Construction of irf4a Transgenic Zebrafish Using Tol2 System and Its Potential Application

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Abstract

Purpose: Interferon regulatory factor 4 (IRF4) is identified as a transcriptional factor and plays an important role in the immune response in mammals; however, there are few reports about the function of zebrafish IRF4.

Methods: We first amplified the coding sequence of irf4a from the testis of zebrafish. Besides, the fragments of irf4a, P2A, EGFP, and Tol2 vector were added for homologous recombination. By sequencing, we can get the Tol2-ef1α-irf4a-EGFP recombinant plasmid and it was microinjected into zebrafish embryos. Fluorescence observation was proceeded at days 3 post fertilization; F0 generations expressing green fluorescence in multiple tissues throughout the body were screened as the founder and raised them to sexual maturity. After mating with WT zebrafish to generate F1 offspring, polymerase chain reaction was used to identify whether irf4a was integrated into the zebrafish genome.

Conclusion: We obtained the systematic overexpressed irf4a transgenic zebrafish with green fluorescence labeled in spine, eyes, heart, brain, and other tissues. The transgenic zebrafish will be used as a tool for the role of IRF4a in the immune response to the inflammation preconditioning in the future study.

Keywords
transgenic, irf4a, Tol2, microinjection, inflammatory preconditioning

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.1 The pathogenesis of sepsis is complex and involves multiple aspects such as inflammation, immune response, and coagulation dysfunction.2-4 The morbidity and mortality of sepsis remain high,5 which makes the search for therapeutic intervention for sepsis an urgent need for innate immune research. Studies have shown that the most common pathogen of sepsis is Gram-negative bacteria, and the most representative is Escherichia coli.6 In early studies, we have established a sepsis model in mice by intraperitoneal injection of lethal dose of E coli. In the process of drug screening, it was found that intraperitoneal injection of a lower dose of E coli into mice in advance allowed it to obtain resistance to the subsequent lethal dose of E coli and survived, and we termed this phenomenon as “inflammatory preconditioning (InP).”7

Interferon regulatory factor (IRF) is an important class of transcription factors which was originally found in the promoter region of the human interferon β gene.8 So far, 10 members of the IRF family have been found in vertebrates (IRF10 are eliminated in humans and mice).9 Studies have shown that IRF plays an important role in the regulation of innate and adaptive immune responses.10 Interferon regulatory factor 4, like other members of IRF family, contains a highly conserved N-terminal DNA-binding domain (DBD) and a non-homologous C-terminal IRF association domain (IAD).11 The expression of IRF4 is restricted to immune cells and is not

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induced by IFNs. As is reported, IRF4 can function as a transcriptional activator or repressor, depending on the ligand with which it interacts.

Zebrafish (Danio rerio), a small tropical fish, has been widely used to study development, homeostasis, and the pathogenesis of human diseases. Compared with rodent models, it has many advantages. For example, adult zebrafish can produce a large amount of embryos once mating, which is convenient for drug screening on certain diseases. Coupled with the optical transparency and mature micromanipulation of zebrafish embryo, real-time imaging and dynamic tracing can be realized in vivo in transgenic zebrafish with fluorescent protein labeling, which provides great convenience for the study of migration of specific cells or proteins in the disease-related signaling pathways.

Previous studies by our team showed that IRF4 was involved in the immune response to the InP in mice. The expression level of IRF4 was increasing with the prolongation of the preconditioning time. Besides, detection of peripheral blood in mice showed increased levels of anti-inflammatory cytokine interleukin 10 (IL-10), intimating that IRF4/IL-10 anti-inflammatory signaling pathway was activated in InP induced by E. coli. As a result, it inhibited the further extension of inflammatory response and avoided excessive tissue damage. To deepen our understanding of the activation of inflammatory responses in vivo, animal models need to be established. In the past few decades, zebrafish has gradually become an indispensable model organism in immunological research due to its fast life cycle, transparent embryos, easy genetic manipulation, large sample size, and other advantages. There are 2 homologues of IRF4 in zebrafish, IRF4a and IRF4b. Phylogenetic analysis revealed that irf4a has higher homology with mammalian irf4. Therefore, we constructed a transgenic zebrafish with systemic overexpression of irf4a to identify the role of IRF4a in InP and related immune signaling pathways in zebrafish.

### Materials and Methods

#### Breeding of Zebrafish

Zebrafish is a wild-type (WT) AB line and was purchased from the China Zebrafish Resource Center. The feeding scheme was described in the Zebrafish Book. Fertilized eggs were obtained by mating females and males in the early morning. Zebrafish embryos were cultured in the aquaculture water in the temperature-light incubator under a 14/10 light/dark regime at 28.5 °C. Microinjected embryos need to be cultured in E2 water. Pick out dead eggs in time after injection to avoid mold growth. The animal protocol was approved by the Scientific Investigation Board of Science and Technology.

#### Construction of the Recombinant Plasmid

First dissect and collect testis from 5 to 10 adult WT zebrafish in 1.5-mL centrifuge tube, drain the fish water, and add 1 mL TRIZol Reagent (Invitrogen) for the extraction of total RNA according to the manufacturer’s instructions. After that, add 8 μL Nuclease-free (NF) water to dissolve the RNA sufficiently and then reverse transcription was performed using the complementary DNA (cDNA) First Strand Synthesis Kit according to the manufacturer’s instructions (TransGen). Using the cDNA as template, the irf4a coding sequence (CDS) was amplified by polymerase chain reaction (PCR) using gene-specific primers (irf4a-F1 and irf4a-R1). Then the recovery product of target gene was used as template to amplify the coding region of irf4a by PCR amplification using primers irf4a-V-F and IRF4a-P2A-R1. The product recovered from the above step was used as template, and the irf4a gene with P2A2 was amplified with primers irf4a-V-F and P2A-R2 to generate a homology arm for homologous recombination with the vector backbone. Finally, the Tol2-egfp-sv40 plasmid we constructed earlier was used as a template to amplify the sequence of EGFP and the vector backbone linked with P2A3 by primers Vector-F and Vector-R. The PCR system and reaction procedure were according to the manufacturer’s instructions (Takara). The sequences of primers are shown in Table 1.

The linearized Tol2 vector and the fragments of target gene were mixed at a ratio of 1:3 using Minerva Super Fusion Cloning Kit (US Everbright), and the homologous recombination was performed at 50 °C for 20 minutes according to the manufacturer’s instructions. Add 10 μL of ligation reaction solution to 100 μL of competent cells, mix gently, and place on ice for 30 minutes. Heat shock at 42 °C for 90 seconds, then quickly put in ice-bath for 2 minutes. Add 200 μL LB liquid medium and shake for 30 minutes at 37 °C with the speed of 200 rpm.

### Table 1. Primers Used in PCR.

| Primer       | Sequence (5′-3′)                              |
|--------------|-----------------------------------------------|
| Irf4a-F1     | ATGAACATTAGATGGGACTGC                         |
| Irf4a-R1     | GTCGATGCAGATGGATTG                           |
| Irf4a-V-F    | CAAAGAATTCCTCGGCACCATGAACTTAGATGGGACTGC      |
| Irf4a-P2A-R1 | GAAATTAGTAGCTCCCGGATCCCTCTGTCAGGTGTAGACT      |
| P2A-R2       | TCAACATCACCAGGTCTTGAAGAACATTAGTGACTCCGGATC    |
| Vector-F     | AAGCAAGCGGCGATGCGTTGGAACGATCCCGTCCGGGAGCAAGGGCGAGGA |
| Vector-R     | GGTGGCCGAGGAATTCTTTGCA                       |

Abbreviation: PCR, polymerase chain reaction.
The transformation product was then spread on LB solid medium containing ampicillin and incubated at 37 °C overnight.

**Sequencing and Screening of Recombinant Plasmids**

Three to 5 single colonies were selected from the transformed plates, and add 100 μL LB liquid medium for amplification culture. Sequencing was performed using M13 universal primers (Sangon). The correct recombinant plasmid was screened by sequence alignment and extracted by Endotoxin-free Plasmid Extraction Kit (Tiangen), according to the manufacturer’s instructions. Finally, add 50 μL NF water to dissolve the Tol2-ef1a-irf4a-EGFP recombinant plasmid. After measuring the concentration, it was diluted to 50 ng/μL for microinjection and stored at −70 °C.

**Microinjection of Tol2-ef1α-irf4a-EGFP Plasmid**

Transcription of the transposase gene in vitro. The pCS-TP plasmid (a generous gift from Xinjia) was linearized with XbaI restriction enzyme, and transcription was performed by the mMESSAGE mMACHINE SP6 Transcription Kit (Ambion), according to the manufacturer’s instructions. Then the product was purified by lithium chloride precipitation method. And finally the transposase messenger RNA (mRNA) was resuspended in 20 μL NF water. Determine the RNA concentration and diluted to 50 ng/μL for microinjection. Store frozen at −70 °C.

Solution preparation. For the experimental group, mix the transposase mRNA with Tol2-ef1α-irf4a-EGFP recombinant plasmid in equal proportions (with the final concentration of both were 25 ng/μL); for the positive control group, mix the transposase mRNA with Tol2-ef1α-EGFP plasmid (same concentration as above); for the negative control group, mix the transposase mRNA with Tol2-ef1α (C0); for the positive control group, mix the Tol2-ef1α-irf4a-EGFP plasmid (same concentration as above); for the negative control group, mix the Tol2-ef1α-irf4a-EGFP plasmid (same concentration as above). About 200 eggs were injected, and the injection volume of each fertilized egg was about 1 nL.

Microscopy and Screening of irf4a Transgenic Zebrafish

The injected embryos were transferred to a petri dish containing E2 water and cultured in a constant temperature incubator at 28.5 °C. The expression of fluorescence was observed at days 2 to 3 post fertilization. Select F0 generations that express EGFP in multiple tissues of the body and maintain them to sexual maturity. F0 was then mated with AB line zebrafish to produce F1 offspring. If F1 larvae can express green fluorescence normally as the parent fish, then F0 can be used as a founder. Furthermore, irf4a should be amplified by PCR to identify whether it is integrated into the F1 zebrafish genome, and finally, we can obtain stably expressed Tg (ef1α: irf4a, EGFP) zebrafish. Microscopy of irf4a transgenic zebrafish larvae was performed using the fluorescence microscope (Olympus V1.18). Images were processed using the Olympus Capture software.

**Results**

Sequence Characterization and Structure Analysis of Zebrafish irf4a

The CDS of *Danio rerio* irf4a (accession no. NM_001122710.1) consists of 1383 nucleotides which encodes 460 amino acids (AAs). Sequence analysis showed 2 conserved domains of protein IRF-4a, called IRF (17aa-122aa) and IRF-3 (247aa-428aa), as shown in Figure 1B. These 2 domains correspond to the DBD and IAD domains of the IRF family, respectively. We can know from the tertiary structure of the 2 conserved domains predicted by the SWISS-MODEL website that both of them contain the basic forms constituting the secondary structure of protein, such as α-helix, β-sheet, and β-turn. The difference is that the IRF-3 domain contains more α helices, and the adjacent α helices are connected by regular β-turn, which is supposed to be more stable, while IRF domain consists of more β turns, which is presumed to be more flexible and easier to bind with other proteins and factors. Studies have shown that each member of IRF family contains a conserved N-terminal region of about 120 AAs, which folds into a structure that binds specifically to the interferon consensus sequence. That is consistent with our speculation.

Then we predicted the secondary structure of IRF4a using Protein software (Figure 1C), in which α-helices and β-sheets are evenly distributed, while β-turns and random coils are permuted in dense fragments at the site of 140 to 220 AAs. Kyte-Doolittle analysis showed that IRF4a had high hydrophilicity and few hydrophobic regions. Notably, most of the regions with high antigen index coincide with the hydrophilic regions, indicating that segments forming the epitope are more hydrophilic. Furthermore, the surface probability plot predicted by the Emini method are mainly distributed at the site of 40 to 60, 160 to 170, 280 to 290, 300 to 310, 350 to 360, and 400 to 410 AAs, indicating that the AA residues in these regions are likely to be located on the surface of protein IRF4a.
The Schematic Diagram of Constructing irf4a Transgenic Zebrafish

The Toll-ef1α-EGFP-SV40 plasmid was used as the vector backbone and should be linearized. As shown in Figure 2, the same sequence as both ends of irf4a was generated on the linearized Tol2 vector by PCR amplification. As well, the target gene should produce a sequence homologous to the ef1α promoter at the 5′-end and a “self-cleaving” 2A peptide (P2A) at the 3′-end, and the P2A is correspondingly amplified on the vector. Then the linearized Tol2 plasmid was mixed with irf4a for homologous recombination. The recombinant products were directly transformed into DH5α competent cells and spread on the LB medium with ampicillin for overnight culture. After that, select several single colonies for sequencing and those without mutations should be reactivated to extract the endotoxin-free irf4a recombinant plasmid.

To increase the efficiency of generating stable irf4a-expressing lines, the Tol2-ef1α-irf4a-EGFP plasmid and Tol2 transposase mRNA were coinjected into zebrafish embryos at one-cell stage. Subsequently, the juveniles expressing green fluorescence was selected and raised to sexual maturity (F0 generation). For further screening the founder fish,22 F0 was mated with WT zebrafish to produce F1 offspring, and the possibility of F0 as a founder was based on the efficiency of EGFP expression in F1 embryos. The zebrafish genome of F1 generation with green fluorescence was used as a template to amplify irf4a partial sequence and sequencing to identify whether target gene was integrated into genome (data not shown). In this way, we can eventually acquire the stably inherited F1 of irf4a transgenic zebrafish.

Cloning of irf4a and Linearization of Tol2 Vector

We employed nested PCR to amplify irf4a and ligated P2A to the 3′-end of the CDS of target gene by overlapping PCR. As shown in Figure 3A, the full length of irf4a coding region is
target gene by PCR for homologous recombination. As shown in Figure 2D, P2A3, which overlaps 20 base pairs with P2A2, was ligated to the Tol2 vector. And the length of the linearized vector with homology arms is 5.5 kb. By the way, the electrophoretic band is affected by the amount of sample loaded, the concentration of agarose gel, and the voltage. For example, there is a deviation in the band width and the swimming rate among each group shown in Figure 3C, which may be due to the unstable voltage. As a result, it leads the sample to form a groove in electrophoresis, resulting in visual deviation.

Homologous Recombination and Identification of Tol2-ef1α-irf4a-EGFP Plasmid

The linearized Tol2 plasmid was homologously recombined with irf4a and transformed into competent cells. Then we selected 1 to 5 single colonies for expanded culture and plasmid extraction. As shown in Figure 4A, the molecular weight of group T2 was larger than that of the original Tol2 plasmid, and it’s most likely a recombinant plasmid. Sequencing alignment revealed no mutation in the coding genes. Therefore, T2 was the correct Tol2-ef1α-irf4a-EGFP recombinant plasmid. Figure 4B depicts the map of the recombinant plasmid. 5’-Tol2 and 3’-Tol2 can transpose in the presence of transposase and integrate the internal genes into the host genome without causing any rearrangement and modification at the target site. P2A mediated the “cleavage” between 2 adjacent proteins, enabling irf4a and EGFP to be expressed separately. SV40 poly (A) was used as a termination signal to terminate the transcription of EGFP, and it can enhance the stability of mRNA and improve translation efficiency. It should be noted that irf4a and the EGFP-encoding gene are ligated in series with no termination, and both of them are driven by the ef1α promoter.

Fluorescent Screening of irf4a Transgenic Zebrafish

The Tol2-ef1α-irf4a-EGFP recombinant plasmid was extracted without endotoxin and mixed with transposase mRNA for microinjection. Among the embryos injected with irf4a recombinant plasmid, about one quarter expressed green fluorescence. And most of them were expressed in spine, tail, eyes, heart, and brain (Figure 5A). As a ubiquitous promoter, ef1α (elongation factor-1 alpha) was reported to promote the expression of the downstream genes in the lens, retina, brain, heart, gill, and other tissues of zebrafish, which is consistent with our results. As a selection marker, EGFP could be expressed normally, indicating that the upstream target gene, irf4a, should have been expressed successfully. In addition, larvae injected with the Tol2-ef1α-EGFP plasmid as positive control were observed to express green fluorescence in multiple tissues throughout the body (Figure 5B), while negative control juveniles did not express green fluorescence (Figure 5C).

Figure 2. Experimental principles and procedures. Construction of irf4a transgenic zebrafish includes 6 steps. First off, amplify the CDS of irf4a and the sequence of “self-cleaving” 2A peptide. Then obtain the linearized Tol2 expression vector with EGFP by PCR. Thirdly, the fragments of irf4a, P2A, and Tol2 vector were ligated using one-step cloning kit. Fourthly, resistance screening and sequencing of irf4a recombinant plasmid. Fifthly, microinjection of Tol2-ef1α-irf4a-EGFP plasmid. Finally, through fluorescence screening, we can obtain EGFP-labeled irf4a transgenic zebrafish driven by ef1α promoter. CDS indicates coding sequence; PCR, polymerase chain reaction.
Discussion

Over the past 2 decades, many studies have focused on the role of IRF4 in the regulation of immune responses, immune cell activation, and immune cell differentiation. A recent study reported that graded expression of IRF4 developmentally coordinates isotype switching with plasma cell differentiation. Besides, Tr1 (type 1 regulatory T) cell development and suppressive function of Itk (IL-2 inducible T-cell kinase)-deficient cells can be restored by the expression of the transcription factor IRF4. The other study use the loss-of-function, RNA interference–based genetic screen to prove that IRF4 inhibition is toxic to myeloma cell lines. Reducing IRF4 expression is reported to restore the functional and metabolic properties of antigen-specific T cells and promoted memory-like T-cell development.

Furthermore, the members of IRF family are closely related to the polarization and typing of macrophages. And IRF5 and IRF4 play important roles in driving the polarization of non-polarized macrophages (M0 phenotype) to classical activated macrophages (M1 phenotype) and alternative activated macrophages (M2 phenotype), respectively. Interferon regulatory factor 5 is required for the expression of IL-6, tumor necrosis factor α, IL-12, and other pro-inflammatory cytokines in Toll-like receptor-mediated macrophages, leading to the formation of M1 phenotype in macrophages. In contrast, IRF4 stimulates the secretion of anti-inflammatory cytokines such as IL-10 and is a key transcriptional factor that controls the initiation and polarization of M2 macrophages. As we know, the balance of M1/M2 is controlled by activity of intracellular signaling mediators activated by exogenous stimuli, which is
important to maintain the stability of the internal environment. Although IRF4 was sufficiently studied in mammals, there were few researches on the function of zebrafish IRF4 and its regulation mechanism.

In earlier study, we found that IRF4 played an important role in monophosphoryl lipid A (MPLA)-induced InP in mice.7 In this study, we constructed the transgenic zebrafish with over-expression of irf4a, for one thing, to determine whether IRF4a could play the same role with mammal IRF4; whether the over-expression of irf4a can improve the survival rate of sepsis in zebrafish induced by lethal dose of E coli; and whether irf4a also plays an anti-inflammatory role in InP in zebrafish by activating the anti-inflammatory cytokines such as IL-10. For another, to investigate the effects of increased expression of IRF4a in zebrafish on the polarization of macrophages as a complement to macrophage typing.

What’s more, we also encountered some problems during the process of constructing Tol2-ef1α-irf4a-EGFP recombinant plasmid. For example, we found it was difficult to acquire irf4a using cDNA of embryos or juvenile zebrafish as template. By contrast, irf5 was more easily obtained under the same conditions. Therefore, we speculated whether this was related to the underdeveloped immune system and low expression of irf4a in juveniles. It was reported that the expression level of irf4a in zebrafish was highest in the testis and moderately high in liver, spleen, trunk kidney, muscle, heart, and intestine.36 In terms of this, we dissected and collected testis from 5 to 10 adult zebrafish to amplify the irf4a target gene. Another problem we solved is the independent expression of irf4a and EGFP. As is mentioned earlier, P2A is a self-cleaving sequence that mediates “cleavage” between 2 proteins and is widely used in eukaryotic expression systems.21 Thereby, we added a P2A sequence between irf4a and EGFP-encoding gene, which largely avoided low protein production and low levels of downstream gene expression when the 2 genes were expressed in fusion.

**Figure 4.** Construction of recombinant plasmids. A, Electrophoresis detection of recombinant plasmid. Groups T1 to P3 are plasmids extracting from 6 different individual colonies, and the Tol2-ef1α-EGFP-SV40 plasmid was used as a negative control. B, The map of recombinant plasmid. The recombinant plasmid is composed of Tol2 vector backbone, ef1α promoter, irf4a, P2A, EGFP encoding gene, and SV40 poly(A).

**Figure 5.** Fluorescent screening of transgenic zebrafish. A, Image of irf4a transgenic juvenile zebrafish at 3 dpf. B, Image of EGFP-expressing juvenile zebrafish at 3 dpf (as positive control). C, Image of juvenile zebrafish microinjected with the fragment of irf4a at 3 dpf (as negative control). To ensure the accuracy of the results, we photographed the juveniles of zebrafish under optical microscope and fluorescence microscope, respectively. dpf indicates days post fertilization.
Conclusion
In conclusion, we constructed a transgenic zebrafish with high expression of irf4a throughout the whole body based on the Tol2 transgenic system, providing a model basis for studying the role of IRF4a in MPLA-induced InP. Subsequent research will be carried out on the identification of target gene and expression levels of mRNA and protein. In addition, it serves as a powerful tool to study the role of IRF4a in the immune response and its effect on macrophage polarization as well. As we all know, transgenic and knockout animals are very effective tools for studying gene functions. In future research, we will proceed to build the irf4a knockout zebrafish to characterize the gene function more comprehensively.

Authors’ Note
Yawei Gou and Wei Sun contributed equally to the work.

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