ZFP36 ring finger protein like 1 significantly suppresses human coronavirus OC43 replication

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CCCH-type Zinc figure proteins (ZFP) are small cellular proteins that are structurally maintained by zinc ions. Zinc ions coordinate the protein structure in a tetrahedral geometry by binding cysteines or cysteines and histidine amino acids. The unique structure of ZFP enables it to interact with a wide variety of molecules including RNA and thus modulate several cellular processes including host immune response and virus replication. CCCH-type ZFPs have shown their antiviral efficacy against several DNA and RNA viruses. However, their role in human coronavirus still needs to be characterized. The current study evaluated the effect of ZFP36L1, a CCCH-type ZFP on human coronavirus (HCoV)-OC43. We overexpressed or knockdown the ZFP36L1 in HCT-8 cells using lentivirus transduction. Wild type, ZFP36L1 overexpressed, or ZFP36L1 knockdown cells were infected with HCoV-OC43 and virus titer in these cells was measured over 96 hours post-infection (p.i.). Results of the current study showed that HCoV-OC43 replication was significantly reduced with overexpression of ZFP36L1 while knockdown of ZFP36L1 significantly enhanced the virus replication. ZFP36L1 knockdown HCT-8 cells started producing the infectious HCoV-OC43 at 48 hours post-infection which was much earlier as compared to wild-type or ZFP36L1 overexpressed HCT-8 cells which started producing infectious virus at 72 hours post-infection. Overall, the current study showed that overexpression of ZFP36L1 suppressed human coronavirus (OC43) production.

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Abstract

CCCH-type Zinc figure proteins (ZFP) are small cellular proteins that are structurally maintained by zinc ions. Zinc ions coordinate the protein structure in a tetrahedral geometry by binding cysteines or cysteines and histidine amino acids. The unique structure of ZFP enables it to interact with a wide variety of molecules including RNA and thus modulate several cellular processes including host immune response and virus replication. CCCH-type ZFPs have shown their antiviral efficacy against several DNA and RNA viruses. However, their role in human coronavirus still needs to be characterized.

The current study evaluated the effect of ZFP36L1, a CCCH-type ZFP on human coronavirus (HCoV)-OC43. We overexpressed or knockdown the ZFP36L1 in HCT-8 cells using lentivirus transduction. Wild type, ZFP36L1 overexpressed, or ZFP36L1 knockdown cells were infected with HCoV-OC43 and virus titer in these cells was measured over 96 hours post-infection (p.i.). Results of the current study showed that HCoV-OC43 replication was significantly reduced with overexpression of ZFP36L1 while knockdown of ZFP36L1 significantly enhanced the virus replication. ZFP36L1 knockdown HCT-8 cells started producing the infectious HCoV-OC43 at 48 hours post-infection which was much earlier as compared to wild-type or ZFP36L1 overexpressed HCT-8 cells which started producing infectious virus at 72 hours post-infection. Overall, the current study showed that overexpression of ZFP36L1 suppressed human coronavirus (OC43) production.

Keywords: CCCH type Zinc finger protein, ZFP36L1, RNA binding protein, human coronavirus OC43

Introduction

ZFPs are small cellular proteins that are structurally maintained by zinc ions. Zinc ions coordinate the protein structure in a tetrahedral geometry [1, 2]. There are more than 40 different types of ZFP that have been annotated so far [2]. The unique structure of ZFP enables it to interact with a wide variety of molecules such as DNA, RNA, PAR (poly-ADP-ribose), and cellular proteins and thus modulate several cellular processes including host immune response and virus replication [3-11]. Among various ZFPs, one family known as CCCH-type ZFP where Zinc ions coordinate the protein structure by binding cysteines or cysteines and histidine amino acids[1, 2], has been characterized for its antiviral [2, 10, 12-21] and immune modulator activity [22-35].

A few of the known mechanisms by which CCCH-type ZFPs exhibit these antiviral or immunomodulatory activities are, that they limit the total mRNA turnover in the cell. CCCH type ZFPs have two tandem zinc finger (TZF) domains that bind to adenyl and uracyl nucleotides rich (AU-rich) elements (AREs) in mRNA and lead to the removal of the poly(A) tail from the mRNA by promoting its deadenylation and degradation [36-41]. CCCH-type ZFPs also have the ability to interact with cellular mRNA decaying enzymes such as exoribonuclease (5 -3 exoribonuclease XRN1 and 3 -5 exoribonuclease exosome complex) or decapping enzymes (DCP1 and DCP2), and these interactions promote the degradation of mRNA[41]. CCCH-type ZFPs also exhibit their antiviral and immunomodulatory activity by inhibiting the translation process. CCCH-type ZFPs disrupt the interaction between eIF4G and eIF4A, preventing protein translation and leading to RNA degradation [42]. As an eIF4G- eIF4F complex is needed for recruiting the 40S ribosomal subunit for translation [43].

CCCH-type ZFPs showed their antiviral efficacy against several RNA viruses including Influenza A virus [10], retrovirus[15, 44, 45], filoviruses [8], and alphavirus such as Sindbis virus, Semliki Forest virus, Ross River virus, and Venezuelan equine encephalitis virus [46]. However, the role

of CCCH-type ZFPs on the human coronavirus is little explored. To fill that knowledge gap current study evaluated the effect of ZFP36L1, a CCCH-type ZFP on human coronavirus (HCoV)-OC43.

Materials & Methods

Cells, Virus Strains and Virus Propagation

The current study used human coronavirus (HCoV)-OC43 (ATCC catalog number VR1558) which was purchased from American Type Culture Collection (ATCC, Manassas, VA). Virus stock was prepared in HCT-8 cells (ATCC catalog number CCL-244, ATCC, Manassas, VA). HCT-8 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and antibiotic-antimycotic (penicillin 100 units /ml, streptomycin 0.10 mg /ml and amphotericin B 0.25 μg /ml) (Sigma-Aldrich, St. Louis, MO). During virus culture, HCT-8 cells were adopted to 1% FBS. HCT-8 cells culture with RPMI 1640 medium supplemented with 1% FBS were used to grow the virus and for virus titration.

Overexpression and knockdown of ZFP36L1

HCT-8 cells were stably overexpressed for ZFP36L1 (NCBI reference sequence: NM_001244701.1) with green fluorescent protein (GFP) marker using lentivirus. The overexpressed sequence for ZFP36L1 contained both tandem zinc finger domains (TZFD) such as TZFD1 and TZFD2. While HCT-8 cells were knockdown for ZFP36L1 using lentivirus with "GTAACAAGATGCTCAACTATA" as a target sequence for ZFP36L1 with upstream mCherry marker. Successful transduction was measured through GFP or mCherry expression for ZFP36L1 overexpression and ZFP36L1knockdown, respectively. Transduced HCT-8 cells were selected with increased concentration of puromycin (2-3 μg/ml) over 7 days.

Western blot analysis for ZFP36L1 overexpression and knockdown confirmation

ZFP36L1 overexpression or knockdown was confirmed by western blot. Wild type, ZFP36L1 overexpressed or ZFP36L1 knockdown HCT-8 cells were seeded in T25 flask. When cells reach 75-80% confluency then cells were lysed using radioimmunoprecipitation assay buffer (RIPA buffer) (Cell Signaling Technology, Danvers, MA) supplemented with Protease-Phosphatase Inhibitor (Cell Signaling Technology, Danvers, MA). Lysate were centrifuged at 3000 X g for 15 minutes at 4 °C. Supernatant was collected and protein concentration in each supernatant was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). A 40 µg cell lysate were run through 12% resolving SDS PAGE gel, after running, protein were transfected to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA). Membrane was blocked with 5% skimmed milk (Sigma-Aldrich, St. Louis, MO) in Tris-buffered saline (TBS) for 1 h at room temperature followed by incubation with anti- ZFP36L1 antibody (1:1000) (Thermo Fisher Scientific, Waltham, MA) and anti- βactin antibody (1:4000) (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Membranes were washed with Tris-Buffered Saline +0.1% Tween 20 (TBST) and incubated with HRP conjugated secondary antibodies (1:2000). Membrane was developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA). Image of western blot was taken by Odyssey XF Imaging System (LI-COR Biosciences, Lincoln, NE).

Measuring the effect of ZFP36L1 expression on virus titration

Wild type, ZFP36L1 overexpressed or ZFP36L1 knockdown HCT-8 cells were infected with HCoV-OC43 with 0.1 multiple of infection (MOI). Supernatant from these cells were collected at 24 hours, 48 hours, 72 hours and 96 hours post infection (p.i.). Collected cell supernatants were centrifuged at 1000Xg at 4°C for 15 minutes to remove cell debris and stored at -80 ° C till

used. Once samples from all time point were collected, virus titer in those samples were estimate for HCoV-OC43 virus titer as per the method described earlier [47].

Statistical analysis

The significant change in HCoV-OC43 titer in wild-type, ZFP36L1 overexpression, or ZFP36L1 knockdown cells was estimated using paired T-test with a 95% degree of freedom. Virus titer in wild-type, ZFP36L1 overexpression or ZFP36L1 knockdown cells were repeated at least three times, and average, standard deviation, and error were calculated.

Results

ZFP36L1 was successfully overexpressed and knockdown in HCT-8 cells.

A stable ZFP36L1 overexpression with GFP marker in HCT-8 cells was performed using a lentivirus system. GFP expression in HCT-8 cells was considered for ZFP36L1 overexpression (Figure 1B), which was further confirmed by western blot (Figure 2). Similarly, ZFP36L1 knockdown where shRNA was located downstream to mCherry was confirmed by mCherry expression (Figure 1C) as well as western blot analysis (Figure 2).

ZFP36L1 overexpression significantly suppressed the human coronavirus (HCoV)-OC43 virus production

Wild type and ZFP36L1 overexpressed HCT-8 cells were infected with HCoV-OC43 with M.O.I. of 0.1. Cell supernatants were collected at 24 hours, 48 hours, 72 hours, and 96 hours p.i. and analyzed for virus titer.

Results showed that ZFP36L1 overexpression in HCT-8 cells significantly reduced virus titer ($p<0.05$) (Figure 3). Virus titer in ZFP36L1 overexpression were 2.24±1.28 log10/ml and

4.32±0.00 log10/ml at 72 hours and 96 hours p.i. respectively. Which was significantly lower than virus titer in wild-type cells at the same time points such as 72 hours p.i. (4.08±0.11 log 10/ml) or 96 hours p.i. (5.42±0.10 log 10/ml) ($p<0.05$) (Figure 3).

3.3 ZFP36L1 knockdown significantly enhanced the human coronavirus (HCoV)-OC43 virus production as compared to control cells

Results with ZFP36L1 knockdown HCT-8 cells showed that ZFP36L1 knockdown significantly enhanced virus titer ($p<0.05$) (Figure 3). Knocking down ZFP36L1 also facilitated the infectious virus production as early as 48 hours post-infection (p.i.) while wild-type cells produced infectious virus at 72 hours p.i. Virus titer in ZFP36L1 knockdown cells was recorded as 00.00±0.00 log 10/ml, 2.86±0.00 log 10/ml, 4.52±0.22 log 10/ml, and 5.85±0.01 log 10/ml at 24 hours, 48 hours, 72 hours and 96 hours p.i. respectively while wild-type HCT-8 cells have virus titer of 0.00±0.00 log 10/ml, 0.00±0.00 log 10/ml, 4.08±0.11 log 10/ml, and 5.42±0.10 log 10/ml at 24 hours, 48 hours, 72 hours and 96 hours p.i. respectively. Virus titer in ZFP36L1 knockdown cells was significantly higher at 48 hours pi. and 96 hours p.i as compared to wild-type cells ($p<0.05$) (Figure 3).

Discussion

The current study was designed to determine the role of ZFP36L1 (a CCCH type ZFP) on HCoV-OC43 replication. Our results showed that overexpression of ZFP36L1 significantly reduced infectious HCoV-OC43 production while ZFP36L1 knockdown significantly enhanced virus titer as compared to wild-type cells.

ZFPS are one of the most abundant proteins in humans which can make up to 5% of total human proteins[48]. ZFPs have an extremely high binding ability. It can bind to cellular DNA, RNA, lipids, proteins, and PAR (poly-ADP-ribose), and therefore can modulate several cellular types of

machinery [3-11, 48]. The diverse binding properties of ZFPs make it difficult to characterize their functional effect in the cells [48]. However, such a challenge is overcome by classifying the ZFP and then identifying their functional characteristics [49]. Classification of ZFP is based on zinc ion, zinc ion interaction with specific amino acids, and folded structure of the protein [50]. Based on such classification CCCH type ZFP is characterized to interact with RNA and thus modulate RNA metabolism in the cell [51] including interfering with RNA virus replication [15, 52].

CCCH type ZFPs including ZFP36L1 have two tandem zinc finger (TZF) domains that interact with AU-rich elements (AREs) of poly (A) tail of mRNA, leading to its removal and thus promoting mRNA degradation [36-41]. The coronavirus genome is 5′-capped with a 3′ poly(A) tail of variable length [53]. The length of the poly (A) tail varies at different stages of the virus replication cycle and the virus with longer poly (A) tail replicates at a faster rate [54]. Therefore, the effect of ZFP36L1 on viral poly (A) may be the reason for reduced virus production with ZFP36L1 overexpression in the current study.

However, there is the possibility that ZFP can affect viral RNA replication with different mechanisms. A study showed that CCCH Type ZFP also targets the non-ARE sequence of 3’ and 5’ (untranslated region) UTR of mRNA [55]. While another study showed that CCCH Type ZFP targets CG-rich viral sequences [5]. The study also showed that ZFP36 (ZFP36L1) suppressed the virus production (influenza A virus) by interfering with viral protein translation/ export from the nucleus to the cytoplasm without affecting viral RNA replication [56]. Therefore, a detailed study to determine the mechanism of action for ZFP36L1 in suppressing coronavirus replication needs to be explored.

Conclusions

The current study showed that overexpression of ZFP36L1, a CCCH type ZFP significantly reduced that HCoV-OC43 production. While ZFP36L1 knockdown significantly enhanced the HCoV-OC43 production. However, the mechanism of such virus reduction is still needs to be explored.

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Figure 1

Overexpression and knockdown of ZFP36L1 in HCT-8 cells

Wild type HCT8 wells (A), ZFP36L1 overexpressed HCT-8 cells with GFP marker (B), and ZFP36L1 knockdown HCT-8 cells with mCherry marker (C). Overexpression and knockdown of ZFP36L1 were performed by lentivirus transduction.

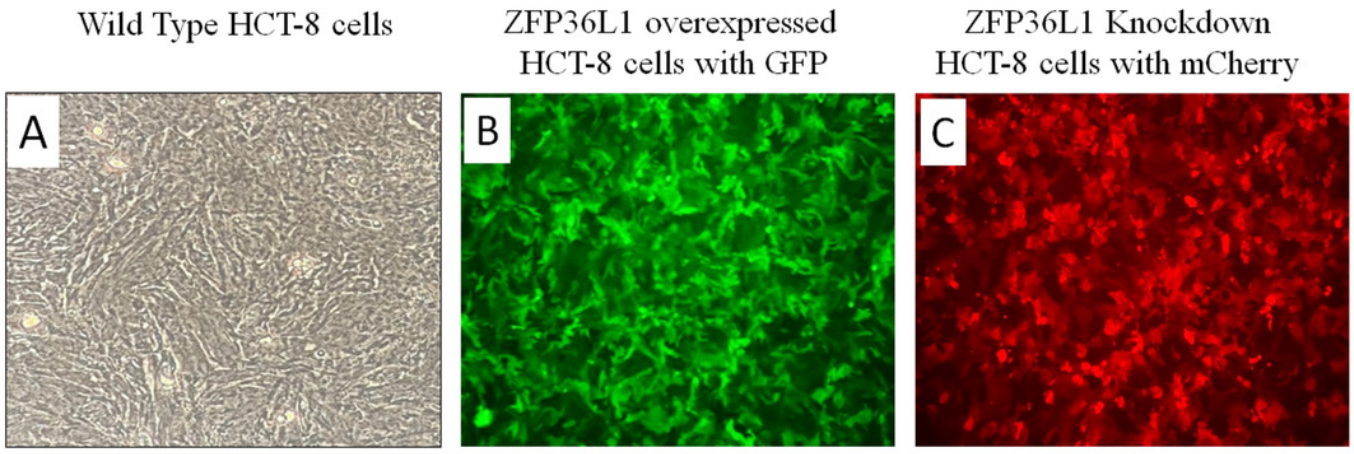

Figure 2

Western blot for confirming ZFP36L1 overexpression and knockdown in HCT-8 cells

Cell lysate for ZFP36L1 knockdown (A), ZFP36L1 overexpressed (B) and wild-type HCT-8 cells were separated with 12% resolving SDS PAGE gel and transferred to PVDF membrane. Proteins on the membrane were detected with anti-ZFP36L1 antibody and anti- βactin antibody with HRP-conjugated secondary antibodies

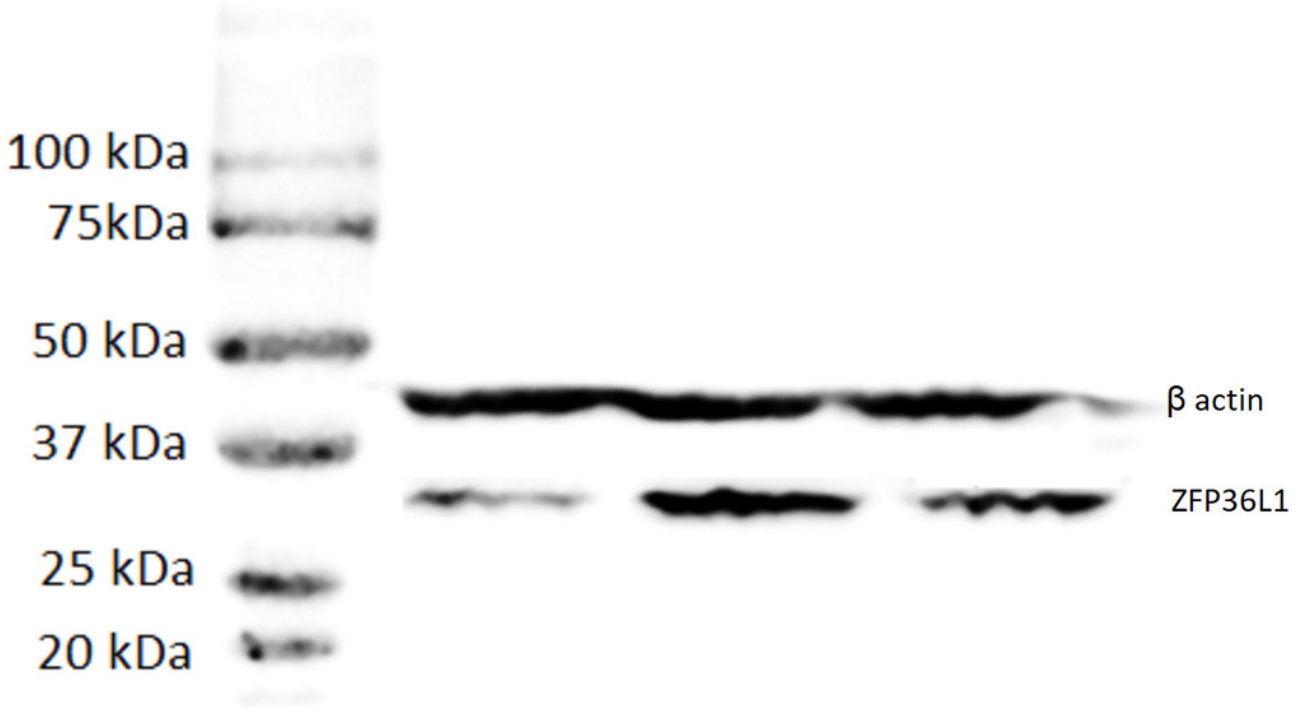

Figure 3

Human coronavirus-OC43 titer in HCT-8 cells

Wild type, ZFP36L1 overexpressed or ZFP36L1 knockdown HCT-8 cells were infected with HCoV-OC43 with 0.1 MOI. Supernatant from these cells was collected at 24 hours, 48 hours, 72 hours, and 96 hours p.i. and analyzed for virus titer. Asterisks are showing significant differences in virus titer