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Design, biological activity and signaling pathway of bovine consensus omega interferon expressed in *Pichia pastoris*

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ABSTRACT

The bovine IFN-ω (BoIFN-ω) multigene family is located on chromosome 8, which has 14 potential functional genes and 10 pseudogenes. After aligning 14 BoIFN-ω subtypes and assigning the most frequently occurring amino acids in each position, one artificial consensus BoIFN-ω (CoBoIFN-ω) gene was designed, optimized and synthesized. Then, CoBoIFN-ω was expressed in *Pichia pastoris*, which was demonstrated to have 3.94-fold and 14.3-fold higher antiviral activity against VSV on MDBK cells than that of BoIFN-ω24 and BoIFN-ω3, respectively. Besides this, CoBoIFN-ω was confirmed to have antiviral activity against VSV on BL, BT, PK-15 cells, and against BEV, BHV-1, BPIV3 on MDBK cells. Additionally, CoBoIFN-ω could bind with bovine type I IFN receptors, and then activate the promoters of NF-κB, ISRE and BoIFN-β, and induce the transcription of ISGs and expression of Mx1 and NF-κB p65, which suggested CoBoIFN-ω exerts antiviral activity *via* activation of the JAK-STAT signaling pathway. Overall, this research on CoBoIFN-ω not only extends and improves consensus IFN research, but also reveals that CoBoIFN-ω has the potential to be used in the therapy of bovine viral diseases.

1. Introduction

Interferon omega (IFN-ω), one kind of cytokine for trigging antiviral responses, is released during innate immune activation and present across mammalian groups, including cats, pigs, and cows (Minagawa et al., 1999; Rodriguez et al., 1998; Zhao et al., 2009). A single functional gene and at least two pseudogenes are present in human IFN-ω, but only a single pseudogene can be identified in mice IFN-ω (Hardy et al., 2004), while the IFN-ω family appears to have expanded in cats, which possess at least 10 variants on the basis of cDNA evidence (Hughes, 1995). IFN-ω has been approved as the veterinary antiviral agent for feline calicivirus infection and canine parvovirus infection (Minagawa et al., 1999; Vischer, 2006), as well as other disease (Domenech et al., 2011; Litzlbauer et al., 2014; Zetner et al., 2004). In bovine IFN-ω (BoIFN-ω), there are 24 potential IFN-ωs and at least 8 pseudogenes, which is significantly more compared with human and mouse IFN-ωs (Walker and Roberts, 2009). Among the members of BoIFN-ω multigene family, BoIFN-ω1 has been demonstrated to be highly secreted by *Pichia pastoris* (*P. pastoris*) showing antiviral activity in different kinds of cell line and an antiluteolytic effect in cyclic ewes, with no detrimental effects to the animals (Rodriguez et al., 1998); BoIFN-ω3 and BoIFN-ω24 were demonstrated to have the typical characteristics of type I IFNs with significant virus-resistance activity (An et al., 2017; Luo et al., 2015).

Artificial consensus IFN was designed to combine the IFN multigene family including many members with different biological activities, which is a kind of artificially engineered protein that links the most common occurring amino acid sequences at each position of natural IFN (Blatt et al., 1996; El-Baky et al., 2015; Ozes et al., 1992). Infergen (also named IFN-alfacon-1, CIFN or IFN-Con1) was the first commercial recombinant consensus IFN-α, which was approved by the Food and Drug Administration (FDA) for use in the treatment of chronic HCV infection (El-Baky et al., 2015). Researches have shown that the antiviral, antiproliferative, and inducing NK cell activities of Infergen are more

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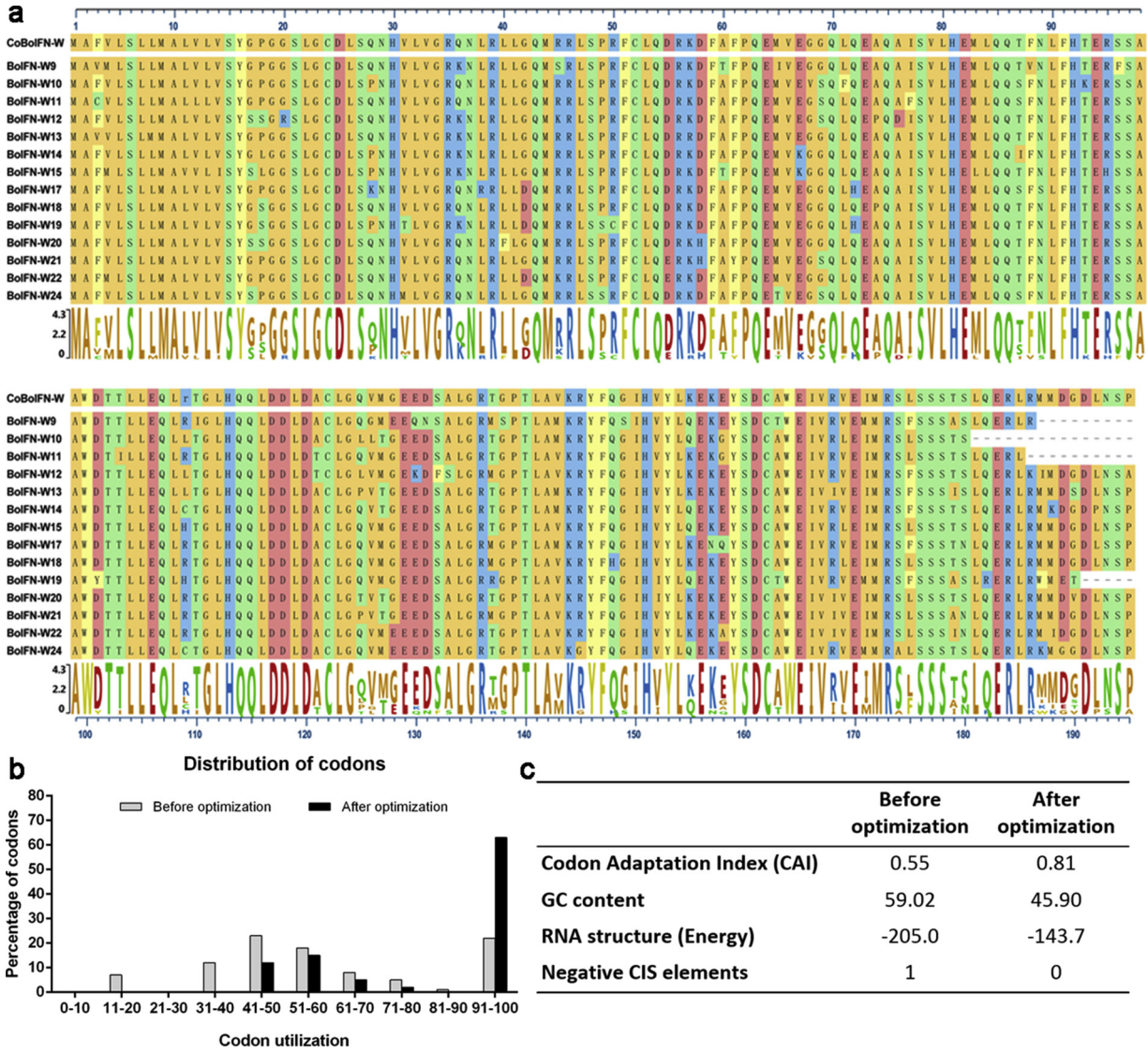

Fig. 1. Design and optimization of CoBoIFN-ω sequence. (a) Design of CoBoIFN-ω sequence. The scale represents the amino acids position, and amino acids at the lowest position represent the utilization percentage among the wild-type BoIFN-ω subtypes. The top sequence is the CoBoIFN-ω sequence designed in this study. (b) Optimization for codon usage rate of the CoBoIFN-ω gene in *P. pastoris*. The percentage distribution of codons is computed in codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. Codons with values lower than 30 are likely to hamper expression efficiency. (c) Comparison of the values of index optimization. The possibility of high protein expression level is correlated with the value of CAI (a CAI of 1.0 is considered to be ideal, whereas a CAI of > 0.8 is rated as good for the desired expression). The ideal percentage range of GC content is between 30% and 70%. Any peaks outside this range will adversely affect transcriptional and translational efficiency.

potent than those of natural IFN-αs, which demonstrates antiviral activity 10-fold to 20-fold higher than that of IFN-α2a and IFN-α2b (Ozes et al., 1992), and is effective and safe in the treatment of chronic hepatitis C and the retreatment of patients who either failed previous IFN treatments or presented relapse after cessation (Heathcote et al., 1998; Heathcote, 1998). Consensus porcine IFN-α (CoPoIFN-α) was demonstrated to have higher antiviral and antiproliferative activities than those of natural PoIFN-α, and enhance the mRNA level of Mx1 and OAS1 (Huang et al., 2012). As mentioned above, BoIFN-ω forms a multigene family with different biological activities (An et al., 2017; Luo et al., 2015; Rodriguez et al., 1998), which makes developing one kind of BoIFN-ω combining the biological activities of BoIFN-ωs possible. Therefore, a consensus BoIFN-ω (CoBoIFN-ω) was designed and characterized in this study, and was demonstrated to have antiviral and antiproliferative activities, transduce signals through binding with the type I IFN receptor complex, induce the transcription of ISGs (including Mx1, OAS, ISG15, ISG56), the expression of Mx1, and the activation of NF-κB, ISRE and BoIFN-β promoter.

2. Materials and methods

2.1. Cells, virus, protein and antibody

Madin–Darby bovine kidney (MDBK) cells, porcine kidney (PK-15) cells, baby-hamster kidney (BHK-21) cells, and primary bovine testicular (BT) cells were preserved in our laboratory. Primary embryo bovine lung (BL) cells were kindly provided by Dr. Fei Xue. VSV (Vesicular Stomatitis Virus), BHV-1 (Bovine Herpesvirus 1), BPIV3 ((Bovine Parainfluenza Virus type 3) were purchased from the China Institute of Veterinary Drug Control. BEV ((Bovine Enterovirus) was isolated and preserved in our laboratory (Zhang et al., 2014). Recombinant protein BoIFN-ω24, BoIFN-ω3 and BoIFN-αA were produced and preserved in our laboratory (An et al., 2017; Luo et al., 2015).

Rabbit polyclonal antibodies (PAb) against Mx1 (GTX110256) and

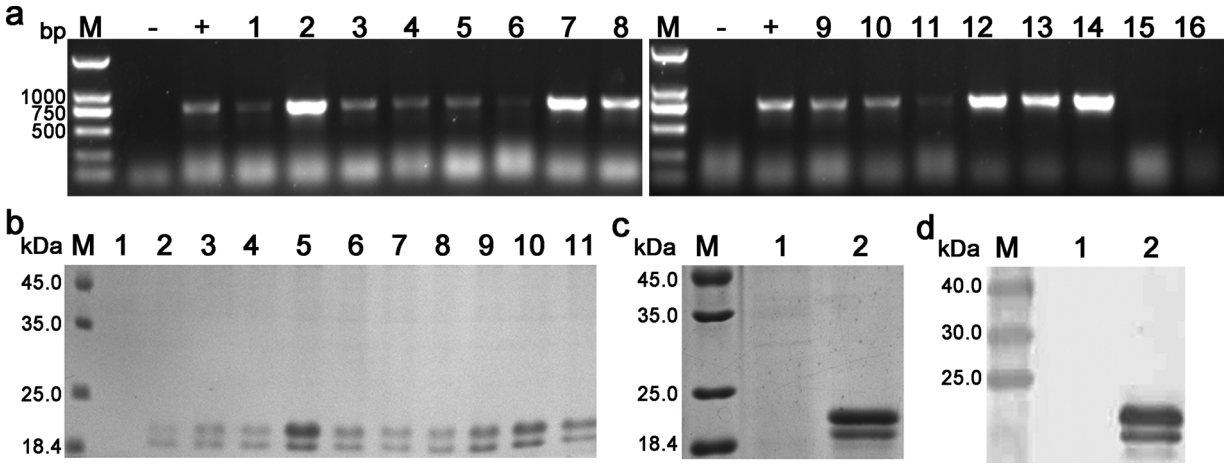

Fig. 2. Identification and expression of CoBoIFN-ω. (a) PCR identification of the *P. pastoris* transformants integrated into the CoBoIFN-ω gene. Lane 1–16, 16 different clones of *P. pastoris* transformants selected randomly. (b) SDS-PAGE analysis of CoBoIFN-ω expressed by *P. pastoris* transformants. 1–10, 10 different positive transformants selected for expressing CoBoIFN-ω. (c) SDS-PAGE analysis of CoBoIFN-ω with large-scale expression. Lane 1, large-scale expression of negative control; Lane 2, large-scale expression of CoBoIFN-ω. (d) Western blot analysis of CoBoIFN-ω. Lane 1, large-scale expression of negative control; Lane 2, large-scale expression of CoBoIFN-ω after purification.

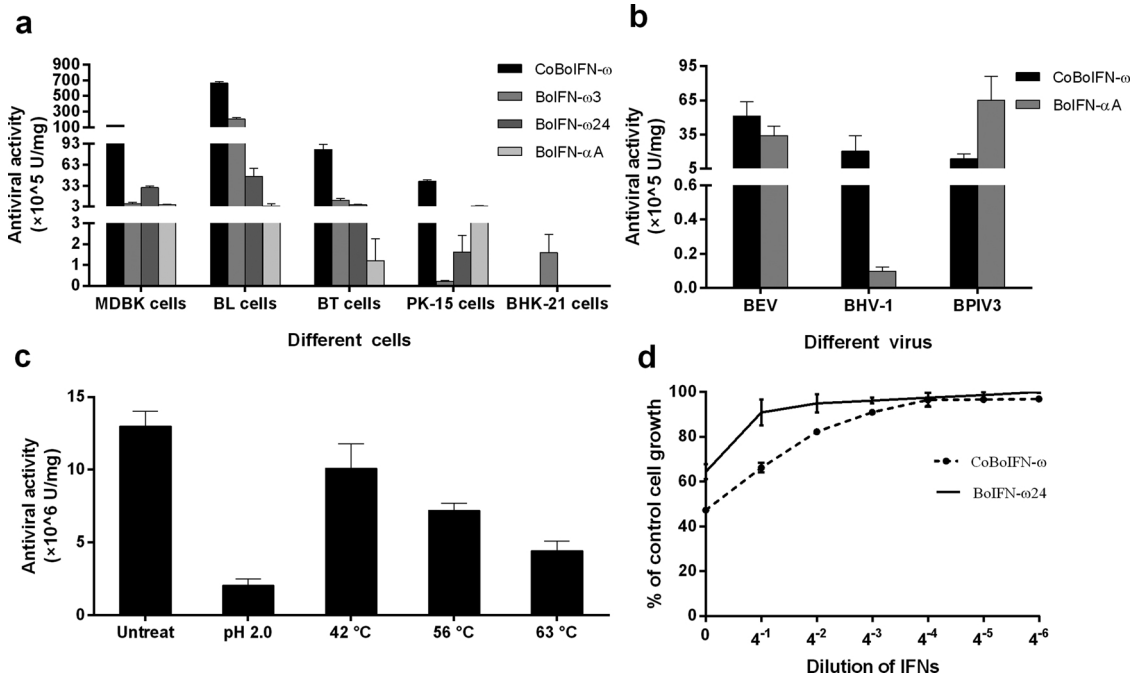

Fig. 3. Biological activity of CoBoIFN-ω. (a) Antiviral activities of CoBoIFN-ω against VSV on different cells. BoIFN-ω3, BoIFN-ω24 and BoIFN-αA were used as control. (b) Antiviral activities of CoBoIFN-ω against BEV, BHV-1 and BPIV3 on MDBK cells. BoIFN-αA were used as control. (c) pH and temperature sensitivity of CoBoIFN-ω. (d) Antiproliferative activities of CoBoIFN-ω on MDBK cells by an MTT assay after 72 h of treatment. Relative proliferation was determined as percentage of the control (0 μg/mL). BoIFN-ω24 was used as a reference.

GAPDH (GTX10 0118) were purchased from GeneTex (CA, USA). Rabbit PAb against NF-κB p65 (AF0246) were purchased from Beyotime (Beijing, China). HRP-conjugated goat anti-rabbit IgG was purchased from ZSGB (Beijing, China). Rabbit PAb against BoIFN-ω24, IFNAR1 and IFNAR2 were prepared and preserved in our laboratory (Guo et al., 2017; Luo et al., 2015).

2.2. Design and synthesis of CoBoIFN-ω

To get the location of BoIFN-ω genes in the bovine genome sequence (*Bos_taurus_UMD_3.1.1*), the BLAST algorithm was used by aligning with BoIFN-ω1 and BoIFN-ω24 (Luo et al., 2015; Rodriguez et al., 1998) with more than 80% identity. After locating the BoIFN-ω genes in the bovine genome, all the potential functional genes were compared, and then the sequence encoding the most frequently occurring amino acids was selected for designing CoBoIFN-ω. On the basis of the CoBoIFN-ω sequence and the codon preference of *P. pastoris*, the CoBoIFN-ω gene was optimized by using the GenScript OptimumGene™ codon optimization tool, and synthesized by BGI (Beijing, China).

2.3. Construction of recombinant expression vector

The synthesized CoBoIFN-ω gene flush with the Kex2 cleavage site was cloned into pPICZαA vector at the 5′ and 3′ sites with *Xho* I and *Eco*R I to obtain the recombinant protein with native N-terminus. After identification with PCR and sequencing, the positive recombinant

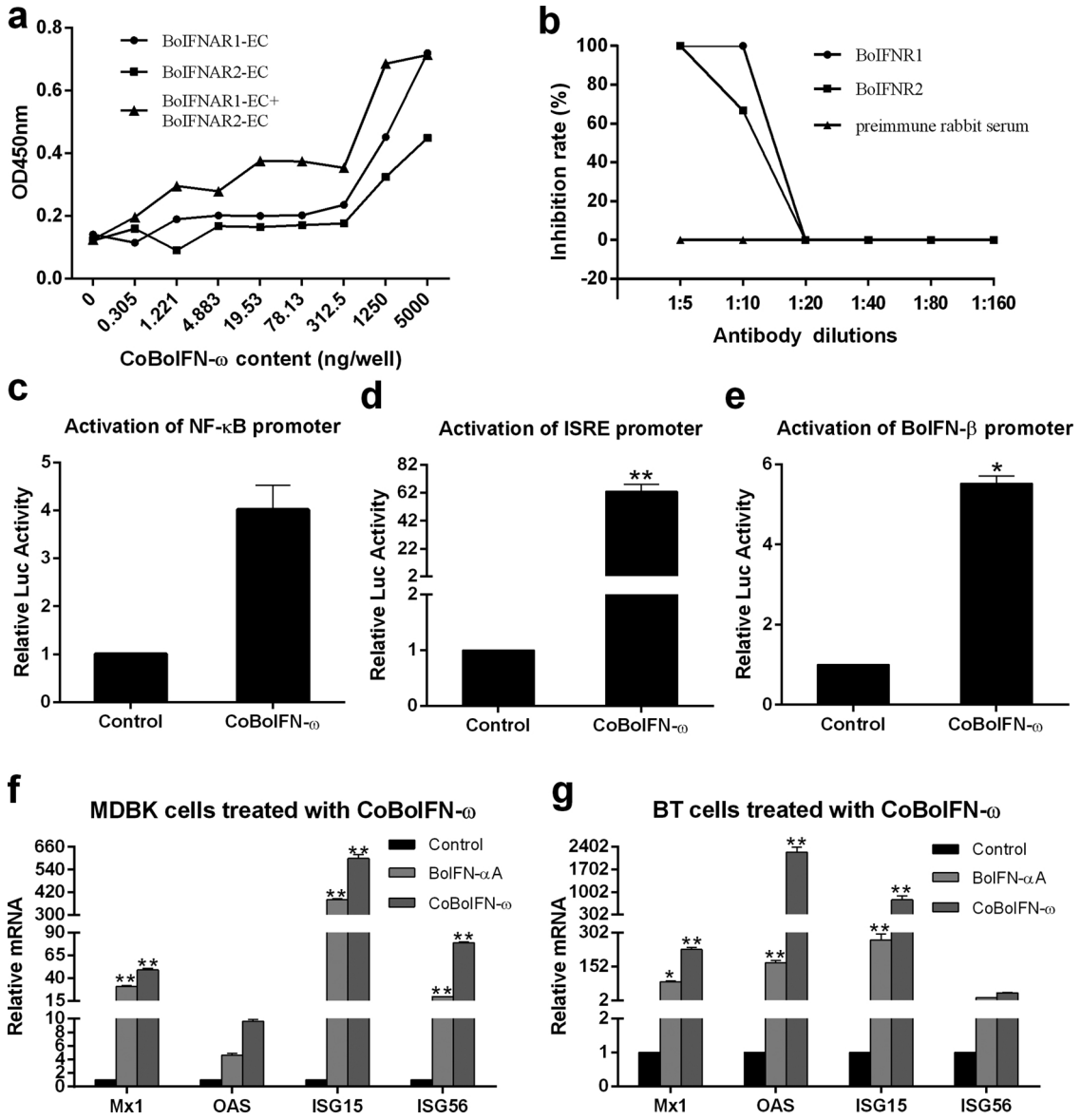

Fig. 4. Signaling analysis of CoBoIFN-ω. (a) ELISA assay for the binding ability of CoBoIFN-ω to BoIFNAR1 and BoIFNAR2. (b) Blocking assay with PAb against BoIFNAR1 or BoIFNAR2 on MDBK cells. (c–e) Activation of NF-κB, ISRE and BoIFN-β promoter activity treated with CoBoIFN-ω on BT cells. (f–g) Real-time RT-PCR detection of Mx1, OAS, ISG15 and ISG56 on MDBK and BT cells for 12 h after CoBoIFN-ω treatment. MDBK and BT cells without IFN treatment were used as the mock-treated control, BoIFN-αA was used as positive control. Data are represented as the mean ± S.D. (n = 3) of one representative experiment. *P < 0.05; **P < 0.01.

plasmid was named as pPICZαA-CoBoIFN-ω. Then the plasmids pPICZαA-CoBoIFN-ω and pPICZαA were linearized with *Pme* I, and transformed into competent *P. pastoris* GS115 cells mediated by electroporation separately. After incubation in YPD (Yeast Extract-Peptone Dextrose) medium at 30℃ for at least 72 h, the gene integration was verified by PCR using yeast genomic DNA as a template, and 5′AOX1 (5′-GACTGGTTCCAATTGAGAAGC-3′) and 3′AOX1 (5′-GCAAATGGCAT TCTGACATCC-3′) as primers, and positive transformants were selected for expression.

2.4. Expression and identification of CoBoIFN-ω

Selected positive transformants transformed in *P. pastoris* GS115 cells were grown in BMGY (Buffered Glycerol-complex Medium) at 30 ˚C until an OD_{600} of between 2 and 6 was reached, and then centrifuged and resuspended with BMMY (Buffered Methanol-complex Medium) for induction with 1% methanol (v/v) every 24 h. After 96 h of induction, the entire culture supernatant was harvested by centrifuging at 3000 × g at 4 ˚C for 15 min. Then SDS-PAGE was performed to analyze the expression of CoBoIFN-ω, and Western blot was used to analyze the specificity of CoBoIFN-ω with rabbit PAb against BoIFNω24. Finally, the expressed CoBoIFN-ω was purified with ammonium sulfate precipitation, and dialyzed with PBS for 48 h. The final protein concentration was determined using a BCA protein assay kit (Beyotime, Beijing, China).

2.5. Antiviral activity analysis of CoBoIFN-ω in vitro

First, virus (VSV, BEV, BHV-1, BPIV3) titers were determined by an endpoint dilution assay and were expressed as TCID50 using the Reed-Muench method (Cheng et al., 2006). Then antiviral activity of CoBoIFN-ω was determined with a standard cytopathic effect (CPE) assay, as previously described (Guo et al., 2015). Cells without viruses were used as mock-treated controls, and cells without IFN were used as CPE

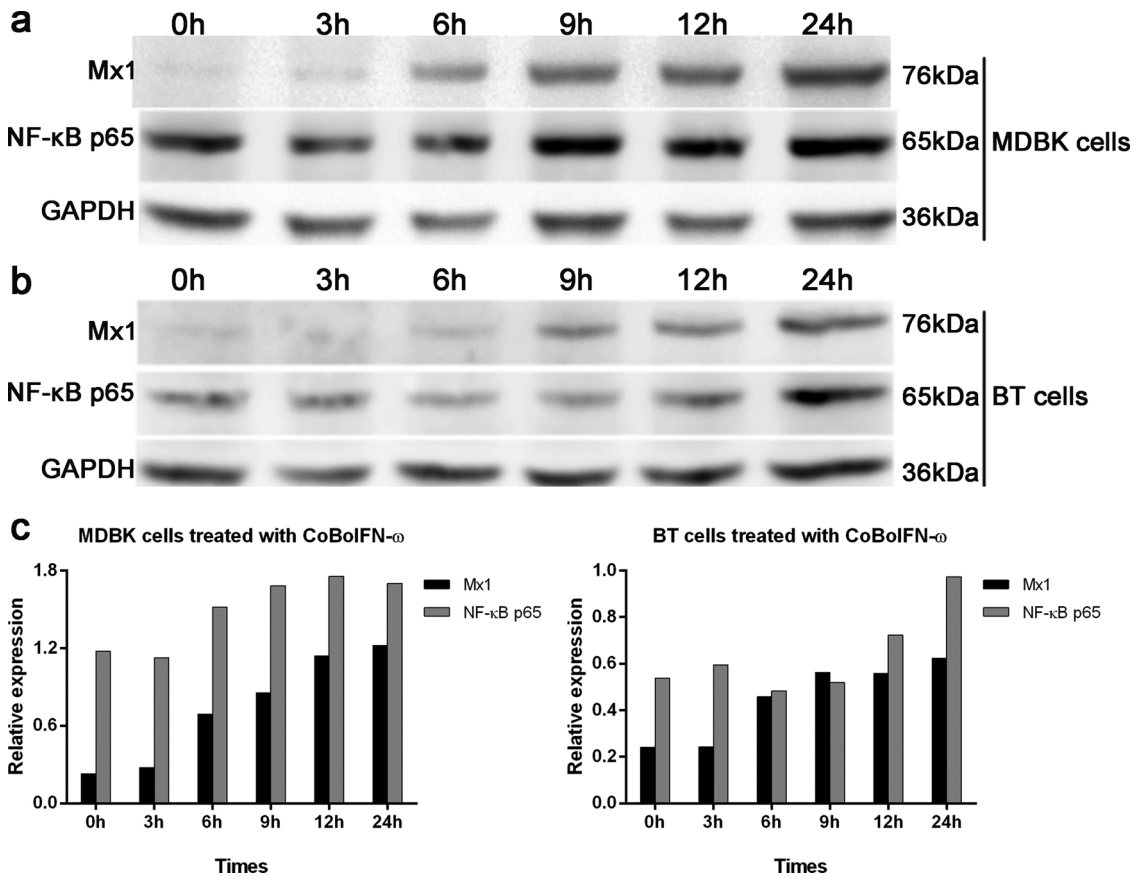

Fig. 5. Western blot analysis of Mx1 and NF-κB p65 after CoBoIFN-ω treatment. (a) Western blot analysis of Mx1 and NF-κB p65 on MDBK cells treated with CoBoIFN-ω. (b) Western blot analysis of Mx1 and NF-κB p65 on BT cells treated with CoBoIFN-ω. (c) Relative expression of Mx1 and NF-κB p65 after CoBoIFN-ω treatment basing on Western blot results with Quantity One software. GAPDH was used as internal reference control. Cells were treated with CoBoIFN-ω (1000U/ml) and harvested at different time points (0 ~ 24 h).

controls.

The specificity of CoBoIFN-ω antiviral activity was analyzed with PAb against BoIFN-ω24 by antiviral activity blocking assay by the method described previously (Shao et al., 2015).

2.6. Physicochemical characteristics analysis of CoBoIFN-ω

The antiviral activity of CoBoIFN-ω treated with 0.25% trypsin, pH 2.0 and temperatures of 42 °C, 56 °C, and 63 °C, respectively, was measured on the MDBK/VSV system according to the methods described previously (Shao et al., 2015).

2.7. Antiproliferative assays

The antiproliferative activity of CoBoIFN-ω on MDBK cells was measured by MTT assay (Luo et al., 2015). MDBK cells seeded in 96-well plates were treated with CoBoIFN-ω and BoIFN-ω24 for 72 h separately, and then incubated with MTT for 4 h. DMSO was added after removing the culture medium, and OD_{570nm} was measured.

2.8. Blocking assay with BoIFNAR or PAb against BoIFNAR on MDBK cells

Plates coated with the extracellular protein of BoIFNAR were blocked with 5% skimmed milk, then serial dilutions of CoBoIFN-ω were incubated before the PAb against BoIFN-ω24 was added to each well. HRP-conjugated goat anti-rabbit IgG was incubated and OD_{450nm} was measured (Gao et al., 2012; Guo et al., 2017).

Blocking assay with PAb against BoIFNAR on MDBK cells were analyzed according to the method reported previously (Guo et al., 2017). Wells without virus were used as blank controls, and wells without PAb were used as mock-treated controls.

2.9. CoBoIFN-ω-induced activation of NF-κB, ISRE, and BoIFN-β promoter

The activities of NF-κB, ISRE, and BoIFN-β promoter on BT cells treated with CoBoIFN-ω were measured by the Dual-Luciferase Reporter Assay System. The results were expressed as relative luciferase activity, which was shown as fold change relative to the mock-treated control.

2.10. CoBoIFN-ω-induced expression of ISGs

Real time PCR was used to analyze the expressions of ISG genes (including Mx1, OAS, ISG15, ISG56) on MDBK or BT cells treated with CoBoIFN-ω according to methods reported previously (Guo et al., 2017).

The expressions of Mx1 and NF-κB p65 on MDBK or BT cells treated with CoBoIFN-ω were analyzed by Western blot according to methods reported previously (Guo et al., 2017).

2.11. Statistical analysis

All experiments were repeated three times. Data are expressed as the mean values standard deviation (SD). Statistical significance was determined by Student's t-test, with values of $P < 0.05$ considered to be statistically significant.

3. Results

3.1. Design and construction of the recombinant plasmid

BLAST analysis in the bovine genome database revealed that BoIFN-ω cluster is distributed over 600 kb of the long arm of chromosome 8, which consists of approximately 24 genes. Then CoBoIFN-ω was designed by aligning the sequences of 14 BoIFN-ω functional genes, which contain 172 amino acid residues, assigning the most frequently observed amino acid in each corresponding position and optimizing for high-level expression in *P. pastoris* (Fig. 1a). As shown in Fig. 1b and c, the Codon Adaptation Index (CAI) reached 0.81, which was much higher than 0.55 before optimization, and the GC content changed from 59.02% to 45.90%. After optimization, the total percentage distribution of codons exceeded 30%.

3.2. Protein expression and identification of CoBoIFN-ω

Positive yeast transformants containing the CoBoIFN-ω gene were confirmed by PCR, which revealed a specific band of 725bp with different brightness (Fig. 2a). Then, ten brighter positive transformants were selected for expression, and the highest expression clone was selected for large scale expression, which showed there are two main bands expressed at the location between 18.4 kDa and 25.0 kDa (Fig. 2b and c), which is consistent with the predicted molecular weight of 19.6 kDa. Western blot confirmed that the two expressed bands were both the specific bands of BoIFN-ω (Fig. 2d), and the upper band could be attributed to the glycosylation of the two putative O-glycosylation sites at amino acid residue 74, and 117 in the mature peptide. The protein concentration of CoBoIFN-ω purified by ammonium sulfate was 1.542 mg/mL.

3.3. Antiviral activities of CoBoIFN-ω in vitro

The antiviral activity of CoBoIFN-ω was tested in five different cell lines against VSV, and BoIFN-ω24, BoIFN-ω3 and BoIFN-αA were used as references. CoBoIFN-ω, BoIFN-ω24 and BoIFN-αA can inhibit the cytopathic effect caused by VSV on MDBK and PK-15 cells, but not on BHK-21 cells. The antiviral activity of CoBoIFN-ω on MDBK and PK-15 cells were approximately 3.94-fold and 12.8-fold higher than that of BoIFN-ω24, and 14.3-fold and 173.6-fold higher than that of BoIFN-ω3 (Fig. 3a). Additionally, CoBoIFN-ω exerted a protective effect against bovine viruses (BEV, BHV-1 and BPIV3) on MDBK cells (Fig. 3b). The antiviral activity of CoBoIFN-ω on MDBK cells against VSV can be completely blocked by PAb against BoIFN-ω24 at a dilution of 1:16. When the dilution of PAb against BoIFN-ω24 is 1:64, there is no blocking of the antiviral activity of CoBoIFN-ω.

3.4. Physicochemical characteristic

After treatment with 0.25% trypsin, the antiviral activity of CoBoIFN-ω completely disappeared. However, the antiviral activity of CoBoIFN-ω was retained after treatment at pH 2.0 and only slightly declined with increasing temperature (Fig. 3c).

3.5. Antiproliferative activity

As shown in Fig. 3d, both CoBoIFN-ω and BoIFN-ω24 exerted a dose-dependent antiproliferative effect on MDBK cells. CoBoIFN-ω was demonstrated to have a lighter antiproliferative effect than BoIFN-ω24, which also showed CoBoIFN-ω has a lower cytotoxicity on MDBK cells, even at high concentration.

3.6. CoBoIFN-ω binding with type I IFN receptor

ELISA results suggest that CoBoIFN-ω can combine with BoIFNAR1, BoIFNAR2 or BoIFNAR1 and BoIFNAR2, which showed a dose-dependent relationship, and the combination of BoIFNAR1 and BoIFNAR2 with CoBoIFN-ω was not in conflict but synergetic (Fig. 4a). In addition, the antibodies against BoIFNAR1 and BoIFNAR2 can both block the antiviral activity of CoBoIFN-ω by binding with the corresponding receptor BoIFNAR1 and BoIFNAR2 on MDBK cells, and this blocking effect displayed a dose-dependent relationship with the dilution of the PAb against BoIFNAR (Fig. 4b).

3.7. CoBoIFN-ω-induced activation of NF-κB, ISRE, and BoIFN-β promoter

The NF-κB, ISRE, and BoIFN-β promoters can be activated with CoBoIFN-ω treatment 3.65-fold, 66.7-fold, and 5.66-fold, respectively, compared with those in mock-treated cells (Fig. 4c–e).

3.8. CoBoIFN-ω-induced expression of ISGs

Similar to BoIFN-αA, CoBoIFN-ω can also induce the expression of Mx1, OAS, ISG15 and ISG56 gene 49.0-fold, 9.62-fold, 599-fold and 79.0-fold on MDBK cells (Fig. 4f), and 227-fold, 2233-fold, 765-fold and 35.5-fold on BT cells (Fig. 4g), respectively.

Mx1 protein was expressed in a time-dependent manner after treatment with CoBoIFN-ω on MDBK and BT cells, which started at 3 h and increased with the time extension (Fig. 5). NF-κB p65 can also be slightly induced by CoBoIFN-ω in a time-dependent manner on MDBK and BT cells, which peaked at 12 h on MDBK cells and at 24 h on BT cells (Fig. 5). The molecular weights of Mx1, NF-κB p65 and GAPDH were 76.0 kDa, 65.0 kDa and 36.0 kDa, respectively.

4. Discussion

IFN constitutes a family of potent naturally occurring cytokines that activate innate and acquired immune responses. Consensus IFN is an artificially engineered protein combining most of the biological features of natural IFN and showing high anticancer and antiviral activities (El-Baky et al., 2015). The first artificial recombinant IFN-α Infergen showed higher antiviral activity than the natural recombinant IFN-α (Blatt et al., 1996; Fish et al., 2008). Currently, Infergen is the only FDA-approved IFN-related product for treatment of patients with no response to other forms of IFN therapy or for treating relapsing patients which has significantly greater potency than conventional IFNs in both *in vitro* and *in vivo* assays (Blatt et al., 1996; Nordstrom et al., 2015; Ozes et al., 1992). Similar to IFN-α family, BoIFN-ω is a member of the type I IFN family that comprises a group of structurally related proteins derived from a multigene family. In this study, 14 functional BoIFN-ω genes and 10 pseudogenes present on bovine chromosome 8 were identified in the latest bovine genome database by BLAST scanning. BoIFN-ω1, BoIFN-ω3 and BoIFN-ω24 have been reported to exhibit antiviral activity on different cell lines with some difference. In order to develop one kind of BoIFN-ω combining the basic characteristics of the BoIFN-ω multigene family, CoBoIFN-ω was designed and developed. CoBoIFN-ω is the first artificial recombinant IFN-ω, designed by scanning 14 natural BoIFN-ω subtypes using the multiple sequence alignment tools followed by assigning the most frequently occurring amino acid in each corresponding position. Similar to the majority of BoIFN-ω subtypes, the coding amino acid sequence of consensus IFN comprises 172 amino acids, which includes four conserved cysteine residues, five alpha helices and two putative O-glycosylation sites.

P. pastoris is widely and successfully used as the eukaryotic expression system for the production of heterologous proteins (Macauley-Patrick et al., 2005). High-level expression of recombinant BoIFN-α, BoIFN-ω, and CoPoIFN-α have been successfully achieved using the *P. pastoris* expression system (Hao et al., 2006; Huang et al., 2012; Shao et al., 2015). On the basis of the above and our objectives, *P. pastoris* was used as the host strain to express CoBoIFN-ω. In order to improve the expression efficiency, the coding gene of CoBoIFN-ω was optimized

based on yeast codon usage preference. In this study, CoBoIFN-ω was successfully expressed in *P. pastoris*, which showed two bands with similar molecular weight. Such additional bands observed previously for *P. pastoris*-derived IFN may be the glycosylated form of CoBoIFN-ω (Ghosalkar et al., 2008; Liu et al., 2001; Salunkhe et al., 2010; Shao et al., 2015). Similar to BoIFN-ω24 and BoIFN-ω3, CoBoIFN-ω has antiviral activity against VSV on MDBK, BL, BT and PK-15 cells. Unlike BoIFN-ω3, CoBoIFN-ω has no antiviral activity against VSV on BHK-21 cells. And the antiviral activity against VSV of CoBoIFN-ω on MDBK and PK-15 cells was much higher than that of BoIFN-ω3 and BoIFN-ω24. What's more, CoBoIFN-ω exerted antiviral activity against bovine virus (BEV, BHV-1 and BPIV3) on MDBK cells, which suggested CoBoIFN-ω has broad antiviral activity. The antiviral activity of CoBoIFN-ω can be neutralized completely by PAb against BoIFN-ω24, which showed that this antiviral activity of CoBoIFN-ω is specific. In addition, CoBoIFN-ω was determined to exhibit physicochemical characteristics similar to other types of I IFN, such as being highly sensitive to trypsin, and insensitive to temperature and stable at pH 2.0. A slight antiproliferative activity was detected on MDBK cells, which also revealed that CoBoIFN-ω has no cytotoxicity on MDBK cells at a reasonable working concentration. The results above suggested that CoBoIFN-ω could be used as a safe therapeutic agent in bovine viral diseases.

Type I IFN binds to IFNAR complex consisting of IFNAR1 and IFNAR2 located on the nucleated cell surface (de Weerd et al., 2007), and then activates the expression of numerous ISGs *via* the JAK-STAT signaling pathway to establish an antiviral state within host cells (Platanias, 2005; Raftery and Stevenson, 2017). PAb against BoIFNAR (BoIFNAR1 and BoIFNAR2) could block the antiviral activity of CoBoIFN-ω suggesting that CoBoIFN-ω exerts antiviral activity by interacting with type I IFN receptors first. After activation of the JAK-STAT pathway, the promoters of NF-κB, ISRE and BoIFN-β are activated by CoBoIFN-ω, leading to the antiviral gene (including Mx1, OAS, ISG15 and ISG56) expression that plays an important role as a mediator of inducible signal transduction in gene regulation. CoBoIFN-ω can obviously induce the expression of these ISGs, especially the ISG15 gene, which can further inhibit viral replication and protein synthesis to exert antiviral activity, such as VSV, BEV, BHV-1 and BPIV3 reported in this study. These findings demonstrate that CoBoIFN-ω signals through binding with the type I IFN receptor and inducing ISG expression to produce antiviral proteins exerting the antiviral activity. CoBoIFN-ω is an artificial BoIFN-ω, which still has similar characterization to the naturally occurring BoIFN-ω, and has much higher antiviral activity than that of BoIFN-ω3 and BoIFN-ω24, indicating CoBoIFN-ω has the potential to be used as an antiviral reagent.

In summary, CoBoIFN-ω was successfully designed and expressed in *P. pastoris,* and then the biological activity and signaling pathway was analyzed. The results not only demonstrated CoBoIFN-ω could be used as a potential therapeutic agent for bovine viral disease, but also provided new ideas for designing the consensus molecular of a multigene family.

Competing interests

The authors declare that they have no competing interests.

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