

Molecular cloning of duck CD40 and its immune function research

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ABSTRACT Cosignal molecules are cell surface molecules that transduce signals to other cells to modulate immune response positively (costimulate) or negatively (cosuppress). Costimulatory signals are key factors in determining whether T/B cells are capable of responding to specific antigens and ultimately mediating an appropriate immune response. In this study, the cDNA sequence containing the complete coding frame of the

costimulatory molecule duck CD40 gene was cloned and reported for the first time, and its mediated antiviral innate immune was verified in vitro. Results suggested duck CD40 molecule plays an important role in the innate immune responsiveness against some viruses. These data will be beneficial for the further understand of the avian immune system.

Key words: duck CD40, molecular cloning, antiviral assay

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INTRODUCTION

CD40 and CD40L are a pair of costimulatory molecules in immune response system. CD40 is primarily expressed on the surface of B cells and can bind to the surface molecule CD40L (CD154) on T cells, resulting in a series of biological effects (Noelle et al., 1992). CD40 belongs to the tumor necrosis factor receptor (TNFR) superfamily and is approximately 270 to 327 amino acid residues in length. It consists of 3 or 4 TNFR domains, a transmembrane domain and an intracellular domain. It's reported that CD40 is glycosylated after translation, forming a type I transmembrane glycoprotein with a relative molecular mass of 40,000 to 45,000. CD40 is widely expressed on a variety of cells, in recent years, they have been found on the surface of various immune cells, including B cells, activated T cells, macrophages, and dendritic cells, as well as on the surface of nonimmune cells, such as epithelial cells, endothelial cells,

fibroblasts, interstitial cells and platelets (Kim et al., 2014; Rau et al., 2013).

CD40L is also a member of the tumor necrosis factor (TNF) family and is approximately 240 to 274 amino acids in length. It consists of a cytoplasmic domain, a transmembrane domain, and an extracellular TNF domain. It is a potential immune-enhanced molecule and is mainly expressed on activated CD4+ T cells (Armitage et al., 1992; Noelle et al., 1992), and monocytes, natural killer cells, B cells, CD8+ T cells, mast cells and basophils (Andre et al., 2002; Carbone et al., 1997; Kwa et al., 2014; Pinchuk et al., 1996; Xie et al., 2013).

CD40/CD40L interaction plays a variety of roles in the host immune response, it exerts different effects depending on the type of cells and differentiation stages, and the powerful biological functions of CD40-CD40L make them widely studied in the fields of biology, including clinical and therapeutic areas (Hassan et al., 2014; Kanzawa et al., 2019; Quezada et al., 2004; Rickert et al., 2011).

In the present study, we successfully cloned duck CD40 (**duCD40**) coding sequence, analyzed its amino acids sequence and secondary structure. We then determined its tissue distribution in ducklings and ducks via RT-qPCR. We also detected its expression profiles in DEF cells infected with duck plague virus (DPV) and

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duck hepatitis A virus type 1 (**DHAV-1**) at the transcriptional level. Finally, we assessed the potential role within the innate immune response against viral invasion of duCD40.

MATERIALS AND METHODS

Cloning and Bioinformatics Analysis of the duCD40

To clone duCD40 complete CDs, 3 primers (CD40-F1, CD40-F2, CD40-R1) were designed based on the predicted sequence (GenBank ID: XM_021276409.1). When we designed this project, the predicted sequence (GenBank ID: XM_027442951.2) was not released, so, to identify the duCD40 3' terminal CDs, a reverse primer (CD40-R2) was designed based on the conserved region in 3'UTR of chicken CD40 (**chCD40**) and goose (**goCD40**), and paired with CD40-F2. CD40-F1/R1 primers were used to identify the 5' terminal coding sequence. Finally, the complete CDs of duck CD40 was amplified using primers CD40-F1/R3 by RT-PCR. Total mRNA isolated from duck blood used as template for all PCR procedures. The PrimeScrip II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) was used for cDNA synthesis per the manufacturer's protocol, and PCR conducted with the PrimeSTAR Max DNA Polymerase (Takara) under the conditions as below: 98°C for 2 min; 35 cycles of denaturation, annealing and extension at 98°C for 10 s, 66°C for 10 s and 72°C for 15 s; and a final elongation step at 72°C for 5 min. The PCR fragments were cloned into the pMD19-T vector (Takara) and verified by sequencing. The primers used in this study were listed in [Table 1](#).

Viruses and Animals

Three of the main viral pathogens for duck industry in China mainland were used in this study. DPV (CHV Strain, GenBank ID: JQ647509.1, belongs to *Mardivirus* genus, *Herpesviridae* family) ([Wu et al., 2012](#)), DHAV-1 (CH Strain, GenBank ID: JQ316452.1, belongs to *Avihepatovirus* genus, *Picornaviridae* family) and duck Tembusu virus (**DTMUV**, CQW1 Strain, GenBank ID: KM233707.1, belongs to *Flavivirus* genus, *Flaviviridae* family) ([Zhu et al., 2015](#)) were all obtained from the Institute of Preventive Veterinary Medicine, Sichuan Agricultural University. All 3 viruses were isolated from clinically infected ducks and the virus stocks were prepared in duck embryo fibroblast (**DEF**) cells. Furthermore, the viral titers were determined by the methods of

tissue culture infective dose 50 (**TCID₅₀**) in DEF cells. Three 5-day-old and three 60-day-old Cherry Valley ducks (purchased from Waterfowl Breeding Center of Sichuan Agriculture University) were euthanatized and indicated organ samples were collected for further analysis.

Cell Culture, Infections, and Transfections

DEF cells, derived from the 11-day-old duck embryo, were cultured in Dulbecco's modified Eagle medium (**DMEM**, Gibco, Shanghai, China) supplemented with 10% new bovine serum (**NBCS**, Gibco) and incubated at 37°C with 5% CO₂. For the infection of the transfected cells, DEF cells were seeded into 24-well plates and cultured in DMEM plus 10% FBS overnight. After 16~24 h, when confluent was 70 to 90%, the DEF cells were transfected with 0.5 μg of plasmids with Lipofectamine 3000 (Invitrogen, Waltham, MA, USA). Then, 36 h later, DEF cells were infected with 200 μL DPV (10⁵ TCID₅₀), DHAV-1(10³ TCID₅₀) and DTMUV (10³ TCID₅₀), respectively.

Construction of Expression Plasmids

Two different eukaryotic expression plasmids of duCD40 were constructed. The coding sequence of His-tagged duCD40 was cloned into pCAGGS plasmid using the one-step cloning kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's protocol. The first construct pACGGS-CD40(A) coding 3 TNFR domains, and pCAGGS-CD40(Z) coding only one TNFR domain. After confirmed by sequencing, plasmids were purified using an Endo-free Plasmid Mini Kit II (Omega Bio-tek, Georgia, USA).

Western Blotting

DEF cells were lysed with RIPA buffer (beyotime Biotech, Shanghai, China) at 36 h post transfection. Cell lysates were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the isolated proteins were electroblotted on PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% milk for 1.5 h in TBST (0.1% Tween-20 in PBS) and then incubated with mouse anti-His monoclonal antibody (Ruiyingbio, Suzhou, China) or mouse anti-β-actin monoclonal antibody (Transgen Biotech, Beijing, China) for 2 h, in 37°C. HRP-conjugated goat anti-mouse IgG (Transgen Biotech) antibody 1:5000 was used as the secondary antibody, incubate in 37°C for 1h. The proteins were visualized by chemiluminescence using an ECL kit (Bio-Rad, USA).

Quantitative Real-Time PCR (RT-qPCR)

The procedure was performed as described previously and modification slightly ([Wu et al., 2011](#)). Briefly, total RNA from tissue or cell cultures was extracted using the

Table 1. The primers for duCD40 amplification.

Name	Sequence (5'-3')
CD40-F1	CTGCTTCCATCCCCTTCA
CD40-F2	CCAAAACCGAGCCGTGCTACCCC
CD40-R1	GATCATCCGAAGTGTTFGTCC
CD40-R2	CGGGCTGTGCTCCTGTCCG
CD40-R3	CCCTCACAGCCCCTCT

RNAiso Plus reagent (Takara) and then reverse transcribed into cDNA using PrimeScript RT Reagent Kit and gDNA Eraser (Takara). Candidate genes for each sample were quantified using TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara) and Bio-Rad CFX96 real-time detection system (Bio-Rad). Relative expression of all genes was calculated and normalized to the duck β -actin gene using the Livak and Schmittgen 2- $\Delta\Delta$ CT methods (Livak and Schmittgen, 2001). The differential mRNA expression levels of candidate genes in each sample were verified in triplicate.

Antiviral Assay

DEF cells were cultured in 24-well plates and were transfected with the duCD40 expression plasmids as described above. The soluble protein duCD40L was added 24 h after transfection, 24 h postinfection, DEF cells were infected with DTMUV, DHAV-1, and DPV, respectively. Cell supernatants were collected at different timepoints postinfection (24 and 36 h). The viral titers in supernatant were determined using TCID₅₀ method. Meanwhile, the monolayer cells were harvested for analyzing the viral copy number using RT-qPCR.

Statistical Analysis

The two-tailed Student's t test method used statistical data to determine whether there was a significant difference. When the p value <0.05, the results were considered to be significant difference, and *, $P < 0.05$, **, $P < 0.01$.

Ethics Statement

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agriculture University in Sichuan, China (Protocol Permit Number: XF2014-18).

RESULTS

Cloning and Analysis of Duck CD40 CDs

Firstly, to confirm the predicted partial duck CD40 sequence from NCBI, we designed 2 pairs of primers (CD40-F1/R1, CD40-F2/R2). Partial 5'-terminal and 3'-terminal nucleotide sequence of duCD40 were successfully amplified and verified by sequencing, and its initiation codon and stop codon are successfully determined (as shown in Figure 1). Finally, the CDs of duCD40 were successfully amplified using primers CD40-F1/R3 and verified by Sanger sequencing. It's worth noting that the cloned duCD40 nucleotide sequence is not totally identical (approximately 70% identity) to any of the predicted duCD40 sequences in GenBank, and the cloned sequence was verified by 3 independent assays by using different template (from different ducks). The

cloned duCD40 CDs data were submitted to GenBank (accession number: MW458946).

Alignment of multiple sequences showed that the mRNA sequences of duCD40, goCD40 and chCD40 are conserved, as shown in Figure 1. Significantly, it seems that a single mutation in the duCD40 sequence corresponding to initiation codon of goCD40, resulted in the CDs of duCD40 is 516 nucleotides in length, encoding 171 amino acids, but is much (100~120 aa) shorter than chCD40 (276 aa) and predicted goCD40 (293 aa) molecules (Figure 1B and Figure 3). The results of secondary structure prediction using SMART (<http://smart.embl-heidelberg.de/>), suggesting that duCD40 only consist of 1 TNFR domain in N-terminus, by contrast, chCD40 and goCD40 containing 3 TNFR domains (Figure 3). The results of multiple protein sequences alignments showed that the transmembrane domain, and intracellular domain are conserved, and duCD40 is more homologous to goCD40 in comparison with chCD40 (Figure 1B). Accordingly, phylogenetic analysis showed that duCD40 together with chicken and goose CD40 protein sequences belong to the same cluster, and CD40 molecule sequences from fishes and mammals form 2 separated clusters, respectively (Figure 2). Altogether, we successfully cloned duCD40 coding sequence in present study, but a single mutation resulted in the N-terminal extracellular domain is much shorter than chCD40 and goCD40.

Tissues Distribution of duCD40

To depict the expression profile of duCD40 at transcriptional level, we performed real-time RT-PCR (RT-qPCR) to detect the transcription levels of CD40 in different tissues of 5-day old ducklings and 60-day adult ducks using duck β -actin as a house-keeping gene. As shown in the Figure 4, duCD40 was expressed in all the tested tissues of ducklings and adult ducks, and was strongly expressed in the harderian gland, lung, kidney, duodenum and pancreas in ducklings and in the blood, pancreas and heart in adult ducks. The extremely high expression of duCD40 in blood consistent with the early studies that CD40 primarily expressed on the surface of B cells (Armitage et al., 1992; Noelle et al., 1992; Rickert et al., 2011).

Expression Changes of Duck CD40 and CD40L at the Transcriptional Level in DEF Cells Postinfected With Viruses

After infected by DHAV-1 and DPV, the transcriptional change of duCD40 and duCD40L in DEF cells were determined by RT-qPCR. As shown in the Figure 5, compared with mock (PBS treated), the expression level of duCD40 was significantly up-regulated (approximately 3-fold) at 24h postinfected with DPV, and further up-regulated (approximately 6-fold,) at 48 h postinfection (Figure 5B). When DEF cells infected with DHAV-1, the expression of duCD40 also upregulated at 24 h and 48 h postinfection (Figure 5A).



Figure 1. Cloning and Analysis of duCD40 CDs. The alignment of nucleotide sequences from duck, chicken (NM_204665.2) and goose (XM_013198462.1) of CD40 as shown in (A). Amino acid alignment of CD40 is shown in (B). Protein sequences of duCD40 was aligned together with goCD40 (XM_013198462.1) and chCD40 (EF554721.1) for comparison. The alignment was performed using DNAMAN 9.0 software. Black shading indicates amino acid identity; The blue frame denotes a semi-conserved amino acid; Tumor necrosis factor receptor domains (TNFR) and transmembrane domains are indicated. Initiation codon and termination codon are marked with red and green rectangles, respectively. Initiation codon of duCD40 is indicated with a yellow rectangle.

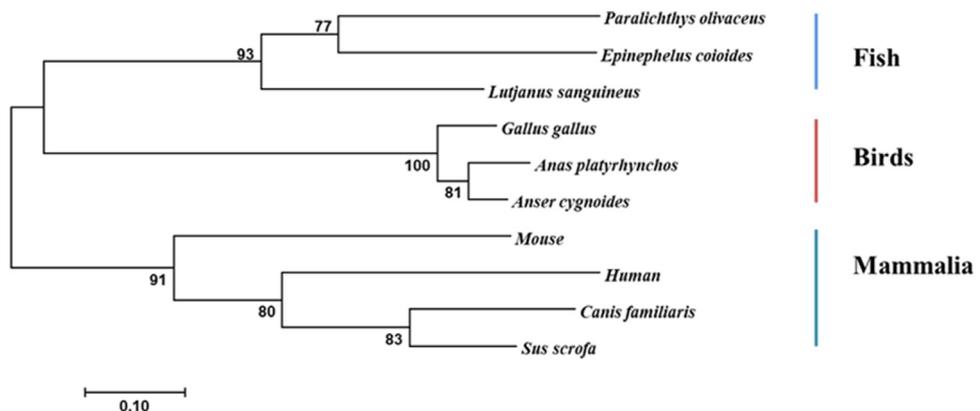


Figure 2. Evolutionary relationships of CD40s. Evolutionary analyses were conducted using MEGA7 and evolutionary history was inferred using the Neighbor-Joining method and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 10 amino acid sequences, including CD40 of *Anser cygnoides*, *Anas platyrhynchos*, *Gallus gallus*, *Paralichthys olivaceus*, *Epinephelus coioides*, *Lutjanus sanguineus*, *Mus musculus*, *Homo sapiens*, *Canis familiaris* and *Sus scrofa*. Evolutionary analyses were conducted using MEGA7.

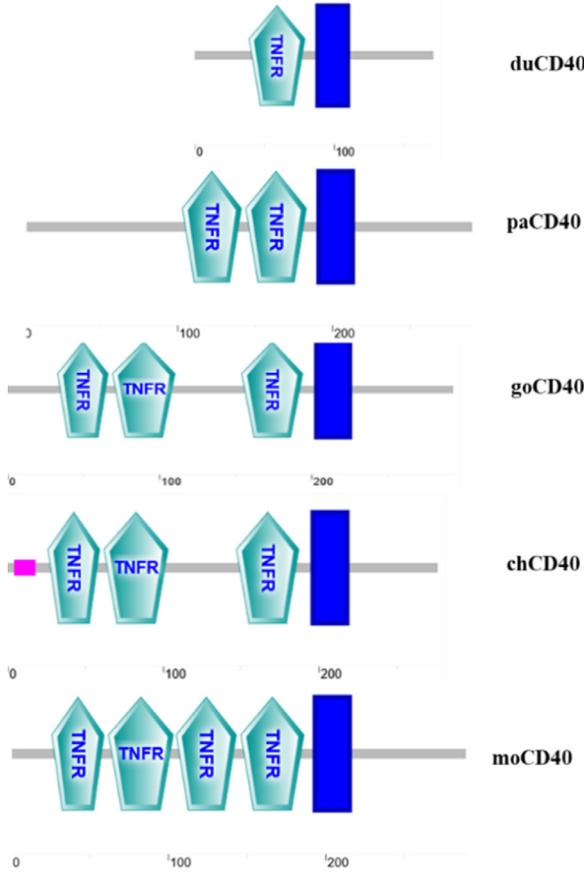


Figure 3. Secondary structure of CD40 protein. Comparison of the predicted protein secondary structures among duCD40, goCD40, chCD40, *Mus musculus* CD40 (muCD40), and *Paralichthys olivaceus* CD40 (paCD40), which was performed using the SMART.

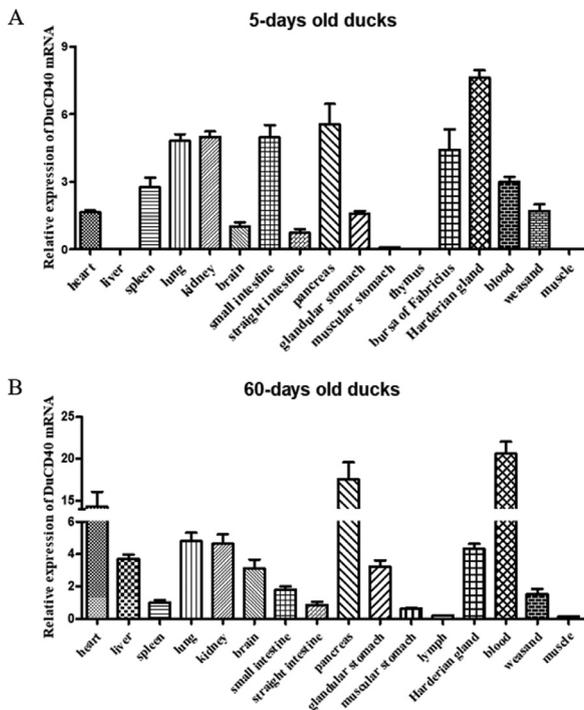


Figure 4. Analysis of duck CD40 tissue distribution in 5-day-old (A) and 60-day-old (B). Tissue distribution of duCD40 were detected by RT-qPCR. The mRNA expression levels were normalized to the expression of the β -actin gene. Calculation method was $2^{-\Delta\Delta Ct}$.

In contrast with duCD40, the duCD40L was extremely upregulated postinfection with either DPV or DHAV-1. At 24 h postinfection with DPV and DHAV-1, the duCD40L was upregulated approximately 65-fold (Figure 5D) and 30-fold (Figure 5C), respectively. At 48 h postinfected with DPV and DHAV-1, the duCD40L was upregulated approximately 140-fold (Figure 5D) and 220-fold (Figure 5C), respectively. All together, these data indicate the CD40/CD40L in DEF cells were significantly upregulated by DPV and DHAV-1 infection, suggesting that CD40/CD40L are likely to play a role in viral infection.

Antiviral Assay

Previous studies demonstrate that the activation of human CD40-CD40L pathway results in inhibition of HCV replication and Herpes Simplex Virus type-1 infection in cells (Rau et al., 2013; Vlahava et al., 2015). To verify whether the duck CD40-CD40L plays a role in innate immune against viral infection, we performed antiviral assays against DPV, DHAV-1 and DTMUV, which cause 3 of the most important viral diseases in duck industry. DEF cells (70 to 90% confluent) were transfected with CD40 plasmid, 24 h post transfection, cells were treated with CD40L proteins for about 4 h, and then infected with DPV, DHAV-1 or DTMUV, and then continuously incubated at 37 °C. The copy number of viral nucleic acid and viral titers were analyzed. As shown in Figure 6B, 6C, 6E, and 6F, when comparing the treated group with the untreated control group, there was no significant difference for copy number of DPV and DHAV-1 at both 24h and 36h postinfection. Surprisingly, only in the group transfected with CD40 (A) but not CD40(Z), and treated with CD40L, the copy number of DTMUV was significantly decreased when compared with the control groups, at 36h postinfection. Consistent with this result, the viral titers only decreased in the group transfected with CD40(A) treated with CD40L (Figure 6G). Altogether, these results indicate that the activation of CD40-CD40L pathway has an effect on DTMUV infection in DEF cells, but not DHAV-1 and DPV, suggesting that CD40-CD40L pathway plays a role in innate immune against some viruses.

DISCUSSION

The important roles of CD40 immune response processes such as Ab production, memory B cell formation and immune globulin class switching have been well elucidated in mammals (Gray et al., 1994; Kawabe et al., 1994; Quezada et al., 2004; van Kooten and Banchereau, 2000). In recent years, it also has been reported that the activation of CD40-CD40L have effects on the innate immune response against viral infection independent of interferon system (Rau et al., 2013). But, the significance of CD40 in avian immune system is rarely reported.

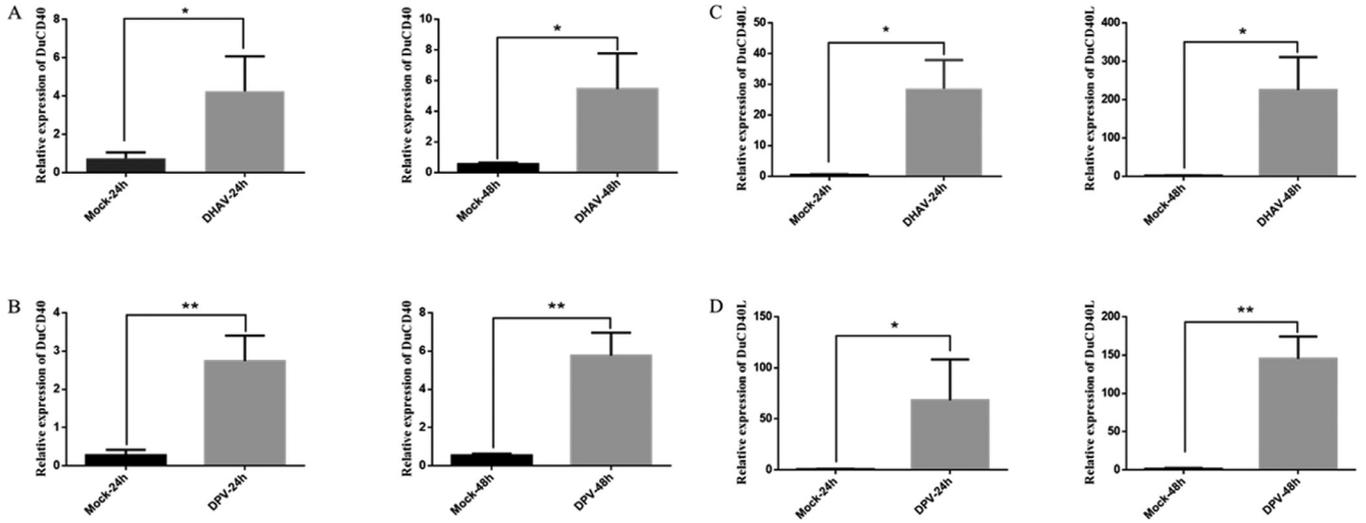


Figure 5. Effects of DPV and DHAV-1 on duCD40 and CD40L transcript levels in DEF. DEF cells were challenged by DPV (200 μ L/well) and DHAV-1 (200 μ L/well), at the indicated timepoints, the mRNA level of duCD40 (A, B) and CD40L (C, D) were measured by RT-qPCR. Mock group treated with equal volume of PBS. Fold change for mRNA levels was standardized by β -actin levels, data are mean \pm SEM (n = 3), significant difference was indicated by asterisks, (*, $P < 0.05$, **, $P < 0.01$).

In this study, the CDs of duCD40 was successfully cloned for the first time. The duCD40 protein is composed of 171 amino acids, which is much (100~120 aa) shorter than goose (292 aa) and chicken (275 aa) CD40 molecules. Phylogenetic analysis of CD40 showed that duck CD40 is closely related to birds, especially with goose, and shown lowest similarity to mammalian CD40. This result indicates the evolutionary conservation

of CD40 in waterfowl. Second structure prediction revealed that duCD40 has only one TNFR domain, while paCD40 contains 2 TNFR domains, chCD40 and goCD40 contain 3 TNFR domains, and moCD40 contain 4 TNFR domains. The TNFR domain is a cysteine-rich region and responsible for CD40-CD40L binding. Only one TNFR domain within duCD40 may result in the deficiency of duck CD40-CD40L pathway

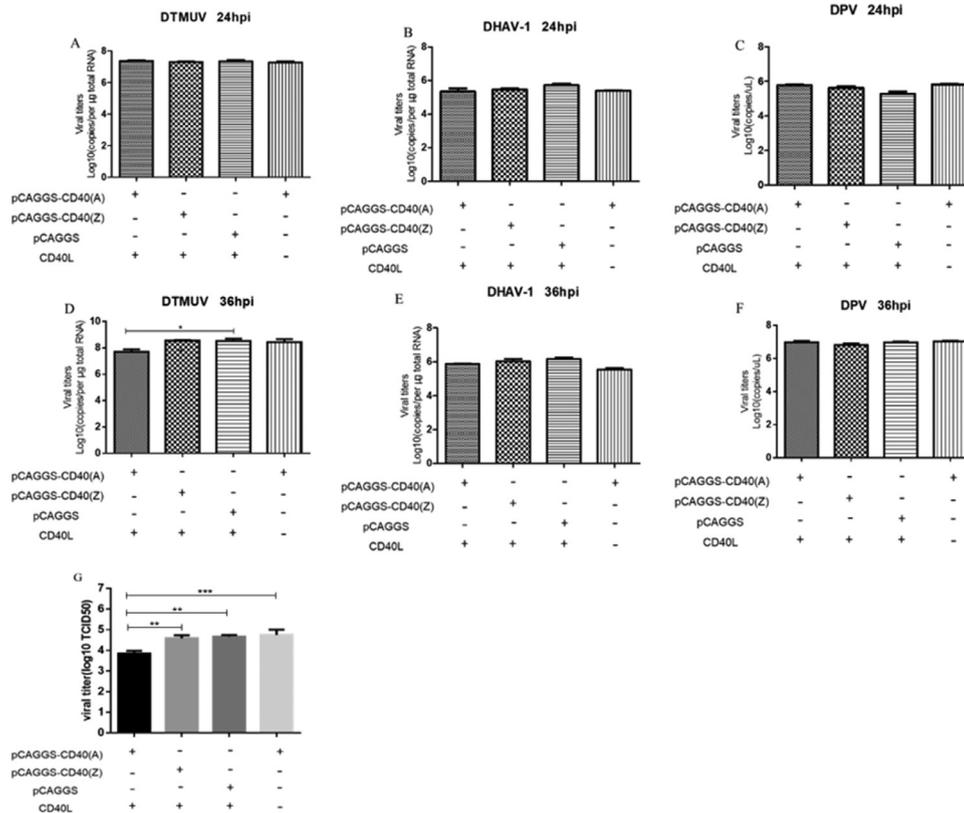


Figure 6. Effect of activation of CD40-CD40L on duck pathogenic virus replication. The DEF cells were transfected with 500 ng/well pCAGGS-CD40(Z), pCAGGS-CD40(A), or pCAGGS. At 24h post transfection, the cells were stimulated with soluble protein duck CD40L 4 μ g/well, after 4h, the cells were infected with DPV, DHAV-1 or DTMUV. Intracellular virus copies were detected by RT-qPCR at 24h and 36h postinfection. Supernatant was collected to determine virus titers. Data are mean \pm SEM (n = 3), significant difference was indicated by asterisks, (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

activation, and its dysfunction. When the duck is attacked by viruses, inefficient CD40 may be one of the reasons why ducks are vulnerable to certain viruses.

CD40-CD40L is widely and thoroughly studied in human tumors (Irenaeus et al., 2019; Li et al., 2019; Vitale et al., 2019; Wang et al., 2019), autoimmune diseases (Li, Cao et al., 2019; Mousa et al., 2019), and organ transplant rejection (Kanzawa et al., 2019; Oura et al., 2019). There are few data on its natural antiviral effect, but in recent years, the role of CD40-CD40L in antiviral immunity has received attention. It was demonstrated that the interaction of CD40-CD40L inhibited the transcriptional activity of the HPV promoter, thereby inhibiting the proliferation of the virus (Altenburg et al., 2016), and affected different stages of HSV-1 replication in U2OS cells stably expressing CD40L (Vlahava et al., 2015). However, in the present study, activation of duck CD40-CD40L did not inhibit DPV replication in DEF cells, although DPV and HSV-1 belong to Herpesviridae. This may be due to the biological effects of CD40-CD40L activation in ducks and humans are not totally the same, or the replication mechanism of DPV and HSV-1 are different, but the specific molecular mechanism needs further experimental verification and elucidation. In addition, it has been reported that in Huh7.5 cells, activation of CD40-CD40L inhibit HCV replication by innate immune mechanism independent from the interferon pathway. This pathway might mediate viral clearance, and disruptions might be involved in the pathogenesis of HCV infection (Rau et al., 2013). DTMUV and HCV belong to the Flaviviridae. In present study, we demonstrated that DTMUV replication was significantly inhibited in DEF cells transfected with CD40(A) plasmid and treated with CD40L, at 36 h postinfection, which suggests that the duck CD40-CD40L signaling pathway plays an important role in the host resistance DTMUV infection. However, in this experiment, only CD40(A) exerted antiviral effects, while CD40(Z) did not exert antiviral activity, probably due to the lack of TNFR domain in CD40 (Z), resulted in the ineffective CD40-CD40L signaling pathway and therefore didn't allow for an effective antiviral effect. This may be one of the reasons why ducks are more susceptible to DTMUV than chickens and geese.

Altogether, we successfully cloned duck CD40 CDs, analyzed its amino acids sequence via bioinformatics analysis, and determined its tissues distribution in ducklings and ducks via RT-qPCR in present study. We also determined its transcriptional expression change in DEF cells infected with DPV and DHAV-1. Finally, our data also revealed that the natural lack of TNFR domains in duck CD40, may help explain why ducks are susceptible to certain diseases.

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DISCLOSURES

The authors declare no conflict of interest.

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