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Original Research

Mosquito CYP4C21 knockout reduces dengue virus and Zika virus replication in *Aedes aegypti* cells

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ABSTRACT

Aedes aegypti (*Ae. aegypti*) is a major vector of dengue virus (DENV) and Zika virus (ZIKV). Understanding the complex interaction mechanisms between mosquito vectors and arboviruses is essential to interrupt virus transmission. This study constructed CYP4C21 knockout (KO) Aag2 cells (*Ae. aegypti* cells) and confirmed that CYP4C21 KO reduced DENV2 and ZIKV copies in Aag2 cells, which suggests that CYP4C21 may play an important role in mosquito infection with arboviruses. Furthermore, it is the first report of the CYP4 family related to viral infection, which lays the foundation for exploring the role of the CYP4C21 in the interaction of *Ae. aegypti* and arbovirus and provides novel insights into the function of cytochrome family proteins.

1. Introduction

Aedes aegypti (*Ae. aegypti*) is the most common endemic mosquito vector in tropical and subtropical regions, transmitting multiple viruses, such as dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), and chikungunya virus (CHIKV) [1]. *Ae. aegypti* mosquito-transmitted DENV is the most widespread arbovirus in the world, with more than 100 dengue-endemic countries and approximately 390 million people infected annually [2]. ZIKV infection has the potential to cause Guillain-Barré syndrome in adults [3] or microcephaly in infants [4]. Due to the antibody-dependent enhancement effect of DENV and ZIKV infection [5], there is no widely used or approved vaccine to prevent infection by these viruses [6].

CYP4C21 may be involved in *Ae. aegypti* infection with DENV2 according to a previous study of transcriptome analysis (unpublished data). CYP4C21 is a member of the cytochrome P4 family of the cytochrome P450 (CYP450) family. CYP450 participates in the synthesizing or catabolizing of exogenous and endogenous physiological substances (pheromones, ecdysteroids, juvenile hormones) [7,8]. The CYP4 family contains numerous cytochrome P450 genes, which have been reported in many species. Most CYP4 family genes are associated with the catabolism and detoxification of exogenous compounds [9,10]. CYP4C21 genes have been reported in the German cockroach, *Blattella germanica* [11], *Ae. aegypti*, *Ae. albopictus*, *Culex quinquefasciatus*, and *Cx. pipiens pallens*. The expression of CYP4C21 was induced after Haedoxan A treating in the transcriptome analysis of *Ae. albopictus* larvae, which indicates the response of *Ae. albopictus* CYP4C21 to exogenous metabolism [12]. A potential relationship between CYP4C21 gene expression and insecticide resistance was also identified by transcriptome analysis in *Ae. aegypti* [13]. CYP4C21 is expressed in the antennae of *Cx. quinquefasciatus* and *Cx. pipiens pallens*, and transcriptomic results suggest that this gene may play a specific role in insecticide resistance differences between mosquito species [14]. Most studies to date on CYP4C21 are associated with insecticide resistance, and there are no studies on this gene and arbovirus infection or innate immunity.

In recent years, genetically modified mosquitoes by CRISPR/Cas9 system is emerging as a new technique for mosquito control [15]. In addition, CRISPR/Cas9 technology is often used for gene function research. Rozen-Gagnon et al. constructed plasmids for AGO1 editing in Aag2 cells (*Ae. aegypti* cells), enabling efficient editing, and immunocompetent mosquito cell lines used widely in arbovirus research [16].

To investigate the function and mechanism of the CYP4C21 gene in response to DENV2 in Aag2 cells in detail, CYP4C21 knockout (KO) Aag2 cells were constructed. Extracellular viral RNA copies were used to evaluate viral replication because viral particles were released into the extracellular after replication and assembly. The KO of CYP4C21 resulted in the decrease of extracellular DENV2 copies by comparing the difference in extracellular DENV2 or ZIKV RNA copies between

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HIGHLIGHTS

Scientific question

The CYP4C21 gene was found to be potentially involved in the interaction of *Aedes aegypti* (*Ae. aegypti*) and dengue virus (DENV). This study's main scientific issue is to verify the correlation between the CYP4C21 gene and the process of DENV and Zika virus (ZIKV) infection.

Evidence before this study

Cytochrome P450 is involved in the metabolism of endogenous or exogenous substances. Mosquito CYP family members are mostly associated with insecticide metabolism, however, there are no published research associated with mosquito CYP family member and arboviral infections.

New findings

CYP4C21 gene expression was decreased in both *Ae. aegypti* and Aag2 cells following infection with DENV2. Moreover, the CYP4C21 knockout Aag2 cell lines were successfully constructed by CRISPR/Cas9 system, which revealed the CYP4C21 deletion reduced the replication of DENV and ZIKV in *Ae. aegypti* cells.

Significance of the study

This study shows that CYP4C21 is involved in the mosquito vector response to arboviruses, and lays the foundation for subsequent exploration of the role of the CYP4C21 gene in arbovirus infection in *Ae. aegypti* but also provides novel insights into the function of cytochrome family proteins.

CYP4C21 KO and wild-type (WT) Aag2 cells. This study contributes to a better understanding of the complicated biological interactions of mosquitoes and arbovirus, as well as establishes the foundation for the further development of novel mosquito control techniques.

2. Methods

2.1. Virus, cells, and mosquito

The DENV2 Guangdong strain was provided by Guangdong Provincial Centers for Disease Control and Prevention, China [17]. ZIKV strain SZ01 (GenBank: KU866423) is stored in the microbial culture collection center of the Institute of Microbiology and Epidemiology in Beijing. *Ae. aegypti* Aag2 cells were cultured in Schneider's Drosophila Medium (SDM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 28 °C with 5% CO_2. C6/36 cells were used to amplify DENV2 and ZIKV by culturing in RPMI Medium 1640 basic (RPMI 1640, Gibco) containing 10% FBS, at 28 °C and 5% CO_2. BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS at 37 °C with 5% CO_2 for DENV2 and ZIKV titers determination. *Ae. aegypti* (the Hainan strain) was fed with 8% sugar water at a temperature of 28 °C, relative humidity of 80%, and a photoperiod of 14:10.

2.2. Oral infection of Ae. aegypti with DENV2 and mosquito dissection

After emergence, 4- to 6-day-old mosquitoes were deprived of sucrose solution for twenty-four hours before being offered the infectious blood meal. The blood meal was prepared by mixing the DENV2 suspension with fresh mouse blood at a ratio of 1:1. At the same time, DMEM replaced the DENV2 suspension in the controls. The virus blood meal was retained at 37 °C by a multifunctional insect blood supply device (Hemotek, PS6A Power Unit) and supplied to the mosquitoes for two hours in the dark. Fully engorged females were then transferred to an 8% sugar solution. The female mosquitoes to be dissected were anesthetized with CO_2 and then placed in a dish maintained at a low temperature. The mosquitoes were placed in phosphate-buffered saline (PBS) in the middle of the slides before the salivary glands and midgut tissue were dissected under the microscope. These tissues were rinsed three times with PBS and transferred to electropolished (EP) tubes. Mosquito tissue RNA was extracted using TRIzol (TAKARA).

2.3. Design and synthesis of sgRNA targets

The DNA sequence of the *Ae. aegypti* CYP4C21 gene (Gene ID: 23687556) was obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/), and sgRNA guide sequences were designed on the first two exons through the benching website (https://benching.com/): sgRNA1 target sequence, 5′-TGCCGACATGCTCAACCATC-3′, sgRNA2 target sequence, 5′-GTGCCCAAAATCTAGTCAAG-3′. sgRNA sequences were synthesized by GenScript Biotech.

2.4. Construction of CYP4C21 KO Aag2 cells

Chemically synthesized sgRNA and Cas9 proteins (Invitrogen) are delivered into the cell by electrotransfer. Ribonucleoprotein (RNP) complexes are formed by sgRNA and Cas9 protein combined in vitro. The collected Aag2 cells were resuspended in a mixture of RNP complex and electrotransfer buffer (Lonza), subsequently transferred to the electrotransfer cup. The electro transformer (Lonza) was run in the K562 program for five minutes. Cells were resuspended in SDM medium containing 10% fetal bovine serum (FBS) after electro-transformation and incubated at 28 °C. Individual cell clones were subsequently selected under a microscope and placed in 96-well plates.

2.5. CYP4C21 KO cells detection at the DNA level

After growing to a specific quantity, cell DNA was extracted (Insect DNA Extraction Kit, Shanghai Enlighten Biotech) and amplified by polymerase chain reaction (PCR) with the designed primer sequences (forward primer: 5′-CGGTTAGTTCCGATACGGTC-3′ and reverse primer: 5′-TCAACTACTAGCCACCTCGT-3′), then the amplified products were subjected to agarose gel electrophoresis and sequencing. The PCR system included the following: 2 × EasyTaq® PCR SuperMix (TransGen Biotech), 12.5 μl, forward primer (PCR system: 2 × EasyTaq® PCR SuperMix, 12.5 μl, forward primer (10 μM), 0.5 μl, reverse primer (10 μM), 0.5 μl, DNA template, 2 μl, nuclease-free water, replenished to 25 μl. The reaction procedure was as follows: 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min 15 s, and 72 °C for 10 min. The PCR product was subjected to agarose gel electrophoresis, and the PCR products were submitted to Tianyi Huiyuan Company for sequencing.

2.6. qRT-PCR detection of CYP4C21 expression in Ae. aegypti and Aag2 cells

KO Aag2 cells, WT Aag2 cells (including DENV2-infected and non-infected cells), and midgut and salivary gland RNA were extracted with TRIzol, and TransScript® Green One-Step qRT-PCR SuperMix (TransGen Biotech, Beijing) was used for quantitative reverse transcription PCR (qRT-PCR) detection with RNA as the template. The qRT-PCR primer sequences used were forward primer (5′-GCCAAGCTGTTTCAAATACGAATG-3′) and reverse

primer (5′-TACAAGAAAGGTTTCTCAAGGCACT-3′). The reaction system included 2 × PerfectStartTM Green One-Step qPCR SuperMix, 10 μl, TransScript® Green One-Step RT/RI Enzyme Mix, 0.4 μl, Forward Primer (10 μM), 0.4 μl, Reverse Primer (10 μM), 0.4 μl, Passive Reference Dye (50 ×), 0.4 μl, RNA template, 2 μl, nuclease-free water, filled to 20 μl. The reaction procedure was as follows: 45 °C for 5 min, 40 cycles of 94 °C for 30 s, 94 °C for 5 s and 60 °C for 30 s. Ribosomal protein S6 (RPS6) was used as the reference gene, and the relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method[17]. The reference gene sequences were forward primer (5′-CGTCGTCAGGAACG TATCC-3′) and reverse primer (5′-TTCTTGGCAGCCTTAGCAG-3′).

2.7. Viral infection of KO and WT Aag2 cells

Five milliliters of Aag2 cell suspension with a 1×10^6 cells/ml density were inoculated into 25-cm^2 cell culture bottles. After incubation for twelve hours, DENV2 was diluted to an appropriate proportion in an SDM medium containing 5% FBS, and the multiplicity of infection (MOI) was 0.01. Next, one milliliter of the diluted virus was added to each cell vial and cultured in a cell incubator at 5% CO_2 and 28 °C. After infection, 200 μl of the cell supernatant was taken daily and stored at −80 °C until the 7th day after infection for viral RNA copy detection. The experiment was designed in two groups, with four biological replicates: the KO group (KO Aag2 cells + DENV2) and the WT group (WT Aag2 cells + DENV2). First, ZIKV was diluted to the appropriate proportions in an SDM medium containing 5% FBS to obtain an MOI of 0.001. Other operations were similar to those for DENV2. The supernatant was taken from cells at 1, 2, 3, 4, 5, 6, and 7 days post-infection and stored at −80 °C.

2.8. Detection of extracellular viral copies in the cell supernatant

Extracellular DENV2 RNA copies were detected using the DENV2 nucleic acid-free detection kit (Beijing Merabo Medical Technology). The reaction system included 2 × DENV2 amplification solution, 10 μl, DENV2 primer and probe mixture, 1 μl, reverse transcription PCR (RT-PCR) enzyme mixture, 1 μl, cell supernatant, 4 μl, and nuclease-free water to 20 μl. The reaction conditions were as follows: 50 °C for 10 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. DENV2 primers and the probe sequence were as follows: forward primer (5′-AATTAGAGAGCAGATCTCTGATGAA-3′) and reverse primer (5′-AGCATTCCAAGTGAGAATCTCTTTGT-3′), DENV2 primers and probe (5′-AGCATTCCAAGTGAGAATCTCTTTGTCA-3′). Extracellular ZIKV RNA copies were detected using a ZIKV nucleic acid-free detection kit (Beijing Meilaibo Medical Technology). The ZIKV primers and probe sequence were as follows: forward primer (5′-CGCTGCCCAACACAAGGT-3′), reverse primer (5′-CCAC TAACGTTCTTTTGCAGACA-3′), and ZIKV probe (5′-AGCCTACCTTGA CAAGCAGTCAGA-3′). The reaction system and conditions were the same as described above for DENV2 amplification.

2.9. Statistical methods

All data were presented as means ± standard deviation (SD). Excel 2019 was used to process the data, GraphPad Prism 8.0 software was used for mapping, and SPSS software was used to conduct an independent sample *T*-test for target gene expression levels and extracellular viral RNA copies in KO and WT cells.

3. Results

3.1. The CYP4C21 gene was downregulated after the DENV2 infection

DENV2 infection and control group relative CYP4C21 expression fold change (FC) were detected in *Ae. aegypti* midgut and salivary glands of. The FCs of the salivary gland and midgut were 0.606 and 0.232, respectively (Table 1), suggesting that the CYP4C21 was downregulated after DENV2 infection in *Ae. aegypti*. To verify the presence of this phenomenon in Aag2 cells, this study confirmed that the CYP4C21 gene was downregulated in Aag2 cells infected with DENV2 ($P < 0.05$), consistent with the results observed in *Ae. aegypti*.

Table 1
CYP4C21 gene Expression in different tissues after dengue virus 2 (DENV2) infection.

Tissue	Fold change (DENV2 infected/noninfected)	*P* value
Salivary gland	0.606	0.003
Midgut	0.232	0.001
Aag2	0.381	0.012

3.2. CYP4C21-KO cells construction by CRISPR/Cas9 system

This study constructed CYP4C21 KO cell lines by CRISPR/Cas9 system. Ribonucleoprotein (RNP) complexes are formed by chemically synthesized sgRNA and Cas9 protein combined in vitro, which increases the stability of sgRNA and decreases the off-target effect [18]. The construction process of CYP4C21 KO cell lines is shown in Fig. 1A, and a total of 85 cell lines were obtained. According to the agarose gel electrophoresis According to the agarose gel electrophoresis of monoclonal Aag2 cell CYP4C21 DNA amplification products (Fig. 1B), seven of the 85 strains selected showed no results, 19 were polyclonal, and 59 were monoclonal. Together with the product sequencing results, 15 of the 59 monoclonal cell strains exhibited sequence alignments compatible with WT Aag2 cells. The CYP4C21 gene was mutated in the remaining 44 cell lines. Compared with WT Aag2 cells, 42 cells were successfully knocked out (the length of the deleted fragment was not a multiple of three). The KO rate (number of monoclonal KO cells/total number of cells) was approximately 49.41%.

3.3. Detection of CYP4C21 gene expression in Aag2 KO cells

CRISPR/Cas9 gene editing strategy is shown in Fig. 2A, where two pairs of sgRNAs were designed at positions 268-290 bp in exon 1 and 589-611 bp in exon 2. There were two main types of deletions in these 42 cell lines. In some of these cells, gene KO was only caused by deleting gene fragments. For example, cell line 44 (the successor is called KO1) carried a deletion of 327 bp at position 279-605 bp of the CYP4C21 DNA fragment. Deleting 263 bp in the coding region successfully caused a frameshift mutation. The sequence of deleted fragments is shown for KO1 in Fig. 2A. In some cell lines, fragments were deleted and caused code-switching mutations by fragment insertion. For example, in cell line 36 (the successor is called KO2), a 120 bp fragment was inserted, and a 321 bp fragment was replaced at 284-604 bp. 257 bp was deleted at the position of the coding region, and a sequence of 120 bp in length was inserted, as shown in KO2 in Fig. 2A. Both KO cell lines caused frameshift mutations at the DNA level. Sequencing peaks of the two KO cell lines with the WT cell line are depicted in Fig. 2B. The red box shows the sequence sites that have changed, and the cell peak map indicates that the KO cell lines are monoclonal cells.

To further verify the deletion at the mRNA level, the expression levels of CYP4C21 mRNA in KO cells were measured by qRT-PCR. We designed primers at the sites of codeletion in two KO cell lines, and the pairing of primers and CYP4C21 mRNA is shown in Fig. 2A. The detection results are shown in Fig. 3A and 3B. The KO and WT groups had three replicates per group. As illustrated, CYP4C21 mRNA was usually expressed in WT Aag2 cells, whereas it was hardly expressed in KO Aag2 cells. The relative CYP4C21 gene expression

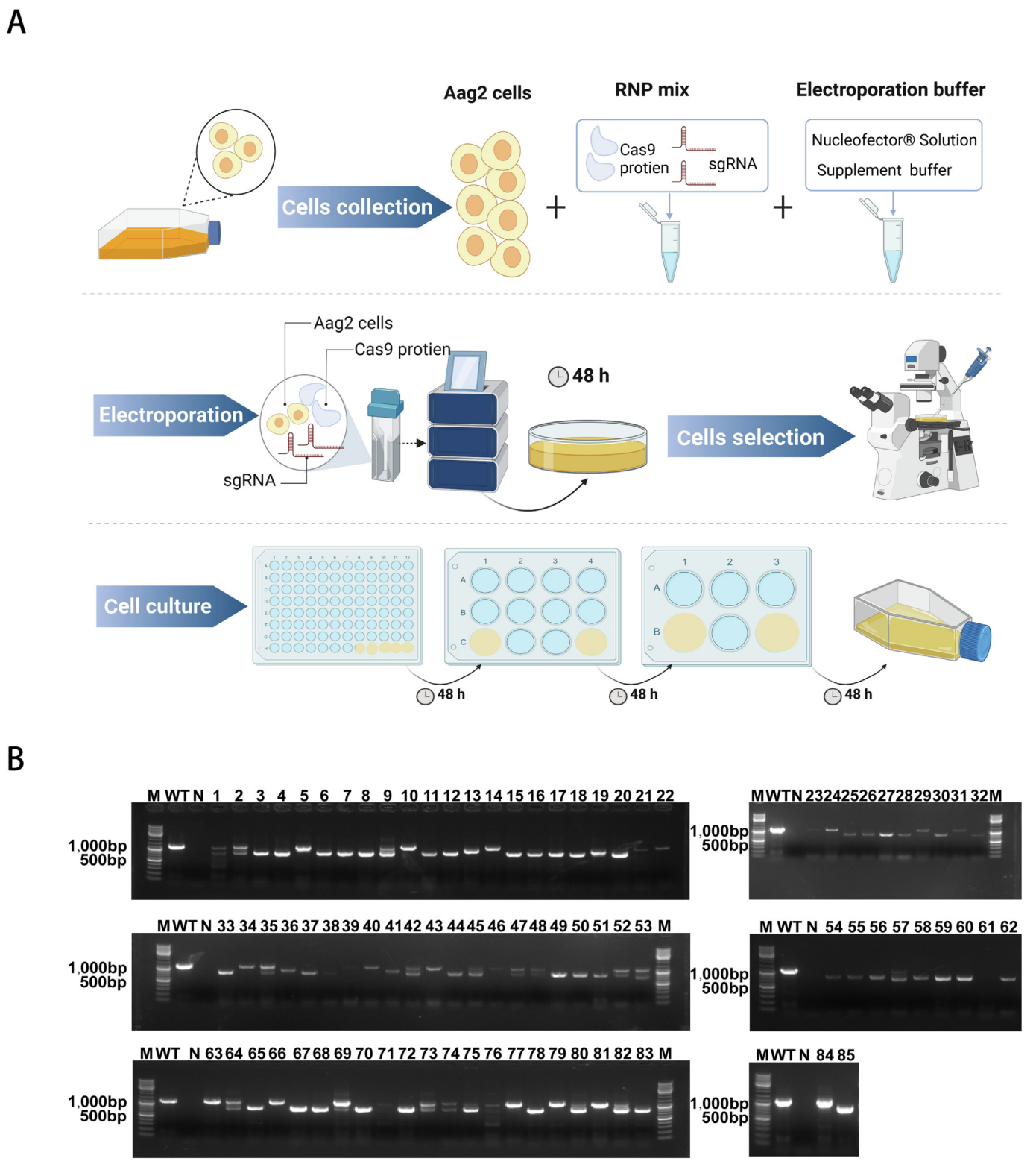

Fig. 1. Construction of CYP4C21-knockout (KO) Aag2 cells. A) The construction process of CYP4C21 gene-KO Aag2 cells. B) The results of agarose gel electrophoresis of CYP4C21 gene amplification in single cells. M indicates the 2,000 bp marker, WT is the CYP4C21 gene of WT Aag2 cells 1,134 bp in length, and N indicates the blank control. The selected monoclonal cells were named cell lines 1–85. Abbreviations: WT, wild-type; RNP, ribonucleoprotein.

level in KO Aag2 cells was significantly decreased. There was a significant difference in the relative expression of the CYP4C21 gene between WT and KO Aag2 cells. In conclusion, CYP4C21 was successfully knocked out in our two KO cell lines at both the DNA and mRNA levels.

3.4. Effect of CYP4C21 gene KO on virus replication in Aag2 cells

To verify the influence of the CYP4C21 gene on virus replication, extracellular viral RNA copies were detected on days 1, 2, 3, 4, 5, 6, and 7 post-DENV2 infections in KO and WT Aag2 cells. The DENV2

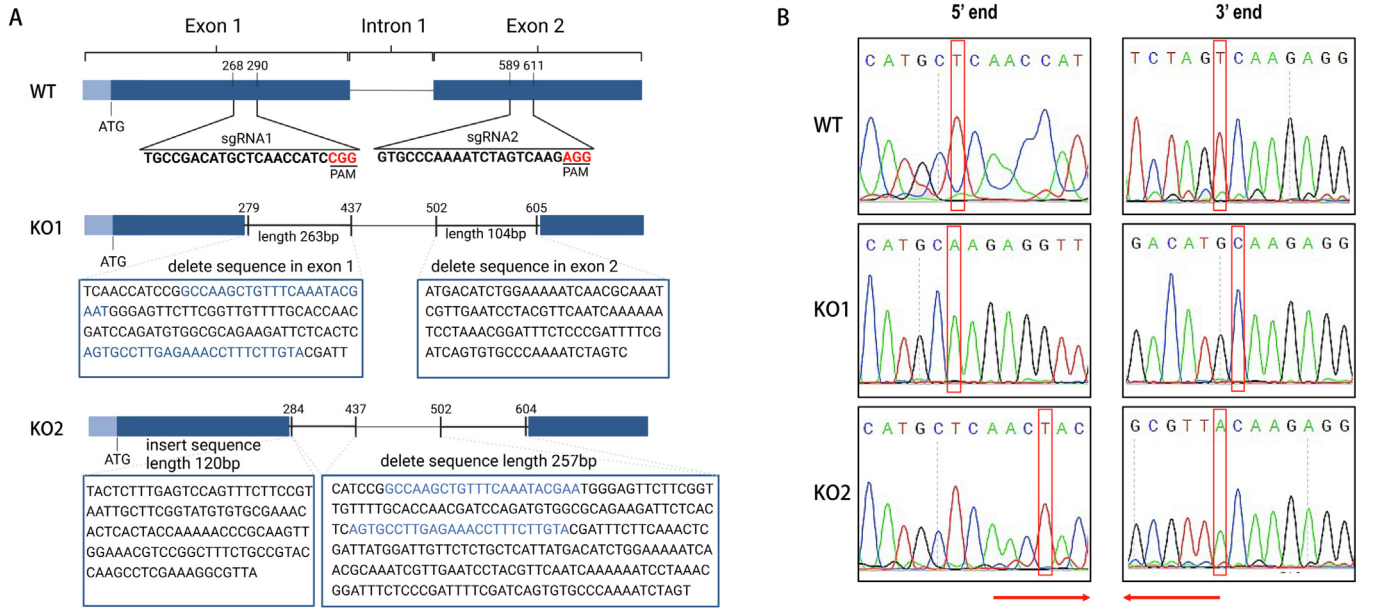

Fig. 2. CYP4C21 deletion in knockout (KO) cells. A) CYP4C21 KO strategy. Two pairs of sgRNAs were designed in the first two exons, and the sgRNA sequences and sites are shown in the figure. KO1 KO cells deleted 263 bp in the CYP4C21 coding region, causing a frameshift mutation. The blue part of the exon 1 deletion sequence is the primer sequence for quantitative reverse transcription polymerase chain reaction (qRT-PCR) detection. KO2 KO cells successfully generated a frameshift mutation by inserting a 120 bp sequence into the CYP4C21 coding region and deleting 257 bp. B) Comparison of peak maps between two KO cell lines and wild-type(WT) cells. The sequence sites that started to change are in the red box, and the cell peak plot indicates that the screened cell line is monoclonal.

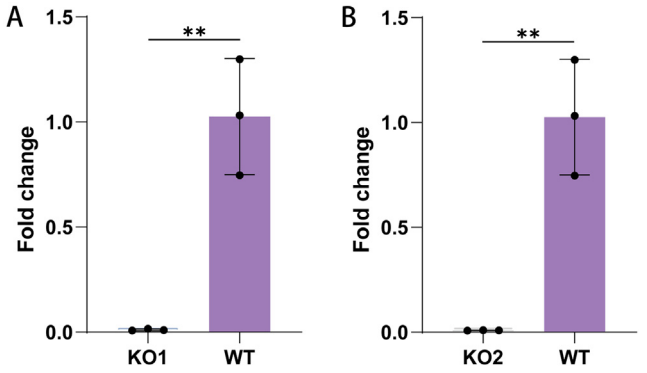

Fig. 3. Quantitative reverse transcription polymerase chain reaction (qRT–PCR) detection of CYP4C21 mRNA expression level in knockout (KO) Aag2 cell lines. A) qRT–PCR primers were used for the CYP4C21 mRNA binding region. The original sequence was jointly deleted in the two KO cell lines. B) CYP4C21 mRNA expression level in KO1 cell lines. The expression of CYP4C21 mRNA in WT Aag2 cells was used as a control, and the ribosomal protein S6 was used as the reference gene. The 2-ΔΔC method was used to determine relative expression. Independent sample *T*-tests were employed, with three biological replicates per group. Data represent means ± standard deviations (SDs), ** $P < 0.01$. Abbreviation: WT, wild-type.

infection results are provided in Fig. 4A and 4C. In general, extracellular viral RNA copies accumulated as the duration of infection increased, with the extracellular viral RNA copies of the KO cell lines being lower than those of the WT cell line. There was a significant difference in DENV2 RNA copies between KO1 and WT at all observation time points except 3 dpi (Fig. 4A). Although there was a significant difference between KO2 and WT only on days 1, 2, and 7 dpi ($P < 0.01$), the extracellular DENV2 RNA copies of KO2 were consistently lower than those of WT during infection (Fig. 4C), which was consistent with the results of KO1. Extracellular ZIKV RNA copies of KO and WT Aag2 cell lines were analyzed on days 1, 2, 3, 4, 5, 6, and 7 post-ZIKV infections to investigate whether the gene has a similar effect on other viruses of the *Flavivirus* genus. As with DENV2, extracellular ZIKV RNA copies increased with increasing duration of infection. The extracellular ZIKV RNA copies of the KO1 cells were significantly lower than those of the WT cells (Fig. 4B), with the most significant difference observed at 6 dpi ($P < 0.001$). Cell numbers were counted at the same time points to exclude the effect of cell growth. The growth curves of KO1, KO2, and WT cells are shown in Fig. 4D. The growth rates of different cells were slightly different: KO1 cells grew marginally slower than WT cells, and KO2 cells grew appreciably faster than WT cells. However, the DENV2 copies in KO cell lines were lower than those in WT cell lines. Therefore, CYP4C21 KO reduced DENV2 replication in Aag2 cells and had the same effect on ZIKV.

4. Discussion

In summary, this study found a decrease in *Ae. aegypti* CYP4C21 expression in response to DENV2 infection, with similar results observed in Aag2 cells, suggesting that CYP4C21 is downregulated after DENV2 infection in *Ae. aegypti* and Aag2 cells. Subsequently, to investigate the role of CYP4C21 in the infection of *Ae. aegypti* with DENV2, CYP4C21 KO cell lines were constructed by CRISPR/Cas9 system, and the deletion of CYP4C21 was identified at the DNA and RNA levels. CYP4C21 KO reduces DENV2 and ZIKV replication, by detecting viral RNA copies and growth rates in KO and WT cells at different time points. Despite the different growth rates, the viral copies of the two KO cell lines were lower than those of the WT cells. This study hypothesizes that this phenomenon may be caused by activating the innate immune response of mosquitoes.

Ae. aegypti mosquitoes acquire the virus by feeding on the blood of an infected individual. The virus first infects the midgut, breaks through the midgut barrier, infects other tissues after replicating in the midgut to some degree, and finally reaches the salivary glands of mosquitoes for transmission [19]. This replication cycle means the virus must infect and replicate in multiple types of mosquito cells, impacting multiple molecules and pathways [20]. DENV infection

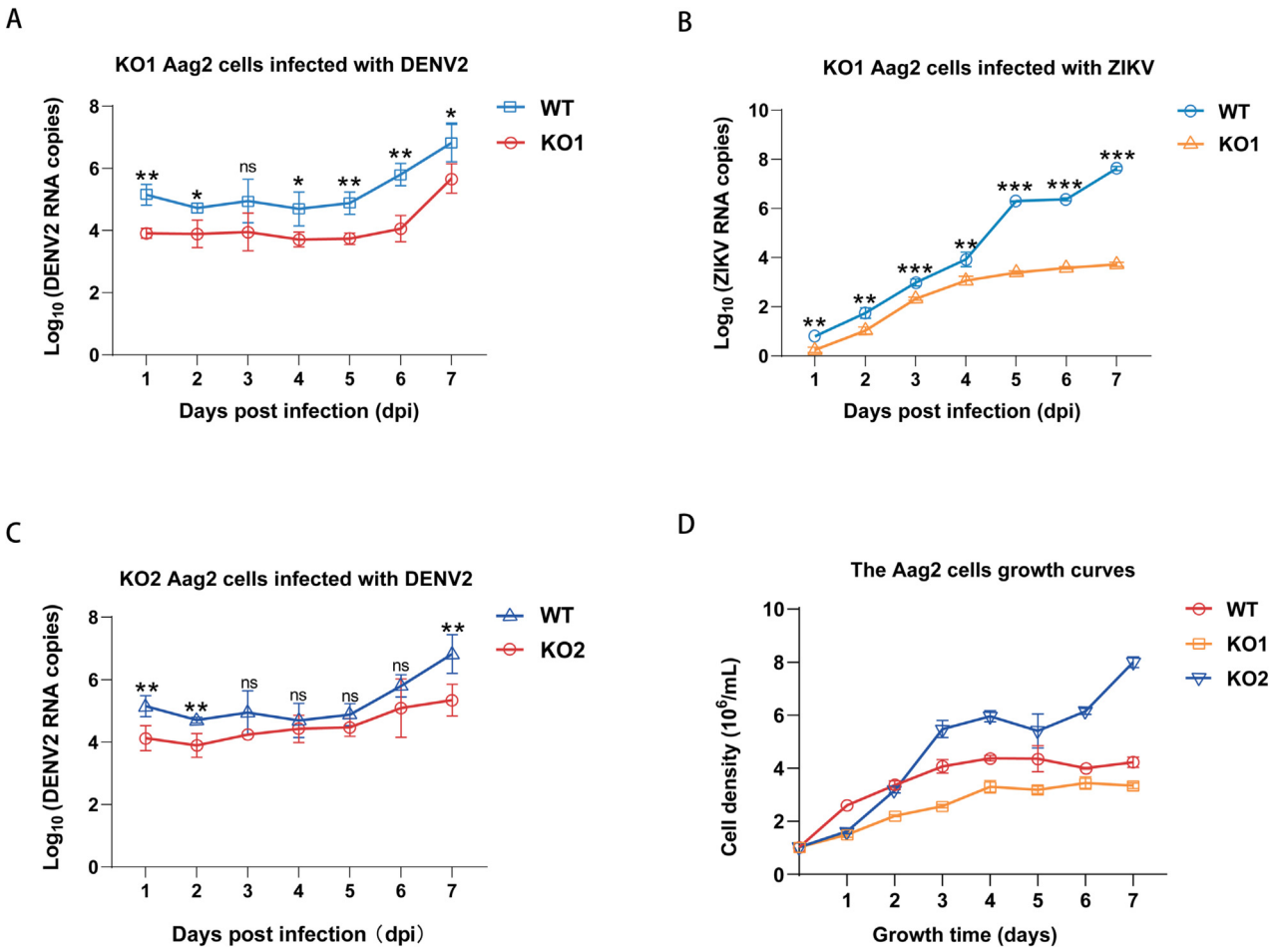

Fig. 4. Detection of extracellular viral RNA in knockout (KO) cell supernatants. A) dengue virus 2 (DENV2) replication curves in KO1. Extracellular DENV2 RNA copies were assayed at 1, 2, 3, 4, 5, 6, and 7 days post-DENV2 infection. B) ZIKV replication curves in KO1. Extracellular ZIKV RNA copies were assayed at 1, 2, 3, 4, 5, 6, and 7 days post-ZIKV infection. C) DENV2 replication curves in KO2. Extracellular DENV2 RNA copies were assayed at 1, 2, 3, 4, 5, 6, and 7 days post DENV2 infection. All the above were tested by independent sample *T*-tests, with four biological replicates per group. Data represent means ± standard deviations (SDs), $^{**}P < 0.01$, $^{***}P < 0.001$, ns means no significance. D) Cell growth curve, indicates number of cells at different growth stages. Abbreviations: WT, wild-type; DEMV2, dengue virus 2; ZIKV, Zika virus.; KO, knockout cell.

induces innate immunity in mosquitoes, and the mosquito innate immune response is a crucial determinant for successful virus transmission [21,22]. Mosquito innate immunity mainly includes activation of signaling pathways and immune effector responses of hemocytes, leading to the synthesis and secretion of soluble molecules such as antimicrobial peptides (AMPs), reactive oxygen species (ROS), and components of the phenoloxidase cascade [23–25]. Genes encoding antimicrobial peptides are transcribed after activation of the Toll signaling pathway, JAK-STAT signaling pathway [26], and immunodeficiency signaling (IMD) pathway [27,28].

Although mosquito infection with DENV usually leads to the activation of innate immunity, producing a strong immune response and promoting the expression of some immune genes, virus replication also leads to the downregulation of some immune responses in mosquito tissues. For example, studies have reported that most differentially expressed genes in the midgut, body, and salivary glands of *Ae. aegypti* are downregulated in the late stage of DENV infection [24,29]. Similar findings in the Aag2 cell line with immune competence were found, whereby the expression levels of many immune signaling molecules and antimicrobial peptides (AMPs) were downregulated after DENV infection [30].

Furthermore, Li et al. found that the infection rate of dengue virus decreased after the KO of C-type lectin, the immune pathways of mosquitoes, such as JAK/STAT, IMD, and Toll, were activated, and antimicrobial peptides were increased, further indicating that our conjecture is possible [31]. These studies suggest that many genes associated with innate immunity are downregulated in the mosquito response to DENV. In our study, CYP4C21 was also downregulated in *Ae. aegypti* and Aag2 cells in response to DENV2 infection. These results suggest that innate immunity may also be activated after CYP4C21 KO and indirectly reduce viral copies.

The CYP4C21 gene belongs to the CYP4 family. Many genes in this family are involved in arachidonic acid metabolism, retinol metabolism, the PPAR signaling pathway, fatty acid degradation, and other metabolic pathways. In the serotonergic synapse, insect hormone biosynthesis (JH) and regulation of inflammatory mediators of TRP channels. For example, CYP4A and CYP4F8 participate in the metabolism of arachidonic acid. CYP4A is also involved in retinol metabolism, fatty acid metabolism, the PPAR signaling pathway, and inflammatory mediator regulation of TRP channels. In addition, CYP4X is related to the serotonergic synapse, and CYP4C7 is related to insect hormone biosynthesis.

Among the above pathways, the PPAR signaling pathway and fatty acid degradation are all related to mosquito innate immunity. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors activated by fatty acids and their derivatives [32], they play

an important role in lipid oxidation and cell proliferation, promoting adipocyte differentiation and enhancing blood glucose absorption [33]. The PPAR signaling pathway regulates fatty acid oxidation and promotes fatty acid metabolism [34,35]. In mosquitoes, fatty acids play an important role in innate immunity [36]. After the Japanese encephalitis virus infection, genes related to the PPAR signaling and fatty acid metabolism pathways were found to be downregulated in dendritic cells (DCs) [37]. In studies on the isolation and identification of human fatty acid homologs in *Ae. aegypti* (AaFAS1), the replication of DENV was significantly inhibited in the midgut of *Ae. aegypti* and in Aag2 cells and after AaFAS1 knockdown [38]. Insect juvenile hormone-binding protein is essential for mosquito innate immunity [39], and a mutant phenotype characterized by immunosuppression at both humoral and cellular levels were identified by constructing insect juvenile hormone-binding protein-deficient mosquito strains. Interestingly, injection of recombinant WT juvenile hormone-binding protein into adult female mosquitoes three days before infection was sufficient to restore normal immune function. Another study showed that the KO of the methoprene-tolerant (Met) gene encoding a juvenile hormone receptor in insects resulted in a black larval phenotype at the L3 and L4 stages and death before pupation [40].

According to the above studies on the function of CYP4 family genes, they are primarily involved in the metabolism and synthesis of substances and participate in the innate immune response of mosquitoes through substance metabolism. Therefore, we hypothesize that the CYP4C21 gene may be involved in metabolism and indirectly participate in innate immunity, thereby regulating the response to arbovirus infection in *Ae. aegypti*.

In summary, the CYP4 subfamily members identified thus far are primarily involved in the metabolism of arachidonic acid, retinol, fatty acids, and insect juvenile hormone synthesis. These factors are also associated with mosquitoes' innate immunity and endostatin environment. However, no study has demonstrated the relationship between viral infection and CYP4 family members. Here, we propose for the first time that CYP4C21 KO reduces the replication of DENV2 and ZIKV in Aag2 cells suggesting that the CYP4C21 gene plays a vital role in the response of *Ae. aegypti* to mosquito-borne viruses and provides new clues for the function of the CYP4C21 gene. However, the specific mechanism of the CYP4C21 gene in this process needs to be further investigated.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

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