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β-cantenin is potentially involved in the regulation of c-Jun signaling following bovine herpesvirus 1 infection

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ABSTRACT

C-Jun, activated by various extracellular signals, is important for cell differentiation, proliferation, apoptosis, and inflammatory responses. We have previously reported that bovine herpesvirus 1 (BoHV-1) infection in MDBK cells stimulates the c-Jun NH2-terminal kinase (JNK)/c-Jun cascade for efficient replication. However, the mechanisms regarding the regulation of c-Jun following BoHV-1 infection remain unknown. In this study, we show that virus infection increases accumulation of p-c-Jun(S73) (phosphorylated c-Jun at Ser73) and p-β-catenin(S552) in the nucleus, resulting in relocalized nuclear p-c-Jun(S73) to assemble in highlighted punctum via a confocal microscope assay. An association between β-catenin and c-Jun in the nucleus was readily detected in virus-infected, but not mock-infected cells. Interestingly, β-catenin was found to be involved in the regulation of c-Jun signaling in virus-infected cells as iCRT14, a β-catenin-specific inhibitor that can inhibit β-catenin-dependent transcriptional activity, was able to decrease protein expression and phosphorylation of c-Jun. Furthermore, we suggest that BoHV-1 infection stimulates c-Jun phosphorylation regulated by β-catenin via both c-Jun NH2-terminal kinase (JNK)-dependent and JNK-independent mechanisms. These data add to our knowledge regarding the regulation of c-Jun following virus infection and further support the important roles of β-catenin signaling playing in BoHV-1 infection.

1. Introduction

Similar to herpes simplex virus 1 (HSV-1), bovine herpesvirus 1 (BoHV-1) is an enveloped DNA virus which belongs to the family *Herpesviridae* and the subfamily *Alphaherpesvirinae* (Muylkens et al., 2007; Tikoo et al., 1995). BoHV-1 can infect cattle of all ages and breeds. Acute infection of cattle with BoHV-1 generally results in inflammatory responses in distinct tissues, including the upper respiratory tract, nasal cavity, and ocular cavity, and leads to erosions in the mucosal surface (Hodgson et al., 2005). BoHV-s1 infection suppresses the immune response, which may result in secondary infection by other pathogens, such as bovine viral diarrhea viruses (BVDV), bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI3V) and bovine coronaviruses, and bacteria including *Mannheimia haemolytica* and *Pasteurella multocida*, ultimately leading to life-threatening pneumonia known as bovine respiratory disease complex (BRDC) (Fulton et al., 2016; Muylkens et al., 2007; Tikoo et al., 1995). BoHV-1 is regarded as a critical co-factor for BRDC development (Neibergs et al., 2014). It has been suggested that BoHV-1 infection costs the US cattle industry approximately 3 billion dollars in losses annually (Jones and Chowdhury, 2007).

As an activator protein 1 (AP-1) family member, c-Jun plays essential roles in multiple cellular processes, including cell proliferation, survival, and death (Angel and Karin, 1991; Jochum et al., 2001; Kovary and Bravo, 1991). C-Jun can be activated by various extracellular signals such as growth factors, cytokines, and extracellular stresses, typically through a serine/threonine kinase c-Jun NH2-terminal kinase (JNK) (Davis, 2000; Liu et al., 2015; Yung and Giacca, 2020). Studies have indicated that c-Jun facilitates the production of numerous inflammatory cytokines and chemokines. For example, c-Jun facilitates H5N1 influenza virus replication in human lung epithelial cells and expression of cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-β, interleukin (IL)-6, and IL-10 (Xie et al., 2014). Additionally, c-Jun is able to mediate inflammatory responses triggered by these cytokines, resulting in establishment of a potential feedback loop to amplify inflammatory effects following virus infection (Riesenberg et al., 2015; Schonthaler et al., 2011). Targeting c-Jun is regarded as an effective avenue for therapeutic interventions of

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inflammatory diseases. As such, studying the interplay between c-Jun and BoHV-1 infection is important to understand mechanisms of virus pathology.

It has been reported that c-Jun-dependent trans-activation supports HSV-1 replication in cell cultures (McLean and Bachenheimer, 1999). Similarly, BoHV-1 infection activates the JNK/c-Jun cascade, with inhibition of this pathway via the chemical inhibitor SP600125 significantly blocking productive infection in cell cultures (Zhu et al., 2016). However, how c-Jun signaling is affected by virus infection remains poorly understood.

The Wnt/β-catenin signaling pathway is required for the regulation of cell proliferation, cell survival, development and tissue regeneration (Clevers and Nusse, 2012; Gough, 2012). Furthermore, it can both regulate the production of multiple inflammatory cytokines, and be activated by inflammatory cytokines to form a feedback loop (Silva-Garcia et al., 2014; Yang et al., 2018). The β-catenin signaling pathway is additionally activated to support BoHV-1 latent infection in sensory neurons and productive infection in cell culture (Liu et al., 2016; Workman et al., 2018; Zhu and Jones, 2018; Zhu et al., 2017a, b).

In quiescent cells, β-catenin in the cytoplasm is constitutively associated with adenoma polyposis coli (APC), axin, glycogen synthase kinase 3β (GSK-3β), and casein kinase I, resulting in the assembly of a β-catenin destruction complex, which leads to polyubiquitination of β-catenin and degradation by the proteasome (Clevers and Nusse, 2012). Upon activation by Wnt, the intracellular phosphoprotein disheveled (DVL) is activated, causing the disassociation of the β-catenin destruction complex. The activated β-catenin is stabilized and accumulates in nucleus, where it interacts with members of the T-cell factor (TCF) family of DNA-binding proteins specifically bound to the consensus site AGATCAAGG (Behrens et al., 1996; van de Wetering et al., 1997). The binding of β-catenin to TCF family members displaces the bound corepressors and recruits coactivators such as cyclic AMP-response element-binding protein (CREB)-binding protein (CBP) and its close relative p300 to the carboxy-terminal transactivation domain of β-catenin to activate Wnt target genes (Clevers and Nusse, 2012; Mosimann et al., 2009). C-Jun is able to heterodimerize with CREB family members such as activating transcription factor-2 (ATF2) and forms a complex which subsequently binds to cyclic AMP-responsive element (CRE), to drive CRE-dependent transcription (Angel and Karin, 1991; McLean and Bachenheimer, 1999). It is likely that CREB/CRE connects the interplay between c-Jun and β-catenin. *C-Jun* promoter can be activated by c-Jun itself because this promoter also contains CRE sites (Gupta et al., 1995; Kappelmann et al., 2014; Karin et al., 1997; McLean and Bachenheimer, 1999). Importantly, it has been reported that nuclear Dvl, c-Jun, β-catenin, and TCF form a complex on the promoters of Wnt target genes and regulate gene transcription by stabilization of β-catenin-TCF interactions (Gan et al., 2008), providing evidence that c-Jun physically associates with β-catenin and regulates β-catenin-dependent transcription. However, whether β-catenin has an effect on c-Jun expression remains unknown. Here, we hypothesized that β-catenin is involved in the regulation of c-Jun expression during BoHV-1 infection in vitro.

In this study, we report that BoHV-1 infection stabilized the association between β-catenin and c-Jun in MDBK cells, and that association was readily detected in the infected nucleus but not in uninfected nucleus. BoHV-1 infection promoted nucleus accumulation of activated c-Jun [p-c-Jun(S73)] and activated β-catenin [p-β-catenin(S552)]. Furthermore, BoHV-1 infection relocalized nucleus p-c-Jun(S73), and stimulated the expression and activation of c-Jun through β-catenin, suggesting that c-Jun signaling is regulated in part via β-catenin.

2. Material and methods

2.1. Cells and viruses

Madin-Darby bovine kidney (MDBK) cells (purchased from Chinese model culture preservation center, Shanghai, China) were cultured in DMEM containing 10 % fetal bovine serum (FBS). BoHV-1 (NJ-16-1 isolated from bovine semen samples (Zhu et al., 2017c) was propagated in MDBK cells. Aliquots of virus stocks were titered in MDBK cells and stored at −80 ˚C.

2.2. Antibodies and chemical reagents

The following chemical reagents were used in this study: iCRT14 (MedChemExpress, cat# HY16665), sp600125 (Cell Signaling Technology, cat#8177). The following antibodies were used in this study: phospho(p)-c-Jun (Ser73) rabbit monoclonal antibody (Cell Signaling Technology, cat# 3270), c-Jun rabbit mAb (Cell Signaling Technology, cat# 9165), p-JNK (Thr183/Tyr185) rabbit mAb (Cell Signaling Technology, cat# 9251), JNK rabbit polyclonal antibody (pAb) (Cell Signaling Technology, cat# 9252). p-β-catenin (Ser552) rabbit mAb (Cell Signaling Technology, cat# 9566). β-catenin (Ser552) rabbit mAb (Abcam, cat# ab32572), mouse control IgG (ABclonal, cat# AC011), rabbit control IgG (ABclonal, cat# AC005), laminA/C mouse mAb (Santa Cruz Biotechnology, cat# sc-376248), β-Tubulin rabbit pAb (Abclonal, cat# AC015), GAPDH mouse mAb (Cell Signaling Technology, cat# 2118), *β*-actin rabbit mAb (Cell Signaling Technology, cat# 4970), Alexa Fluor 488®-conjugated goat anti-rabbit IgG (H + L) (Invitrogen, cat# A-11008), HRP- (horseradish peroxidase-) conjugated goat anti-mouse IgG (Cell Signaling Technology, cat# 7076), and goat anti-rabbit IgG (Cell Signaling Technology, cat# 7074). Goat anti-BoHV-1 serum was purchased from VMDR Inc (cat# 20PAB-IBR).

2.3. Western blot analysis

MDBK cells were seeded into 60 mm dishes and cultured overnight. Cell cultures were treated with either DMSO vehicle or iCRT14 at a concentration of 10 μM for 1 h at 37 ˚C in a humidified incubator with 5% CO_2. Cells were infected with BoHV-1 (MOI = 0.1) for 1 h in the presence of the chemicals indicated. After washing three times with PBS, fresh medium containing either DMSO or iCRT14 was replaced. At 24 h post infection (hpi), cell lysates were prepared using lysis buffer (1% Triton X-100, 50 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.5 g/mL leupeptin, 1 mM benzamidine, and 1 mM sodium orthovanadate in 20 mM Tris-HCl, pH 8.0). After centrifugation at 13,000 rpm for 10 min at 4 ˚C, clarified supernatants were collected and boiled together with Laemmli sample buffer for 10 min; samples were subsequently separated by 8 % or 10 % SDS-PAGE and proteins were transferred onto PVDF membranes (Bio-Rad, cat# 1620177). After blocking with 5 % nonfat milk in PBS for 1 h (h) at room temperature, the membranes were incubated with primary antibodies diluted in 5 % bovine serum albumin in PBS, overnight at 4 ˚C. After extensive washing with PBST (0.1 % Tween-20 in PBS), membranes were incubated with either anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature. After extensive washing with PBST, protein bands were developed onto film by using Clarity Western ECL Substrate (Bio-Rad, cat# 1705061).

2.4. Immunoprecipitation assay

MDBK cells grown in 60 mm dishes were mock infected or infected with BoHV-1 (MOI = 0.1) for 1 h. After washing three times with PBS, fresh medium was replaced. At 24 hpi, cells were lysed with 600 μL of RIPA buffer (1x PBS, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) supplemented with the aforementioned protease inhibitors. Cell lysates were then clarified by centrifugation at 13,000 rpm for 10 min and incubated with Dynabeads protein A beads (Life Technologies, cat# 10001D), which were precoated with primary antibodies or isotype IgG by incubation for 1 h at room temperature with rotation. After

overnight incubation at 4 °C with rotation, beads were collected using a magnet (DynaMag™) (Life Technologies, cat# 12321D). After three washings with PBS, beads were boiled in SDS-loading buffer and Western blots were performed using the indicated antibodies.

2.5. Immunofluorescence assay

MDBK cells seeded into 8-well chamber slides (Nunc Inc., IL, USA) were mock infected or infected with BoHV-1 (MOI = 0.1) for 24 h. Cells were fixed with 4 % paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.25 % Triton X-100 in PBS for 10 min at room temperature, and blocked with 1 % BSA in PBST for 1 h followed by incubation with the antibodies indicated in 1 % BSA in PBST overnight at 4 °C. After three washings, cells were incubated with Alexa Fluor 488®-conjugated goat anti-rabbit IgG (H + L) (Invitrogen, cat# A-11008, 1 : 1500 dilution) for 1 h in the dark at room temperature. After three washings, DAPI (4′,6-diamidino-2-phenylindole) staining was performed to visualize nuclei. Slides were covered with coverslips by using antifade mounting medium (Electron Microscopy Sciences, cat# 50-247-04). Images were captured using a confocal microscope (Leica).

3. Results

3.1. BoHV-1 infection promoted nucleus accumulation of p-β-catenin (S552) and p-c-Jun(S73)

Phosphorylation of β-catenin at Ser552(S552) increases transcriptional activity (Fang et al., 2007; Hino et al., 2005; Taurin et al., 2006). C-Jun protein has a C-terminal DNA-binding domain and an N-terminal transactivation domain. The phosphorylation of serine 73(S73) in the transactivation domain is closely associated with its transcriptional activity (Pulverer et al., 1991; Zhu et al., 2014). To address our hypothesis, we initially investigated the effects of BoHV-1 infection on the accumulation of β-catenin, p-β-catenin(S552), c-Jun, and p-c-Jun(S73) in the cytosol and nucleus. MDBK cells were mock infected or virus-infected for 24 h, after which fractions of both cytosol and nucleus were isolated by using a commercial kit, and subjected to Western blotting. We found that relative to the mock infected control, higher levels of p-β-catenin(S552) were readily detected in virus infected cells (whole cell extracts) (Fig. 1A), as well as in fractions of both the cytosol (Fig. 1A) and nucleus (Fig. 1C). Steady state β-catenin protein expression was decreased in whole cell extracts (Fig. 1B) and cell fractions of both cytosol (Fig. 1B) and nucleus (Fig. 1D) following virus infection. In addition, laminA/C in the cytosol fraction and Tubulin in the nucleus fraction were rarely detected, indicating that these cellular fractions were not contaminated (Figure E). The host-shutoff effects of BoHV-1 at late stages of virus infection may account for the decreased protein levels of β-catenin. Increased levels of p-β-catenin(S552) were not due to the increased protein expression of total β-catenin. P-c-Jun(S73) was readily detected at higher levels in whole cell extracts (Fig. 1A) and the nucleus fractions following virus infection (Fig. 1C), but not in the cytosol fractions of either mock-infected or virus-infected cells (Fig. 1A). C-Jun was detected at higher levels in whole cell extracts (Fig. 1B) and cell fractions of both cytosol (Fig. 1B) and nucleus (Fig. 1D) following virus infection. Increased c-Jun steady state protein expression may partially account for the increased phosphorylation levels of c-Jun.

To validate the association between virus infection and these identified modulations in protein expression and cellular relocalization, the protein expression of virion-associated proteins was detected by Western blot. As can be seen in Fig. 1F, three bands indicative of virion-associated proteins were readily detected after infection for 24 h. Collectively, these data suggest that BoHV-1 infection activates β-catenin and c-Jun, and promotes nuclear accumulation of these phosphorylated proteins.

3.2. BoHV-1 infection led to relocalization of nucleus p-c-Jun (S73)

Since virus infection promoted nucleus accumulation of both p-β-catenin(S552) and p-c-Jun(S73), we next investigated if they were relocated. IFA assay indicated that following virus infection, β-catenin (S552) partly translocated into the nucleus, in agreement with results obtained via Western blot (Fig. 1C). In the mock infected cells, pronounced staining of p-β-catenin(S552) was mainly located at sites of cell-cell contacts, with faint staining observed in the nucleus (Fig. 2A). P-β-catenin(S552) was readily detected in virus infected cells, and highlighted staining was located at sites of cell-cell contacts (Fig. 2B). Interestingly, following virus infection p-c-Jun(S73) predominantly located in the nucleus, and clearly formed foci which was not readily observed in uninfected cells (Fig. 2C–D). Of note, to readily detect p-β-catenin(S552) (Fig. 2A) and p-c-Jun(S73) (Fig. 2C) in uninfected cells, a longer exposure time was required. As a result, direct comparisons between protein levels in mock infected versus infected cells with these images is not possible. The highlighted p-c-Jun(S73) foci may indicate that agglutination of p-c-Jun(S73) is driven during virus infection to execute specific biological functions.

3.3. BoHV-1 infection stabilized the association between c-Jun and β-catenin

It has been reported that nuclear c-Jun and β-catenin form a complex in HEK293 T cells (Gan et al., 2008). To detect whether this association was affected by BoHV-1 infection, immunoperecipitation (IP) was performed by using antibody against c-Jun and β-catenin, respectively. When c-Jun was immunoprecipitated from whole cell lysates, β-catenin was consistently detected, but levels of β-catenin were reduced in precipitates from BoHV-1-infected cells in comparison to that of uninfected control (Fig. 3A, left panel). Vice versa, c-Jun could be detected in immunoprecipitates when IP was performed by using β-catenin antibody (Fig. 3A, middle panel). Specific binding to either β-catenin or c-Jun by individual antibody in these IP assays was confirmed by isotype IgG (Fig. 3B). Since virus infection increased c-Jun steady state expression, but reduced β-catenin protein levels (Fig. 1), lower levels of β-catenin protein in the c-Jun IP products does not indicate that virus infection disrupted the association between c-Jun and β-catenin (Fig. 3A, left panel) because the increased c-Jun molecules attributable to virus infection led to a reduced probability of capturing c-Jun-bound β-catenin by c-Jun antibody in the IP assay. While the β-catenin IP products could correctly reflect the binding ability of β-catenin to c-Jun, quantitative analysis indicated that the amount of β-catenin-bound c-Jun was increased by virus infection to approximately 1.48-fold relative to that in the uninfected control (Fig. 3A, middle panel), suggesting that the association between β-catenin and c-Jun was stabilized following BoHV-1 infection. These IP assay data confirmed the presence of an association between c-Jun and β-catenin as reported elsewhere (Gan et al., 2008), with this association stabilized by virus infection. Of note, these antibodies against either β-catenin or c-Jun were generated with rabbits, with an different species suit for bovine cells currently unavailable. Subsequent colocalization studies through IFA would be of benefit to validate the association between β-catenin and c-Jun, once a proper antibody is available in the future.

After infection for 24 h, the activated molecules of both p-c-Jun (S73) and p-β-catenin(S552) were promoted to translocate into the nucleus (Fig. 1). We further investigated whether the association between Jun and β-catenin could be detected in the nucleus, by performing IP assays using nucleus fractions. These experiments revealed that β-catenin could be clearly detected in the c-Jun immune precipitates from virus infected nucleus fractions but not in those of the uninfected nucleus (Fig. 4), suggesting that the activated β-catenin was also associated with the activated c-Jun in the nucleus. It is likely that undetectable associations between β-catenin and c-Jun in the mock-infected nucleus are attributed to the relatively lower levels of c-Jun

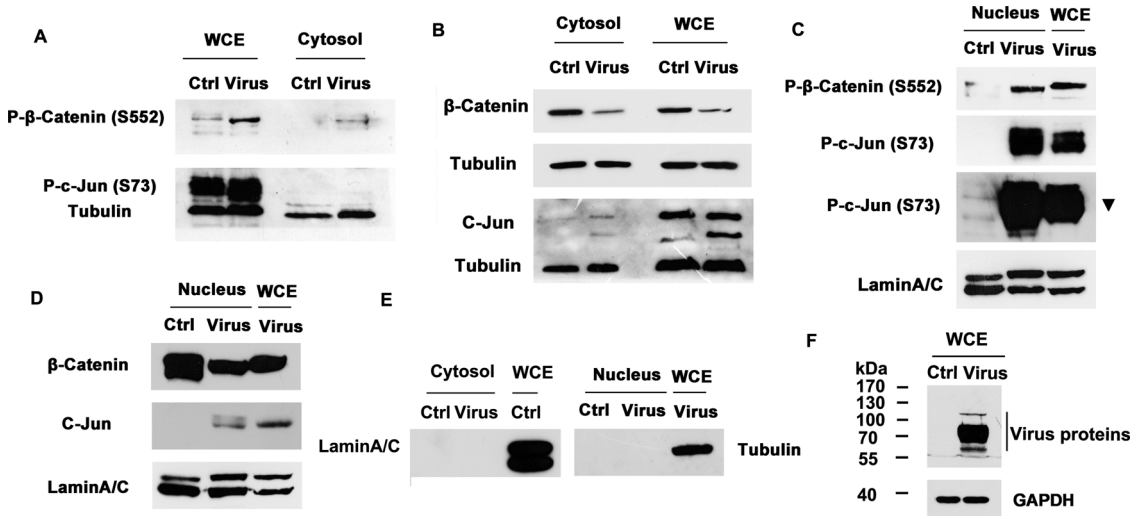

Fig. 1. BoHV-1 infection increased nucleus accumulation of p-c-Jun (S73) and p-β-catenin(S552). MDBK cells in 60 mm dishes were mock infected or infected with BoHV-1 (MOI = 0.1) for 24 h, after which cells were collected for isolation of nuclear proteins and cytosol using a commercial nuclear protein purification kit (Beyotime Biotechnology, cat# P0027). (A) P-β-catenin(S552) and p-c-Jun (S73) in cytosol fractions and whole cell extracts (WCE) were detected by Western blot. Tubulin was detected and used as a protein loading control. (B) β-catenin and c-Jun in the cytosol fractions and whole cell extracts (WCE) were detected by Western blot. Tubulin was detected and used as protein loading control. (C) P-β-catenin(S552) and p-c-Jun (S73) in the nuclear fractions were detected by Western blot. LaminA/C was detected and used as protein loading control. (D) β-catenin and c-Jun in the nucleus fractions were detected by Western blot. LaminA/C was detected and used as protein loading control. (E) LaminA/C in the cytosol fractions and Tubulin in the nucleus fractions were detected by Western blot to determine if these fractions were contaminated by the counterpart fractions. Data shown are representative of three independent experiments.

that are nearly undetectable in the uninfected nucleus fractions via Western blotting (Fig. 1). Taken together, the association between β-catenin and c-Jun was maintained in the BoHV-1-infected nucleus, which further confirms the physical interaction between β-catenin and c-Jun, providing supporting evidence that β-catenin may affect c-Jun signaling.

3.4. β-catenin was involved in the regulation of c-Jun signaling following BoHV-1 infection

In order to understand the effects that β-catenin had on the activation of c-Jun signaling stimulated by BoHV-1 infection, we detected the protein levels of both c-Jun and p-c-Jun(S73) in the presence of β-catenin-specific inhibitor iCRT14 via Western blot. Using this approach, we found that c-Jun protein expression levels were reduced by iCRT14 either in the presence or absence of virus infection in comparison to the individual control (Fig. 5A). It is known that iCRT14 inhibits β-catenin-dependent transcription by interfering with the interaction between β-catenin and TCF family members (Gonsalves et al., 2011; Zhu et al., 2017a). It has been reported that iCRT14 blocks β-catenin-dependent transcription stimulated by BoHV-1 infection (Zhu et al., 2017a). As such, it is reasonable that iCRT14 reduces c-Jun protein expression by disruption of β-catenin-dependent transcriptional activity essential for c-Jun transcription. However, we found that the phosphorylation of c-Jun at Ser73 was significantly inhibited by the treatment of iCRT14 (10 μM) in the absence or presence of virus infection (Fig. 5B). This was unexpected because broad spectrum anti-kinase activity of iCRT14 has not been documented, and was not associated with β-catenin phosphorylation (Fig. 5C). ICRT14 at a concentration of 10 μM was not cytotoxic in uninfected cell cultures analyzed by Trypan-blue exclusion test as described elsewhere (Fiorito et al., 2008) (Fig. 5D). When cell morphology was examined, 10 μM iCRT14 reduced the effects of virus infection-induced cytopathology (Fig. 5E), suggesting that treatment with 10 μM iCRT14 did not exert cytotoxicity in virus-infected cells. Collectively, these data indicate that the observed inhibitory effects of iCRT14 on either c-Jun expression or phosphorylation in the virus infected cells was not due to cytotoxicity effects. Taken together, we suggest a role for β-catenin in the activation of c-Jun signaling pathway stimulated during late-stage BoHV-1 infection.

3.5. β-catenin was potentially involved in the regulation of c-Jun signaling through JNK

JNK is a canonical upstream activator of c-Jun phosphorylation. To understand how c-Jun signaling was inhibited by iCRT14, the effects of iCRT14 on JNK signaling activity were investigated. There are three JNK genes (*JNK1, JNK2 and JNK3*), each of which undergoes alternative splicing, resulting in numerous isoforms, which allows for different JNK activities in specific tissue types (Gupta et al., 1996; Kyriakis and Avruch, 2001). Two isoforms of JNK, JNK-p54 and JNK-p46, could be detected with the antibody used in this study. Treatment with iCRT14 led to the depletion of JNK-p46 expression in uninfected cells but not in virus-infected cells (Fig. 6A). Phosphorylation of both JNK-p54 and JNK-p46 at Thr183/Tyr185 stimulated by virus infection was blocked by iCRT14 in cell cultures (Fig. 6B), corroborating our results that iCRT14 inhibited the phosphorylation of c-Jun stimulated by virus infection.

JNK specific inhibitor SP600125 could efficiently block JNK phosphorylation. To validate the inhibitory effects of iCRT14 on JNK phosphorylation, virus-infected cells were treated with either iCRT14 or SP600125, and the phosphorylation of c-Jun was detected by Western blotting. We found that the phosphorylation of both JNK-p54 and JNK-p46 stimulated by BoHV-1 infection could be blocked by both iCRT14 (10 μM) and SP600125 (25 μM) (Fig. 6C). In addition, the phosphorylation of c-Jun at Ser73 was also inhibited by both iCRT14 and SP600125 with distinct intensity (Fig. 6D). As determined by a Trypan-blue exclusion text, SP600125 at a concentration of 25 μM was not cytotoxic to uninfected cell cultures (Fig. 6E). When cell morphology was examined, treatment with 25 μM SP600125 reduced the effects of virus infection-induced cytopathology (Fig. 6F). As such, it was unlikely that the observed inhibitory effects of SP600125 on the phosphorylation of JNK and c-Jun in the virus-infected cells was due to cytotoxicity effects. Of note, even though both SP600125 and iCRT14 possessed a similar capacity to depress JNK phosphorylation, higher levels of p-c-Jun(S73) were still observed following SP600125 treatment, but not following iCRT14 treatment (Fig. 6D). Therefore, iCRT14 may inhibit c-

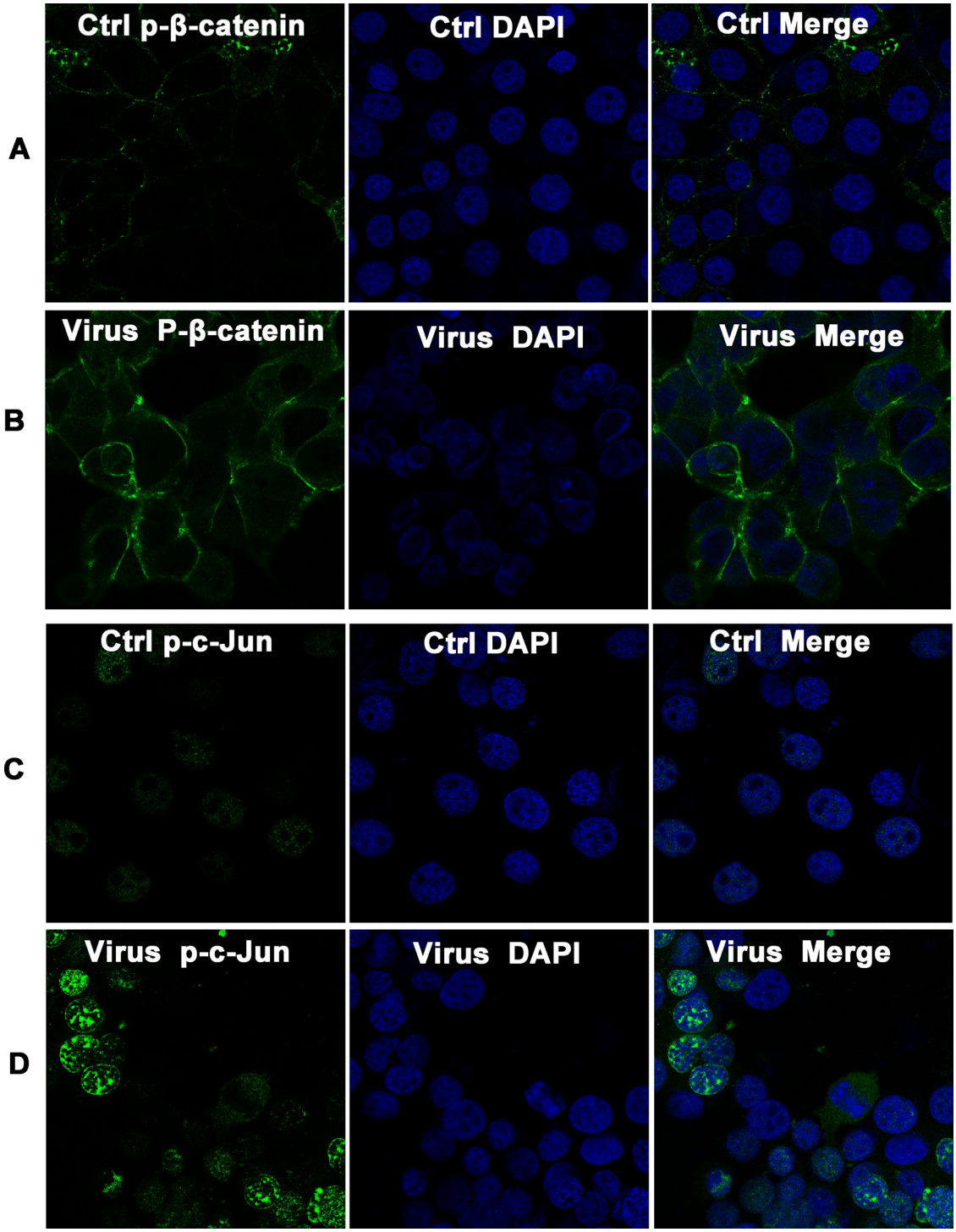

Fig. 2. Localization of p-β-catenin(S552) and p-c-Jun (S73) in the absence or presence of BoHV-1 infection.
MDBK cells were mock infected (A and C) or infected with BoHV-1 (MOI = 0.1) (B and D) for 24 h. After three washings with PBS, cells were fixed with 4% formaldehyde, and p-β-catenin(S552) and p-c-Jun (S73) were detected by IFA. Nuclei were stained with DAPI. Images were obtained by performing confocal microscopy. The images shown are representative of three independent experiments.

Jun phosphorylation with either JNK-dependent or JNK-independent mechanisms. Taken together, these data suggest that in BoHV-1-infected MDBK cells, β-catenin is potentially involved in the phosphorylation of c-Jun signaling partially through the activation of JNK.

4. Discussion

C-Jun, a known transcriptional activator, regulates gene expression via binding to a CRE motif in the promoter region of targeted genes, which is implicated in a diversity of virus infections. For instance, the increased phosphorylation of c-Jun in Epstein-Barr virus (EBV)-infected gastric AGS cells stimulates the EBV IE BZLF1 promoter via a CRE motif, which is important for EBV lytic infection (Feng et al., 2007). The binding of c-Jun to the U3 region of long terminal repeats (LTR) in human foamy virus (HFV) maintains the optimal activity of the HFV promoter (Maurer et al., 1991). C-Jun together with another AP-1

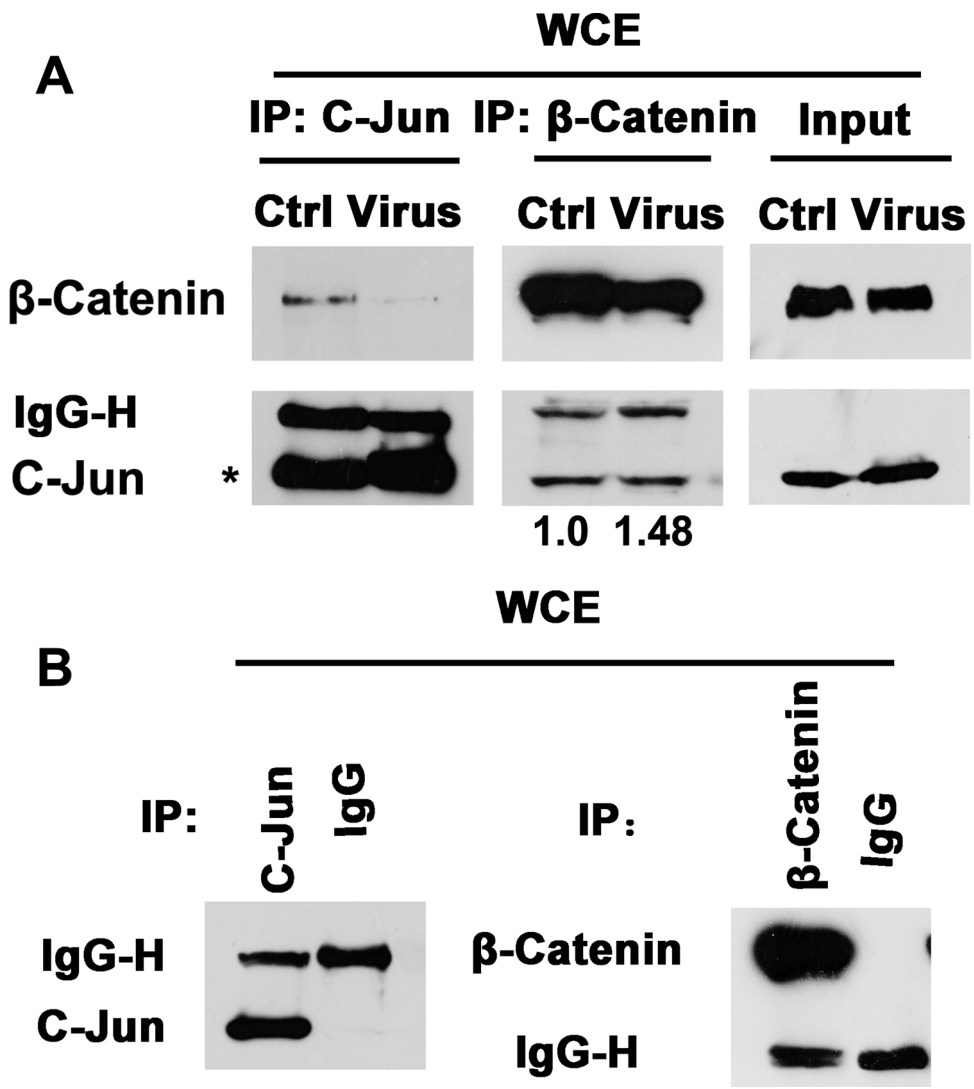

Fig. 3. BoHV-1 infection affected the association between β-catenin and c-Jun. MDBK cells in 60 mm dishes were mock infected or infected with BoHV-1(MOI = 0.1) for 24 h. Cell lysates were subjected to IP using antibody against β-catenin and c-Jun (A) or isotype IgG (B) as a control. Western blots were performed by using the antibodies indicated. Data shown are representative of two independent experiments.

family member, c-Fos, binds to early and late promoters of Human Polyomavirus JC (JCV), resulting in the activation of these promoters. Consequently, the phosphorylation and protein expression of c-Jun are substantially increased at the late phases of JCV infection cycles (Sadowska et al., 2003). These reports suggest a possible versatile mechanism whereby c-Jun directly associates with the viral genome to regulate viral gene expression. C-Jun has been shown previously to physically bind to the consensus sequence (5′-TGAC/GTCA-3′ and 5′-TGACGTCA-3′) in K562 cells (Li et al., 2011). An analysis of the BoHV-1 genome sequence (Acc. No. AJ004801.1) indicated that it contains three potential c-Jun binding sites, located at 69595–69602 nt, 48802–48808 nt and 60681–60687 nt, respectively (data not shown). Here we found that nuclear p-c-Jun(S73) was relocalized following BoHV-1 infection, and formed numerous c-Jun foci (Fig. 2B), reminiscent of the virus replication compartment as characterized in HSV-1 by either bromodeoxyuridine (BrdU)-pulse-labeled DNA synthesis initiation sites or ICP8 staining (Xu and Roizman, 2017; Zhong and Hayward, 1997). It is possible that c-Jun can bind to the BoHV-1 genome in the virus replication compartment to regulate virus replication and gene transcription, which is an interesting question that warrants further study.

Apart from the canonical stimulator JNK, c-Jun can be activated by various extracellular signals such as growth factors, cytokines and extracellular stresses, employing diverse mechanisms (Davis, 2000; Liu et al., 2015; Yung and Giacca, 2020). For example, c-Jun can be activated by the protein kinase C (PKC)/p38MAPK cascade in a JNK-independent manner, in human primary T lymphocytes (Humar et al., 2007). Epidermal growth factor (EGF) induces c-Jun expression via both extracellular signal-regulated kinases 1/2 (ERK1/2) and ERK5 in mouse embryonic fibroblasts (MEFs) (Kayahara et al., 2005). Radiation-induced c-Jun activation depends on the mitogen-activated protein kinase kinase1 (MEK1)-ERK1/2 signaling pathway in microglial cells (Deng et al., 2012). Hepatitis C virus infection stimulates c-Jun signaling via protein kinase R to promote proliferation of hepatocellular carcinomas (Watanabe et al., 2013). We have previously shown that BoHV-1 infection stimulates the JNK/c-Jun cascade in growth arrested MDBK cells for efficient replication (Zhu et al., 2016). In this study, we showed that β-catenin was involved in the regulation of c-Jun expression following BoHV-1 infection in MDBK cells (Fig. 5A), which provides a novel mechanism underlying the regulation of the c-Jun pathway. Moreover, our data show that β-catenin is potentially involved in the phosphorylation of both JNK and c-Jun stimulated by virus infection (Fig. 5B and 6B). Broad spectrum anti-kinase activity of iCRT14 has not been documented, and in this study it did not appear to

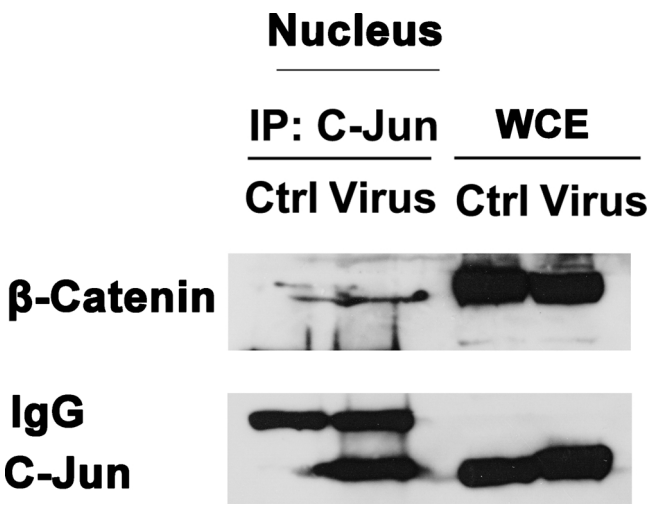

Fig. 4. BoHV-1 affected the association between β-catenin and c-Jun in the nucleus. MDBK cells in 60 mm dishes were mock infected or infected with BoHV-1 (MOI = 0.1) for 24 h. Nucleus proteins were purified using commercial nuclear protein purification kit (Beyotime Biotechnology, cat# P0027). IP was performed using antibodies against c-Jun. Then, Western blotting was performed using corresponding antibodies. Data shown are representative of two independent experiments.

modulate the phosphorylation of either β-catenin itself (Fig. 5C) or PLC-γ1 (data not shown). To our knowledge, similar anti-phosphorylation activity of iCRT14 has not be documented previously. While the precise mechanism of how the β-catenin inhibitor iCRT14 depresses phosphorylation of JNK/c-Jun stimulated by virus infection remains unclear which need further study in the future, these data nonetheless extend our understanding on the biological functions of β-catenin signaling pathway.

It is well known that activated β-catenin is transported into the nucleus to activate β-catenin-dependent transcription (Herbst et al., 2014). Here, we found that accumulation of nuclear p-β-catenin(S552) was increased following BoHV-1 infection (Fig. 1C), which corroborates a previous report that BoHV-1 infection enhanced β-catenin-dependent transcriptional activity (Zhu et al., 2017a). Since c-Jun transcription is autoregulated by its own product (Angel et al., 1988), the physical association between β-catenin and c-Jun in the BoHV-1-infected nucleus (Fig. 4) provides a possibility for increased c-Jun transcriptional activity by β-catenin, as validated by our observation of increased protein expression of c-Jun in response to virus infection (Fig. 1B and D). We noticed that in our previous report that BoHV-1 infection lead to an increased phosphorylation of c-Jun, consistent with our data demonstrated here (Fig. 1A), while increased expression of c-Jun was not observed (Zhu et al., 2016). In that study, MDBK cells were of different origination, cultured in horse serum, and subjected to serum starvation overnight before infection with a high MOI of 10. In this study, MDBK cells were cultured in fetal bovine serum without serum starvation and infected at a MOI of 0.1. It is highly possible that the distinct cell origins, different serum used for cell culture, and with or without serum

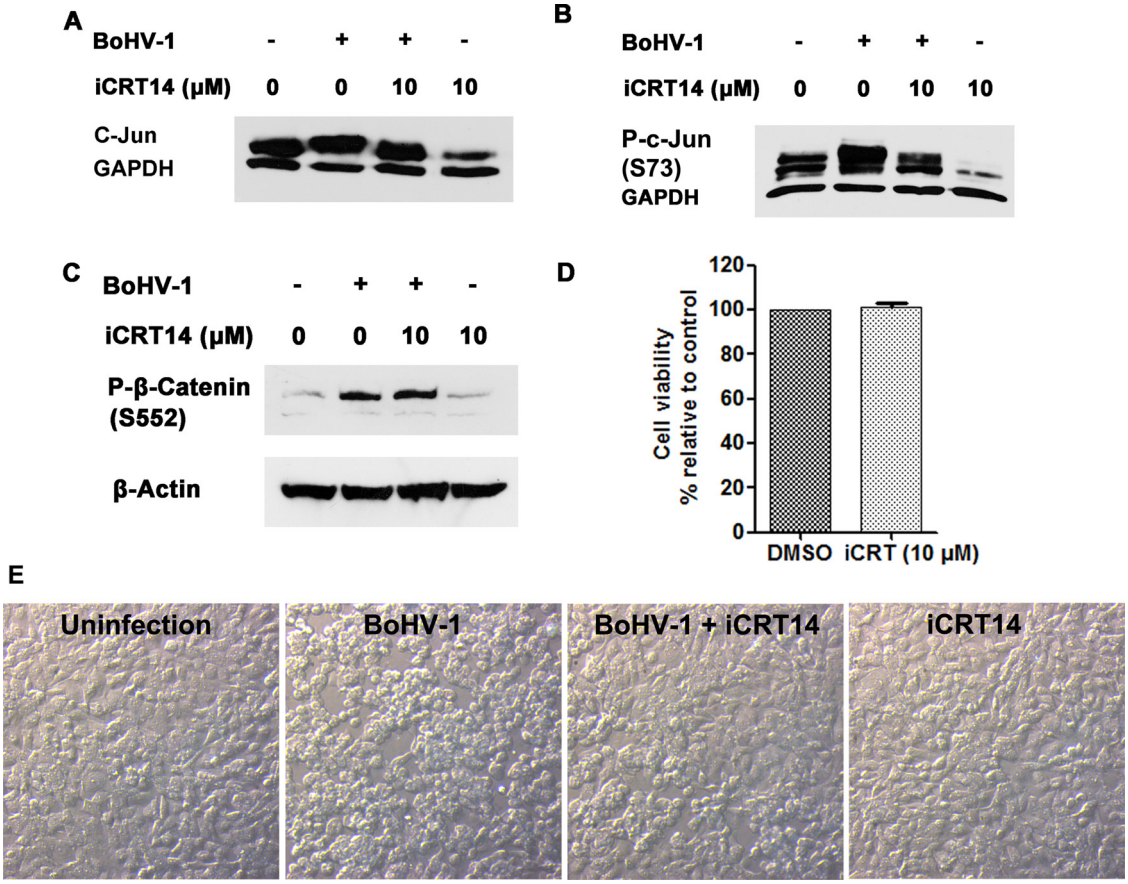

Fig. 5. The effects of β-catenin inhibitor iCRT14 on c-Jun signaling. MDBK cells in 60 mm dishes were pretreated with iCRT14 (10 u M) for 2 h, after which they were mock infected or infected with BoHV-1 at an MOI of 0.1 in the presence of iCRT14 (10 u M) or DMSO control. After infection for 24 h, cell lysates were prepared for Western blotting to detect the expression of c-Jun (A), p-c-Jun (S73)(B), and p-β-catenin(S552). (C), Cell morphology was observed under a light microscope. Images shown are representative of three independent experiments (Magnification ×200) (E). (D) The cytotoxicity of iCRT14 (10 u M) in MDBK cells for 24 h was analyzed by Trypan-blue exclusion test. Data shown are representative of three independent experiments.

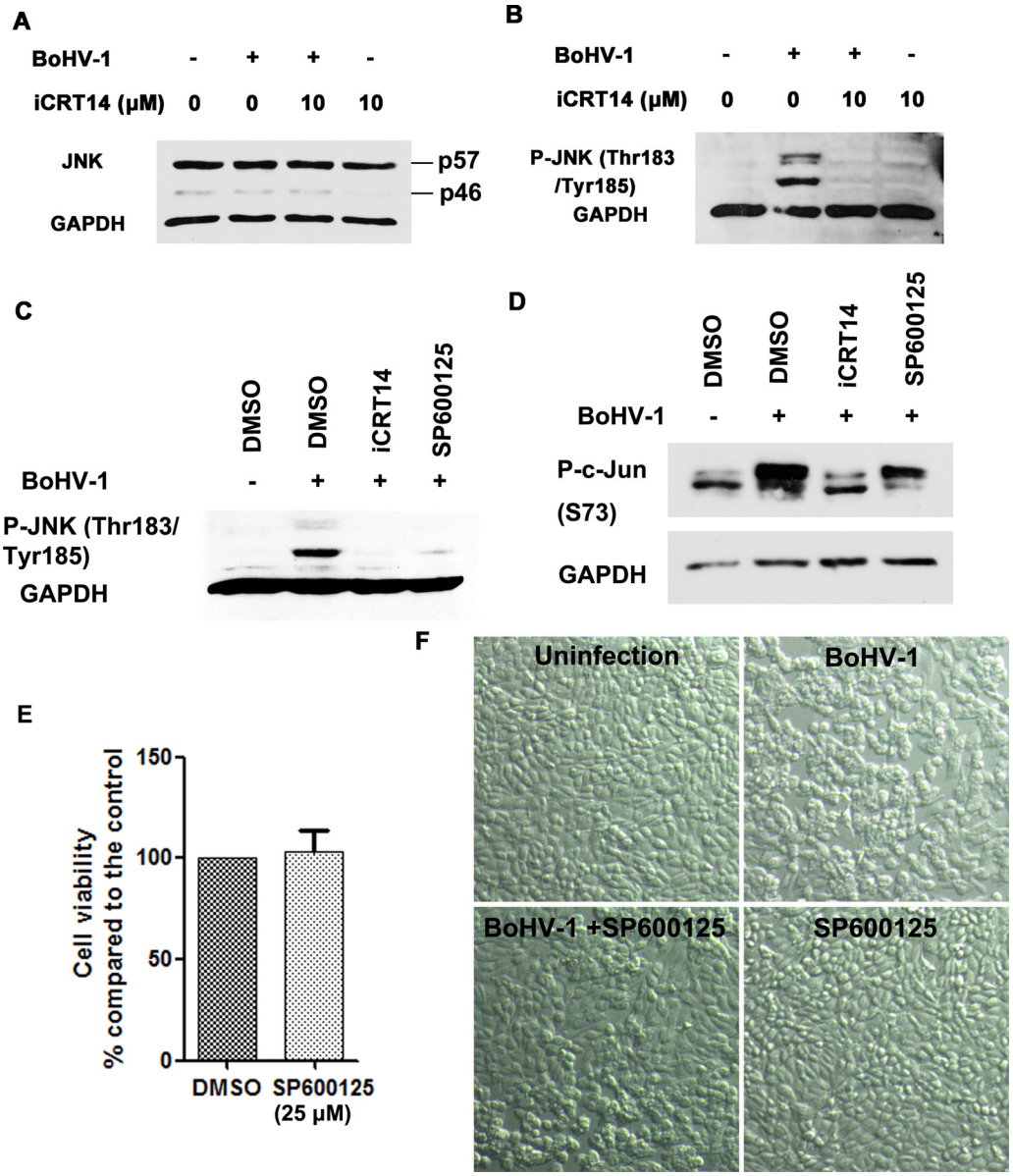

Fig. 6. The effects of β-catenin inhibitor iCRT14 on JNK signaling. MDBK cells in 60 mm dishes were pretreated with iCRT14 (10 u M) for 2 h, after which they were mock infected or infected with BoHV-1 at an MOI of 0.1 in the presence of iCRT14 (10 u M) or DMSO control. After infection for 24 h, the cell lysates were prepared for Western blotting to detect the expression of JNK (A) and p-JNK(Thr183/Tyr185) (B). MDBK cells in 60 mm dishes were pretreated with either iCRT14 (10 u M) or SP600125 (25 u M) for 2 h, after which they were mock infected or infected with BoHV-1 at an MOI of 0.1 in the presence of indicated inhibitors or DMSO control. After infection for 24 h the cell lysates were prepared to detect both p-JNK(Thr183/Tyr185) (C) and p-c-Jun(S73) (D), respectively. (E) The cytotoxicity of SP600125 (25 u M) in MDBK cells for 24 h was analyzed by Trypan-blue exclusion test. (F) After a pretreatment for 2 h with SP600125 (25 u M), MDBK cells were mock infected or infected with BoHV-1(MOI = 0.1) in the presence of either SP600125 or DMSO control for 24 h, and cell morphology was observed under a light microscope. Magnification ×200. Data shown are representative of three independent experiments.

starvation contributed to this discrepancy in results.

Taken together, in this study for the first time we show that in BoHV-1-infected MDBK cells, β-catenin was associated with c-Jun, β-catenin was involved in regulation of c-Jun expression and phosphorylation, and nucleus c-Jun was relocalized to form highlighted foci in an IFA assay. This study expands our understanding of how c-Jun signaling is regulated in response to BoHV-1 infection, and provides a novel cross talk between β-catenin and c-Jun, which further addresses the implications of β-catenin in BoHV-1 infection and pathogenesis.

Authors contributions

LC generated the figures, WFY performed data analysis. LQZ designed and supported this study and prepared the manuscript.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108804.

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