01$ and $P < 0.001$ are considered significantly different and indicated by *, ** and ***, respectively. The heatmap of gene expression was generated using the Heatmap Illustrator 1.0 programme.

embryos and adult tissues (Figs. 4 and 5). Notably, the S100W and ICN2 are highly expressed in embryos 24 h after fertilization but weakly in tissues including gills, gut, head kidney and spleen. Conversely, the opposite expression patterns were observed for the S100A1. These results indicate that the S100 genes are regulated differentially in embryos and adult fish. Although the biological activities of the S100 family remain to be characterized, it is reasonable to speculate that they are likely to function at different developmental stages.

Constitutive expression of S100 genes were examined in 4 major immune tissues in zebrafish (Fig. 5). Gills showed highest expression levels of S100 genes relative to other tissues. This finding perhaps could explain the fact that gills serve as the central organ for gas and ion exchange in fish and that as sensors for ions such as calcium, S100 proteins could play vital roles in regulating gill osmoregulation and hemostasis. At the individual gene level, relatively high expression was seen for S100A1, S100ICN1 and S100T, and S100A10b, suggesting that they are the key players. The results are consistent with the findings in humans that S100A2-4 and S100A6-12 are expressed primarily in the epidermis or keratinocytes [23–26].

The S100 proteins are known to play important roles in immune response to infections in mammals. Studies in humans have shown that they are overexpressed under inflammatory conditions and are linked with disease pathogenesis [27]. Some S100 family members such as S100A8, S100A9 and S100A12 can be modulated by bacterial and viral infection and are shown to be directly or indirectly involved in pathogen killing, either as antimicrobial effectors or as damage-associated molecular pattern molecules [27,28]. Furthermore, the S100 proteins are found to be secreted and act as chemoattracts to co-ordinate migration and chemotaxis of immune cells to infection sites through activation of cell surface RAGE or TLRs. The present study, for the first time, demonstrates that the S100 genes can be modulated by bacterial or viral infections and supports the notion that their immunological functions may be conserved in teleost fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.051.

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