INTRODUCTION

Since its inception more than 30 years ago, the baculovirus expression system (BES) has been widely employed for recombinant protein expression at massive levels (van Oers, Pijlman, & Vlak, 2015; Smith, Summers, & Fraser, 1983). Depending on posttranslational modifications in insect cells and larvae, the BES is markedly suitable for eukaryotic protein expression (Kidd & Emery, 1993). In recent years, multigene expression employing the BES has been reported (Berger, Fitzgerald, & Richmond, 2004; Kanai, Athmaram, Stewart, & Roy, 2013; Yao et al., 2012). Multigene expression in a single recombinant baculovirus has advantages in expression applications. The expression of double-chain antibodies...
and packaging of recombinant adeno-associated virus (rAAV) are a few examples of this expression system (Furuta, Ogawa, Katsuda, Fuji, & Yamaji, 2010; Negrete, Yang, Mendez, Levy, & Kotin, 2007).

Bombyx mori nucleopolyhedrovirus (BmNPV) is one of the most widely employed baculoviruses for gene expression. In the last 3 decades, BmNPV has undergone modifications in many ways to generate recombinant BmNPV that is more convenient and enhances the expression efficiency of foreign genes (Kato, Kajikawa, Maenaka, & Park, 2010; Maeda et al., 1985). Compared with the AcMNPV-cell expression system, the BmNPV-silkworm system possesses better posttranslational processing and greater expression efficiency (Dojima et al., 2009; Usami et al., 2011). Previously, we successfully constructed a reBmBac system for recombinant BmNPV with increased efficiency (Liu et al., 2016). By utilizing this system, researchers can proficiently and rapidly obtain recombinant baculoviruses and target proteins in silkworms.

Interferons (IFNs) were first discovered in the 1950s, and since then, they have been researched deeply in many fields. Interferons are categorized into three major classes. Type I IFNs are generated by almost any type of cell in response to invading pathogens (Alsharifi, Mullbacher, & Regner, 2008). They can induce the expression of specific antiviral proteins and related physiological responses by binding with specific receptors on the cell membrane (Levy, Marie, & Durbin, 2011). Type II IFNs possess strong immune regulation ability and can regulate the activity of lymphocytes (Muller et al., 1994; Platianias, 2005). Type III IFNs have been recently discovered. They have functions similar to those of type I IFNs (Kotenko, 2011; Sheppard et al., 2003). Some studies indicate that type I and II IFNs demonstrate synergy in the establishment of an antiviral state (Muller et al., 1994; Platianias, 2005; Sekellick, Lowenthal, O’Neil, & Marcus, 1998).

Chicken IFN-α (chIFN-α) is a type I IFN. Research has revealed its antiviral potential against Rous sarcoma virus, Newcastle disease virus, infectious bursal disease virus, and avian influenza virus in vitro and in vivo (Jiang, Yang, & Kapczynski, 2011; Marcus, van der Heide, & Sekellick, 1999; Meng et al., 2011; O’Neil, Livant, & Ewald, 2010). Chicken IFN-γ (chIFN-γ) is a type II IFN. It demonstrates avian virus inhibition both in vitro and in vivo and has the capability to prevent poultry coccidiosis (Cardenas-Garcia et al., 2016; Khatri & Sharma, 2008; Shah et al., 2010). Studies also illustrate that chIFN-γ enhances the growth performance of reared broilers (Lowenthal, 2001). These two IFNs have an immune synergism effect. The combination of chIFN-α and chIFN-γ can significantly enhance viral inhibition and elicit an antiviral state (Plachy et al., 1999; Sekellick et al., 1998).

In the current study, recombinant BmNPV simultaneously carrying the chIFN-α and chIFN-γ genes at distinct gene sites was constructed using the reBmBac system. This recombinant baculovirus was employed for the coexpression of two types of IFN and can provide a foundation for the combination of IFNs and their possible future therapeutic application.

### 2 | MATERIALS AND METHODS

A Bombyx mori-derived cell line, Bm5, was cultured in TC100 insect cell culture medium (Applichem) with 10% fetal bovine serum (FBS, Gibco, USA) at 27°C according to published procedures (Summers & Smith, 1987). DMEM (Dulbecco’s modified Eagle’s medium) and Trypsin-EDTA were obtained from Thermo Fisher Scientific. Specific pathogen-free (SPF) fertilized eggs (8–10 days) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. The recombinant vesicular stomatitis virus VSV-GFP was acquired from Lanzhou Veterinary Research Institute, CAAS.

Escherichia coli BW25113/pKD46 was obtained from the Molecular, Cellular and Developmental Biology Department, Kline Biology Tower 830, Yale University. The reBmBac vector was constructed in our laboratory. The pVL1393 transfer vector and Lipofectin were acquired from Invitrogen. Rabbit anti-chicken IFN-α and IFN-γ antibodies were obtained from RayBiotech. HRP-conjugated goat anti-rabbit IgG was obtained from Abcam.

#### 2.1 | Construction of the gene-targeting vector

The pP10 vector (GenBank ID: MN702625) was used to transfer the target gene into the baculovirus at the p10 gene site. Homologous targeting arms 232 bp upstream and 118 bp downstream of the p10 gene were amplified and inserted into the pMD18-simple vector.

The pP10-rpsL-neo vector (GenBank ID: MN702626) was utilized to knock out the p10 gene. The counterselection cassette rpsL-neo (GenBank ID: GU084141.1) was synthesized and inserted into the pP10 vector.

The pVL1393-Cα and pVL1393-Cγ vectors were employed to individually transfer the chicken interferon-α gene and interferon-γ gene into the recombinant baculovirus genome at the polyhedron site. The chIFN-α and chIFN-γ genes were codon optimized according to amino acid sequences (GenBank IDs: ADU60333.1 and ABI83735.1) and synthesized. These two genes were individually inserted into the pVL1393 transfer vector, in which there are baculovirus recombination sequences on both sides of the MCS. The pP10-Cγ vector was used to transfer the chIFN-γ gene into the recombinant baculovirus genome at the p10 gene site. The chIFN-γ gene was inserted into the pP10 vector via BamHI I/Not I digestion.

#### 2.2 | Construction of the recombinant baculovirus

The reBmBac vector was modified from BmNPV. An E. coli CopyControl origin of replication was inserted into the genome at chi-cat gene site. And tetracycline resistance gene was inserted at polyhedrin gene site. These two make sure the baculovirus DNA can be edited and amplified in E. coli. The essential ORF1629 gene was also partial deleted to make sure the highly recombination efficiency.

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According to a published protocol (Datsenko & Wanner, 2000; Liu et al., 2016), the p10 gene of reBmBac baculovirus genomic DNA was replaced by the ρsl-neo cassette by phage λ-Red recombinase in E. coli. Then, the ρsl-neo cassette was replaced by the chIFN-γ gene. The recombinant baculovirus shuttle vector containing the chIFN-γ gene at the p10 gene site was named reBmBac-p10Cγ.

According to published procedures (Liu et al., 2016), the pVL1393-Cα or pVL1393-Cγ vector was mixed with the reBmBac vector. The mixture was used to cotransfect Bm5 cells. chIFN-α and chIFN-γ genes were inserted into the baculovirus genome at the polyhedron gene site. The recombinant baculoviruses were named reBm-Cα and reBm-Cγ. A mixture of the pVL1393-Cα vector and reBmBac-p10Cγ was then utilized to cotransfect Bm5 cells. chIFN-α was transferred into the reBmBac-p10Cγ genome at the polyhedron gene site. The recombinant baculovirus, which contained the chIFN-α gene at the polyhedron site and the chIFN-γ gene at the p10 site, was named reBm-Cαγ. The recombinant baculoviruses reBm-Cα, reBm-Cαγ, and reBm-Cγ were purified by plaque screening (Pen, Welling, & Welling-Wester, 1989).

2.5 | Inactivation of chicken interferon γ

IFN-α is both acid and heat resistant. However, IFN-γ is easily deactivated by both acid (pH 2.0) and temperature (56°C) (Ho, Armstrong, & Breinig, 1975). Thus, the antiviral activity of IFN-α in the coexpression product was studied. The pH value of larval hemolymph or pupa product was adjusted to 2.0 ± 0.2 using a hydrochloric acid solution (0.1 M). After 24 hr of static incubation at 4°C and filtration, the pH value was adjusted to 7.0 ± 0.1 using a sodium hydroxide solution (1 M). After 1 hr of static incubation at 4°C and filtration, the sample was heat treated (56°C) for 30 min. After filtration with a 0.22 μm syringe filter, IFN-γ of the product was deactivated.

The expression of IFN was detected by western blotting according to the Protein Blotting Guide (Bio-Rad). Relative expression levels of IFN-α and IFN-γ in these products were detected by indirect ELISA.

3 | RESULTS

3.1 | Generation of the recombinant baculoviruses reBm-Cαγ, reBm-Cα, and reBm-Cγ

Two transfer plasmids, pVL1393-Cα and pVL1393-Cγ, were used to transfer the chIFN-α and chIFN-γ genes into reBmBac for single expression of IFN. The recombinant baculoviruses reBm-Cα and reBm-Cγ, constructed though cotransfection of transfer plasmids and reBmBac genome DNA (Figure 1a), were capable of expressing chIFN-α and chIFN-γ, respectively. In the IFN coexpression recombinant baculovirus reBm-Cαγ, the chIFN-γ gene was inserted into the reBmBac genome downstream of the p10 promoter sequence through homologous recombination in E. coli. The chIFN-α gene was inserted into the same reBmBac genome downstream of the polyhedron promoter sequence through cotransfection in cells (Figure 1b).

3.2 | Expression and antiviral activity analyses of coexpression IFNs and single-expression IFNs

The expression products of chIFN-α and chIFN-γ were analyzed by Western blotting (Figure 2). Figure 2 demonstrates that an approximately 22 kDa protein band that reacted with an anti-chIFN-α antibody was detected in reBm-Cαγ and reBm-Cα expression samples. Likewise, an approximately 19 kDa protein band that reacted with an anti-chIFN-γ antibody was observed in reBm-Cαγ and reBm-Cγ expression samples. No corresponding immunoreactive protein was detected in the negative control sample from larval hemolymph infected with control BmNPV.
Antiviral activity of recombinant IFNs was assayed utilizing a CPE inhibition assay with CEF cells. Recombinant IFN products could inhibit VSV-GFP infection in CEFs (Figure 3). The antiviral activity assay results indicated that the antiviral potential of reBm-Cα, reBm-Cγ, and reBm-Cαγ products were $3.26 \pm 0.61 \times 10^6$ IU/mL, $5.08 \pm 0.43 \times 10^6$ IU/mL, and $3.27 \pm 0.50 \times 10^7$ IU/mL in hemolymph, respectively. These recombinant baculovirus expression products were then acid (pH 2.0) and heat treated (56°C). IFN-α is acid and heat resistant, but IFN-γ is acid and heat labile (Ho et al., 1975). Therefore, the antiviral activity of the reBm-Cα product remained almost the same before and after treatment. The antiviral activity of the reBm-Cγ product declined to an undetectable level. The antiviral activity of the reBm-Cαγ product was still $5.78 \pm 0.88 \times 10^6$ IU/mL, which was due to IFN-α activity. This activity was 2 times greater than that of the reBm-Cα product. This enhanced expression level of IFN-α must be due to p10 gene deletion in coexpression recombinant baculovirus (Hitchman et al., 2010).

The ELISA results for chIFN-γ illustrate that the expression level of chIFN-γ in reBm-Cαγ-infected larval hemolymph was approximately 2.5 times greater than that in reBm-Cγ-infected larval hemolymph (Figure A1).

3.3 | The synergistic antiviral effect of interferon type I and II

The reBm-Cα and reBm-Cγ products were diluted to $10 \times 10^4$ IU/mL and mixed in different proportions. The antiviral activity of the mixtures was detected. The results revealed that chIFN-α and chIFN-γ (at a ratio of 1:2) enhanced the maximum antiviral activity, that is $17.92 \pm 1.07 \times 10^4$ IU/mL (Table 1). This result indicated that an increased ratio of IFN type II caused an obvious synergistic antiviral effect of IFN type I and II.

3.4 | Inhibitory effect of coexpression interferons on in vitro replication of Marek’s disease virus

The highly oncogenic strain RB1B of Marek’s disease virus (MDV) has the ability to replicate and develop plaques in CEFs. Chicken embryo fibroblasts treated with co- or single-expression chIFNs were employed to determine the inhibitory effect on the in vitro replication of MDV. Mean PFU is listed in Table 2. The results revealed that 800 IU of coexpression product completely inhibited MDV replication in CEFs. The
same titer of single-expression IFN products containing one of the two types of IFN could only partially inhibit MDV replication. This result indicated that chicken IFNs expressed in our baculovirus-silkworm system have excellent anti-MDV activity. This study further validated the synergistic antiviral potential of type I and type II IFNs.

4 | DISCUSSION

In our previous studies, the reBmBac recombinant baculovirus construction strategy was successfully established, and recombinant porcine IFN-α was efficiently expressed (Liu et al., 2016). Then, IFNs from several other species were successively expressed utilizing this system. All of the IFN products exhibited prominent antiviral activity.

In the present study, the coexpression of type I and II IFNs at different gene sites was successfully achieved using a single recombinant baculovirus. The type I IFN chIFN-α gene was introduced at the polyhedron gene site. The chIFN-γ gene, a type II IFN-encoding gene, was inserted at the p10 gene site. The antiviral potential of the coexpression product was five to ten times higher than that of any single-expression product. After heat and acid treatment, the remaining IFN-α antiviral activity of the coexpression product was approximately 22 kDa. ChIFN-γ proteins were detected in reBm-Cα and reBm-Cγ samples at approximately 19 kDa. No corresponding immunoreactive protein was detected in control samples that multi-IFN expression in the coexpression product was higher than that in the single-expression product. This result was consistent with the antiviral assay. The increase in expression level is due to deletion of the p10 gene. Just like polyhedrin gene, p10 is a nonessential gene for baculovirus replication and budded virus production. And also, it is nonessential for recombinant protein expression, but with high expression level (Hitchman et al., 2010). In recombinant baculovirus, polyhedrin promoter is the preference for foreign gene expression. The polyhedrin gene is replaced by a foreign gene. The saved cost of polyhedrin expression provides for the recombinant protein expression. In reBm-Cαγ, the deletion of p10 gene can also save a certain degree of gene expression, even though the p10 promoter is weaker than the polyhedrin promoter. And these saving costs could be used for foreign gene expression.

### TABLE 1 Antiviral activity of a mixture of two types of interferon

| Ratio of the two types interferon | Theoretical value (×10^4 IU/ml) | Measured value (×10^4 IU/ml) |
|----------------------------------|---------------------------------|-----------------------------|
| chIFN-α | chIFN-γ |                             |                             |
| 4      | 1      | 10                           | 10.04 ± 0.75                |
| 2      | 1      | 10                           | 10.22 ± 0.59                |
| 1      | 1      | 10                           | 11.41 ± 0.78                |
| 1      | 2      | 10                           | 17.92 ± 1.07                |
| 1      | 4      | 10                           | 14.05 ± 0.99                |
expression. Thus, in reBm-Cαγ, the deletion of p10 gene enhances the chIFN-α expression which driven by polyhedrin promoter, indirectly. This finding also provides an optimal direction for enhanced expression levels. However, the antiviral assay against VSV and MDV showed that the antiviral potential of the coexpression product was more than two times greater than that of any single-expression product. This difference must be due to the synergistic effect of the two types of IFN. Type I and II IFNs display diverse antiviral mechanisms. Here, coadministration of these two IFNs exhibited synergistic effects and elevated antiviral potential. Hence, the coexpression of the two IFNs by employing the BES is significant and beneficial. The coexpression products displayed greater antiviral activity and synergistic effects than the single-expression products. This strategy of combined administration of chicken IFNs can suppress viral diseases in the poultry industry.

The insertion of the chIFN-γ gene at the p10 gene site was mediated by the counterselection cassette rpsL-neo and phage λ-Red recombinase. There was no fundamental sequence or antibiotic resistance gene residue in the target gene site of the baculoviral genome. By employing this method, multiple gene insertion into various gene sites in one recombinant baculovirus can be achieved easily. This multigene baculovirus expression system can be a potential tool in many research fields. The BES is advantageous for antibody expression (Verma, Boleti, & George, 1998). Previously, heavy- and light-chain genes were inserted in the same gene site (Furuta et al., 2010). Using the multigene expression strategy in the present study, heavy- and light-chain genes can be introduced into different sites in one recombinant baculovirus, and enhanced expression levels will be achieved. rAAV packaging is another application area of the BES. In preceding studies, rAAV packaging required two or three recombinant baculoviruses, which individually contained cap, rep, and target genes (Aslanidi, Lamb, & Zolotukhin, 2009; Negrete et al., 2007). By employing our multigene expression strategy, rep, cap, and target genes can be inserted separately into the same recombinant baculovirus at the egt, p10, and polyhedron gene sites, respectively. The packaging efficiency of rAAV would thus be greater than that when using two or three recombinant baculoviruses (Galibert & Merten, 2011).

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| Sample                  | CK     | reBm-Cα | reBm-Cγ | reBm-Cαγ |
|-------------------------|--------|---------|---------|----------|
| Interferon dose (IU/well) | 0      | 800     | 800     | 800      |
| Plaque (PFU/well)       | 18.5 ± 1.8 | 11.8 ± 0.9 | 10.3 ± 1.4 | 0       |
|                          | 16.0 ± 2.5 | 9.8 ± 1.8   | 9.5 ± 1.5  | 0       |
|                          | 20.0 ± 4.0 | 12.5 ± 1.5  | 12.8 ± 1.7 | 0       |

**TABLE 2** Inhibitory effect of interferons on MDV

**CONFLICT OF INTEREST**

None declared.

**AUTHOR CONTRIBUTIONS**

Xingjian Liu: Conceptualization-Lead, Data curation-Lead, Formal analysis-Lead, Investigation-Lead, Methodology-Lead, Project administration-Lead, Resources-Equal, Validation-Lead, Writing-original draft-Lead, Writing-review & editing-Lead.

Xin Yang: Data curation-Equal, Investigation-Equal, Validation-Equal, Writing-review & editing-Supporting.

Arslan Mehboob: Investigation-Equal, Methodology-Supporting, Validation-Equal, Writing-review & editing-Supporting.

Xiaoyuan Hu: Investigation-Equal, Resources-Equal, Writing-review & editing-Supporting.

Yongzhu Yi: Investigation-Supporting, Writing-original draft-Supporting.

**ETHICS STATEMENT**

None required.

**DATA AVAILABILITY STATEMENT**

All data generated or analyzed during this study are included in this published article.

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**REFERENCES**

Alsharifi, M., Mullbacher, A., & Regner, M. (2008). Interferon type I responses in primary and secondary infections. *Immunology and Cell Biology, 86*(3), 239–245. https://doi.org/10.1038/sj.icb.7100159

Aslanidi, G., Lamb, K., & Zolotukhin, S. (2009). An inducible system for highly efficient production of recombinant adeno-associated virus (rAAV) vectors in insect SF9 cells. *Proceedings of the National Academy of Sciences of the United States of America, 106*(13), 5059–5064. https://doi.org/10.1073/pnas.0810614106

Berger, I., Fitzgerald, D. J., & Richmond, T. J. (2004). Baculovirus expression system for heterologous multiprotein complexes. *Nature Biotechnology, 22*(12), 1583–1587. https://doi.org/10.1038/nbt1036
APPENDIX

**FIGURE A1** ELISA assay of chIFN-γ expression in reBm-Cαγ and reBm-Cγ product. The reBm-Cαγ sample is larval haemolymph infected with coexpression recombinant baculovirus reBm-Cαγ, reBm-Cγ sample is larval haemolymph infected with the single-expression recombinant baculovirus reBm-Cγ. The mean absorbance (450 nm) of reBm-Cαγ sample was around 2.5 times as much as that of reBm-Cγ sample.