

The long noncoding RNA MIR122HG is a precursor for miR-122-5p and negatively regulates the TAK1-induced innate immune response in teleost fish

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Weiwei Zheng^{1,‡} , Renjie Chang^{1,‡}, Qiang Luo¹, Guiliang Liu¹, and Tianjun Xu^{1,2,3,4,*}

From the ¹Laboratory of Fish Molecular Immunology, College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China; ²Laboratory of Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China; ³Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources (Shanghai Ocean University), Ministry of Education, Shanghai, China; ⁴National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai, China

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Long noncoding RNAs (lncRNAs) are a diverse subset of RNA species of noncoding transcripts that are usually longer than 200 nt. However, the biological role and function of many lncRNAs have not been fully identified. It has been shown that one potential function of lncRNAs is to act as a precursor miRNA and promote the production of multiple miRNAs. However, the function of the miiuy croaker lncRNA MIR122HG has not been explored. In the present study, we show that this differentially expressed teleost fish lncRNA can act as the host gene of miR-122-5p, regulate its expression, and indirectly regulate the expression of potential inflammatory target protein transforming growth factor- β -activated kinase 1. We show that MIR122HG can negatively regulate the transforming growth factor- β -activated kinase 1-triggered NF- κ B and interferon regulatory factor 3 signaling pathways and subsequently attenuate the innate immune response. In addition, MIR122HG can promote the replication of *Siniperca chuatsi* rhabdovirus and exacerbate the pathological effects caused by viral infection. We conclude that the study of lncRNA-miRNA-mRNA interaction through bioinformatics analysis or experimental-supported analysis can provide information for further elucidation of the functions of fish lncRNAs in innate immunity.

Pathogen infection of organisms induces a series of immune responses that naturally occur for the survival of the organism and the maintenance of immune homeostasis. In these processes, pattern recognition receptors (PRRs) are responsible for detecting pathogen-associated molecular patterns in the host immune system (1, 2). PRRs mediate the activation of different downstream signaling pathways by recognizing the specific molecular characteristics of different pathogenic microorganisms. The activated signaling pathway induces immune response through the production of various inflammatory factors and type 1 interferons (IFN-1) to resist

microbial infection or the entry of foreign objects. PRRs mainly include toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain-containing protein-like receptors, C-type lectins, and some DNA sensors (3). TLRs can recognize both extracellular and intracellular viruses, whereas RLRs are intracellular virus sensors, which are mainly responsible for identifying viral nucleic acid that invades the cytoplasm (4, 5). When the virus infects the host, the virus-specific recognition receptor TLRs detects the pathogen signal and then recruits the toll-interleukin (IL)-1 receptor domain-containing adapter-inducing interferon- β adaptor protein. Toll-IL-1 receptor domain-containing adapter-inducing interferon- β interacts with tumor necrosis factor receptor-associated factor (TRAF) 6 and TRAF3 (6). Activated TRAF6 recruits the kinase receptor-interacting protein 1, which then interacts with the protein and activates the transforming growth factor- β -activated kinase 1 (TAK1) complex, activates NF- κ B and mitogen-activated protein kinases (MAPKs), and induces inflammatory factors (7). TAK1 also plays an important role in the RLR pathway. After RIG-I recognizes the virus, crucial adaptor protein mitochondrial antiviral signaling protein is recruited, and then interferon regulatory factor 3 (IRF3) is activated by the I κ B kinase complex, thus promoting the mass production of interferon-stimulating genes (ISGs) and IFN-1 (8, 9). The TAK1 molecule is indispensable in these two pathways, indicating that TAK1 is essential in the immune pathway.

TAK1, a serine/threonine kinase, is an evolutionarily conserved member of the MAPK family. TAK1 is an important molecule both in TLR and RLR signaling pathways; it could be activated by various proinflammatory cytokines, and it mediates downstream signal transduction (10). It contains an N-terminal and C-terminal domain, and the C-terminal conformation changes to bind to TAK1-binding protein 1, which is a significant step in kinase activation and complex formation (10). TAK1 plays a key role in innate immune pathway, and it can be modified through different ways to regulate the intensity of immune response, including phosphorylation (11) and

[‡] These authors contributed equally to this work.

* For correspondence: Tianjun Xu, tianjunxu@163.com.

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ubiquitination (12). In mammals, several regulatory molecules regulate the activity of the innate immune response by TAK1. For example, apoptosis signal-regulating kinase 1 inhibits IL-1-induced NF- κ B activity by blocking TRAF6-TAK1 interactions (13). Moreover, S6K1 interacts with TAK1, thereby inhibiting the association of TAK1-binding protein 1 and p38 α with TAK1, and finally induces the activation of NF- κ B and AP-1 by inhibiting the activation of I κ B kinase and p38/Jun N-terminal kinase, respectively (14). In lower vertebrates such as fish, black carp TAK1 upregulates IRF7-mediated antiviral signal during the activation of host innate immunity (15). In addition, miiuy croaker miR-217 inhibits pathogen-induced antimicrobial and antiviral innate immune responses by targeting the 3'UTR of TAK1 (12). Considering that TAK1 is a core kinase that mediates innate immune response in the organism, the regulatory mechanism of TAK1 antiviral pathway in fish should be determined.

Long noncoding RNA (lncRNA), which is a noncoding RNA (ncRNA), is greater than 200 nt with mRNA-like structure, and after splicing, it consists of a polyadenylic acid tail and promoter structure (16). lncRNA is widely distributed, and lncRNA is found in animals, plants, yeast, and other organisms. It is less conservative in sequence, and only approximately 12% of the lncRNA of humans can be found in other organisms (17). The function of lncRNA involves almost all biological processes of biological physiology and pathology. It can not only regulate cell proliferation, differentiation, metabolism and other physiological processes but also participate in the regulation of various pathological processes of the body. Several important functions of lncRNAs have been identified, including acting as miRNA sponges, binding to RNA-binding protein, and involving in protein translation (18, 19). Some lncRNAs can also be used as the host genes of miRNA, and these lncRNAs can produce the mature miRNA and have corresponding biological functions (20, 21). For example, in humans, lncRNA H19 is the host gene of miR-675-3p and miR-675-5p (22, 23), and it acts as a precursor miRNA to coordinate with mature miRNA to regulate the expression of targeted genes, which is the most classic case that lncRNA functions as a host gene to regulate miRNA. Moreover, lncRNA Gm2044 as the miR-202 host gene indirectly regulates Rbfox2 expression and modulates NCCIT cell proliferation, providing new clues for the pathogenesis and treatment of male infertility in humans (24). Although this regulatory mechanism of lncRNA has been very mature in humans and mice, the research of lncRNA in lower vertebrates such as teleost fish is still unclear.

miRNA is another important member of the ncRNA family and has attracted much attention because of its important regulatory role in the past several decades. miRNA mainly affects the expression of the entire gene through base complementary pairing with the seed sequence to the 3'-UTR of the target mRNA (25). Generally, miRNA has two modes of action, such as degrading the stability of mRNA and inhibiting the translation process of coding genes. In view of the interaction between miRNA and coding genes, the function of miRNA can be used to determine drug treatment targets for

various diseases. The process of miRNA formation is well known, and the pre-miRNA undergoes a series of steps such as splicing in the nucleus and finally being transferred to the cytoplasm to obtain a mature miRNA (26, 27). lncRNAs can also produce miRNAs in humans, and these lncRNAs can also obtain functions through miRNAs, similar to precursor miRNAs (28–30). H19 is dependent on miR-675 to enhance the proliferation and invasion of AGS cells in gastric cancer, and the expression of miR-675 is positively correlated with the host gene H19 of miR-675 in patients with gastric cancer (31). The knockdown of the lncRNA H19/miR-675 axis regulates myocardial ischemic and reperfusion injury by targeting peroxisome proliferator-activated receptor alpha to protect the heart against myocardial ischemia/reperfusion injury (32). However, the study of miRNA derived from lncRNA in fish is still in a blind spot.

Generally, fish is an excellent biological model for studying the origin of the immune system. Here, we studied a new immune regulation mechanism of teleost fish after virus infection. Through high-throughput sequencing, we identified that the expression of lncRNA MIR122HG was significantly upregulated under viral stimulation. MIR122HG can regulate the production of miR-122-5p, similar to the function of pre-miR-122. We predicted that TAK1 is one of the potential targets of miR-122-5p and confirmed that miR-122-5p can inhibit TAK1 expression at different levels. Moreover, MIR122HG, which is the host gene of miR-122, can down-regulate the activity of TAK1 by deriving miR-122-5p. In addition, TAK1 could negatively regulate the innate immune response induced by *Siniperca chuatsi* rhabdovirus (SCRV), thereby promoting the replication of SCR. Our research provides information for the in-depth understanding of the relationship of lncRNA-miRNA-mRNA and reveals the important position of lncRNA in the immune response of fish.

Results

Identification of MIR122HG

Recent studies have revealed the regulatory potential of many lncRNAs, such as participation in antiviral response. To investigate the function of lncRNA in teleost antiviral immune responses, we treated miiuy croaker with SCR for 48 h and then performed RNA-Seq analysis by comparing the differential expression of lncRNA between SCR untreated and treated groups. Approximately 897 differentially expressed lncRNAs have been found (33), including MIR122HG. To further verify the RNA-Seq results of the significant abundance of MIR122HG, we performed quantitative real-time PCR (qPCR) analysis of MIR122HG expression in miiuy croaker SCR-treated spleen and *Müichthys müiuy* spleen cell (MSPc). The results showed that SCR remarkably increases the expression of MIR122HG, both in spleen tissue (Fig. 1A) and MSPCs (Fig. 1B), suggesting that MIR122HG may be an immune-related ncRNA. We then evaluated the expression levels of MIR122HG in *M. müiuy* intestine cells (MICs), MSPCs, *M. müiuy* kidney cells, *M. müiuy* muscle cells, and *M. müiuy* brain cells (Fig. 1C). Among the aforementioned cell

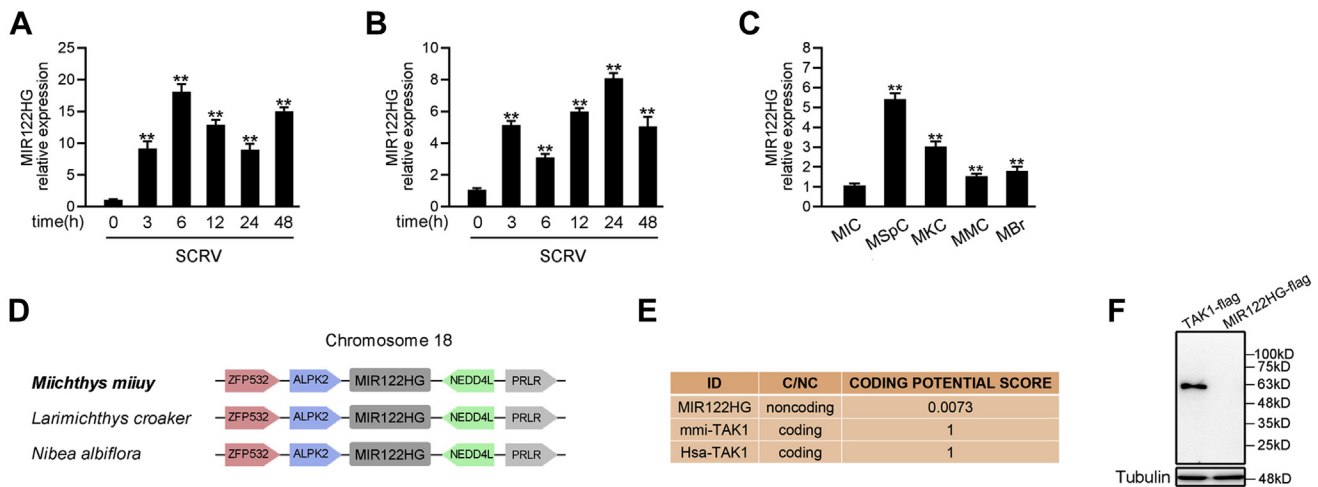


Figure 1. Identification of MIR122HG. A, expression patterns of MIR122HG at the indicated time after SCR infection in miyu croaker spleen samples measured by qRT-PCR. B, expression patterns of MIR122HG at the indicated time after SCR infection in MICs measured by qRT-PCR. C, relative expression of MIR122HG in indicated cell lines was determined by qRT-PCR, and the expression of MIR122HG in MIC cell line was used as control. D, schematic of the MIR122HG locus. MIR122HG locates on miyu croaker chromosome 18. E, MIR122HG was predicted to be ncRNAs. The RNA sequences of MIR122HG were put into the coding potential calculator (CPC) program, which was predicted to be ncRNAs. F, MIR122HG was verified to be ncRNA by Western blotting. TAK1-FLAG plasmid and MIR122HG-FLAG plasmid were transfected into MICs, respectively, and the expression was detected by Western blotting. All data presented as the means \pm SD from at least three independent triplicated experiments. $**p < 0.01$; $*p < 0.05$. hsa-TAK1, *Homo sapiens* TAK1 gene; MIC, *Miichthys miyu* intestine cell; mmi-TAK1, *Miichthys miyu* TAK1 gene; ncRNA, noncoding RNA; qRT-PCR, quantitative RT-PCR; SCR, *Siniperca chuatsi* rhabdovirus.

lines, MSpCs and MICs showed the highest and the lowest expression of MIR122HG, respectively. Therefore, MIR122HG is an immune-related lncRNA. Then, we used single-molecule full-length transcript sequencing, and the results showed that the length of MIR122HG was 945 bp, which is consistent with that of the *Larimichthys crocea* and *Nibeal biflora*, located in chromosome 18 between genes ALPK2 and NEDD4L (Fig. 1D). Finally, the coding potential calculator computational algorithm was used to predict the coding ability of MIR122HG (Fig. 1E). To further verify that MIR122HG does not have the potential to encode protein, we constructed MIR122HG expression plasmid with FLAG tag (MIR122HG-FLAG). The experimental results showed that MIR122HG-FLAG plasmid did not express protein (Fig. 1F). Therefore, MIR122HG has very low coding ability, which supports that MIR122HG is an ncRNA.

MIR122HG is associated with the host innate antiviral immunity

To further investigate the biological functions of MIR122HG, we verified the differential upregulation of MIR122HG *in vivo* and conducted *in vitro* experiments. As shown in Figure 2A, the full-length expression plasmid of MIR122HG was constructed and named MIR122HG. To determine whether MIR122HG acts as an lncRNA to exert its function, we designed three siNAs (siRNA1, siRNA2, and siRNA3) of MIR122HG and found that siRNA1 had a better knockdown effect (~40% reduction) than si-negative control (si-NC). siRNA1 was designed on the MIR122HG sequence, except the pre-miR-122 sequence. Then the siRNA1 of MIR122HG was used in all subsequent experiments and written as si-MIR122HG (Fig. 2B). Then, we investigated the effect of MIR122HG on the regulation of immune response

through the overexpression of MIR122HG in MICs and knockdown of MIR122HG in MSpCs with SCR stimulation, respectively. Considering the important role of IFN, ISGs, and inflammatory cytokines in the clearance of invading viruses, we investigated the role of MIR122HG in regulating the expression of IFN, ISGs, and inflammatory cytokines. As shown in Figure 2C, the overexpression of MIR122HG decreased the expression levels of tumor necrosis factor alpha, IL-8, IFN-2, and Mx1 in MICs under SCR stimulation. By contrast, knockdown of MIR122HG significantly increased this indicated gene expression in MSpCs with SCR stimulation (Fig. 2D). Moreover, 5-ethynyl-2'-deoxyuridine (EdU) assays were performed to detect cell proliferation rates for further exploring the role of MIR122HG in innate immunity. As shown in Figure 2, E and F, the overexpression of MIR122HG plasmid could decrease the cell proliferation rate, whereas transfection with si-MIR122HG could increase cell proliferation. Therefore, MIR122HG can inhibit cell proliferation. Overall, MIR122HG acts as a negative regulator to modulate the antiviral response and cell proliferation.

MIR122HG regulates the release of miR-122-5p

Many lncRNAs function as primary miRNA transcripts for miRNA to regulate mRNA expression (34). MIR122HG, an lncRNA with a dramatically increased expression upon SCR stimulation, may participate in immune response in teleost fish. Through sequence alignment, we found that miR-122-5p is matched to MIR122HG precisely; thus, we further investigated the relationship between MIR122HG and miR-122-5p. To confirm the relationship between MIR122HG and miR-122-5p, we examined the expression of miR-122-5p in SCR-infected spleen tissue and MICs at the same time. As shown in Figure 3, A and B, miR-122-5p levels could be dramatically

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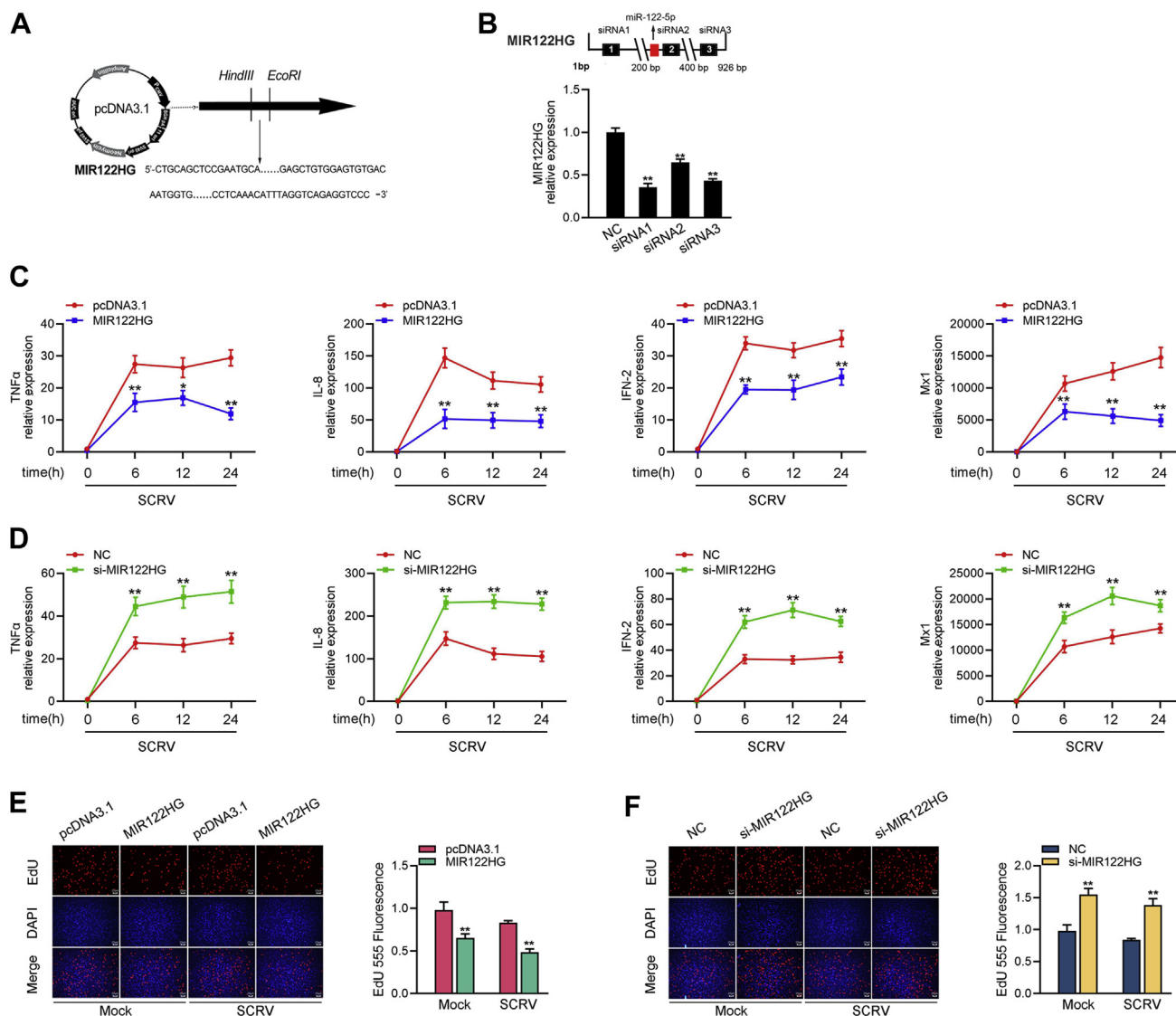


Figure 2. MIR122HG suppresses host antiviral immunity. *A*, schematic diagram of MIR122HG expression plasmid construction. *B*, location schematic diagram of si-MIR122HG and efficient si-MIR122HG was screened. The three siRNAs of MIR122HG was transfected into MSpCs for 48 h, respectively. *C*, effect of the expression plasmid of MIR122HG on TNF- α , IL-8, IFN-2, and Mx1 expression during SCRv infection. MICs were transfected with pcDNA3.1 vector or MIR122HG expression plasmid for 24 h and then treated with SCRv for 6, 12, or 24 h; subsequently, expression of the indicated genes was determined by qPCR. *D*, MSpCs were transfected with si-NC or si-MIR122HG for 24 h. The cells were treated with SCRv for different times. Then, the expression of TNF- α , IL-8, IFN-2, and Mx1 were analyzed by qPCR. *E* and *F*, cell proliferation was assessed by EdU assays in MICs transfected with pcDNA3.1 vector or MIR122HG expression plasmid (*E*) and si-NC or si-MIR122HG (*F*) to determine the effect of MIR122HG after SCRv infection. MICs were transfected with si-NC, si-MIR122HG, pcDNA3.1 vector, or MIR122HG expression plasmid for 24 h and then treated with SCRv for 24 h. A cell proliferation assay was performed. The scale bar represents 20 μ m. All data presented as the means \pm SD from at least three independent triplicated experiments. ** $p < 0.01$; * $p < 0.05$. EdU, 5-ethynyl-2'-deoxyuridine; IFN-2, interferon 2; IL-8, interleukin 8; MIC, *Miichthys miiuy* intestine cell; MSpC, *Miichthys miiuy* spleen cell; NC, negative control; qPCR, quantitative PCR; SCRv, *Siniperca chuatsi* rhabdovirus; TNF- α , tumor necrosis factor alpha.

increased in miiuy croaker SCRv-infected spleen tissue (Fig. 3A) and MICs (Fig. 3B).

We have demonstrated in Figure 2, A and B that MIR122HG and si-MIR122HG increased or inhibited the expression levels of MIR122HG, respectively. To further verify the relationship between MIR122HG and miR-122-5p, we detected the effect of gain and loss of MIR122HG on miR-122-5p expression. Figure 3C shows that increasing the levels of MIR122HG by transfecting MICs with the MIR122HG expression vector specifically induced the expression levels of miR-122-5p (Fig. 3C), suggesting that the processing of miR-122-5p from MIR122HG was increased by overexpressing

MIR122HG. We also found that the expression of miR-122-5p was significantly reduced when MIR122HG was silenced, thus supporting that MIR122HG overexpression led to increased miR-122-5p levels. Therefore, MIR122HG may be the host gene of miR-122-5p. At the same time, we mutated all the sequences of the mature miR-122-5p on the MIR122HG overexpression plasmid to construct the mutant type (MT) of MIR122HG (MIR122HG-MT). Consequently, the plasmid no longer produced the mature miR-122-5p. We found that transfection of this MIR122HG-MT plasmid into MICs could not increase the level of miR-122-5p (Fig. 3C). This result confirmed that MIR122HG is the host gene of miR-122-5p and

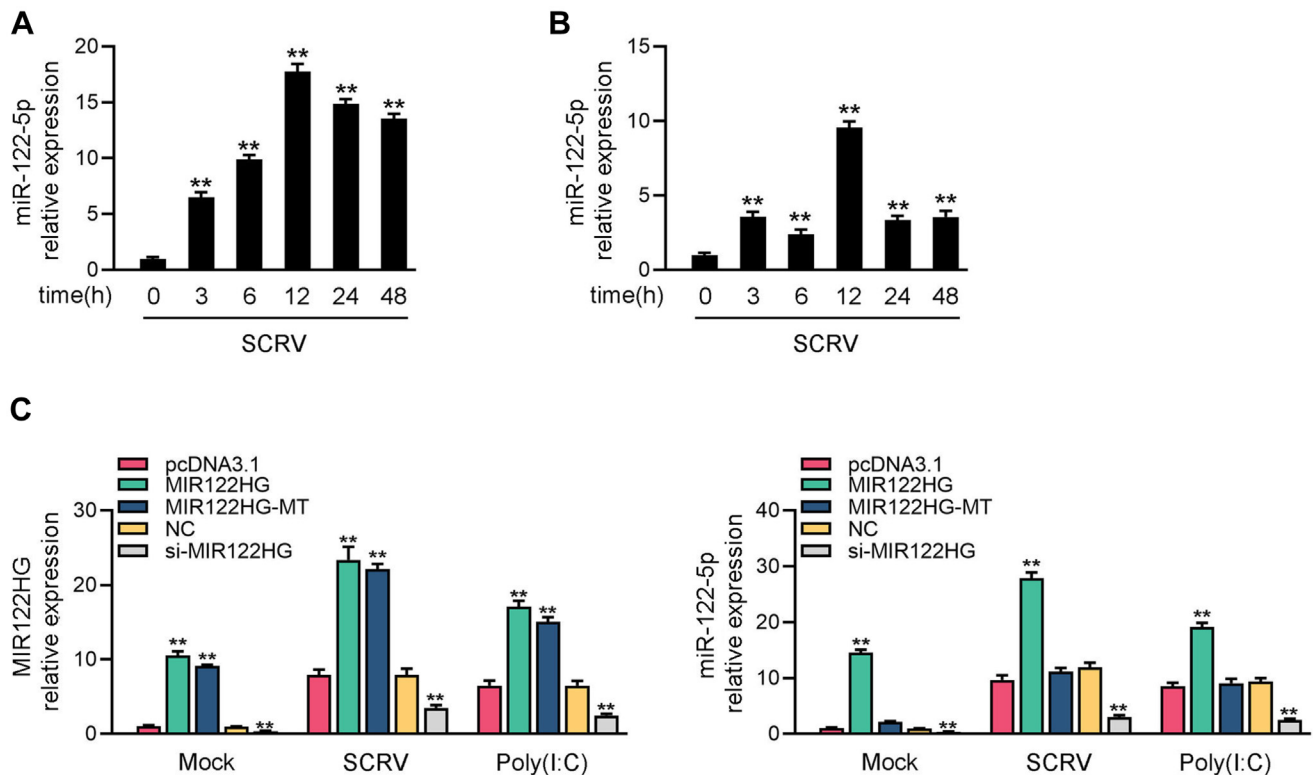


Figure 3. MIR122HG regulates the expression of miR-122-5p. A and B, expression patterns of miR-122-5p at the indicated time after SCR infection in miiuy croaker spleen samples (A) and MSpCs (B) measured by qRT-PCR. C, MICs were transfected with pcDNA3.1, MIR122HG, MIR122HG-MT, NC, or si-MIR122HG for 48 h, and then the expression profiles of MIR122HG and miR-122-5p were detected by qRT-PCR, respectively. All data were presented as the means \pm SD from at least three independent triplicated experiments. ** $p < 0.01$; * $p < 0.05$. MIC, *Michthys miiuy* intestine cell; MSpC, *Michthys miiuy* spleen cell; NC, negative control; qRT-PCR, quantitative RT-PCR; SCR, *Siniperca chuatsi* rhabdovirus.

positively regulates miR-122-5p expression level. We also detected the expression of MIR122HG and miR-122-5p after transfection of pcDNA3.1, MIR122HG, MIR122HG-MT, NC, or si-MIR122HG in MICs for 24 h and treatment with SCR infection for 24 h or treated poly (I:C) 18 h. As shown in Figure 3C, miR-122-5p and MIR122HG were highly expressed after transfection with MIR122HG in MICs under SCR infection or poly (I:C) treatment. Importantly, upon SCR infection or poly (I:C) treatment, the miR-122-5p level did not change when MIR122HG-MT was transfected into MICs. Therefore, regardless of viral infection, miR-122-5p is derived from MIR122HG.

TAK1 is a direct target of miR-122-5p

miRNAs affect various biological processes at the post-transcriptional level by targeting the 3'-UTR of mRNA; thus, we probed the potential target genes of miR-122-5p. Bioinformatics software programs were applied to seek the miR-122-5p targets, and we found that its potential target gene TAK1 is related to antiviral immunity. To obtain more direct evidence that miR-122-5p targets TAK1, we constructed the WT and MT of TAK1 3'-UTR reporter vector, as shown in Figure 4A. To detect the effect of miR-122-5p on the protein expression level of TAK1, we constructed the expression plasmid of TAK1 and transfected it with miR-122-5p, MIR122HG, and their respective controls into epithelioma papulosum cyprini (EPC) cells. As shown in

Figure 4B, the results revealed that increased miR-122-5p expression led to a decrease in TAK1 protein. We also transfected miR-122-5p in different concentrations of the RNA or overexpressed MIR122HG in a concentration gradient manner in MICs. After 48 h, we detected by qRT-PCR, and the results showed that both miR-122-5p and MIR122HG reduced the expression level of TAK1 at the mRNA level in a dose-dependent manner (Fig. 4C). These results preliminarily demonstrated the inhibitory effect of miR-122-5p and MIR122HG expression on TAK1 at the post-transcriptional level.

To further clarify that this negative mechanism is realized through the complementation of miR-122-5p with seed sequences in the 3'UTR region, we performed dual luciferase reporting analysis. EPC cells were transfected with NC or miR-122-5p, along with TAK1 3'-UTR-WT or TAK1 3'-UTR-MT. After 24 h, the luciferase reporter assays showed that the overexpression of miR-122-5p led to a marked decrease of luciferase activity of the TAK1 3'-UTR-WT, whereas no change was observed in the luciferase activity of TAK1 3'-UTR-MT (Fig. 4D). In addition, we inserted the WT or a mutated form of TAK1 3'-UTR into mVenus-C1 vector and examined whether cotransfected with miR-122-5p could suppress the levels of GFP. As shown in Figure 4, E and F, miR-122-5p could significantly inhibit the levels of GFP, indicating the interaction between miR-122-5p and TAK1. To determine the function of MIR122HG

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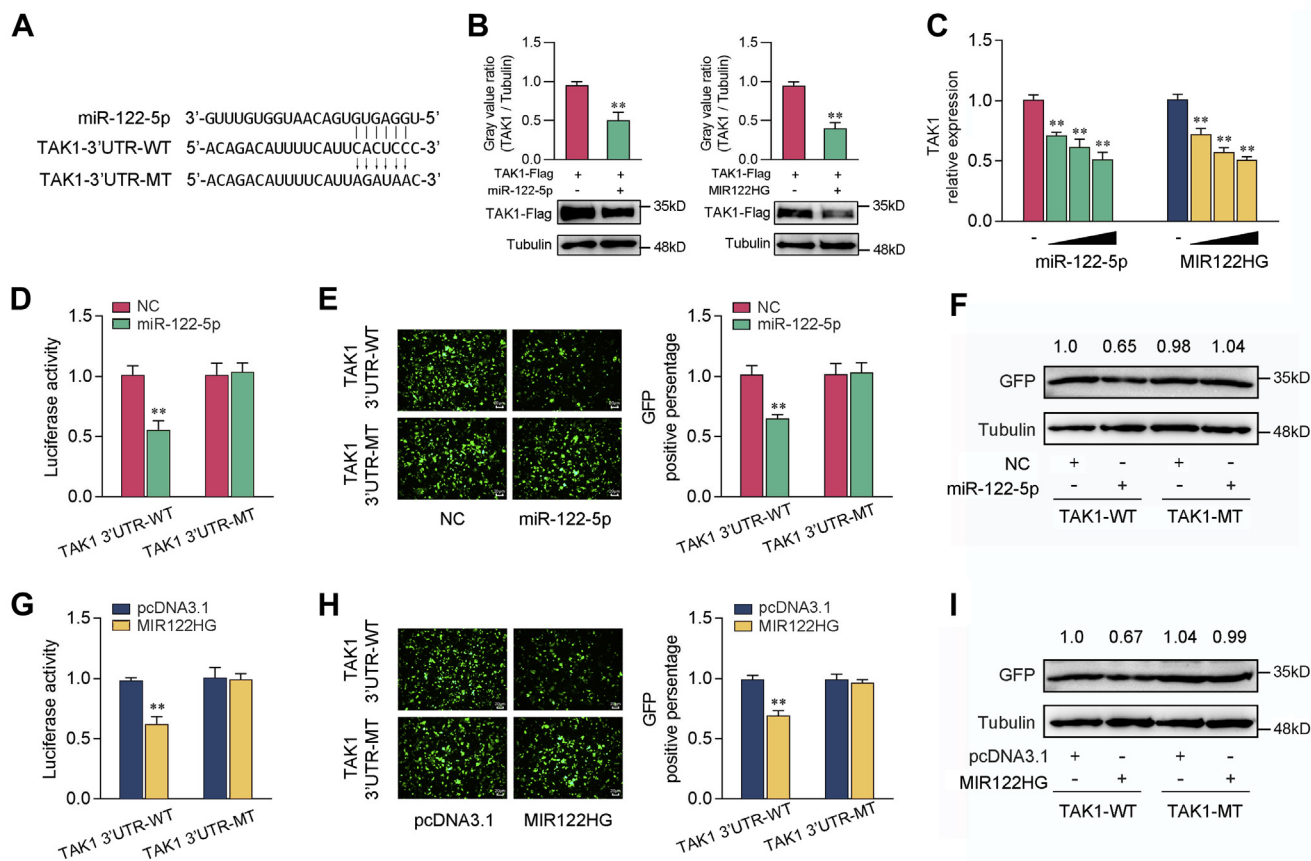


Figure 4. miR-122-5p targets miiuy croaker TAK1 gene. A, schematic diagram of the predicted target sites of miR-122-5p in the 3'-UTR of TAK1. miR-122-5p-binding sites in the WT of TAK1 and the mutant type were shown. B, miR-122-5p regulates TAK1 expression. EPC cells were cotransfected with the TAK1 expression plasmid, along with miR-122-5p or NC, pcDNA3.1 or MIR122HG. After 48 h, TAK1 expression was determined by Western blotting (*lower panel*), and the gray value ratio about TAK1/tubulin is shown in the *upper panel*. C, MICs were cotransfected with the concentration gradient of NC (100, 75, 50, and 0 nM) or miR-122-5p (0, 25, 50, and 100 nM), pcDNA3.1 (200, 150, 100, and 0 ng) or MIR122HG (0, 50, 100, and 200 ng) for 24 h. Then the levels of TAK1 were measured by qRT-PCR. D, EPC cells were transfected with the WT or the mutant type of TAK1 3'-UTR, along with NC or miR-122-5p for 24 h, and then the luciferase activity was measured, respectively. E and F, EPC cells were cotransfected with the WT or mutant type of mVenus-TAK1-3'UTR with the NC or miR-122-5p, respectively. After 48 h of transfection, the fluorescence intensity was evaluated by an enzyme-labeled instrument (E), and the GFP expression (F) was measured by Western blotting, respectively. The scale bar represents 20 μ m; original magnification $\times 10$. G, EPC cells were transfected with the WT or the mutant type of TAK1 3'-UTR, along with pcDNA3.1 or MIR122HG for 24 h, and then the luciferase activity was measured, respectively. H and I, EPC cells were cotransfected with the WT or mutant type of mVenus-TAK1-3'UTR with the pcDNA3.1 or MIR122HG, respectively. After 48 h of transfection, the fluorescence intensity was evaluated by an enzyme-labeled instrument (H), and the GFP expression (I) was measured by Western blotting, respectively. The scale bar represents 20 μ m; original magnification $\times 10$. All data were presented as the means \pm SD from at least three independent triplicated experiments. ** $p < 0.01$; * $p < 0.05$. EPC, epithelioma papulosum cyprini; MIC, *Miichthys miiuy* intestine cell; NC, negative control; qRT-PCR, quantitative RT-PCR; TAK1, transforming growth factor- β -activated kinase 1.

on the luciferase activity of TAK1, we performed a similar experiment, and similar results were observed with MIR122HG (Fig. 4, G–I). Our results strongly indicate that TAK1 is the direct target of miR-122-5p.

MIR122HG exerts functions from miR-122-5p

Considering that miR-122-5p could regulate TAK1-mediated antiviral pathways, we investigated whether MIR122HG, as the primary precursor of miR-122-5p, can modulate the NF- κ B and IRF3 signaling pathways through the functionality obtained from miR-122-5p. First, MICs were transfected with pcDNA3.1 or MIR122HG, and MSpCs were transfected with NC or si-MIR122HG and infected with SCR for 6, 12, and 24 h. Then, qRT-PCR analysis was performed, and we found that the increased expression of TAK1 induced by SCR was attenuated by MIR122HG, and the inhibitory effect was most obvious when SCR was

stimulated for 12 h (Fig. 5A, *left panel*). Knockdown of MIR122HG caused an increase in the expression level of TAK1 after intracellular infection of SCR (Fig. 5A, *right panel*). Protein detection showed that MIR122HG inhibited the expression of TAK1 after SCR infection, whereas MIR122HG silencing promoted the expression of TAK1 at the protein level (Fig. 6B). To determine whether MIR122HG could reduce TAK1-mediated signaling, we performed dual-luciferase reporter assay. TAK1 expression plasmid, pcDNA3.1, MIR122HG, MIR122HG-MT, pRL-TK vector, together with reporter genes, such as NF- κ B, IL-8, IFN-2, and IRF3, were transfected into EPC cells. As expected, MIR122HG inhibited the luciferase reporter activity but not MIR122HG-MT (Fig. 6C). Then the concentration (Fig. 6D) and time gradient (Fig. 6E) assays were carried out. Results showed that MIR122HG could inhibit the luciferase activity in a dose-dependent manner.

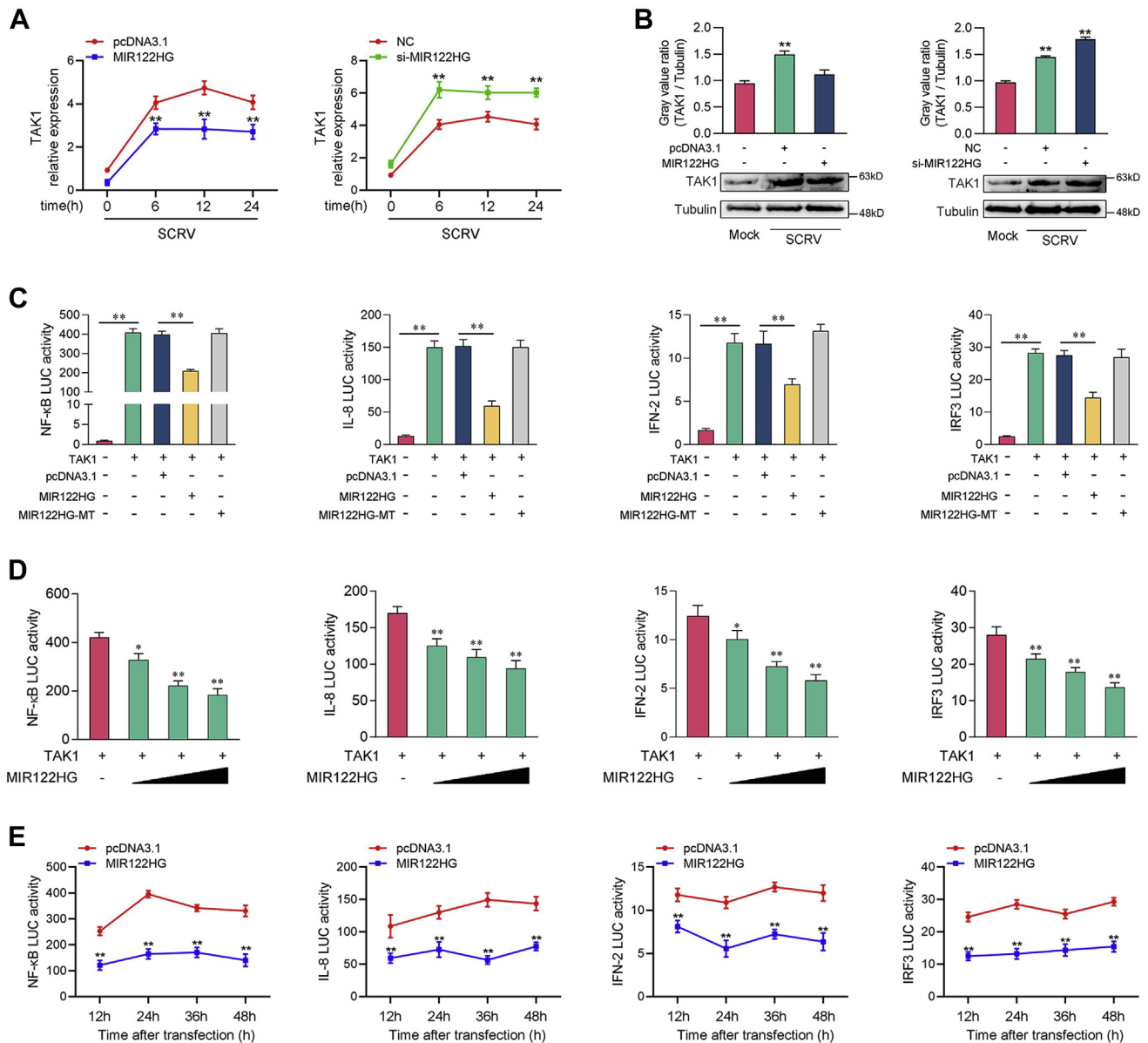


Figure 5. MIR122HG negatively regulates TAK1-mediated antiviral signaling. **A**, MIR122HG inhibits the mRNA level of endogenous TAK1 upon SCR V infection. qPCR assays were performed to determine the expression levels of TAK1 in MICs transfected with pcDNA3.1, MIR122HG, NC, or si-MIR122HG at different SCR V infection times. **B**, MIR122HG suppresses the protein expression of endogenous TAK1 upon SCR V infection. MICs were transfected with pcDNA3.1 or MIR122HG at different concentrations, and MSpCs were transfected with NC or si-MIR122HG for 24 h. Then the cells were treated with SCR V for 24 h, and the expression of TAK1 was determined by Western blotting (lower panel), and the gray value ratio about TAK1/tubulin is shown in the upper panel. **C**, MIR122HG inhibits TAK1-activated luciferase activity. EPC cells were cotransfected with pcDNA3.1, MIR122HG, or MIR122HG-MT, a TAK1 expression plasmid, and a pRL-TK vector, along with an NF-κB, IL-8, IFN-2, or IRF3 reporter gene, to investigate the regulatory effect of MIR122HG on NF-κB and IRF3 signals. **D**, relative luciferase activity of the indicated reporters in EPC cells after cotransfection with pcDNA3.1 (200, 150, 100, and 0 ng) or MIR122HG (0, 50, 100, and 200 ng) and TAK1 expression plasmid, pRL-TK vector, together with NF-κB, IL-8, IFN-2, or IRF3 luciferase reporter genes, and then the luciferase activity was measured after 24 h. **E**, relative luciferase activity of the indicated reporters in EPC cells after cotransfection with pcDNA3.1 or MIR122HG was determined. EPC cells were cotransfected with pcDNA3.1 or MIR122HG plasmid, TAK1 expression plasmid, pRL-TK vector, together with NF-κB, IL-8, IFN-2, or IRF3 luciferase reporter genes, and then the luciferase activity was measured at different time points, as indicated. All data were presented as the means ± SD from at least three independent triplicated experiments. ***p* < 0.01; **p* < 0.05. EPC, epithelioma papulosum cyprini; IFN-2, type 2 interferon; IL-8, interleukin 8; IRF3, interferon regulatory factor 3; MIC, *Micropterus dolomieu* intestine cell; MSpC, *Micropterus dolomieu* spleen cell; MT, mutant type; NC, negative control; qPCR, quantitative PCR; SCR V, *Siniperca chuatsi* rhabdovirus; TAK1, transforming growth factor-β-activated kinase 1.

miR-122-5p inhibits TAK1-mediated antiviral signaling pathways

Considering that miR-122-5p targets and regulates TAK1, we then tested whether miR-122-5p can regulate TAK1 upon SCR V infection. As shown in Figure 6A, overexpression of miR-122-5p led to a decrease in the expression levels of TAK1

in MSpCs treated with SCR V, whereas inhibition of miR-122-5p enhanced TAK1 expression in MICs. We then tested whether miR-122-5p regulates TAK1 expression at the protein level under SCR V treatment. Accordingly, we transfected with NC, miR-122-5p, miR-122-5p inhibitor (miR-122-5p-i), NC inhibitor, or miR-122-5p-i into MICs, respectively. Western

LncRNA MIR122HG modulates antiviral innate immunity

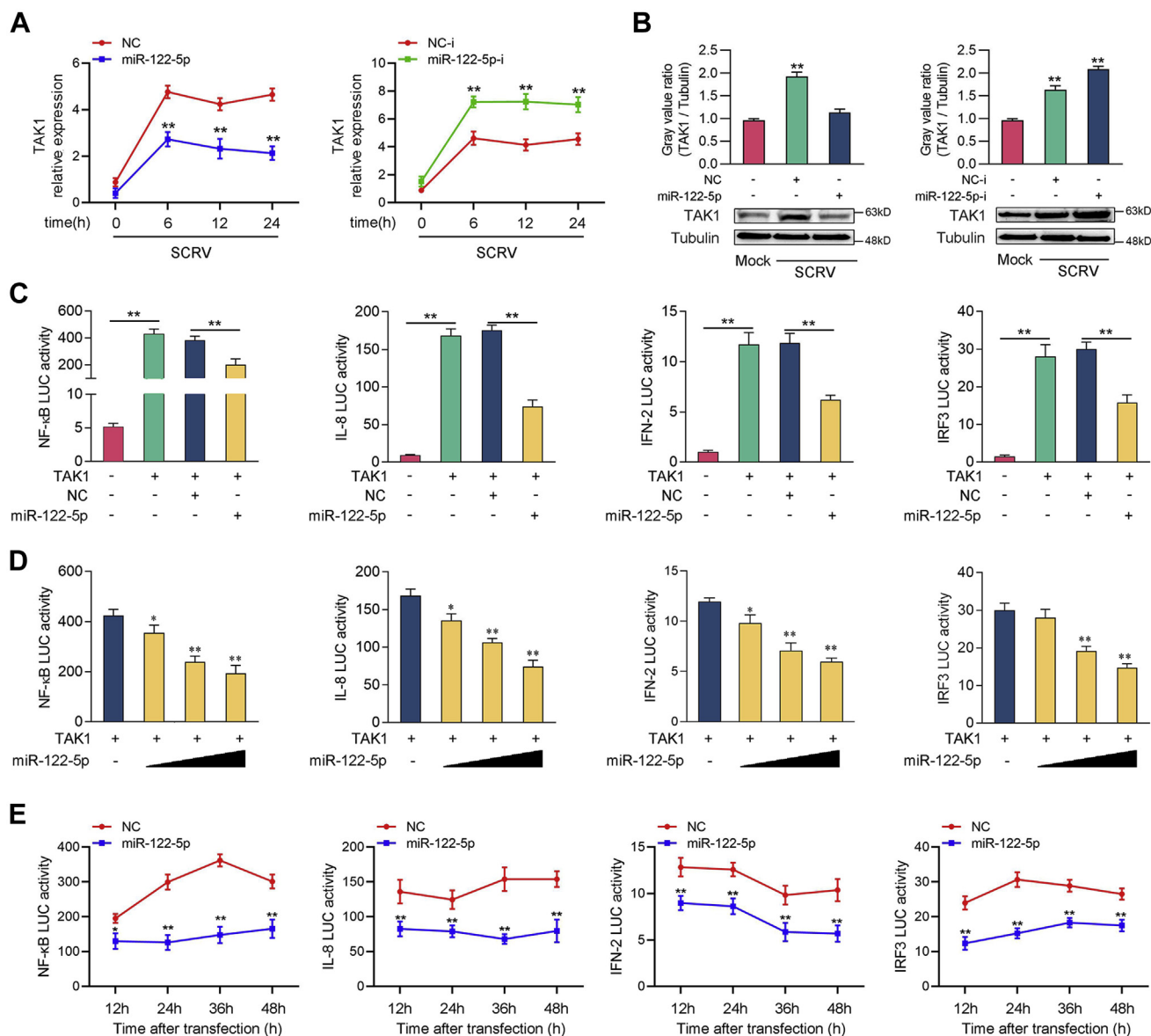


Figure 6. miR-122-5p is able to negatively regulate NF-κB and IRF3 signaling pathway. *A*, miR-122-5p inhibits the mRNA level of endogenous TAK1 upon SCR_V infection. qPCR assays were performed to determine the expression levels of TAK1 in MICs transfected with NC, miR-122-5p, NC-i, or miR-122-5p-i at different SCR_V infection times. *B*, miR-122-5p suppresses the protein expression of endogenous TAK1 upon SCR_V infection. MICs were transfected with NC or miR-122-5p at different concentrations, and MSPCs were transfected with NC-i or miR-122-5p-i for 24 h. Then the cells were treated with SCR_V for 24 h, and the expression of TAK1 was determined by Western blotting (*lower panel*), and the gray value ratio about TAK1/tubulin is shown in the *upper panel*. *C*, miR-122-5p inhibits TAK1-activated luciferase activity. EPC cells were cotransfected with NC, miR-122-5p, a TAK1 expression plasmid, and a pRL-TK vector, along with an NF-κB, IL-8, IFN-2, or IRF3 reporter gene, to investigate the regulatory effect of miR-122-5p on NF-κB and IRF3 signals. *D*, relative luciferase activity of the indicated reporters in EPC cells after cotransfection with NC (100, 75, 50, and 0 nM) or miR-122-5p (0, 25, 50, and 100 nM) and TAK1 expression plasmid, pRL-TK vector, together with NF-κB, IL-8, IFN-2, or IRF3 luciferase reporter genes, and then the luciferase activity was measured after 24 h. *E*, relative luciferase activity of the indicated reporters in EPC cells after cotransfection with NC or miR-122-5p, TAK1 expression plasmid, pRL-TK vector, together with NF-κB, IL-8, IFN-2, or IRF3 luciferase reporter genes, and then the luciferase activity was measured at different time points, as indicated. All data were presented as the means ± SD from at least three independent triplicated experiments. ***p* < 0.01; **p* < 0.05. EPC, epithelioma papulosum cyprini; IFN-2, interferon 2; IL-8, interleukin 8; IRF3, interferon regulatory factor 3; MIC, *Microthys miiuy* intestine cell; miR-122-5p-i, miR-122-5p inhibitor; MSPC, *Microthys miiuy* spleen cell; NC, negative control; NC-i, NC inhibitor; qPCR, quantitative PCR; SCR_V, *Siniperca chuatsi* rhabdovirus; TAK1, transforming growth factor-β-activated kinase 1.

blot analysis results showed that miR-122-5p could exert the inhibitory effect on TAK1, whereas miR-122-5p-i promoted TAK1 expression by counteracting miR-122-5p (Fig. 6B). Next, we investigated the influence of miR-122-5p on the TAK1-mediated signaling pathway. The results showed that TAK1 could activate NF-κB and IRF3 signaling, but the increased fluorescence activity decreased after transfection

with miR-122-5p (Fig. 6C). Then, the concentration gradient of miR-122-5p was determined, and the results showed that the inhibitory effect was significantly increased with the increased transfection of miR-122-5p (Fig. 6D). Furthermore, the time gradient assays were constructed, and the luciferase activity was measured at indicated time points. The results showed that miR-122-5p inhibited TAK1-mediated NF-κB

and IRF3 signaling pathways (Fig. 6E). Therefore, miR-122-5p plays a negative modulator in regulating signaling pathway.

MIR122HG enhanced SCR V replication

To explore the biological significance of MIR122HG upregulation in SCR V-induced host cells, we tested the effect of MIR122HG on SCR V replication in MICs. Previous studies on the involvement of miR-122-5p in antiviral immune response have reported that miR-122-5p promotes viral replication. Here, we investigated whether MIR122HG, a precursor of miR-122-5p, can exert a similar function. We examined the effect of MIR122HG on SCR V replication to explore the biological significance of MIR122HG in SCR V-induced host cells. Based on the detection of SCR V RNA level by qPCR, we found that MIR122HG knockdown significantly inhibited SCR V replication after 24 h of SCR V infection (Fig. 7A, left panel). Transfected MIR122HG plasmid significantly promoted SCR V replication but not the MIR122HG-MT plasmid after 24 h of SCR V infection (Fig. 7A, right panel). By measuring the SCR V 50% tissue culture infectious dose (TCID₅₀) levels in the supernatant from the infected MSpCs and MICs, we found that MIR122HG knockdown significantly inhibited SCR V replication after 48 and 72 h of SCR V infection (Fig. 7B), and MIR122HG overexpression significantly promoted SCR V replication after 48 and 72 h of SCR V infection. Then, we

investigated whether TAK1 can inhibit viral replication. Contrary to the effect of MIR122HG, the result shows that TAK1 can significantly inhibit SCR V replication (Fig. 7, C and D). Therefore, the host MIR122HG can promote SCR V replication.

MIR122HG regulating TAK1 is widely found in other teleost fish

To address the generality of our findings, we first examined the sequence alignment of MIR122HG from different lower vertebrate species. Interestingly, as shown in Figure 8A, mature miR-122-5p displayed high conservation in multiple fishes. The miR-122-5p-binding site in TAK1 3'-UTR also displayed a high conservation in other fish (Fig. 8, B and C, left panel). Then, we constructed the concentration gradient assays of *Lcr*MIR122HG and *Ndi*MIR122HG, and we found that *Lcr*MIR122HG and *Ndi*MIR122HG showed an obvious inhibition on TAK1 3'UTR luciferase activity in a dose-dependent manner (Fig. 8, B and C, middle). To obtain direct evidence that MIR122HG could regulate TAK1 3'-UTR across species, we generated luciferase report genes by cloning the TAK1 3'-UTR of *L. crocea* and *N. diacanthus* into pmir-GLO vector, within the devoid of miR-122-5p-binding site as an NC. Strikingly, *Lcr*MIR122HG and *Ndi*MIR122HG were sufficient to decrease luciferase activities when cotransfected with the

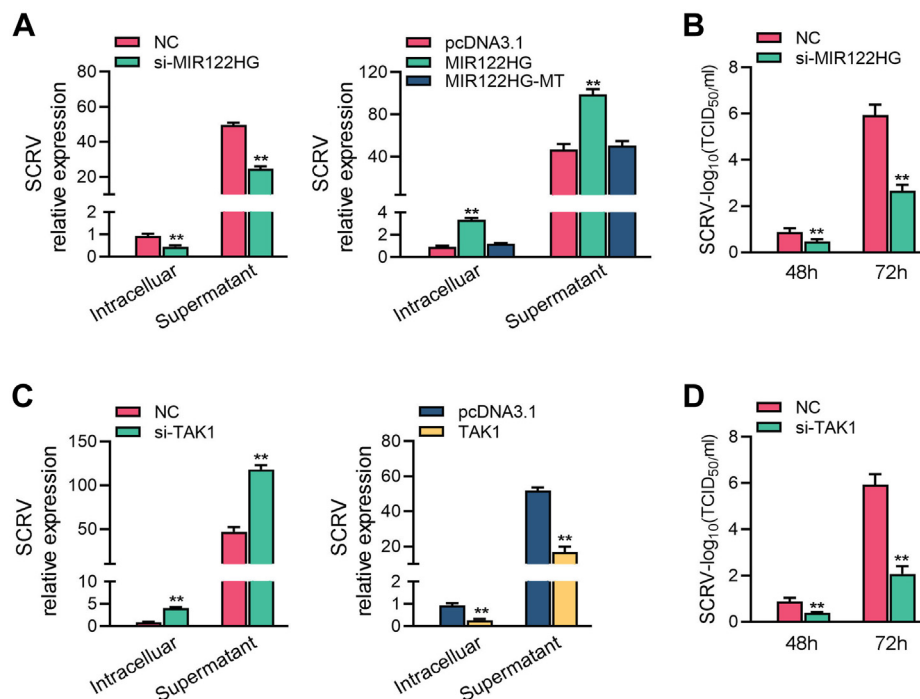
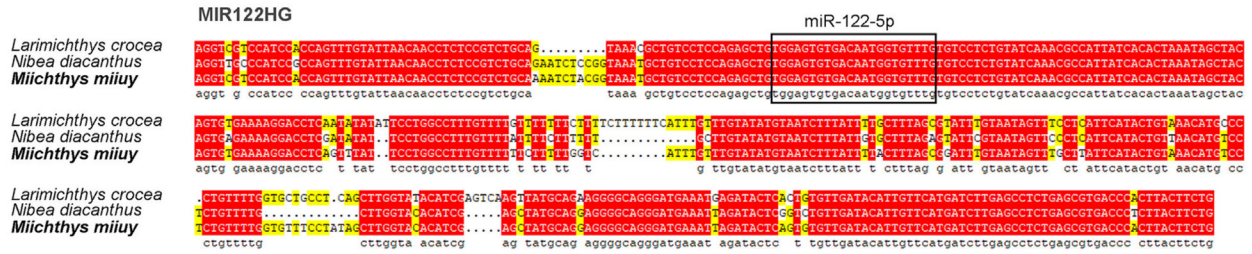


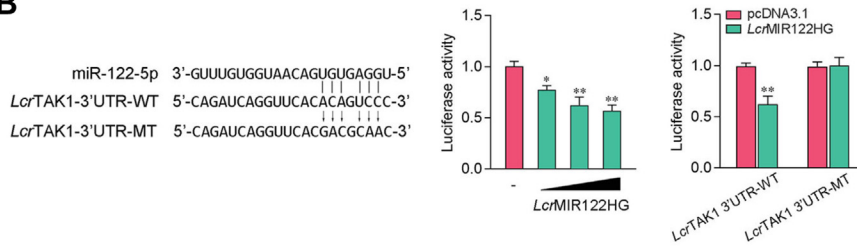
Figure 7. LncRNA MIR122HG promotes SCR V replication. A, MIR122HG promotes SCR V replication. MSpCs transfected with NC or si-MIR122HG and with pcDNA3.1 or MIR122HG or MIR122HG-MT for 24 h, respectively, then infected with SCR V at 24 h. The qPCR analysis was conducted for intracellular and supernatant SCR V RNA expression. B, MSpCs were transfected with NC or si-MIR122HG, and MICs were transfected with pcDNA3.1 or MIR122HG for 24 h and infected with SCR V at MOI 5 for 1 h and washed and then added with fresh medium. After 48 and 72 h, SCR V TCID₅₀ in cultural supernatants was measured with MSpC and MICs. C, miR-122-5p promotes SCR V replication. MSpCs transfected with NC or miR-122-5p and with NC-i or miR-122-5p-i for 24 h, respectively, then infected with SCR V at 24 h. The qPCR analysis was conducted for intracellular and supernatant SCR V RNA expression. D, MICs were transfected with NC or miR-122-5p, and MSpCs were transfected with NC-i or miR-122-5p-i for 24 h and infected with SCR V at MOI 5 for 1 h and washed and then added with fresh medium. After 48 and 72 h, SCR V TCID₅₀ in cultural supernatants was measured with MSpCs and MICs. All data are presented as the means ± SD from at least three independent triplicated experiments. ***p* < 0.01; **p* < 0.05. LncRNA, long noncoding RNA; MIC, *Miichthys miiuy* intestine cell; miR-122-5p-i, miR-122-5p inhibitor; MOI, multiplicity of infection; MSpC, *Miichthys miiuy* spleen cell; MT, mutant type; NC, negative control; NC-i, NC inhibitor; qPCR, quantitative PCR; SCR V, *Siniperca chuatsi* rhabdovirus; TCID₅₀, 50% tissue culture infectious dose.

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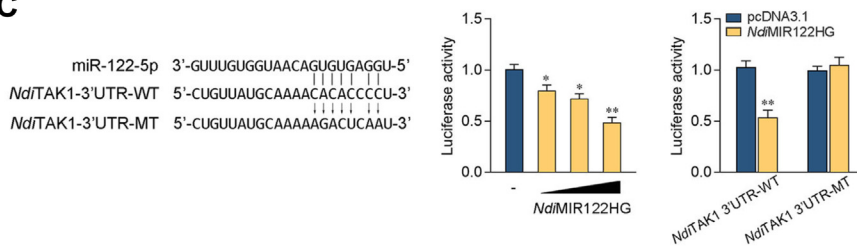
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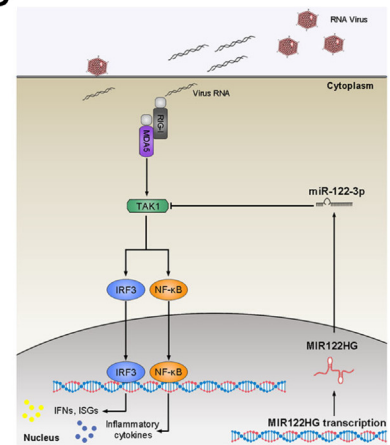


Figure 8. MIR122HG regulating TAK1 gene is widely found in fish. A, sequence alignment of MIR122HG from various species where it is present; miR-122-5p sequences are shown in the black box. B and C, schematic diagram of the predicted target sites of miR-122-5p in the 3' UTRs of *Larimichthys crocea* TAK1 (B) (left) and *Nibeia diacanthus* TAK1 (C) (left). EPC cells were transfected with pcDNA3.1 (200, 150, 100, and 0 ng) or *LcrMIR122HG* (0, 50, 100, and 200 ng) along with the WT *L. crocea* TAK1 3' UTR (*LcrTAK1* 3'-UTR-WT) (B) (middle). EPC cells were transfected with pcDNA3.1 or *LcrMIR122HG* along with the WT *L. crocea* TAK1 3' UTR (*LcrTAK1* 3'-UTR-WT) or the mutant (*LcrTAK1* 3'-UTR-MT) for 24 h, and then the luciferase activity was determined (B) (right). EPC cells were transfected with pcDNA3.1 (200, 150, 100, and 0 ng) or *NdiMIR122HG* (0, 50, 100, and 200 ng) along with the WT *N. diacanthus* TAK1 3' UTR (*NdiTAK1* 3'-UTR-WT) (C) (middle). EPC cells were transfected with pcDNA3.1 or *NdiMIR122HG* along with the WT *N. diacanthus* TAK1 3' UTR (*NdiTAK1* 3'-UTR-WT) or the mutant (*NdiTAK1* 3'-UTR-MT) for 24 h, and then the luciferase activity was determined (C) (right). D, mechanism diagram of TAK1 regulatory network and their function. MIR122HG could regulate the release of miR-122-5p and act as the precursor of miR-122-5p to indirect suppress the expression of TAK1, thus may promote the escape of bacteria. All data were presented as the means \pm SD from at least three independent triplicate experiments. ** $p < 0.01$; * $p < 0.05$. EPC, epithelioma papulosum cyprini; MT, mutant type; TAK1, transforming growth factor- β -activated kinase 1.

WTs of *L. crocea* TAK1 3'-UTR and *N. diacanthus* TAK1 3'-UTR into EPC cells, respectively, but it showed no effect on the luciferase activity of cells transfected with their MTs (Fig. 8, B and C, right). In summary, these data indicate that miR-122-5p and MIR122HG could also target *L. crocea* and *N. diacanthus* TAK1 3'-UTR, miR-122-5p is highly conserved among in other teleost fish, and its precursor MIR122HG can also exert an inhibitory effect to modulate the expression of TAK1 genes in other species (Fig. 8D).

Discussion

The study on the immunity of teleost fish, as an invertebrate to vertebrate transition, not only is an important part of vertebrate innate immunity but also is a key part of the evolution of innate immunity. Studies in the past decades have shown that teleost fish mainly rely on TLR3 and RIG-I to recognize RNA viruses, which invade the cells, recruit corresponding receptor proteins, and activate downstream signal cascades (35, 36). TAK1 is essential for the response to the

innate immune response mediated by these two PRRs. The activated TAK1 mediates the activation of NF- κ B and IRF3 signaling pathways, leads to the production of inflammatory cytokines, chemokines, and type I IFN, and induces antiviral immune events (12). Although the host's antiviral immunity is the most direct and effective way to resist virus invasion, excessive production of inflammatory factors and excessive induction of immune response can cause chronic inflammatory diseases or autoimmune diseases, which is detrimental to the body. Therefore, different levels of regulatory factors and mechanism networks are required to ensure the maintenance of the immune system's homeostasis. Under normal circumstances, the maintenance of immune homeostasis is jointly regulated and supervised by many biological molecules, forming a complex immune regulatory network. In this article, we found that MIR122HG plays a negative regulatory role in teleost antiviral immunity. SCR122HG enhanced the expression of MIR122HG, and the upregulation of MIR122HG deteriorated inflammatory cytokines and ISGs. We further confirmed that MIR122HG can control the release of miR-122-5p and

predicted TAK1 as a direct target of miR-122-5p. The assays demonstrated that both MIR122HG and miR-122-5p inhibited TAK1-mediated NF- κ B and IRF3 signaling pathways by targeting TAK1, thus promoting SCR_V replication (Fig. 8H). miR-217 can negatively regulate the antiviral immune response of miiuy croaker by targeting TAK1 (12). The regulation of TAK1 by miR-122-5p and miR-217 is both independent and coordinated because miRNA can regulate the translation of mRNA by combining its own seed sequence with the 3'UTR of mRNA, whereas the sequences of miR-122-5p and miR-217 are completely different. However, the regulation of TAK1 by the two miRNAs is coordinated with each other because the two miRNAs play a role through different sites on the 3'UTR of TAK1. The two miRNAs we found, miR-122-5p and miR-217, inhibit TAK1 after transcription. Because they both play an important regulatory role after SCR_V infection, it shows that the two miRNAs can regulate TAK1 coordinately.

miRNA is a class of regulatory molecules that conserves sequence and has conserved function. The important regulatory functions of miRNAs are well known earlier than lncRNA. For example, miR-122 is dysregulated in various tumors, such as gastric, renal, and cervical cancer cells in human. miR-122-5p inhibits the proliferation, migration, and invasion of cancer cells by targeting LYN (an Src family tyrosine kinase) in gastric cancer cells (37). In renal cancer cell, miR-122-5p can target pyruvate kinase M2 as a tumor-promoting factor, thereby promoting cell viability, proliferation, and migration (38). In cervical cancer, miR-122-5p enhances the radiosensitivity of cervical cancer cells by targeting CDC25A (39). Recently, miR-122-5p inhibits charged multivesicular body protein 3 through MAPK signaling in breast cancer cells to promote aggressiveness and epithelial-to-mesenchymal transition in triple negative breast cancer (40). Although miR-122-5p plays a regulatory role in various cancer types, a few studies have revealed its effect on the innate immunity of viral infections. The present study revealed that fish miR-122-5p also can suppress the host's antiviral immune response, indicating its important role in avoiding excessive immune responses.

Considering that the innate immunity of teleost fish is an important part of the innate immunity of vertebrates, the immune-related regulatory factors and regulatory mechanism of teleost fish should be studied. At present, the role of lncRNA as a fine-tuning regulator of different biological processes in humans and higher vertebrates has gradually been elucidated. The mechanism of competing endogenous RNA is one of the most studied hotspots, revealing that lncRNA slows down the inhibitory effect of mRNA by competitively binding miRNA. This mechanism was revealed in fish studies, and our previous research shows that antiviral-associated lncRNA (41), MAVS antiviral-related lncRNA (33), and IRL (42) act as molecular sponges to bind miRNAs and slow down the adverse consequences caused by excessive miRNA suppression of the immune response. In addition, lncRNA can be used as the host gene of miRNA to regulate the production of miRNA to indirectly regulate mRNA. For example, in colorectal cancer cells, MIR100HG, which has a higher expression level, is

inhibited by the gene GATA6, whereas miR-125b is derived from MIR100HG target GATA6, thereby lifting the inhibition of MIR100HG (43). Moreover, lncRNA MIR155HG showed cancer-promoting activity through miR-155-5p/-3p, and NSC141562 is an inhibitor molecule of the MIR155HG-miR-155 axis, which provides a new method for the treatment of glioma through a silent combination (44). The current view about the mechanism to form a mature miRNA from lncRNA is that this kind of lncRNA that can produce miRNA can mediate the termination of transcription through RNase III in mammals. Most of this lncRNA do not use the canonical cleavage-and-polyadenylation pathway but instead use microprocessor cleavage to terminate transcription, thus suggesting that splicing in lncRNA transcripts is not functionally important (21). In the present study, we found that fish lncRNA MIR122HG can produce mature miR-122-5p for the first time. We call it the MIR122HG-miR-122-5p axis, and this axis coordinately regulates innate immune response. We found that the MIR122HG-miR-122-5p axis showed high expression after viral infection and could negatively regulate the antiviral immunity mediated by SCR_V. miR-122-5p targets TAK1 and inhibits the activity of TAK1. Moreover, MIR122HG down-regulates the expression of TAK1. Therefore, MIR122HG, as an important negative regulator, controls the strength of immunity and plays an important role in fish antiviral immunity.

In the present study, we found a new lncRNA called MIR122HG in miiuy croaker and found that it can negatively regulate the antiviral immune response. Subsequently, we found that miR-122-5p can negatively regulate TAK1-triggered NF- κ B and IRF3 signaling pathways. Further studies found that MIR122HG can produce miR-122-5p by acting as a precursor of miR-122-5p and then indirectly inhibit the TAK1-mediated antiviral immune response. Aside from mediating signaling, MIR122HG could be involved in SCR_V-triggered antiviral immunity. In addition, the inflammatory cytokines and IFN γ were suppressed by the enhancement of MIR122HG, thereby increasing SCR_V replication. Our data provide new insights into the negatively feedback regulation of antiviral innate response in fish by lncRNA and the network enrichment on the host-virus interaction.

Experimental procedures

Animals and immune challenge

Miiuy croaker (~50 g) was obtained from Zhoushan Fisheries Research Institute. Fish was acclimated in aerated seawater tanks at 25 °C for 6 weeks before experiments. Experimental procedures for SCR_V infect was performed as described (45). All animal experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Research Ethics Committee of Shanghai Ocean University (no. SHOU-DW-2018-047).

Cell culture and treatment

MICs, MSpC, *M. miiuy* kidney cells, *M. miiuy* muscle cells, and *M. miiuy* brain cells were cultured in L-15 medium

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(HyClone) supplemented with 15% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin at 26 °C (45). EPC cells were maintained in Medium 199 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 28 °C in 5% CO₂ (45). Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium, which contained the 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, and under humidified conditions with 5% CO₂ at 37 °C (46). For stimulation experiments, MSpCs and MICs were challenged with SCRv at a multiplicity of infection (MOI) of 5 and harvested at different times for RNA extraction (45).

Plasmid construction

To construct the TAK1 3'UTR reporter vector, the 3'UTR region of miiuy croaker TAK1 gene as well as *L. crocea* and *N. diacanthus* TAK1 3'UTR was amplified using PCR and cloned into pmir-GLO luciferase reporter vector (Promega). To construct MIR122HG expression plasmid, the full-length sequence of MIR122HG in aforementioned species was cloned into pcDNA3.1 vector or pcDNA3.1 vector with FLAG tag, respectively. The mutated forms with point mutations in the miR-122-5p-binding site were synthesized using Mut Express II Fast Mutagenesis Kit V2 with specific primers (Table S1). Meanwhile, miiuy croaker TAK1 3'UTR luciferase gene was inserted into the mVenus-C1 (Invitrogen), which included the sequence of enhanced GFP. Besides, the TAK1 expression plasmid was constructed as previously described (12). The correct construction of the plasmids was verified by Sanger sequencing and extracted through Endo Free Plasmid DNA Miniprep Kit (Tiangen Biotech).

miR-122-5p target gene identification and the conservation analysis

The potential downstream targets of miR-122-5p were predicted using the software computational algorithms of the TargetScan (47), miRanda (48), and microInspector (49). The predictions were ranked according to the predicted efficacy of targeting as calculated by applying the context and scores of the sites. Multiple sequence alignment of MIR122HG among the different species was conducted using DNAMAN8.0 (Lynnon Biosoft).

RNA oligoribonucleotides

The miR-122-5p mimics are synthetic dsRNAs with stimulating naturally occurring mature miRNAs. The miR-122-5p mimics sequence was 5'-UGGAGUGUGACAAUGGUGUUUG-3'. The NC mimics was 5'-UUCUCCGAACGUGUCACGUTT-3'. miRNA inhibitors are synthetic ssRNAs that sequester intracellular miRNAs and block their activity in the RNA-interfering pathway. The miR-122-5p-i sequence was 5'-CAAACACCAUUGUCACACUCCA-3'. The NC inhibitor was 5'-CAGUACUUUUGUGUAGUACAA-3'. And the MIR122HG siRNA was synthesized and purified from GenePharma. Sequences are as follows: si-MIR122HG-1: 5'-GGUAUGAAUGAAUGAAUCUTT-3'; si-MIR122HG-2: 5'-GUCAUUUG

UUU GUAUAUGUTT-3'; and si-MIR122HG-3: 5'-CUGUAUGUAUAGAUAGAATT-3'.

Cell transfection

Transient transfection of cells with miRNA mimic, miRNA inhibitor, or siRNA was performed in 24-well plates using Lipofectamine RNAiMAX (Invitrogen), and cells were transfected with DNA plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. For functional analyses, the overexpression plasmid (500 ng per well) or control vector (500 ng per well) and miRNA mimics (100 nM), miRNA inhibitor (100 nM), or siRNA (100 nM) were transfected into cells in culture medium and then harvested for further detection. For luciferase experiments, miRNA mimics (100 nM) or miRNA inhibitor (100 nM) and pmirGLO (500 ng per well) containing the WT or mutated plasmid of TAK1-3'UTR were transfected into cells.

Dual-luciferase reporter assays

To miRNA target identification, miR-122-5p mimics or NC, MIR122HG plasmid or pcDNA3.1 vector was transfected with TAK1 3'-UTR-WT or TAK1 3'-UTR-MT into EPC cells, respectively. At 48 h post-transfection, the activity of luciferase was detected using a dual-luciferase reporter assay system (Promega). To analyze the functional regulation of miR-122-5p, EPC cells were cotransfected with NF-κB, IL-8, IL-1β, or IRF3 luciferase reporter gene plasmids, TAK1 expression plasmid, and pRL-TK *Renilla* luciferase plasmid, together with miR-122-5p mimics, NC, MIR122HG expression plasmid, or empty vector, respectively. Then, the cells were lysed for reporter activity. All the luciferase activity values were achieved against the *Renilla* luciferase control. Transfection of each construct was performed in triplicate in each assay. Ratios of *Renilla* luciferase readings to firefly luciferase readings were taken for each experiment, and triplicates were averaged.

RNA extraction and real-time PCR

Total RNA was isolated with Trizol Reagent (Invitrogen), and the complementary DNA was synthesized using the FastQuant RT Kit (Tiangen), which includes DNase treatment of RNA to eliminate genomic contamination. The expression patterns of each gene were performed by using SYBR Premix ExTaq™ (Takara). The small RNA was extracted by using miRcute miRNA Isolation Kit (Tiangen), and miRcute miRNA First-strand cDNA Synthesis Kit (Tiangen) was applied to reverse transcription of miRNAs. The expression analysis of miR-122-5p was executed by using the miRcute miRNA qPCR Detection Kit (Tiangen). Real-time PCR was performed in an Applied Biosystems QuantStudio 3 (Thermo Fisher Scientific). β-actin and 5.8S rRNA were employed as endogenous controls for mRNA/lncRNA and miRNA, respectively (50). Primer sequences are displayed in Table S1.

Western blotting

Cellular lysates were generated by using 1× SDS-PAGE loading buffer. Proteins were extracted from cells and

measured with the BCA Protein Assay kit (Vazyme), then subjected to SDS-PAGE (10%) gel, and transferred to polyvinylidene difluoride (Millipore) membranes by semidry blotting (Trans Blot Turbo System; Bio-Rad). The membranes were blocked with 5% bovine serum albumin. Protein was blotted with different antibodies. The antibody against TAK1 was diluted at 1:500; anti-FLAG and antitubulin monoclonal antibody was diluted at 1:2000 (Sigma); and the anti-GFP monoclonal antibody was diluted at 1:1000 (Sigma). The results were the representative of three independent experiments. The immunoreactive proteins were detected by using WesternBright TMECL (Advansta). The digital imaging was performed with a cold charge-coupled device camera.

EdU cell proliferation assay

The EdU dye assays were performed with BeyoClick EdU cell Proliferation Kit following the manufacturer's instructions (Beyotime). All the experiments were performed in triplicate.

Statistical analysis

Data are expressed as the mean \pm SD from at least three independent triplicated experiments. Student's *t* test was used to evaluate the data. The relative gene expression data were acquired using the $2^{-\Delta\Delta CT}$ method, and comparisons between groups were analyzed by one-way ANOVA followed by Duncan's multiple comparison tests (51). A value of $p < 0.05$ was considered significant.

Data availability

All data are contained within the article.

Supporting information—This article contains supporting information.

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Author contributions—T. X. conceptualization; T. X. methodology; R. C., W. Z., and T. X. formal analysis; R. C., W. Z., and T. X. investigation; R. C., W. Z., G. L., Q. L., and T. X. resources; R. C., W. Z., and T. X. writing—original draft.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EdU, 5-ethynyl-2'-deoxyuridine; EPC, epithelioma papulosum cyprini; FBS, fetal bovine serum; IFN-1, type 1 interferon; IL, interleukin; IRF, interferon regulatory factor; ISG, interferon-stimulating gene; lncRNA, long noncoding RNA; MAPK, mitogen-activated protein kinase; MIC, *Miichthys miiuy* intestine cell; miR-122-5p-i, miR-122-5p inhibitor; MOI, multiplicity of infection; MSpC, *Miichthys miiuy* spleen cell; MT, mutant type; NC, negative control; ncRNA, noncoding RNA; PRR, pattern recognition receptor; qPCR, quantitative real-time PCR; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor; SCRNV, *Siniperca chuatsi* rhabdovirus; TAK1, transforming growth factor- β -activated kinase 1; TCID₅₀, 50% tissue

culture infectious dose; TLR, toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor.

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