Molecular cloning and expression analysis of *TRAF3* in chicken

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ABSTRACT. Tumor necrosis factor receptor-associated factor 3 (TRAF3) is a crucial regulator that suppresses c-Jun N-terminal kinase and non-canonical nuclear factor-κB signaling, but facilitates type I interferon production. To determine TRAF3 function in innate immune responses among birds, particularly chicken, we cloned and characterized the chicken TRAF3 gene (*chTRAF3*) and detected its tissue expression profile in chicken. We also detected the differential expression of *chTRAF3* and its downstream gene interferon-β (*IFN-β*) upon different stimuli in primary chicken embryo fibroblast cells. Two *chTRAF3* gene products, *chTRAF3-1* and *chTRAF3-2*, can be produced by alternative splicing. The full-length coding sequence of *chTRAF3* (*chTRAF3-1*) was 1704 base pairs and encoded a protein of 567 amino acids with high identity to TRAF3 homologs from mammals and other birds. The deduced amino acid sequence showed typical characteristics of TRAFs, with a RING finger domain, 2 zf-TRAF motifs, and a MATH domain. Quantitative real-time polymerase chain reaction analysis revealed broad expression of *chTRAF3* in all detected tissues, with abundant expression in the spleen, thymus, lung, and

small intestine. Expression of *chTRAF3* was significantly upregulated in a time- and concentration-dependent manner in chicken embryo fibroblast cells challenged with poly I:C or poly dA-dT. Furthermore, *chTRAF3* and *IFN-β* mRNA expression from chicken embryo fibroblast cells challenged with Newcastle disease virus F48E9 suffered intense suppression compared with Newcastle disease virus Mukteswar infection. Our results indicate that *chTRAF3* plays important roles in defending against both RNA and DNA virus infection.

Key words: Chicken; Cloning; Gene expression; Newcastle disease virus; Tumor necrosis factor receptor-associated factor 3

INTRODUCTION

Tumor necrosis factor receptor-associated proteins (TRAFs) in mammals are intracellular signaling molecules involved in tumor necrosis factor receptor family, the Toll-like receptor (TLR) family, the interleukin-1 receptor family, and the RIG-I-like receptor (RLR) family signal transduction (Hacker et al., 2011). Seven members of the TRAF family have been characterized in mammals, including TRAF-1-7. All TRAFs possess a similar protein structure, a C-terminal TRAF domain, and N-terminal region (Rothe et al., 1994). The TRAF domain contains an amino-terminal coiled-coil subdomain (TRAF-N) and a C-terminal subdomain (MATH) which contributes to TRAF trimerization and interact with various upstream regulators (CD40, interleukin-1 receptor-associated kinase family members) (Hsu et al., 1995; Wesche et al., 1997) or downstream effectors (cellular inhibitor of apoptosis and nuclear factor-κB-inducing kinase, NIK) (Sanjo et al., 2010; Zheng et al., 2010). The N-terminus of TRAFs contains a RING finger domainand several zinc finger motifs (zf-TRAF) that mediate protein ubiquitylation and activation of downstream effectors (Takeuchi et al., 1996).

Despite its structural similarity with other TRAFs, the function of TRAF3 in the immune response is distinct. TRAF3 was initially described as an adaptor of CD40 (Hu et al., 1994), but was then found to negatively regulate the c-jun N-terminal kinases. An alternative NF-κB pathway inhibits the release of transforming growth factor β-activated kinase 1 and NIK from the receptor into the cytoplasm in unstimulated cells. Following CD40 activation, the cellular inhibitor of apoptosis acts as a lysine 48-specific ubiquitin ligase that targets TRAF3 for proteasomal degradation and then counteracts its inhibitory function (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008). A recent study demonstrated that TRAF3 positively regulates the type I interferon and anti-inflammatory cytokine interleukin-10 production (Hacker et al., 2006). Upon recognition of viral invasion, TLR and RLR trigger recruitment of the adaptor protein Toll/ IL-1 receptor domain-containing adaptor inducing interferon-beta and mitochondrial antiviral signaling protein (Oganesyan et al., 2006; Saha et al., 2006), respectively, which in turn recruits TRAF3 to activate the downstream kinases TANK-binding kinase 1 (TBK1) and IκB kinase-ε and ultimately phosphorylate interferon regulatory factor 3 to induce the IFN-β production. Importantly, the lysine 63-linked ubiquitination of TRAF3 is a key mechanism for TRAF3 activation of downstream signaling (Fitzgerald et al., 2003; Tseng et al., 2010).

Over the past few decades, the poultry industry has suffered various viral diseases and large economic losses worldwide. Although the importance of TRAF3 in response to viral infection in mammals is well-characterized, its role in the innate immune system among avians

is poorly understood. In the present study, we cloned and characterized 2 splice variants of the chicken TRAF3 gene (*chTRAF3-1* and *chTRAF3-2*). We also determined the *chTRAF3* expression profile in different tissues and examined its response upon poly I:C, poly dA-dT stimuli and Newcastle disease virus (NDV) infection in primary chicken embryo fibroblast (CEF) cells.

MATERIAL AND METHODS

Chicken, cells, and viruses

Experimental animals were sacrificed in accordance with prescribed ethical policies and approved by Sichuan Agricultural University Animal Care and Use Committees. To detect the tissue distributions of *chTRAF3*, 11 tissues were collected from 3 14-day-old specific pathogen-free White Leghorn chickens, including the heart, liver, spleen, lung, kidney, brain, thymus, pancreas, small intestine, pectoral muscle, and leg muscle. Samples were snap-frozen in liquid nitrogen and stored at -70°C. CEF cells were prepared from 9-day-old embryonated chicken eggs. All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) on 12-well plates ($1x10^6$ cells/well) at 37°C in a humidified atmosphere containing 5% CO_2. NDV F48E9 and Mukteswar were obtained from College of Veterinary Medicine. Sichuan Agricultural University (Ya'an, China).

Primer design and chicken TRAF3 gene cloning

Based on the published predicted sequence of *TRAF3* from *Gallus gallus* (GenBank accession No.: NC_006092.3), a pair of gene-specific primers (Table 1) for the amplification of the complete coding region were designed using the Primer Premier 5 software (Premier BioSoft, Palo Alto, CA, USA). Spleen cDNA was used as a template for amplification. Polymerase chain reaction (PCR) was carried out with 1cycle of denaturalization at 94°C for 3 min, 36 cycles of 94°C for 10 s, 56°C annealing for 30 s, and 72°C for 150 s, and extension at 72°C for 10 min. PCR products were gel-purified, cloned into the pMD-18T vector (Takara, Shiga, Japan), and sequenced by the Shenzhen BGI Biotechnology Company (Beijing, China).

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')	Application
TRAF3-F1	CAGGATGCCACCTTCTCTCAC	Cloning
TRAF3-R1	AGGATGGTGTCGTTGAAGGAG	
GAPDH-F	AGGACCAGGTTGTCTCCTGT	Real-time PCR
GAPDH-R	CCATCAAGTCCACAACACGG	
TRAF3-F2	CAGGATGCCACCTTCTCTCAC	Real-time PCR
TRAF3-R2	AGGATGGTGTCGTTGAAGGAG	
IFNβ-F	CCTCAACCAGATCCAGCATTAC	Real-time PCR
IFNβ-R	CCCAGGTACAAGCACTGTAGTT	

F = forward primer; R = reverse primer.

Bioinformatic analysis

The nucleotide sequence obtained was edited and analyzed against the sequence database using the WWW BLAST server (http://www.ncbi.nlm.nih.gov/blast). Conserved domain

prediction was carried out using the SMART program (http://smart.emblheidelberg.de/). Multiple amino acid sequences alignment was performed using ClustalX 1.83. A phylogenetic tree was constructed using the MEGA 5.05 software. GenBank accession numbers of *TRAF3* from different species for phylogenetic analysis are listed in Table 2.

Table 2. GenBank accession numbers of TRAF3 homologs used in this study.

Name of species	Accession No.
Anas platyrhynchos	XP_005020762.1
Bos taurus	NP_001192515.1
Canis lupus familiaris	XP_854615.1
Capra hircus	XP_005695478.1
Columba livia	XP_005506142.1
Equus caballus	XP_001490050.1
Homo sapiens	NP_003291.2
Meleagrisgallopavo	XP_003206786.1
Mus musculus	NP_035762.2
Ornithorhynchusanatinus	XP_001507440.1
Pan troglodytes	XP_001164813.1
Sus scrofa	XP_005666500.1
Taeniopygiaguttata	XP_002200611.1

Cell treatment with different stimuli

To examine the temporal expression of *chTRAF3* following different stimuli, CEF cells were transfected with poly (deoxyadenylic-thymidylic) (poly dA-dT) orpolyinosinic-polycytidylic acid (poly I:C) (Invivogen, San Diego, CA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. At the indicated time points, cells were harvested and resolved in TRIzol reagent (Invitrogen) for RNA extraction.For virus challenge, cells were infected with NDV F48E9 or Mukteswar at a multiplicity of infection of 1, respectively. At 4, 6, 8, 12, and 16 h post-infection, cells were collected for further analysis.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from chicken immune tissues or CEF cells using TRIzol reagent and diluted in DNase/RNase-Free Deionized Water (Tiangen, Beijing, China). The concentration and purity of total RNA were assessed by the A_{260}/A_{280} and A_{260}/A_{230} ratio, and RNA integrity was tested by electrophoresis using a 1% formaldehyde denaturing agarose gel. First-strand cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). Total RNA extraction and cDNA synthesis were performed following the manufacturer protocol.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed to detect the relative mRNA expression levels of *chTRAF3*, as well as chicken *IFN-β* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as an endogenous reference gene. Each qRT-PCR reaction contained 5μL SYBR Green II, 20 ng cDNA, and 5 pM primer pairs in a final volume of 10 μL.

The cycling parameters were as follows: 98°C for 3 min, 30 cycles of 98°C for 10 s, 30 s at optimum temperatures, 72°C for 10 s, and a final extension for 5 min along with a temperature increment of 0.5°C/s from 65-95°C. The specificity of qRT-PCR products were confirmed by melting curve analysis. Relative expression data was analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008). Statistical analysis was performed using SAS 9.0 (SAS, Inc., Cary, NC, USA) by 1-way analysis of variance. The results were plotted and values are reported as means ± standard deviation using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Bioinformatic analysis of chicken *TRAF3*

Two types of chicken *TRAF3* transcript variant sequences, *chTRAF3-1* and *chTRAF3-2*, were cloned by reverse transcription PCR (RT-PCR) using total RNA isolated from chicken spleen. The full-length coding sequence of chicken *TRAF3* (*chTRAF3-1*) was 1704 bp encoding a putative protein of 567 amino acids and was significantly similar to the *TRAF3* transcript variant X24 (GenBank accession No.: XM_004936344.1) mRNA sequence from *Gallus gallus* (Figure 1). *chTRAF3-2* was absent 168 nucleotides from positions 646-813 in the coding sequence of *chTRAF3-1* and encoded a putative polypeptide of 511 amino acids and shared the highest similarity to the *TRAF3* transcript variant X26 (GenBank accession No.: XM_004936346.1) mRNA sequence from *Gallus gallus* (Figure 1).

Figure 1. Nucleotide and deduced amino acid sequences of *chTRAF3-1*. The translation start (ATG) and stop (TGA) codons are shown in color. The absent amino acid sequences of *chTRAF3-2* (from nucleotide position 648-813) are underlined.

The deduced protein structure prediction results were similar to those for the other members of TRAF family containing a RING finger domain, 2 TRAF-type zinc finger motifs (zf-TRAF), and a conserved C-terminal TRAF domain (MATH). Multiple sequence alignments showed that the deduced protein of chTRAF3-1 shared 90.1, 87.7, and 87.4% amino acid identity with chTRAF3-2, human and mouse TRAF3, respectively (Figure 2).

Based on the amino acid sequence of TRAF3 from other species, a phylogenetic tree of TRAF3 was constructed using the MEGA software 5.05 with the neighbor joining (NJ) method and 1000 replicates (Figure 3). The TRAF3 amino acid sequence from mammals and birds segregated into 2 separate clusters and the intermediate species *Ornithorhynchus anatinus* formed 1 branch in the mammal clade. Moreover, chicken TRAF3 showed the highest homology with *Meleagris gallopavo*, followed by *Columba livia* and *Anas platyrhynchos*.
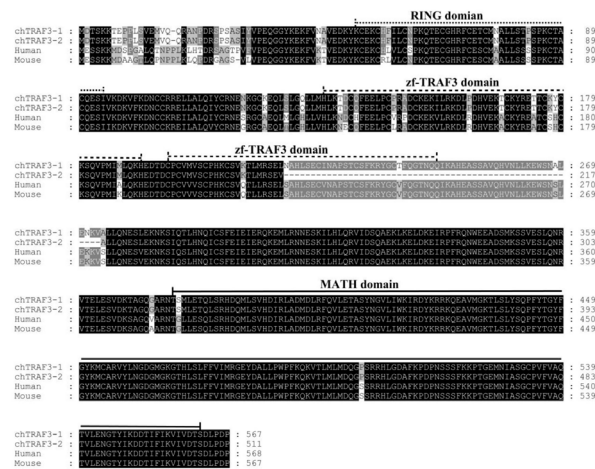
Figure 2. Multiple alignment of TRAF3 amino acid sequences from chTRAF3-1, chTRAF3-2, human TRAF3, and mouse TRAF3. Sequence alignment was performed using CLUSTALX 1.83. The dotted, dashed, and solid lines indicate, respectively, the RING, zf-TRAF, and MATH domains.
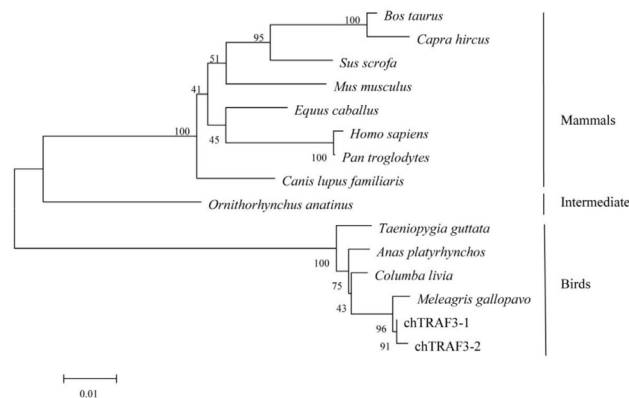
Figure 3. Phylogenetic analysis of TRAF3s from different species. The phylogenetic tree was constructed using the neighbor-joining (NJ) method within MEGA 5.05. The scale bar indicates the branch length, and the bootstrap confidence values are shown at the nodes of the tree.

Tissue distribution of chicken *TRAF3*

The tissue distribution of *chTRAF3* was detected by qRT-PCR in 2-week-old specific pathogen-free White Leghorn chicken. *chTRAF3* expression was highest in the lung, followed by the spleen, thymus, and small intestine; a weak signal was detected in the heart, liver, pancreas, brain, and kidney; and the lowest in the muscle (Figure 4A).

Figure 4. Analysis of the expression patterns of *chTRAF3*. (**A**) Spatial expression of *TRAF3* in different tissues of chicken. (**B**) Temporal expression analysis of *chTRAF3* mRNA in response to poly I:C or poly dA-dT transfection. Each hour post-transfection is marked with different letters (a, b, c) indicating significant differences (P < 0.05). Error bars represent the means ± standard deviation (N = 3).

Time-course analysis of chicken *TRAF3* expression under virus analog challenge

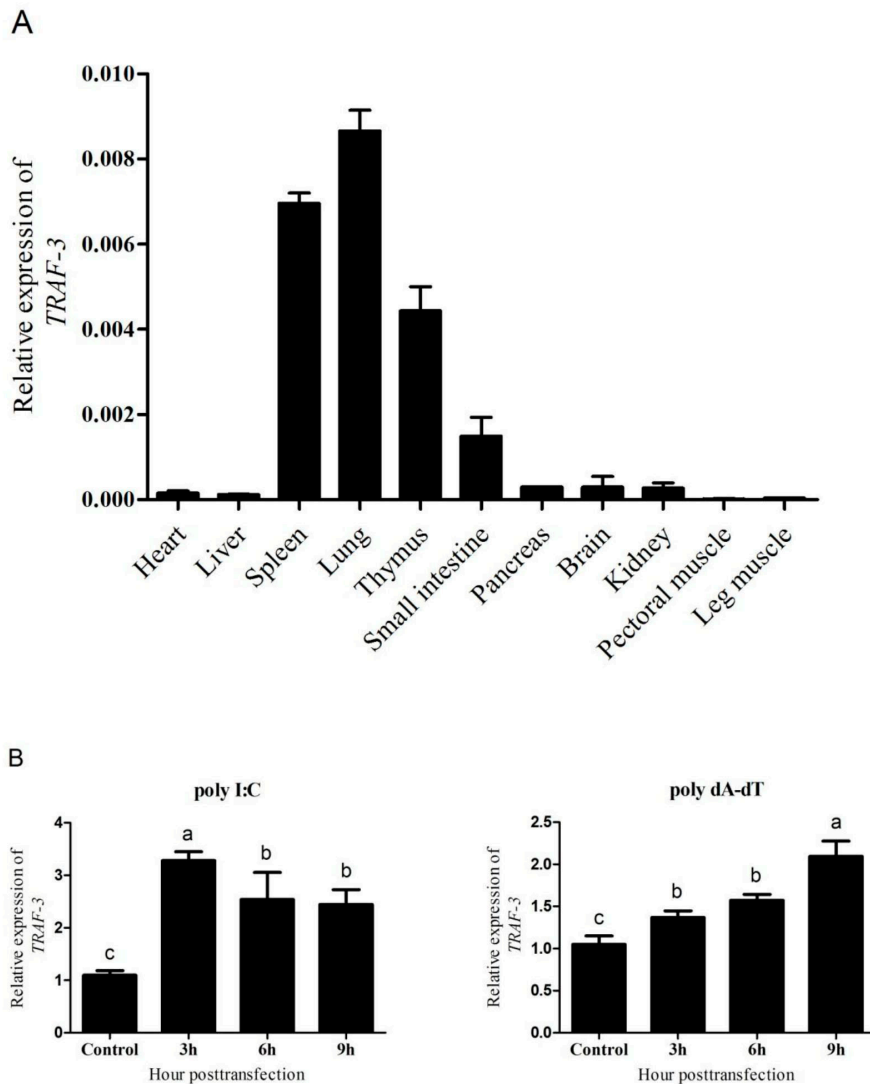To explore the temporal expression of *chTRAF3* regulated by virus mimic stimulation in CEF cells, the transcription level of *chTRAF3* and *IFN-β* were determined by qRT-PCR. As shown in Figure 4B, the *chTRAF3* transcript was significantly upregulated and reached a maximum at 3 h post-transfection by 0.5 μg/mL poly I:C and decreased thereafter. However, the expression of *chTRAF3* mRNA steadily increased over 9 h of induction with 0.5 μg/mL poly dA-dT.

Poly I:C and poly dA-dT induce chicken *TRAF3* in a concentration-dependent manner

To explore the effect of concentration of virus analogues on *chTRAF3* mRNA expression, we used a series of concentration of poly I:C or poly dA-dT to stimulate CEF cells and detected *chTRAF3* and *IFN-β* by qRT-PCR. An increase in the concentration of poly I:C led to significantly increased *chTRAF3* and *IFN-β* mRNA between control and 1 μg/mL at 3 h post-transfection, reaching a maximum at 1 μg/mL and then decreasing thereafter (Figure 5). The CEF cells treated with poly dA-dT also revealed that *chTRAF3* mRNA expression was gradually upregulated among different treatment concentrations at 9h post-transfection. However, *IFN-β* mRNA expression was significantly decreased after reaching a maximum at 2 μg/mL (Figure 5).
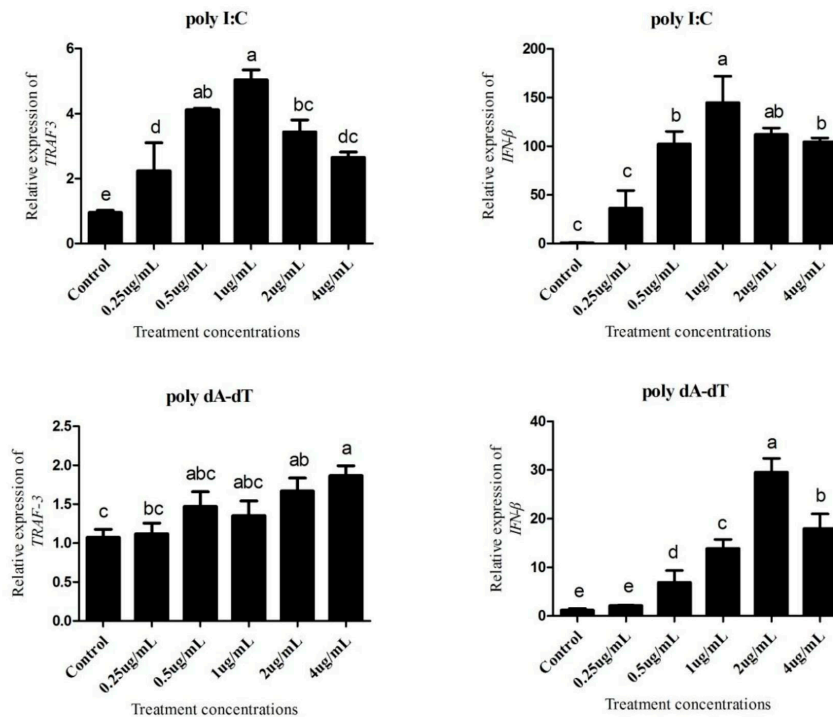

Figure 5. Poly I:C and poly dA-dT induce *chTRAF3* in a concentration-dependent manner. The absence of a common letter in each chart indicates that the values showed a significant difference (P < 0.05) among different treatment concentrations. Error bars represent means ± standard deviation (N = 3).

Differential expression of chicken *TRAF3* in various virulence of NDV-challenged CEF cells

To explore the effect of virulence of NDV infection on *chTRAF3* mRNA expression, we challenged CEF cells with NDV virulent strains F48E9 and vaccine strains Mukteswar. The expression of *chTRAF3* and *IFN-β* mRNA were determined by qRT-PCR. The results show that the *chTRAF3* transcript was significantly upregulated by 24-and 8-fold at 16 h post-infection compared with control levels, challenged with NDV Mukteswar and F48E9, respectively. Additionally, *IFN-β* mRNA expression in CEF cells challenged with NDV vaccine strains Mukteswar was significantly upregulated by 4419-fold at 16 h post-infection, but F48E9 treatment resulted in upregulation by 45-fold compared with control levels (Figure 6).
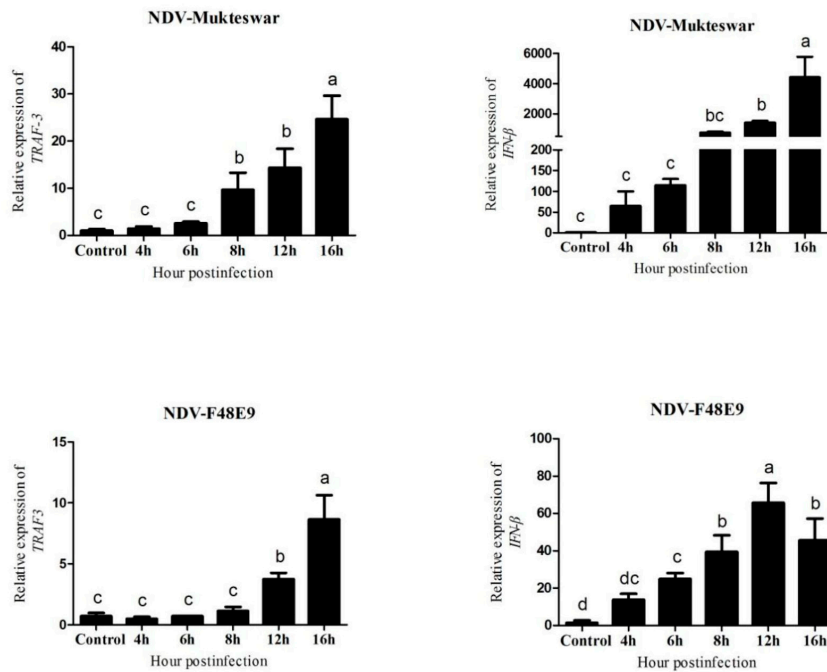
Figure 6. Differential expression of *chTRAF3* in various pathogenicity of NDV-challenged CEF cells. The absence of a common letter in each chart indicates that the values showed a significant difference (P < 0.05) at different times post-infection. Error bars represent means ± standard deviation (N = 3).

DISCUSSION

TRAF3 is a multifunctional protein that not only acts as an adaptor protein in the assembly of signaling complexes, but also as an E3 ubiquitin ligase that activates downstream signaling events. In this study, we cloned 2 types of chicken TRAF3 gene products, *chTRAF3-1* and *chTRAF3-2*. The *chTRAF3-1* was 1704 bp and encoded a protein of 567 amino acids, while chTRAF3-2 was 1536 bp and encoded a protein of 511 amino acids, both of which showed high identity with TRAF3 from human and mouse. Structural analysis revealed that chicken TRAF3 shared typical characteristics of TRAFs, with a RING finger domain, 2

zf-TRAF motifs, and a MATH domain. Over the past decade, chicken TLRs and RLRs have been identified and were found to have some functional similarity to mammalian TLRs and RLRs (Fukui et al., 2001; Yilmaz et al., 2005; Karpala et al., 2011; Liniger et al., 2012). These results suggest that chicken TRAF3 plays a similar role with TRAF3 in mammals.

The expression of TRAFs has been investigated in many studies. Previous studies have shown that *TRAF2*, *TRAF3*, and *TRAF6* are constitutively expressed in most cell types, while *TRAF5* expression is mainly restricted to immune cells (Hacker et al., 2011). In the present study, the *chTRAF3* transcript was observed in all examined tissues at different expression levels, which is similar to *TRAF3* from murine (Cheng et al., 1995) and pearl oyster *Pinctada fucata* (Huang et al., 2012). The broad expression spectrum is in accordance with its multiple functions in the immune system. To control invasion by various pathogens, host cells developed a rapid and effective innate immune after pathogens escaped from mucosa and skin defense. The *chTRAF3* mRNA was found to be abundant in the spleen, thymus, lung, and small intestine, suggesting that chicken TRAF3 has innate immune functions for controlling infection by pathogens in these organs.

Typically, double-stranded (ds) RNA does not exist in uninfected cells and is detected to have non-self-pathogen-associated molecular patterns. In mammalian cells, RIG-I, MDA5, and TLR3 play a major role in the recognition of virus dsRNA and poly I:C, a synthetic dsRNA analogue (Takeda and Akira 2004; Kato et al., 2006). Following the recognition of viral dsRNA, TRAF3 acts as a lysine 63-specific ubiquitin ligase that targets activates TBK1 to trigger antiviral responses (Sun et al., 2010; Tseng et al., 2010). In avians, the chicken *MDA5* homolog has been identified as an sensor of viral dsRNA and regulated IFN-β production in the absence of RIG-I function (Karpala et al., 2011; Hayashi et al., 2014). In our study, *chTRAF3* and *IFN-β* from CEF cells were induced in response to poly I:C transfection. Notably, the transfected poly I:C was sensed by RIG-I/MDA5 in a cell-type-specific manner, but not recognized by TLR3 (Kato et al., 2005; Gitlin et al., 2006). Poly dA-dT is a synthetic dsDNA analog. *chTRAF3* and *IFN-β* were upregulated by poly dA-dT treatment as well, suggesting that chicken TRAF3 may play an important role not only in chicken resistance against RNA virus infection, but also in DNA virus infection. Additionally, *chTRAF3* was induced by transfected poly I:C or poly A:T in a time- and concentration-dependent manner in CEF cells.

Interferons are potent antiviral factors that mediate innate and adaptive immune defense. Pathogens limit the secretion of IFN-β to replicate and survive in the host. Recently, an increasing number of studies has indicated that TRAF3 is a potential target for impairing innate immunity. It is likely that herpes simplex virus 1 or chlamydia pneumoniae expresses a unique protein for preventing TRAF3 from activating TBK1 (Wang et al., 2013; Wolf and Fields 2013). In the present study, *chTRAF3* and *IFN-β* transcripts from CEF cells challenged with NDV vaccine strain Mukteswar were much stronger than NDV F48E9 treatment, suggesting that chicken TRAF3 plays an important role in NDV F48E9 disarm innate immune responses.

In conclusion, 2 types of chicken TRAF3 gene products, *chTRAF3-1* and *chTRAF3-2*, were identified and characterized in this study. *TRAF3* was broadly expressed in most detected tissues in chicken, and at higher levels in the spleen, thymus, lung, and small intestine compared to in other tissues. The expression of *chTRAF3* in CEF cells was upregulated with poly I:C and poly dA-dT as well as NDV challenge. These results indicate that chicken TRAF3 plays an important role in chicken defense against RNA and DNA virus infection. In addition, chicken TRAF3 may be a candidate target for NDV F48E9 to impair immune responses.

ACKNOWLEDGMENTS

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