CircARHGEF15 promotes innate antimicrobial immune response by modulating the miR- 214/*hepcidin*/NF-κB signaling axis.

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Abstract： Circular RNA (circRNAs) can be used as biomarkers and therapeutic targets for various human diseases through competitive endogenous RNA (ceRNA). Previous studies in our laboratory have shown that circRNAs can act as ceRNA to sponge miRNA and regulate the innate immune response of blunt snout bream(*Megalobrama amblycephala*)after *A. hydrophila* infection, but the specific regulatory mechanism is still unclear. In this study, we discovered a circRNA termed circARHGEF15, which was upregulated after *A. hydrophila* infection and played a positive regulatory role in host antibacterial immunity. Moreover, through functional rescue experiments, we found that miR-214 mimics can restore the activation of the NF-κB signaling pathway due to circARHGEF15 overexpression. Our data indicated that circARHGEF15 can act as a ceRNA of miR-214 in the immune response induced by *A. hydrophila* infection of blunt snout bream, reduce the inhibitory effect of miR-214 on hepcidin, activate the NF-κB signaling pathway ,and promote the expression of downstream inflammatory factors *il-1β, il-6, il-8, tnf-α*, and *ifn-γ*. The overall results of this study suggested that circARHGEF15 plays a crucial role in regulating the antibacterial immunity of blunt snout bream, and provides new insights into the function of circRNAs.

Result

1.*A.hydrophila* infection enhanced circARHGEF15 expression

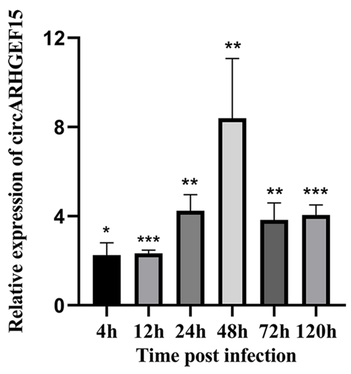


Fig.1 Relative expression of circARHGEF15 in *M. amblycephala* liver post *A. hydrophila* infection. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: *, P <0.05; **, P <0.01; ***, P <0.001.

2.Characterization of circARHGEF15

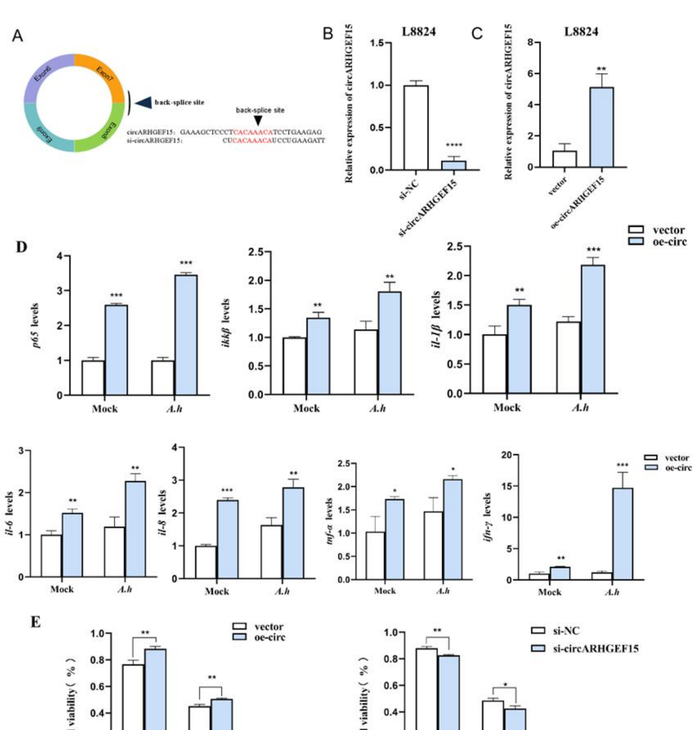


Fig.2 Characterization of circARHGEF15. (A) circARHGEF15 amplified by divergent primers and convergent primers in cDNA and gDNA. (B) Results of Sanger sequencing (red arrow represents the back-splicing site). (C) Relative expression of circARHGEF15 and liner ARHGEF15 mRNA in L8824 cells (liver cells of grass carp) detected by PCR assay followed by nucleic acid electrophoresis or qPCR assay in the presence or absence of RNase R. (D) Relative expression of circARHGEF15 in different tissues of *M. amblycephala*. (E) Relative expression of circARHGEF15 in cytoplasm and nucleus. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: **P < 0.01, and the different letters on the bars indicate the statistical difference (P < 0.05).

3.CircARHGEF15 can enhance the host innate immune response

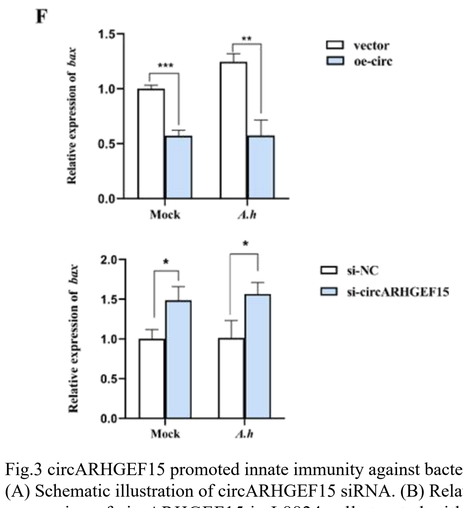
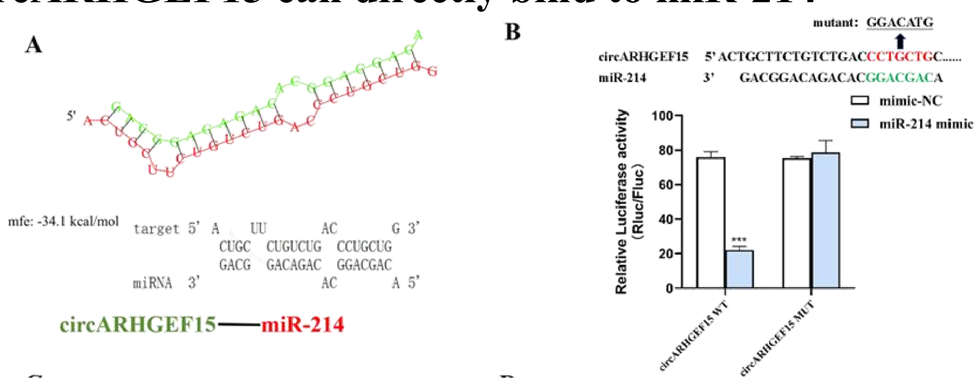


Fig.3 circARHGEF15 promoted innate immunity against bacteria. (A) Schematic illustration of circARHGEF15 siRNA. (B) Relative expression of circARHGEF15 in L8824 cells treated with si-circARHGEF15. (C) Relative expression of circARFGEH15 in L8824 cells after transfected with circARHGEF15 overexpression plasmid (oe-circARHGEF15). (D) Expression levels of *p65, ikkβ, il-1β, il-6, il-8, tnf-α* and *ifn-γ* in L8824 cells transfected with oe-circARHGEF15 (oe-circ) plasmid or control (vector). Mock indicates L8824 cells not treated with *A. hydrophila*. *A.h* indicates L8824 cells treated with A. hydrophila. (E) Cell proliferation assessed by CCK-8 assays in L8824 cells transfected with oe-circ or vector and si-circARHGEF15 or si-NC. (F) Relative expression of bax in L8824 cells transfected with oe-circ or vector and si-circARHGEF15 or si-NC. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: *, P <0.05; **, P <0.01; ***, P <0.001.

4.circARHGEF15 can directly bind to miR-214



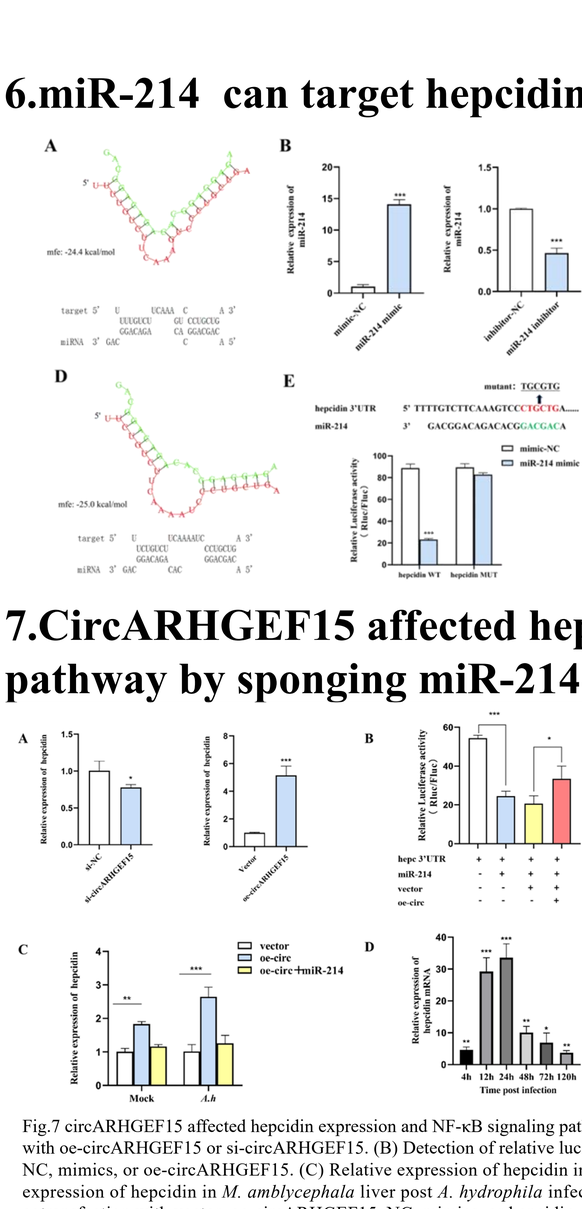
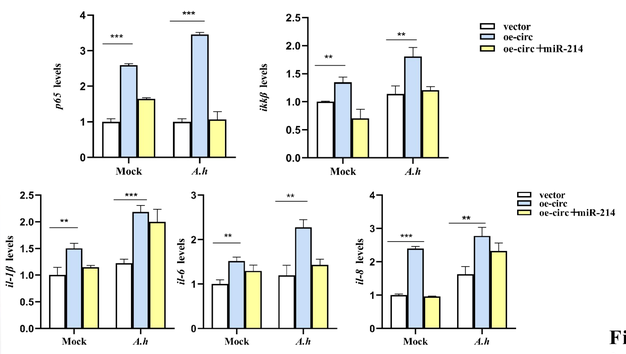


Fig.4 circARHGEF15 can directly bind to miR-214. (A) Predicted binding sites of circARHGEF15 and miR-214. Red indicates the sequence of circAHGEF15, and green indicates the sequence of miR-214. (B) Dual-luciferase activity assay of luciferase reporter verified the binding sites of circARHGEF15 and miR-214. (C) Relative expression of miR-214 in *M. amblycephala* liver post *A. hydrophila* infection. (D) Relative expression of miR-214 in L8824 cells transfected with oe-circARHGEF15 or vector and si-circARHGEF15 or si-NC. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: * P < 0.05，***P < 0.001.

5.CircARHGEF15 promoted the immune response to *A.h* infection by sponge miR-214



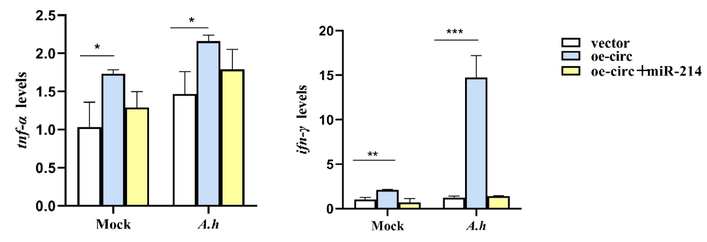


Fig.5 circARHGEF15 sponged miR-214 to promote immune response to *A. hydrophila* infection. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: *, P <0.05; **, P <0.01; ***, P <0.001.

6.miR-214 can target hepcidin

Fig.6 miR-214 can target hepcidin. (A and D) Prediction of binding sites of hepcidin and miR-214 in *M. amblycephala* (A) and grass carp (D). Red indicates the sequence of hepcidin, and green indicates the sequence of miR-214. (B) Relative expression of miR-214 in L8824 cells after cotransfection with mimic-NC or miR-214 mimic (mimic) and inhibitor-NC or miR-214 inhibitor (inhibitor). (C and E) Dual-luciferase activity assay of luciferase reporter verified the binding sites of hepcidin and miR-214 in *M. amblycephala* (C) and grass carp (E). (F) Relative expression of hepcidin in L8824 cells transfected with miR-214mimic/inhibitor/NC. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: **, P <0.01; ***, P <0.001.

7.CircARHGEF15 affected hepcidin expression and NF-κB signaling pathway by sponging miR-214

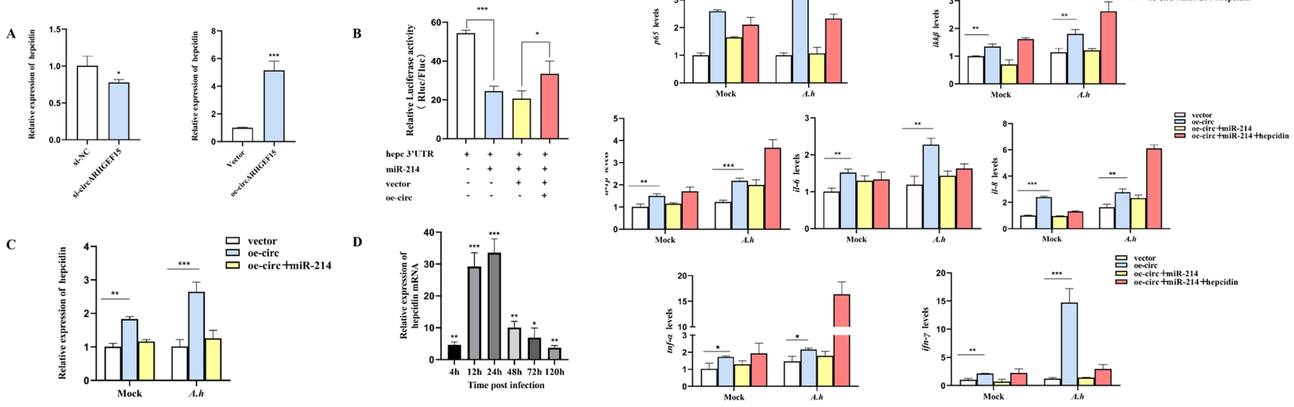


Fig.7 circARHGEF15 affected hepcidin expression and NF-κB signaling pathway by sponging miR-214. (A) Relative expression of hepcidin in L8824 cells after transfection with oe-circARHGEF15 or si-circARHGEF15. (B) Detection of relative luciferase activity in Hela cells after cotransfection with hepcidin 3' UTR luciferase reporter vector, NC, mimics, or oe-circARHGEF15. (C) Relative expression of hepcidin in L8824 cells after cotransfection with vector, NC, oe-circARHGE15, or mimics. (D) Relative expression of hepcidin in *M. amblycephala* liver post *A. hydrophila* infection. (E) Expression levels of *p65, ikkβ, il-1β, il-6, il-8, tnf-α*, and i*fn-γ* in L8824 cells after cotransfection with vector, oe-circARHGEF15, NC, mimics, or hepcidin overexpression plasmid. Values are given as mean ± SD, n = 3. Asterisks indicate significant differences: * P < 0.05， ** P < 0.01, ***P < 0.001.

Conclusion： circARHGEF15 as a ceRNA for miR-214 to enhance hepcidin expression, activate the NF-κB signaling pathway, promote the expression of downstream inflammatory cytokine genes, and thus regulate the innate antimicrobial immune response of *M. amblycephala* after infection by *A.h.*