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Characterization, functional and signaling elucidation of pigeon (*Columba livia*) interferon-α: Knockdown p53 negatively modulates antiviral response

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ABSTRACT

The regulation of interferon-α signaling pathways is essential to protect the host from infection with a broad range of viruses. However, information regarding antiviral response and the specific molecular mechanism of *Columba livia* interferon-α (CoIFN-α) has not been reported to date. In this study, we cloned a 723bp complete ORF of CoIFN-α gene. The specific antiviral activity of CoIFN-α in VSV ($TCID_{50} = 10^{-5.87}$/100 μL)-infected CEFs reached 5.5×10^5 U/mg. Moreover, our result indicated that the anti-VSV efficient of CoIFN-α might depend on the expression of NF-κB. CoIFN-α also showed high sensitivity to trypsin and relatively stable after acid, alkali or heat treatment. Moreover, CoIFN-α activated STAT/Jak signaling and autophagy to inhibit VSV-induced apoptosis. Although the expression of p53 was further increased, apoptosis was not involved in CoIFN-α against VSV. Notably, although STAT signaling was efficiently activated, knockdown p53 did inhibit the antiviral activity of the CoIFN-α via decreasing the expression of Mx1 but not weakened Jak phosphorylation. Moreover, VSV aggravated the apoptosis and the expression of cleaved Mdm2 in knockdown p53 under preincubated CoIFN-α. Taken together, p53 might as a highly interconnected regulator in IFN-α antiviral response and cleaved Mdm2 might as a dominant-negative regulator by competing with full length Mdm2 for p53 binding in virus infection. Overall, our research not only enriches CoIFN-α antiviral features but also helps explain that p53 enhance the CoIFN-α antiviral response against pigeon viral diseases.

1. Introduction

During virus infection, normal homeostasis of the immune system can regulate signal transduction pathways and cytokine production to control the balance of production and death. Interferon-α (IFN-α) is an essential cytokines for antiviral immunity and referred to as 'negative growth factors', and manifests anti-oncogenic activities. IFN-α, which belongs to the type I IFNs, interacts with IFN-α/β receptor on the cell surface to induce activation of JAK1 and TYK2 by cross-phosphorylation, leading to phosphorylation and activation of signal transducers and activators of transcription 1 (STAT1). The activated heterodimer STAT1/STAT2 leaves the receptor and translocates into nucleus and subsequently bind specific promoter regions, associates with IRF-9, resulting in ISGF3 complex formation. ISGF3 then migrates to the nucleus to binds the ISRE of the IFN-stimulated genes (ISGs) and promote their transcription to regulate transcription of the genes responsible for antiproliferative effects (Bonjardim, 2005). The IFN system navigates the innate antiviral defense of mammalian hosts through induction of ISGs, which mediates the biological effects of IFN, inhibiting all steps of the viral cycle and protecting neighboring uninfected cells from incoming viral progeny (Schoggins and Rice, 2013).

IFN as adjuvant with vaccine can enhance humoral response (Asif et al., 2004). Chicken IFN-α (ChIFN-α) has been studied since the 60th of the last century and was cloned in 1994 (Novak et al., 2001). Interest in IFNs of birds has recently sparked from increasing problems with viral diseases in the poultry industry. Because pigeon meat is a traditional food, definite characterization and signaling pathway of *Columba livia* IFN-α (CoIFN-α) can bring a large economic interest. However, the CoIFN-α has not been cloned and producing the transduces signals are not yet well described. Therefore, understanding the properties of CoIFN-α would propose valuable targets for the development of potential therapeutics for a broader range of viruses of both avian and zoonotic importance.

Type I IFN and p53 are both required for immune responses against tumor development and viral infection. The tumor suppressor p53, activated in response to DNA damage, induces cell cycle arrest or apoptosis through transcriptional activation of its target genes, hence having a central role in tumor suppression. p53 directly transactivates

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the expression of several ISGs involved type 1 IFN signaling in response to stimuli (Rivas et al., 2010). However, ISGs promoter do not generally contain p53 consensus binding sites. Previous study identified that IRF9, as a p53 target gene, directly induces the expression of ISRE-containing genes. Moreover, IRF5, ISG15 and Toll-like receptor 3 (TLR3) have been identified as direct p53 target genes (Hummer et al., 2001; Lichtenstein et al., 2004; Taura et al., 2008). A review propose that antiviral effect of p53 are not incidental to its function as a tumor suppressor, and that in fact the inverse in true: the loss of innate immunity caused by p53 inactivation incidentally confers an immune-privileged status that allows tumors to grow (Miciak and Bunz, 2016). Moreover, IFN-α subtypes induced cells to undergo apoptosis through p53 activation directly and indirectly in HepG2, HLE and PLC/PRF/5 cells (Fujioka et al., 2006). p53 is induced in response to viral infection as a downstream transcriptional target of IFN signaling. Knockdown of p53 expression by RNAi enhanced influenza A virus replication, associated with reduced expression of antiviral ISGs, such as interferon regulatory factor 7 (IRF7), IRF9, ISG15, ISG20, GBP1, Rig-I and OAS1 (Zhu et al., 2014). Activation of p53 and induction of its pro-apoptotic target genes in virally infected cells indicate that these events lead to an apoptotic response of the infected cells, an event that can be considered as altruistic suicide that limits virus replication. Induction of Fas ligand (FasL) by IFN-α can be viewed as and efficient mechanism to potentiate NK cell cytotoxicity in the presence of virally infected (Kirou et al., 2000). Moreover, STAT1 can promote p53 protein level through downregulating mouse double minute 2 homolog (Mdm2) and increase apoptosis in response to DNA damage (Thomas et al., 2004; Townsend et al., 2004). In this context the p53 can be viewed as a nodes and interconnecting pathways. However, what remains poorly understood is how p53 modulates IFN signaling at the apoptotic network level.

Autophagy is uniquely suited for degradation of sizeable cytoplasmic targets, and represents a recently recognized cell-autonomous defense against intracellular pathogens (Deretic et al., 2012). The induction of autophagy in immune cells serves an innate immune response to defend against intruding pathogens (Deretic, 2005). Viruses can enter cells by endocytosis resulting in the delivery of viral RNA in the endosome. Once in the endosome, dsRNA can be recognized by TLR3 and ssRNA by TLR7 or TLR8, triggering type I IFN production (Rey-Jurado et al., 2015). However, PI3K-AKT-mTORC1 signaling pathway may modulate IFN-induced autophagy in multiple human cell lines (Schmeisser et al., 2013). Moreover, Herpes simplex virus 1 (HSV-1), influenza virus, human immunodeficiency virus and viruses from Coronaviridae were shown to inhibit autophagy by directly blocking Beclin1 (Rey-Jurado et al., 2015). Global genomic profiling reveals an extensive p53-regulated autophagy program in response to apoptosis (Kenzelmann et al., 2013). The activation of p53 downregulates mTOR activity, resulting in increased levels of autophagy (Feng et al., 2005). The induction of the autophagy transcriptional program in response to p53 activation correlates with increased levels of autophagy in a variety of setting and autophagy is important for p53-dependent apoptosis and transformation suppression (Kenzelmann et al., 2013). Autophagy machinery can deliver the endogenously synthesized antigens for presentation on MHC class II to $CD4^+$ cells, enhance antigen donor cell cross-presentation to $CD8^+$ T cell, dendritic cell cross-presentation of phagocytosed antigens to $CD4^+$ T cells (Paludan et al., 2005; Munz, 2010). Moreover, p53 was shown to regulate autophagy in a dual manner depending on its location side the cells. Its nuclear leads to the activation of autophagy whereas its cytoplasmic location leads to suppression of autophagy (Green and Kroemer, 2009; Maiuri et al., 2010). Therapeutic interventions that modulate p53 expression may prove beneficial in curtailing maladaptive autophagy and cell death after injury (Wang et al., 2013). People pay more and more attention to the role of interferon-α on cell survival of the p53 roles, but p53 knockdown on the regulation of apoptosis, autophagy and interferon-α antiviral pathway in *Columba livia* is not yet clear, less reported, to be indepth. Therefore, understanding the role of autophagy and p53 may explain mechanisms of IFN-α's antiviral ability in immunology.

Taking these literature into consideration, IFN-α is the most profound innate immune response against viruses. Nowadays, people pay more and more attention to the regulatory roles of p53 on the IFN-α-induced antiviral response and its metabolism mechanism. Keeping in view, first, we cloned a full-length IFN-α gene sequence and expressed the mature CoIFN-α protein in *Escherichia coli* and determined the antiviral effects of CoIFN-α using cytopathic inhibition assays. Then, we investigate the role of p53 in functional connectivity ISGs with a particular focus on autophagy and apoptosis. In this study, we show here that knockdown of p53 expression markedly decreased the autophagy response to CoIFN-α, suggesting that p53 function is required in CoIFN-α-induced autophagy Importantly, we found that knockdown p53 not weakened Jak2 phosphorylation, but might inhibit mRNA translation of ISGs or generation of the antivrial effects of CoIFN-α. Altogether, these studies establish an important complement role for p53 to the function of the CoIFN-α antiviral response.

2. Materials and methods

2.1. Cell, virus and antibodies

Vesicular stomatitis virus (VSV) was kindly provided by Dr. Qu Liandong from Harbin Veterinary Research Institute, China. SPF chick embryo were bought from Harbin Veterinary Research Institute, China.

Rabbit polyclonal antibodies against p53 (WL02384), STAT1 (WL00665), JAK2 (WL02188), *p*-JAK2 (WL02997), Mdm2 (WL01926), caspase9 (WL01551), Bcl2 (WL01556), Bax (WL01637), caspase3 (WL02117), LC3I/II (WL01506), Beclin1 (WL02508), NF-κB (WL0866) were purchased from Wanleibio (Shenyang, China). Mouse monoclonal antibodies against caspase8 (66093-1-Ig), β-actin (60008-1-Ig) and Rabbit polyclonal antibodies against AKT (10176-2-AP), PI3K (20583-1-AP), Mx1 (13750-1-AP) were purchased from Proteintech (Wuhan, China). Rabbit polyclonal antibodies Dynein (bs-14469R), TORC1 (bs-3588R) were purchased from Biosynthesis biotechnology (Beijing, China). Rabbit polyclonal antibodies ATG4B (A2837), ATG5 (A0203) were purchased from ABclonal (USA). HRP-conjugated goat anti-rabbit IgG was purchased from Biosharp (USA). HRP-labeled Goat Anti-Mouse IgG (H + L) was purchased from Beyotime (Shanghai, China).

2.2. Cloning of the gene encoding CoIFN-α

Pigeon genomic DNA extracted from American king pigeons (*Columba livia*; 2 months old) heart was applied as template. Amplification of the 5′ and 3′ DNA end sequences was conducted according to the Genome Walking Kit protocol (Takara, Japan). Genespecific primers used in the PCR were designed according to Columba livia IFN partial sequence from GenBank (AB618534). The primers 3-Sp1 and 3-SP2 were used in the first run of the 3′ gene walking. Moreover, Primers 3-Sp3 and 3-SP4 were used in the second and third run of the 3′ gene walking, respectively. The primers 5-Sp1 and 5-SP2 were used in the first run of the 5′ gene walking. Moreover, Primers 5-Sp3 and 5-SP4 were used in the second and third run of the 5' gene walking, respectively. The PCR product obtained using the degenerate primers (Table 1) was cloned into a pMD 18-T Vector (Takara, Japan) and sequenced. The gene walking PCR results was performed using ContigExpress software. The full-length sequences of CoIFN-α was subsequently compiled and submitted to the GenBank database.

2.3. Sequence analyses of CoIFN-α

The open reading frame (ORF) was predicted using translate program on ExPAsy server (http://web.expasy.org/translate/). The signal peptide was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). the glycosylation sites were predicted using the NetNGlyc website (http://www.cbs.dtu.dk/services/NetNGlyc/). The 3D