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Short communication

Infection of grass carp reovirus induced the expressional suppression of pro-viral Fibulin-4 in host cells

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ABSTRACT

Fibulin-4 is not only involved in connective tissue development and elastic fiber formation, but also plays critical neoplastic roles in tumor growth by activating Wnt/β-Catenin signaling in human. Recently, Fibulin-4 was shown to associate with grass carp reovirus (GCRV) outer capsid proteins and might relate to viral hemorrhagic disease in grass carp *Ctenopharyngodon idella*. Here, we monitored the expression pattern of Fibulin-4 during the infection course of GCRV at both translational and transcriptional levels, and found that Fibulin-4 was significantly suppressed upon the viral challenge in grass cap GCO cells. Over expression of Fibulin-4 was achieved by transduction of pEGFP-Fibulin-4 plasmids into GCO cells, which was confirmed by both Western blot and Real time RT-PCR analysis. In GCO cells with over-expression of Fibulin-4, significantly increase of viral protein synthesis and progeny virus production was detected. Our study indicated that Fibulin-4 displayed pro-viral function and was inhibited during viral challenge. Thus, repression of Fibulin-4 expression seemed to be involved in anti-viral response in grass carp *Ctenopharyngodon idella*.

Grass carp *Ctenopharyngodon idella* is currently the largest globally-cultured freshwater fish species with an annual output of over 5 million tons in China alone [1], and it has a high potential for sustainable development worldwide due to its low requirement on fish meal [2]. Grass carp reovirus (GCRV), the causing pathogen for grass carp hemorrhagic disease in cultured grass carp with high mortality, belongs to the *Aquareovirus* genus and the family *Reoviridae* [3]. The genome of GCRV consists of 11 dsRNA segments encoding seven structural proteins (VP1-VP7) and five nonstructural proteins (NS80, NS38, NS31, NS26 and NS16) [4]. Depending on the existence of spike protein, similar to the Sigma-1 protein of mammalian reovirus (MRV), GCRV strains could be categorized into two subgroups [5]. Type I GCRV, represented by strains like GCRV-873 and JX-01, is considered the type strain for aquareovirus due to its high virulence both *in vivo* and *in vitro* [6]. GCRV induced anti-viral response in susceptible grass carp cells have been extensively studied recently, which is generally characterized by a systematic induction of a mild host immune response that includes a variety of classical antiviral signal transduction pathways [7–9].

Fibulin-4, also known as endothelial growth factor (EGF)- containing Fibulin-like extracellular matrix protein 2 (EFEMP2), is one of the extracellular matrix proteins (ECM) [10]. Fibulin-4 in particular is associated with elastic fiber formation and connective tissue development, and is an essential component of ECM [11]. Expression of a 2.0-kb Fibulin-4 transcript in all adult tissues was detected by Northern blot analysis, with highest levels in heart [12]. Fibulin-4 has also been implicated in cell differentiation and development. Fibulin-4 plays critical neoplastic roles in tumor growth of human osteosarcoma (OS) by activating Wnt/β-Catenin signaling [13]; and TGF-β signaling is gradually enhanced in Fibulin-4 deficient smooth muscle cells in a Fibulin-4 dose-dependent manner and influences proliferation of these cells [14]. Similar to the finding that Fibulin-1 is associated specifically with cancer-related HPV E6s [15], we have identified that grass carp Fibulin-4 interact with outer capsid proteins of grass carp reovirus [16]. The ORF of grass carp Fibulin-4 gene encodes 440 amino acids, and the protein consists of (in order) an N-terminal signal peptide, six calcium-binding epidermal growth factor (cbEGF) modules in a tandem arrangement, and one C-terminal fibulin-like domain, which is similar to Fibulin-3 and -5. Furthermore, transcriptional distribution analysis of grass carp Fibulin-4 in various tissues of healthy grass carp showed that Fibulin-4 was highly expressed in muscle, moderately expressed in the intestine and brain, and slightly expressed in other examined tissues which correlated with the tissue tropism of GCRV [15]. However, the biological significance of the interaction remains unknown for both GCRV and its host.

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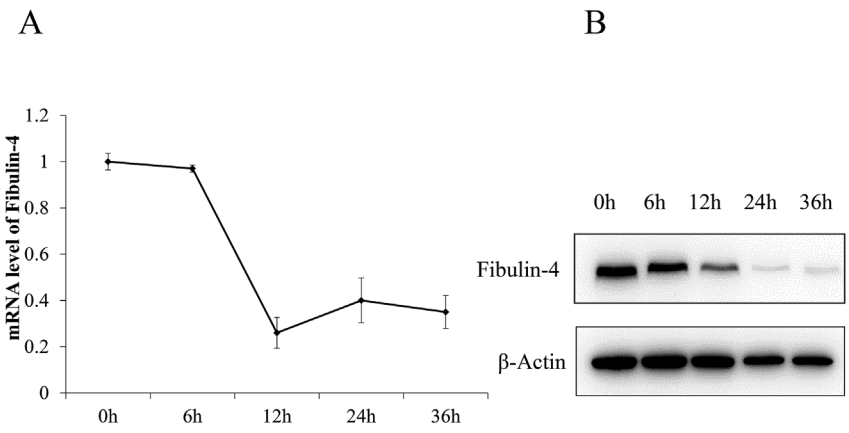

Fig. 1. Suppression of Fibulin-4 by GCRV infection. (A) Transcriptional repression of Fibulin-4 by GCRV in GCO cells. The GCO cells were infected by GCRV-JX01 at a MOI of 1, and the relative mRNA levels of Fibulin-4 was determined by real time RT-PCR and normalized to 18S rRNA level and calculated by comparing to the expression levels of 0 h group by the 2 -ΔΔCT method. (B) Detection of Fibulin-4 expression during GCRV-JX01 infection by Western blot. GCO cells were infected at a MOI of 1, and protein extracts from infected cells were probed with in-house mouse anti-Fibulin4 polyclonal antibody; β-Actin served as the internal reference protein in the IB assays with a commercial mouse anti-β-Actin monoclonal antibody.

The aim of this study was to provide a comprehensive insight into the response of cellular Fibulin-4 to GCRV challenge and to elucidate the mechanism underlying its response. For this purpose, transcriptional pattern of Fibulin-4 in GCRV-infected grass carp ovary cells (GCO) cells was monitored by real time RT-PCR (Fig. 1A). GCO cells were grown in M199 medium with 10% fetal bovine serum (Gibco BRL, USA). GCRV JX-01 was propagated by infecting monolayers of GCO cells and then incubating the cells for 2–3 days at 27 °C. Infected supernatants or cells were harvested when greater than 90% CPE (Cytopathic Effect) was observed. GCRV titer was determined using a standard end-point dilution assay, and the titers were expressed as median tissue culture infection dose ($TCID_{50}$) per mL using the Reed–Muench method [17]. The GCO cells in 6-well plate were infected by GCRV-JX01 at a multiplicity of infection (MOI) of 1. At different time points as indicated in Fig. 1A, cells were harvested for total RNA extraction using a Trizol method (Invitrogen). 200 ng of total RNA was reverse transcribed into cDNA in a 20 μL reaction, following the manufacturer's protocol with the PrimeScript™ RT Master Mix (TaKaRa, Japan). Quantitative PCR was performed in triplicate using the CFX96 real-time PCR system (Bio-rad, USA), and the SYBR®Premix Ex Taq II (TaKaRa, Japan). The Fibulin-4 reaction conditions with a primer pair of TCAT TCAACTTGTGCCCTCC and GCCCACTTTGCGATACCC were: 95 °C for 10 min; followed by 39 cycles of 10 s at 95 °C, 15 s at 56 °C, and 20 s at 72 °C [16]. The relative transcriptional expression levels of Fibulin-4 were normalized to 18S rRNA level with a primer pair of ATTTCCGA CACGGAGAGG and CATGGGTTTAGGATACGCTC [18]. Overall, the mRNA level of Fibulin-4 was immediately suppressed upon GCRV challenge, with an observable sharp reduction period of 6–12 h p.i. resulting in minimum expression level at 12 p.i. (Fig. 1A). Limited recovery of mRNA expression was noticed after the 12 h time point in a significantly-lower level than that of the starting time point.

Taking advantage of the homemade polyclonal antiserum against grass carp Fibulin-4, we were able to monitor its translational expression pattern in response to GCRV infection (Fig. 1B). The GCO cells in 6-well plate were infected by GCRV-JX01 at a MOI of 1. At different time points indicated in Fig. 1B, cells were washed twice with PBS and harvested by scraping for total protein preparation with 2 × Protein Loading Buffer (Tanon, China). Immunoblotting assay was performed as described previously [6]. Briefly, the protein samples were resolved by 10% or 12% SDS-PAGE and then transferred onto 0.45 μm polyvinylidene fluoride (PVDF) membrane (Merck millipore, Darmstadt, Germany), then the membrane were blocked for 2 h at room temperature in 5% non-fat milk dissolved in PBST (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.1% Tween-20). The homemade primary antibody (anti-Fibulin-4, 1:3000) and secondary antibody (HRP conjugated anti-mouse IgG, 1:5000, Abmart, China) were used to probe the target protein. Expression of β-Actin (anti-β-Actin, 1:4000, Abclonal, China) was used as an internal control. Color development was carried out using Tanon™ High-sig ECL Western Blotting Substrate (Tanon, China). In consistence with its transcriptional expression pattern post viral challenge, translational expression levels of Fibulin-4 was significantly inhibited following the onset of virus infection until reaching the minimum level at 24 h p.i. (Fig. 1B).

Why the grass carp cells respond to the GCRV challenge by reducing the expression of Fibulin-4? To answer this question, we continued to study the effect of overexpressing Fibulin-4 on viral replication. The ORF of Fibulin-4, amplified from the total mRNA of grass carp GCO cells with a primer pair of CCGCTCGAGATGCGGCCCGGGTGTGTTT TGG and CCGGAATTCCCCAAAAAGCATAAGGCCCAACGTAG, was cloned into the pEGFP-N1 vector via XhoI/EcoRI restriction sites to

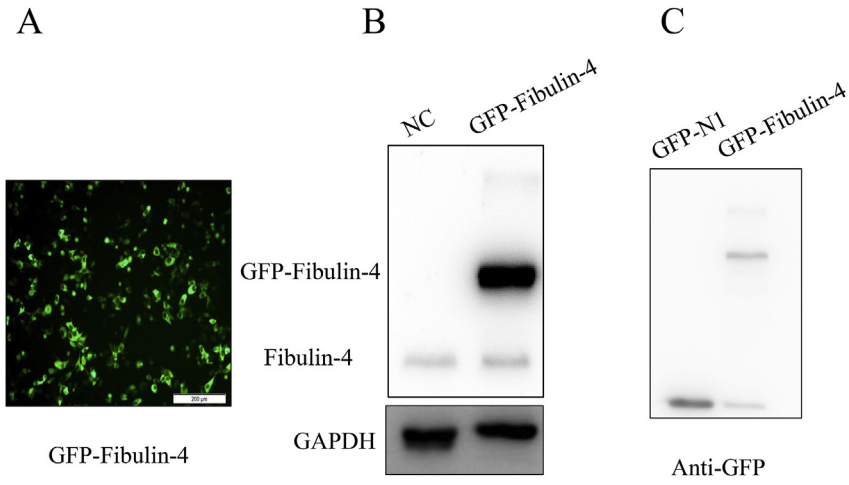

Fig. 2. Construction of GCO cells that constantly express EGFP-Fibulin4. (A) Overexpression of GFP-Fibulin-4 was confirmed by fluorescence microscopy. Scale bars = 200 μm. (B) Fibulin-4 expression was measured in GCO cells transducted with pEGFP-Fibulin4 by Western blotting assay. Anti-Fibulin4 was used to recognize both endogenic Fibulin-4 and overexpressed EGFP-Fibulin-4. Fibulin-4 expression was normalized to GAPDH expression that was probed with a commercial anti-GAPDH monoclonal antiserum. (C) Western blotting assay of GFP-Fibulin-4 and GFP-N1 (as an control) using an anti-GFP antibody.