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Type I interferon-mediated immune response against influenza A virus is attenuated in the absence of p53

Zixiang Zhu [a,b,1], Yifan Yang [a,1], Jianchao Wei [a], Donghua Shao [a], Zixue Shi [a], Beibei Li [a], Ke Liu [a], Yafeng Qiu [a], Haixue Zheng [b], Zhiyong Ma [a,*]

[a] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 518, Ziyue Road, Shanghai 200241, PR China
[b] Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 1, Xujiaping Road, Lanzhou 730046, PR China

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ABSTRACT

Influenza A virus (IAV) infection induces secretion of type I interferon (IFN) and activation of p53, which play essential roles in the host defense against tumor development and viral infection. In this study, we knocked down p53 expression by RNA interference. The expression levels of IFN-stimulated genes (ISGs) including IFN regulatory factor (IRF) 5, IRF9, ISG15, ISG20, guanylate-binding protein 1, retinoic acid-inducible gene-I and 2′-5′-oligoadenylate synthetase 1 were significantly attenuated in response to IAV infection and IFN-α stimulation in p53-knockdown cells. This attenuated expression of ISGs was associated with enhanced replication of IAV. Pretreatment of p53-knockdown cells with IFN-α failed to inhibit IAV replication, indicating impaired antiviral activity. These findings indicate that p53 plays an essential role in the enhancement of the type I IFN-mediated immune response against IAV infection.

1. Introduction

Influenza A virus (IAV) is a single-stranded, negative-sense, segmented RNA virus responsible for annual global epidemics and sporadic pandemics [1]. Host immunity plays a critical role in the clearance of IAV infection. Notably, the type I interferon (IFN)-mediated immune response confers an antiviral state in the host and prevents the replication and spread of IAV. IAV infection elicits type I IFN secretion, which in turn up-regulates the transcription of several IFN-stimulated genes (ISGs) to restrict viral replication [2]. Some ISGs, such as ISG15 [3], ISG20 [4], guanylate-binding protein 1 (GBP1) [5], retinoic acid-inducible gene-I (RIG-I) [6] and 2′-5′-oligoadenylate synthetase (OAS) [7], have been shown to inhibit IAV replication.

The tumor suppressor p53 is an ISG that is known to regulate cell cycle arrest and apoptosis [8]. p53 is transcriptionally up-regulated in response to type I IFN stimulation. It is involved in the host defense against viral infection, as well as protecting against the development of cancer [9]. As a transcription factor, activated p53 binds to a DNA sequence in the promoter region of its target genes to induce their transcription [10]. Some ISGs, including interferon regulatory factor (IRF) 5, IRF9, ISG15, RIG-I and GBP1, are p53 target genes [11–13]. p53 directly transactivates the expression of several ISGs involved in type I IFN signaling in response to stimuli, including viral infection [11].

Type I IFN possesses preventive and therapeutic antiviral abilities in relation to IAV infection [14,15]. p53 is activated during IAV infection and plays an essential role in regulating the innate and adaptive immune responses against IAV infection [16,17]. IRF9 is a central component of the ISG factor 3 complex [18] and a direct target of p53 in response to viral infection [19]. IRF9 expression was attenuated in p53-deficient cells during IAV infection [20]. This observation implied a possible collaboration between p53 and type I IFN in the regulation of the immune response against IAV infection. In this study, we therefore investigated the role of p53 in the expression of ISGs and the anti-IAV response mediated by type I IFN.

2. Materials and methods

2.1. Cells, viruses and antibodies

Influenza viruses A/Puerto-Rico/8/34 (H1N1 subtype; PR8), A/Swine/Jiangsu/2/2006 (H3N2 subtype) and A/Swine/Gangxi/7/07 (H9N2 subtype) were propagated in Madin–Darby canine kidney cells (MDCK) (NBL-2) or in the allantoic cavities of 9-day-old embryonated specific-pathogen-free chicken eggs. Human lung epithelial A549 (wild-type p53) cells and MDCK cells were

* Corresponding author. Fax: +86 21 54081818.
E-mail address: zhiyongma@shvri.ac.cn (Z. Ma).
[1] These authors contributed equally to this work.

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purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in F-12K Nutrient Mixture (Kaighn's modification) (Invitrogen, Carlsbad, CA, USA). MDCK cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen). All cells were cultured in media supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C under 5% CO_2. Commercial anti-p53 monoclonal antibody (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin monoclonal antibody (AC-15, Sigma, St. Louis, MO, USA) were used and an anti-nucleoprotein (NP) polyclonal antibody was generated in our laboratory (unpublished data).

2.2. Viral infection and plaque assay

For viral infection, A549 cells were grown in plates, washed with phosphate-buffered saline (PBS) and incubated with IAV at a multiplicity of infection of 1 at 37 °C for 1 h. The inocula were removed and the cells were cultured in viral maintenance medium containing 1% FBS and 1 μg/ml TPCK trypsin at 37 °C for the indicated times. Mock infection was performed using PBS as the inoculum. Viral titers were determined using plaque assays in MDCK cells, as described previously [12].

2.3. Quantitative RT-PCR and Western blot analysis

For quantitative RT-PCR (qRT-PCR) analysis, total RNA was isolated from cells using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. cDNA was synthesized using 2 μg of total RNA and M-MLV reverse transcriptase (Invitrogen). PCR was performed using equal amounts of cDNA and SYBR Premix Ex Taq (Takara, Kyoto, Japan) in an ABI 7500 PCR system (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's protocol. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Primer sequences are listed in Supplementary Table 1. For Western blot analysis, samples were harvested at the indicated times and the cells were lysed in lysis buffer (1% sodium dodecyl sulfate, 1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 4 mM Pefabloc SC, 2 mg/ml leupeptin, 2 mg/ml aprotinin). The lysates and following treatments were performed as described previously [21].

2.4. RNA interference

Small interfering RNA (siRNA) (5′-AAGACTCCAGTGGTAATCTAC-3′) targeting human p53 (p53 siRNA) [22] was synthesized chemically. A negative control siRNA (NC siRNA) with no known target genes was synthesized in parallel. RNA interference (RNAi)-mediated knockdown of endogenous p53 and transfection were performed as described previously [22].

2.5. IFN-α treatment

Human IFN-α (2a) was purchased from PBL Assay Science (PBL, Piscataway, NJ, USA) and diluted in PBS containing 0.1% bovine serum albumin (BSA) to a concentration of 100 U/μl. For IFN-α treatment, cells were washed three times with PBS and IFN-α solution was added to the medium at 1 U/μl for the indicated incubation times. Mock-treated cells were incubated with 0.1% BSA instead of IFN-α.

2.6. Statistical analysis

All measured values were expressed as means and standard errors. Statistical significance was analyzed using Student's *t*-test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Knockdown of p53 expression enhances IAV replication

Human lung epithelial A549 cells harbor wild-type p53 and are susceptible to IAV infection [16]. We evaluated replication of IAV A/Puerto-Rico/8/34 (H1N1 subtype; PR8), A/Swine/Jiangsu/2/2006 and A/Swine/Gangxi/7/07 strains in p53-knockdown A549 cells silenced by RNAi. Similar results were observed with all the tested IAV strains and only the results obtained with the PR8 strain are therefore shown. A549 cells were transfected with p53 siRNA to silence p53 expression, or with NC siRNA, and then infected with IAV (Fig. 1A). p53 expression was silenced by RNAi as confirmed by Western blot analysis (Fig. 1B). Viral titers in p53 siRNA and NC siRNA cells were measured at 6, 12, 24 and 48 h post-infection (hpi). Viral titers were significantly increased in p53 siRNA cells compared with NC siRNA cells at 24 and 48 hpi (Fig. 1C). To confirm this result, we compared the expression of viral hemagglutinin (HA) and NP mRNAs in p53 siRNA and NC siRNA cells by qRT-PCR analysis. Viral HA and NP mRNA levels were significantly increased in p53 siRNA cells at 12, 24 and 48 hpi compared with levels in NC siRNA cells (Fig. 1D). Viral replication was also analyzed at the protein level. IAV infection induced p53 protein accumulation in NC siRNA cells, consistent with previous observations (Fig. 1E) [20,21]. In contrast, no p53 was detected in p53 siRNA cells, indicating that p53 expression was effectively silenced by RNAi. Viral NP protein levels were significantly higher in p53 siRNA cells compared with NC siRNA cells (Fig. 1E). Overall, these results suggest that p53 inhibited IAV replication, as indicated by previous observations [16].

3.2. ISG expression is attenuated in p53-knockdown cells in response to IAV infection

p53 transactivated IRF9 and regulated ISG expression during vesicular stomatitis virus infection [19]. We therefore analyzed ISG expression to explore the regulatory role of p53 in the IFN signaling pathway activated by IAV infection. p53 siRNA and NC siRNA cells were infected with IAV (Fig. 1A) and the mRNA expression levels of antiviral ISGs were detected by qRT-PCR. IAV infection resulted in significant up-regulation of IRF7, IRF9, ISG15, ISG20, GBP1, RIG-I and OAS1 mRNA levels in both p53 siRNA and NC siRNA cells. However, expression levels were notably impaired in p53 siRNA cells, especially at 24 and 48 hpi, compared with the levels in NC siRNA cells (Fig. 2). These results indicate that p53 was involved in regulating ISG expression during IAV infection.

3.3. ISG expression is impaired in p53-knockdown cells in response to IFN-α stimulation

The attenuated expression of ISGs in p53-knockdown cells suggested an essential role for p53 in regulating IFN signaling during IAV infection. Given that IAV infection induces the secretion of IFN-α [23], we stimulated A549 cells with IFN-α to confirm our earlier findings. p53 siRNA and NC siRNA cells were treated with IFN-α and ISG expression levels were determined by qRT-PCR 12 h post-treatment (Fig. 3A). p53 is a target gene of type I IFN and induced by IFN-α treatment [9]. Treatment of NC siRNA cells with IFN-α led to a significant increase in p53 mRNA expression (Fig. 3B), suggesting that IFN-α effectively upregulated p53. IFN-α also up-regulated the expression of the ISGs IRF7, IRF9, ISG15, ISG20, GBP1 and RIG-I mRNAs in NC siRNA cells, but this response was remarkably impaired in p53 siRNA cells (Fig. 3C). These results further confirmed that p53 was essential for up-regulation of the type I IFN signaling pathway.

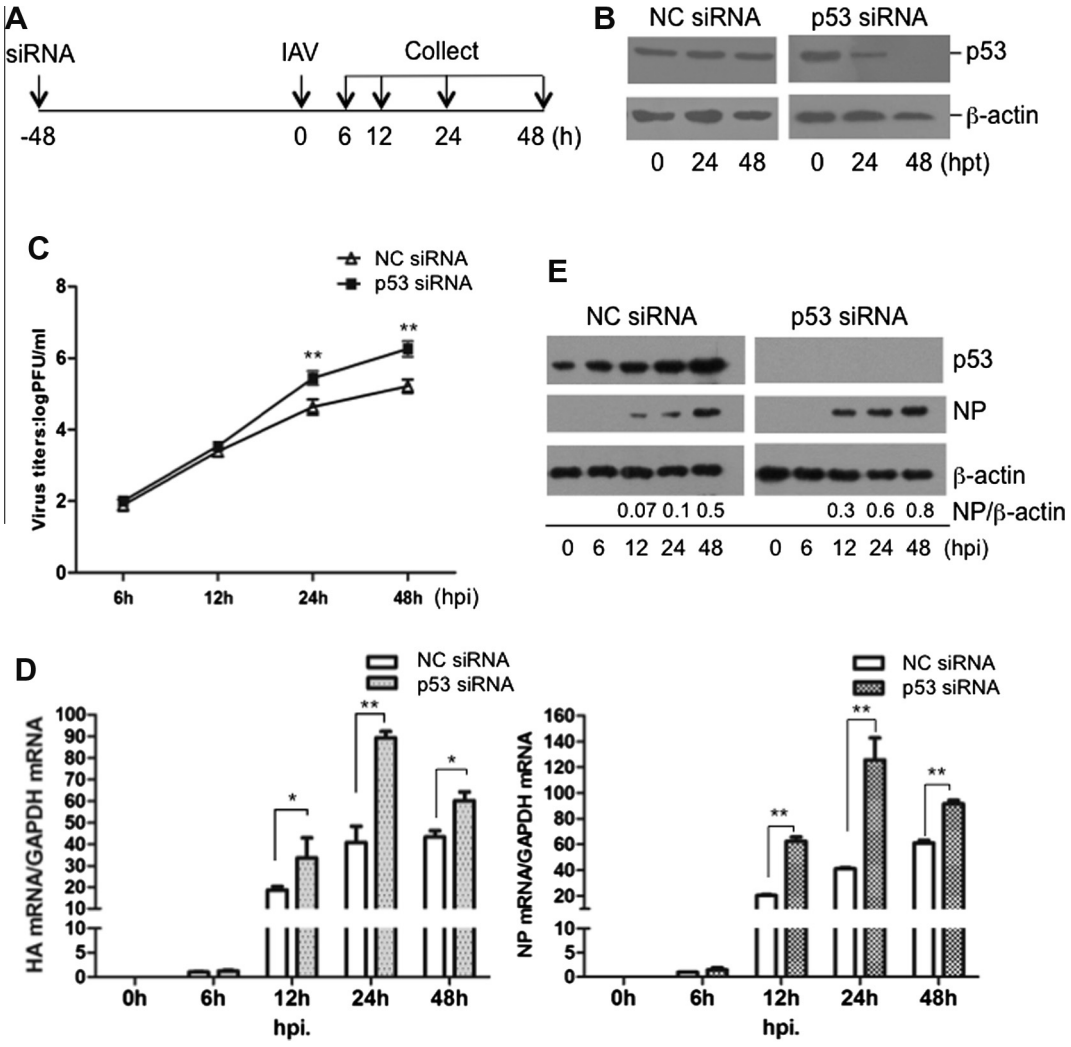

Fig. 1. Enhanced replication of IAV in p53-knockdown cells. (A) Schematic representation of the experimental design. A549 cells were transfected with small interfering RNA (p53 siRNA) to silence p53 expression, or non-targeting small RNA (NC siRNA), for 48 h and subsequently infected with IAV for the indicated times. (B) p53 protein expression levels before IAV infection were analyzed by Western blot. (C) Viral titers were detected by plaque assays at the indicated times. (D) Viral HA and NP mRNA levels were analyzed by qRT-PCR. (E) p53 and viral NP protein expression levels during IAV infection were determined by Western blot analysis. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. hpt, hours post-transfection. hpi, hours post-infection.

3.4. Antiviral effect of IFN-α is impaired in p53-knockdown cells

Type I IFN is an important cytokine for combating viral infection [24]. Pretreatment with IFN-α helps to prevent IAV infection [14,15]. The above results indicated that p53 plays an essential role in type I IFN signaling, and we therefore evaluated its role in regulating IFN-α-mediated antiviral activity against IAV. p53 siRNA and NC siRNA cells were pretreated with IFN-α or control buffer for 12 h and subsequently infected with IAV and incubated for an additional 12 h (Fig. 4A). The expression of viral HA mRNA was analyzed. IFN-α treatment significantly inhibited the expression of viral HA mRNA in NC siRNA cells, but not in p53 siRNA cells, indicating that IFN-α inhibited IAV replication in a p53-dependent manner (Fig. 4B). We also analyzed p53 and ISG (ISG15, GBP1 and IRF9) mRNA levels. IFN-α treatment significantly up-regulated ISG15, GBP1 and IRF9 gene expression levels in NC siRNA cells, but not in p53 siRNA cells, during IAV infection (Fig. 4C). These results indicate that p53 plays an essential role in IFN-α-mediated antiviral activity against IAV infection.

4. Discussion

Type I IFN and p53 are both required for immune responses against tumor development and viral infection. There is increasing evidence for collaborations between type I IFN and p53 in terms of their tumor suppression and antiviral activities [25]. Type I IFN induces p53 expression [9], which transcriptionally regulates the expression of IRF5 and IRF9, indicating a positive feedback loop between p53 and type I IFN signaling pathways [11]. We previously found that the expression pattern of IRF9 was similar to that of p53 during IAV infection [20], implying possible collaboration between type I IFN and p53 in regulating the immune response against IAV infection. In this study, we analyzed the expression of ISGs and the antiviral effect of type I IFN in p53-knockdown cells during IAV infection. We demonstrated an essential role for p53 in type I IFN-mediated antiviral activity against IAV infection.

p53 deficiency has been shown to lead to significant increases in IAV replication *in vitro* and *in vivo* [16,17]. Similar results were

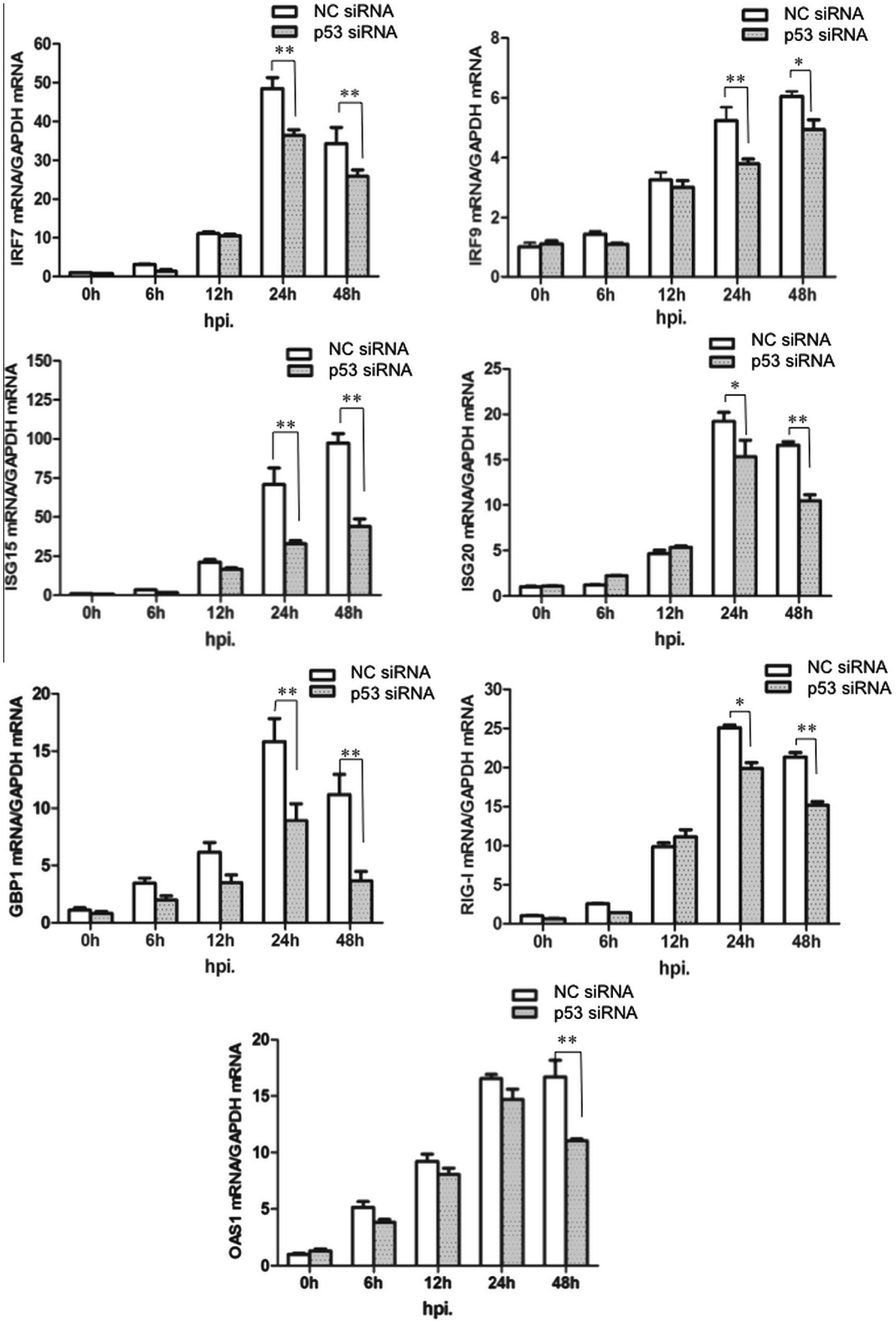

Fig. 2. Expression of ISGs during IAV infection. A549 cells were transfected with small interfering RNA (p53 siRNA) to silence p53 expression, or non-targeting small RNA (NC siRNA), for 48 h and subsequently infected with IAV for the indicated times. ISG mRNA levels were analyzed by qRT-PCR. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. hpi, hours post-infection.

observed in this study. Viral titers were significantly higher in p53-knockdown cells silenced by RNAi than in control cells (Fig. 1). We investigated the basis of this enhanced replication of IAV in p53-deficient cells by analyzing the expression of ISGs in p53-knockdown cells in response to IAV infection. mRNA levels of ISGs including IRF7, IRF9, ISG15, ISG20, GBP1, RIG-I and OAS1 were significantly attenuated in p53-knockdown cells during IAV infection (Fig. 2). ISG15, ISG20, GBP1, OAS and RIG-I have

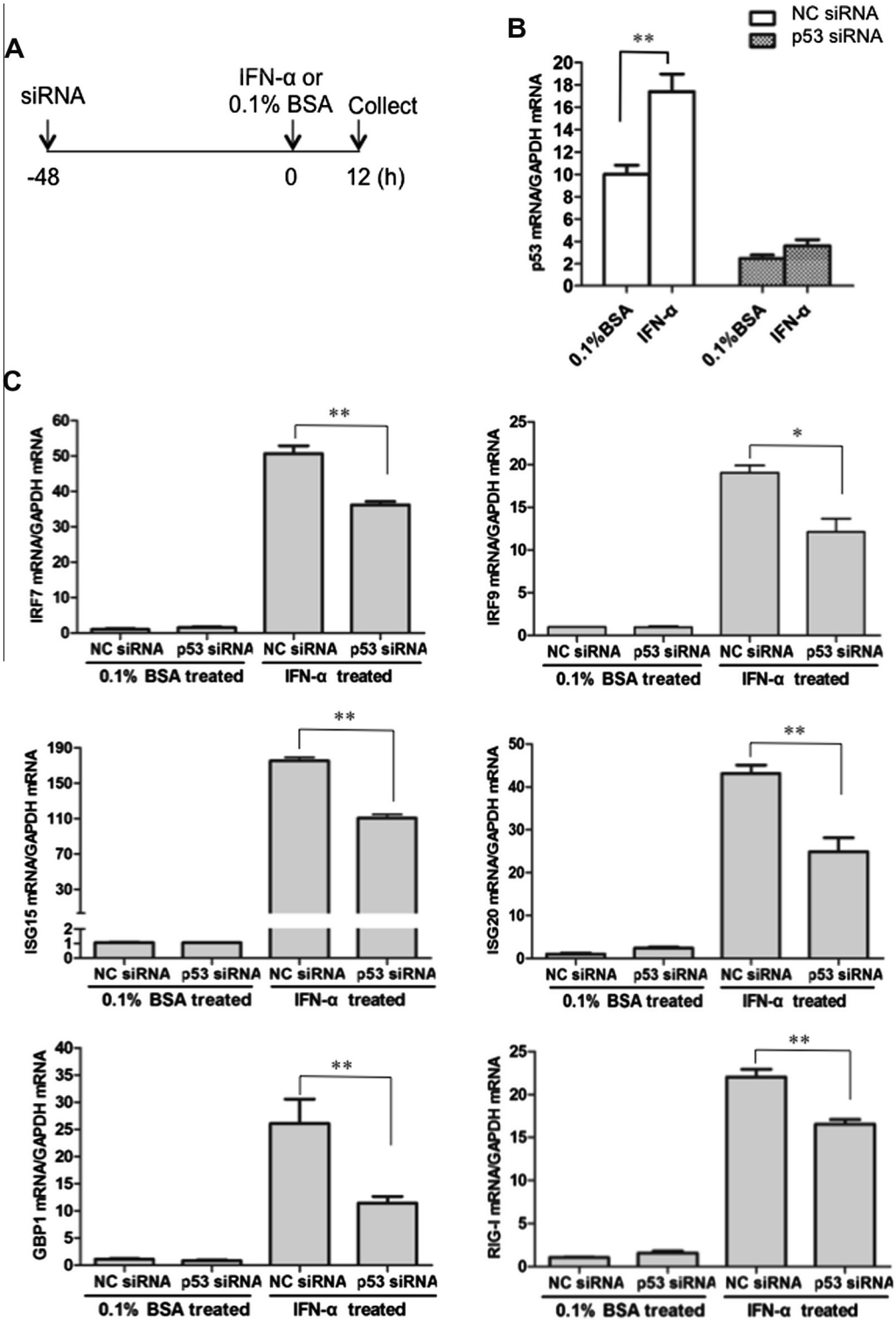

Fig. 3. Expression of ISGs in IFN-α treated cells. (A) Schematic representation of the experimental design. A549 cells were transfected with small interfering RNA (p53 siRNA) to silence p53 expression, or non-targeting small RNA (NC siRNA), for 48 h and subsequently treated with 1 U/μl IFN-α or control buffer (0.1% BSA) for 12 h. (B) p53 mRNA levels were analyzed by qRT-PCR. (C) ISG mRNA levels were analyzed by qRT-PCR. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

previously been shown to inhibit IAV replication [3–7], while IRF7 and IRF9 are essential for regulating expression of IFN and ISGs [26]. IRF7 in particular is the master regulator of type I IFN-dependent immune responses [27] and was significantly up-regulated in IAV-infected mice [28]. Enhanced IAV replication in p53-knockdown cells was thus associated with attenuated ISG expression. These findings indicate that p53 is involved in the regulation of the type I IFN signaling pathway during IAV infection.

Type I IFN induces the expression of numerous ISGs [29], and pretreatment of cells with type I IFN inhibited IAV replication

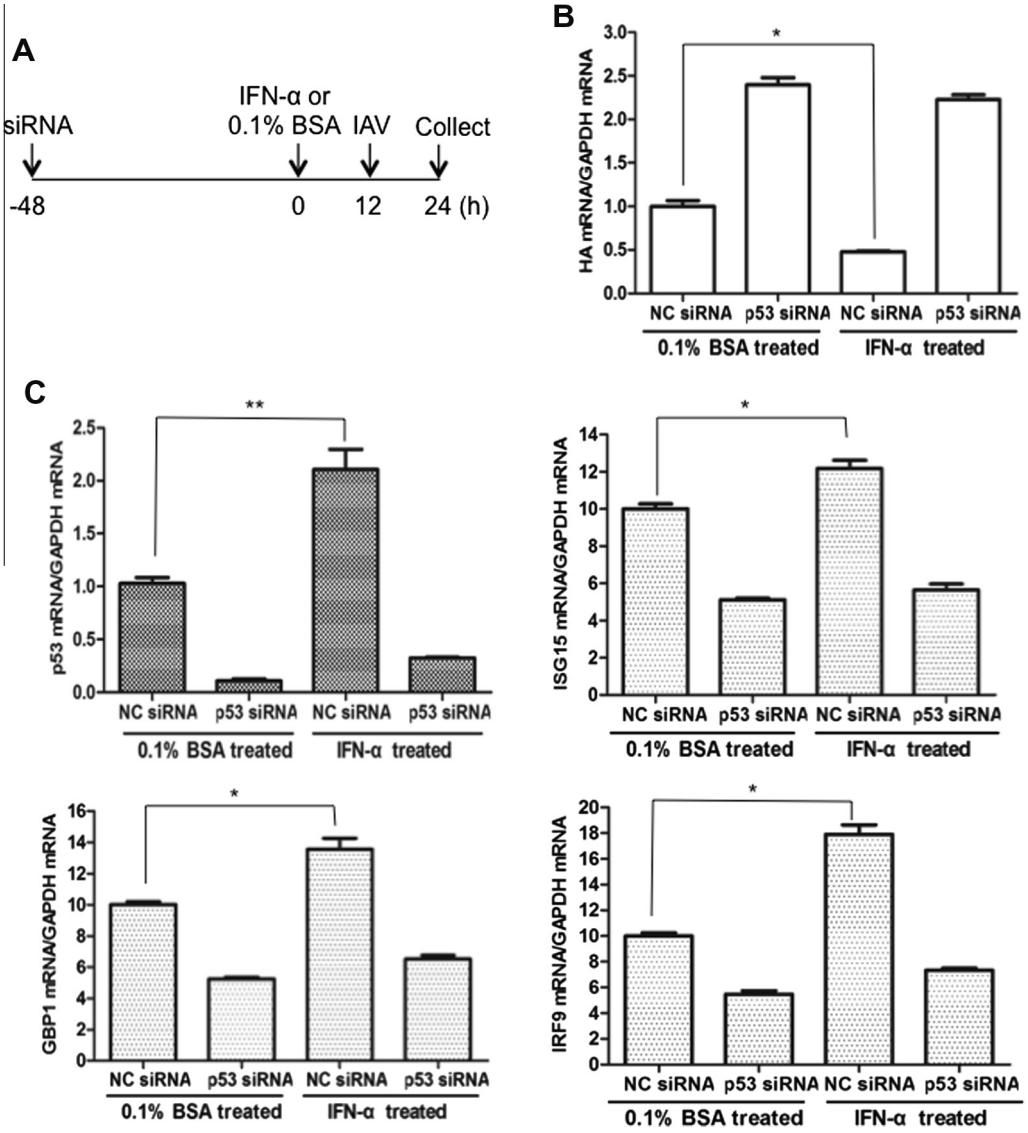

Fig. 4. Antiviral effect of IFN-α in p53-knockdown cells. (A) Schematic representation of the experimental design. A549 cells were transfected with small interfering RNA (p53 siRNA) to silence p53 expression, or non-targeting small RNA (NC siRNA), for 48 h and subsequently treated with 1 U/μl of IFN-α or control buffer (0.1% BSA) for 12 h. The cells were infected with IAV for 12 h. (B) Viral HA expression was analyzed by qRT-PCR. (C) p53 and ISGs mRNA levels were analyzed by qRT-PCR. Results are presented as the mean ± standard error from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

[14,15]. We therefore analyzed the effects of type I IFN treatment on ISG expression and antiviral activities in the absence of p53. As expected, IRF7, IRF9, ISG15, ISG20, GBP1 and RIG-I expression levels were attenuated in p53-knockdown cells compared with control cells (Fig. 3C), further confirming the regulatory role of p53 in type I IFN signaling. Interestingly, however, pretreatment of p53-knockdown cells with IFN-α failed to inhibit IAV replication, and viral HA mRNA in p53-knockdown cells showed a slight but insignificant decrease, compared with control cells (Fig. 4B). These findings indicate that p53 was essential for enhancing type I IFN-mediated antiviral activity.

In conclusion, knockdown of p53 expression by RNAi enhanced IAV replication, associated with reduced expression of antiviral ISGs, such as IRF7, IRF9, ISG15, ISG20, GBP1, RIG-I and OAS1. In addition, pretreatment of p53-knockdown cells with IFN-α failed to inhibit IAV replication, showing an impaired antiviral activity. These findings indicate that p53 plays an essential role in enhancing the type I IFN-mediated immune response against IAV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.067.

References

[1] L. Manzoli, J.P. Ioannidis, M.E. Flacco, et al., Effectiveness and harms of seasonal and pandemic influenza vaccines in children, adults and elderly: a critical review and re-analysis of 15 meta-analyses, Hum. Vaccin. Immunother. 8 (2012) 851–862.
[2] A. García-Sastre, Induction and evasion of type I interferon responses by influenza viruses, Virus Res. 162 (2011) 12–18.
[3] D.J. Lenschow, C. Lai, N. Frias-Staheli, et al., IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 1371–1376.

[4] L. Espert, G. Degols, C. Gongora, et al., ISG20, a new interferon-induced RNase specific for single-stranded RNA, defines an alternative antiviral pathway against RNA genomic viruses, J. Biol. Chem. 278 (2003) 16151–16158.
[5] Z. Zhu, Z. Shi, W. Yan, et al., Nonstructural protein 1 of influenza A virus interacts with human guanylate-binding protein 1 to antagonize antiviral activity, PLoS ONE 8 (2013) e55920.
[6] R. Le Goffic, J. Pothlichet, D. Vitour, et al., Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells, J. Immunol. 178 (2007) 3368–3372.
[7] J.Y. Min, R.M. Krug, The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2′–5′ oligo (A) synthetase/RNase L pathway, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 7100–7105.
[8] B. Rao, S. Lain, A.M. Thompson, p53-Based cyclotherapy: exploiting the 'guardian of the genome' to protect normal cells from cytotoxic therapy, Br. J. Cancer 109 (2013) 2954–2958.
[9] A. Takaoka, S. Hayakawa, H. Yanai, et al., Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence, Nature 424 (2003) 516–523.
[10] O. Laptenko, C. Prives, Transcriptional regulation by p53: one protein, many possibilities, Cell Death Differ. 13 (2006) 951–961.
[11] C. Rivas, S.A. Aaronson, C. Munoz-Fontela, Dual role of p53 in innate antiviral immunity, Viruses 2 (2010) 298–313.
[12] Z. Zhu, J. Wei, Z. Shi, et al., Identification of human guanylate-binding protein 1 gene (hGBP1) as a direct transcriptional target gene of p53, Biochem. Biophys. Res. Commun. 436 (2013) 204–211.
[13] T. Mori, Y. Anazawa, M. Iiizumi, et al., Identification of the interferon regulatory factor 5 gene (IRF-5) as a direct target for p53, Oncogene 21 (2002) 2914–2918.
[14] M.W. Beilharz, J.M. Cummins, A.L. Bennett, Protection from lethal influenza virus challenge by oral type 1 interferon, Biochem. Biophys. Res. Commun. 355 (2007) 740–744.
[15] E. Haasbach, K. Droebner, A.B. Vogel, et al., Low-dose interferon type I treatment is effective against H5N1 and swine-origin H1N1 influenza A viruses in vitro and in vivo, J. Interferon Cytokine Res. 31 (2011) 515–525.
[16] E. Turpin, K. Luke, J. Jones, et al., Influenza virus infection increases p53 activity: role of p53 in cell death and viral replication, J. Virol. 79 (2005) 8802–8811.
[17] C. Muñoz-Fontela, M. Pazos, I. Delgado, et al., p53 serves as a host antiviral factor that enhances innate and adaptive immune responses to influenza A virus, J. Immunol. 187 (2011) 6428–6436.
[18] A.R. Bluyssen, J.E. Durbin, D.E. Levy, ISGF3 gamma p48, a specificity switch for interferon activated transcription factors, Cytokine Growth Factor Rev. 7 (1996) 11–17.
[19] C. Muñoz-Fontela, S. Macip, L. Martínez-Sobrido, et al., Transcriptional role of p53 in interferon-mediated antiviral immunity, J. Exp. Med. 205 (2008) 1929–1938.
[20] Y. Shen, X. Wang, L. Guo, et al., Influenza A virus induces p53 accumulation in a biphasic pattern, Biochem. Biophys. Res. Commun. 382 (2009) 331–335.
[21] X. Wang, X. Deng, W. Yan, et al., Stabilization of p53 in influenza A virus-infected cells is associated with compromised MDM2-mediated ubiquitination of p53, J. Biol. Chem. 287 (2012) 18366–18375.
[22] X.X. Sun, Y.G. Wang, D.P. Xirodimas, et al., Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation, J. Biol. Chem. 285 (2010) 25812–25821.
[23] T. Sareneva, S. Matikainen, M. Kurimoto, et al., Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells, J. Immunol. 160 (1998) 6032–6038.
[24] S.Y. Liu, D.J. Sanchez, G. Cheng, New developments in the induction and antiviral effectors of type I interferon, Curr. Opin. Immunol. 23 (2011) 57–64.
[25] S. Pestka, A dance between interferon-alpha/beta and p53 demonstrates collaborations in tumor suppression and antiviral activities, Cancer Cell 4 (2003) 85–87.
[26] B. Huang, Z.T. Qi, Z. Xu, et al., Global characterization of interferon regulatory factor (IRF) genes in vertebrates: glimpse of the diversification in evolution, BMC Immunol. 11 (2010) 22.
[27] K. Honda, H. Yanai, H. Negishi, et al., IRF-7 is the master regulator of type-I interferon-dependent immune responses, Nature 434 (2005) 772–777.
[28] R. Alberts, B. Srivastava, H. Wu, et al., Gene expression changes in the host response between resistant and susceptible inbred mouse strains after influenza A infection, Microbes Infect. 12 (2010) 309–318.
[29] S.D. Der, A. Zhou, B.R. Williams, et al., Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 15623–15628.