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Full Length Research Paper

Antiproliferative activity of recombinant human interferon-λ2 expressed in stably transformed BmN cells

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This study aimed at the generation of a stable transformed silkworm BmN cell line which can continuously express human interferon-λ2 (IFN-λ2) gene, and investigated the antiproliferative activity of this recombinant human IFN-λ2. Silkworm BmN cells were transfected with the recombinant vector pIZT/V5-His harboring the human IFN-λ2 gene. After the BmN cells were transfected with the pIZT/V5-His- hIFN-λ2 vector, the stably transformed BmN cells expressing hIFN-λ2 gene were selected using Zeocin. Following two months of screening, the transformed BmN cell line was obtained. Stable transformed BmN cell line can be maintained at a lower Zeocin concentration. The representing 26 kDa protein band of IFN-λ2 was detected by SDS-PAGE and Western blotting. The expression level of hIFN-λ2, determined by ELISA, was about 8.142 ng in 4×10^5 cells. The antiproliferative activity of hIFN-λ2 was determined by MTT assay. The 50% inhibitory concentrations (IC_{50}) of the recombinant hIFN-λ2 on A549 (lung cancer cells), HL60 (acute promyelocytic leukemia cells), BEL-7402 (liver cancer cells) and M231 cells (breast cancer cells) were approximately 3.21, 2.84, 6.29 and 9.32 ng/ml, respectively. In summary, Human IFN-λ2 can be stably expressed in the transformed BmN cell line, and the expressed recombinant hIFN-λ2 demonstrated antiproliferative activity to tumor cells *in vitro*.

Key words: Human interferon-λ2 protein, gene expression, antiproliferative activity.

INTRODUCTION

Interferons (IFN) can be divided into three major types: I, II and III. Human interleukin (IL)-28A, also known as IFN-λ2, belongs to type III IFNs (Meager et al., 2005). Like type I IFNs, IFN-λ has similar biological activities, such as antiviral activity, antiproliferative activity and *in vivo* antitumor activity (Kontsek et al., 2003; Sheppard et al., 2003; Kotenko et al., 2003; Langer et al., 2004). IFN-λ1 and IFN-λ2 share a common heterodimeric receptor, in which one subunit is a novel member of the class II cytokine receptor family (IL-28R or IFN-λR), and the other is identical to the second chain of the IL-10 receptor (IL-10R) (Coccia et al., 2004; Renauld, 2003; Kotenko, 2002; Langer et al., 2004; Dumoutier and Renauld, 2002). Both receptor subunits are constitutively expressed in a variety of human cells and tissues, after IFN-λs and its receptors binding, induce the heterodimers of receptors, activate the Janus kinases-signal transducers and activator of transcription (JAK-STAT) signaling pathway. It is well documented that this receptor-ligand system is likely to contribute to antiproliferative or other immune defense activities (Langer et al., 2006; Ihle and Kerr, 1995). With regard to IL-28 activity, a number of studies have been conducted by Li et al. (2006) and Bhushan et al. (2007). However, the expression levels of IFNs are very low, and the expression products were delivered in serum, due to the complexity of their com-position. These systems are not conducive to massive production and further therapeutic applications. The *Escherichia coli* expression system is a prokaryotic system, therefore

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post-translational modification of the protein may be inadequate or absent. The study resorted an alternative way to express the IFNs. Using insect cells to express exogenous genes is an important tool in bio-technology owing to the lower expense. Furthermore, the insect cell can grow without serum; purification of the expression product can be easily achieved under the serum-free insect cell culture condition. These techniques and expression technology have been utilized by Zhao et al. (2009) and Li et al. (2009), who have successfully expressed hIGF-1 (insulin-like growth factor-I) and hGM-CSF (granulocyte-macrophage colony-stimulating factor) in transformed silkworm cells.

To further understand the potential physiological and pathophysiological roles of IFN-λ2, recombinant insect vectors containing IFN-λ2 cDNA was constructed, investigated the feasibility of liposome transfected on BmN cells and whether they could express functional recombinant human IFN-λ2 and at what levels it could be expressed. This study aimed to construct stable IFN-λ2 expressing cell lines and also conduct further studies investigating its anti-tumor activity.

MATERIALS AND METHODS

IFN-λ2 amplification

The DNA template used was pUC57 (IFN-λ2), which was constructed in the laboratory. The primer sequences employed in the amplification of IFN-λ2 were designed with Primer 5.0 software. The primers were synthesized by Bioasia Bioengineering (Shanghai, China) and their sequences were 5'-TAG AAT TCA TGA AAC TAG ACA TGA C-3' (P1; *Eco*RI site underlined) and 5'-TTG ATA TCG ACA CAC AGG TCC CCA CTG-3' (P2; *Eco*RV site underlined). The polymerase chain reaction (PCR) was carried out on a TC-48/T/H (a) Peltier Thermal Cycler (Hangzhou, China). The amplification profile included an initial denaturation step at 95°C for 4 min, followed by 32 cycles at 94°C for 50 s, then 50°C for 50 s and 72°C for 1 min, with a final extension step at 72°C for 10 min.

Generation of recombinant vectors

The PCR products were separated on 1% (w/v) agarose gels. The relevant bands containing IFN-λ2 cDNA were extracted and recovered from the agarose gels using a UNIQ-10 DNA gel extraction kit (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd, China). The purified IFN-λ2 cDNA fragments were inserted into the pIZT/V5-His vector (Invitrogen, USA) using the pIZT cloning technique according to the manufacturer's instructions. The pIZT/V5-His vector and the PCR product were digested with the restriction enzymes *Eco*RI and *Eco*RV, and then ligated at 14°C with T4 DNA ligase. The vector was transformed into competent *E. coli* TOP10 using a $CaCl_2$ method and then transformed clones were selected on agar plates containing Zeocin. The positive recombinant clones were confirmed by colony PCR and restriction enzyme digestion. The sequences of the inserted fragments were confirmed by digestion with *Eco*RI and *Eco*RV.

Cell culture and transfection experiments

The BmN cell line, a type of oocyte cell from silkworm, was obtained from the School of Basic Medical and Bioscience of Suzhou University (Suzhou, China).The BmN cells were routinely grown in TC-100 medium (Invitrogen) supplemented with 10% (v/v) FCS at 26°C. Large scale plasmid DNA preparations and extraction of genomic DNA from cells were carried out using conventional methods. Transfection of pIZT/V5-His-IFN-λ2 was conducted with Lipofectamine 2000 (Invitrogen) in 6-well plates according to the manufacturer's instructions. The pIZT/V5-His vector (Invitrogen) was used as a control to check transfection efficiency and to optimize transfection conditions. Untransfected BmN cells and liposome DNA were added to wells to serve as controls. The day before transfection, BmN cells were collected and seeded into 6-well plates at 1×10^6 cells/well in order to achieve 70 to 80% confluence on the day of transfection. Lipofectamine 2000, diluted in TC-100, was added to the plasmid mixture and incubated for 20 min at room temperature. The BmN cells were transfected by adding 2 µg plasmid and 8 µl of the original concentration of Lipofectamine 2000 per well. After 24 h, the transfected BmN cells were selected with Zeocin (300 to 400 µg/ml) for eight weeks. The transfection efficiencies were estimated by directly observing green fluorescence under a microscope. Stable transformed BmN cell line may reduce the concentration of Zeocin to 50 µg/ml for maintenance. The resistant clones containing IFN-λ2 proteins were identified by Western blotting and ELISA.

A549, HL60, M231 and BEL7402 cell lines were routinely grown in RPMI 1640 complete medium containing 10% (v/v) FCS in a humidified atmosphere of 5% CO_2 at 37°C.

BmN cells transfected with pIZT/V5-His-IFN-λ2 cDNA were harvested after three weeks. Cells were suspended in a 0.65% (w/v) NaCl solution and centrifuged at 8 000 ×g. The cell pellet was resuspended in 1 × PBS (pH 7.4) with the same amount of 2 × SDS sample buffer and boiled at 100°C. The proteins were then separated by SDS-PAGE before being transferred to PVDF membranes (Millipore, MA, USA). Membranes were incubated in 3% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 in PBS at room temperature for 1 h to block non-specific protein binding sites, and then incubated with a 1:1000 dilution of mouse anti-His antibody (Invitrogen) at 37°C for 30 min. Following this, the membranes were washed and then incubated with a 1:1000 dilution of peroxidase conjugated goat anti-mouse IgG for 30 min at 37°C. Visualization of a positive signal was conducted with diaminobenzidine (DAB). Each band was analyzed on a Tanon GIS analysis system.

ELISA

BmN cells transfected with pIZT/V5-His-IFN-λ2 cDNA were harvested after eight weeks. Cells were suspended in 1 × PBS (pH 7.4) solution and centrifuged at 8 000 ×g to collect the cells. The supernatant was discarded and the cell pellet resuspended using 1 ml ELISA sample diluent. An ultrasonic disrupter was used to break open the cells, then samples were centrifuged and the resulting supernatant stored at -20°C for later use in the ELISA. The ELISA was performed using an IFN-λ2 assay kit (Uscn and Life Technology Company, China).

Antiproliferative assay

The antiproliferative activity of IFN-λ2 was assessed by determining how it inhibits tumor cell growth. The cells transfected with blank vectors pIZT/V5-His was as the negative control. BmN cells transfected with pIZT/V5-His-IFN-λ2 and pIZT/V5-His were disrupted ten times by an ultrasonic processor (Newtown, USA) and supernatants collected by centrifugation (8 000 ×g, 5 min, 4°C). The protein concentration was determined using the IFN-λ2 assay kit according to the manufacturer's instructions. IFN-λ2 was diluted in

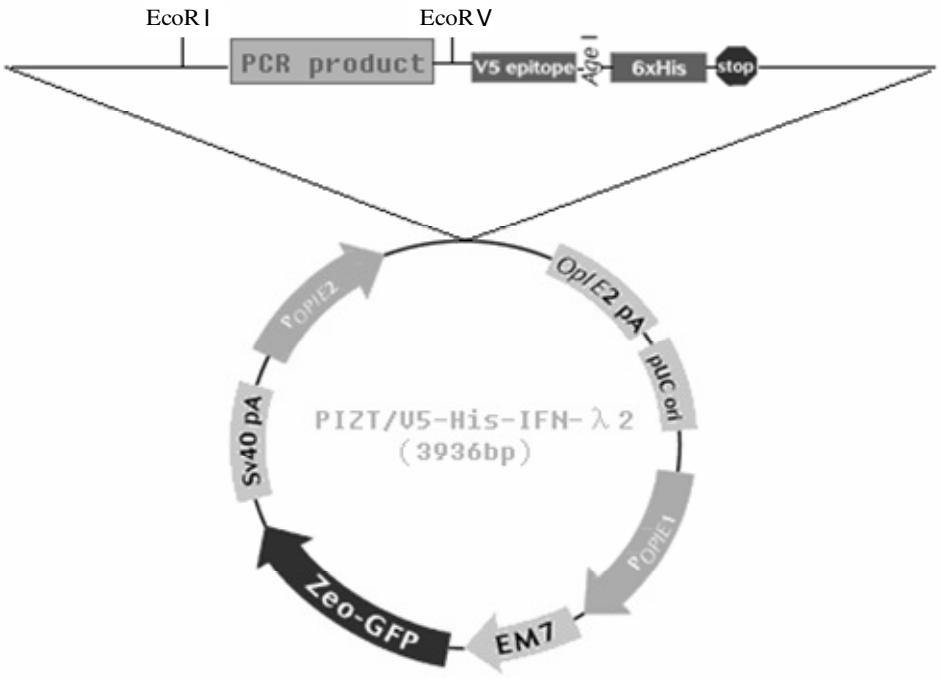

Figure 1a. Map of the constructed vectors, pIZT/V5-His-hIFN-λ2. pUC or1, pUC origin; POpIE1, POpIE1 promoter; EM7, EM7 promoter; Zeo-GFP, Zeocin resistance and GFP gene; SV40 pA, SV40 pA polyadenylation signal; POpIE2, POpIE2 promoter; PCR product, PCR product of IFN-λ2 gene; 6×His, polyhistidine tag; OpIE2 pA, OpIE2 pA polyadenylation signal.

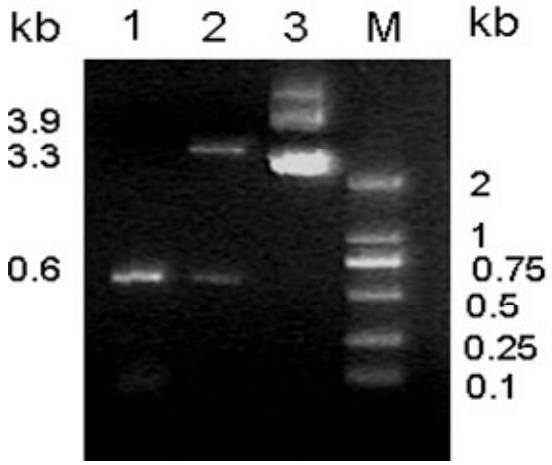

Figure 1b. Identification of the constructed vectors pIZT/V5-His-hIFN-λ2. 1, PCR product of pUC57 (hIFN-λ2 primers); 2, pIZT/V5-His-hIFN-λ2/ EcoRI + EcoRV; 3, pIZT/V5-His-hIFN-λ2; M, DNA maker.

PBS to a final concentration of 8 μg/L. An equal number of A549, HL60, M231 and BEL7402 tumor cells in logarithmic phase were diluted with RPM1640 complete medium containing 10% FCS to a concentration of 1×10^5 cells/ml. Approximately 100 μl each of cell suspension was plated into each well of a 96-well plate for 24 h and treated with two-fold serial dilutions of IFN-λ2 and blank vectors. To all the wells, 10 μl methyl thiazolyl tetrazolium (MTT) was added and incubated for 4 h. The reaction was terminated by adding 100 μl 10% SDS-HCl into every well and then plates were incubated at 37°C overnight. The next day, the absorbance value (A value) of samples was measured using a spectrophotometer at wavelength 570 nm. The growth inhibiting curve was plotted with the A value on the y-axis and time on the x-axis. The cell growth inhibition ratio was calculated to detect the growth inhibitory effect of IFN-λ2 on A549, HL60, BEL7402 and M231 tumor cells. Inhibition ratio (%) = [(1-A value of experiment groups)/ (A value of cell control group)] × 100. The 50% inhibitory concentrations (IC_{50}) were calculated as described previously (Jiang, 1999). The experiments were repeated three times and carried out in duplicate.

RESULTS

Construction of recombinant vectors

PCR products were inserted into the vector pIZT/V5-His efficiently and a map of the constructed pIZT/V5-His-IFN-λ2 plasmid is shown in Figure 1a. The protein was expressed as a fusion protein with a V5 epitope and a polyhistidine tag for detection or purification. Digestion of pIZT/V5-His-IFN-λ2 with *Eco*RI and *Eco*RV resulted in two DNA fragments approximately 2.7 kb and 600 bp, respectively, as expected (Figure 1b).

Screening, identification of stability in transfected cells and expression level of hIFN-λ2

BmN cells transfected with pIZT/V5-His-IFN-λ2 were

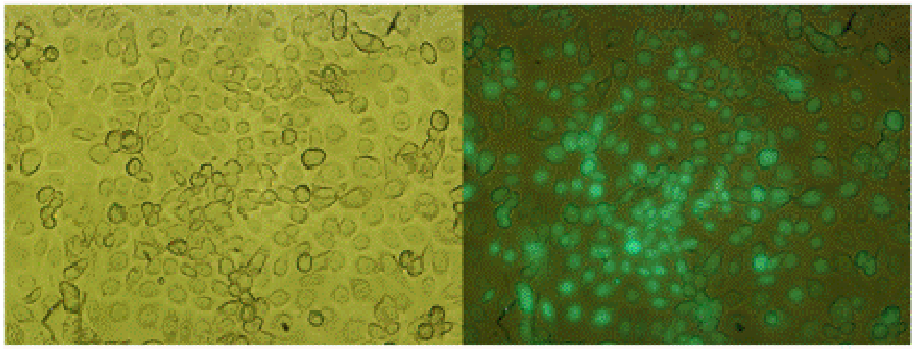

Figure 2. Transfected BmN cells with pIZT/V5-His-hIFN-λ2 (100×) . a, BmN-hIFN-λ2 cell (under natural light vision); b, BmN-hIFN-λ2 cell (under fluorescent light vision).

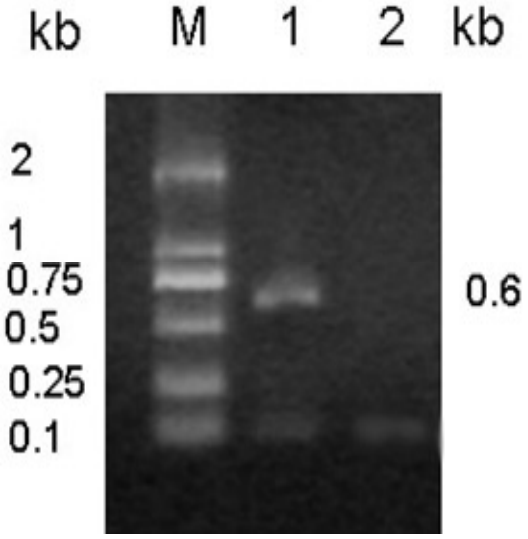

Figure 3. PCR identification of Transfected BmN cells with pIZT/V5-His-hIFN-λ2. M, DNA maker; 1, PCR product of transfected BmN cells with pIZT/V5-His-hIFN-λ2 (hIFN-λ2 primers); 2, Transfected BmN cells with pIZT/V5-His (hIFN-λ2 primers).

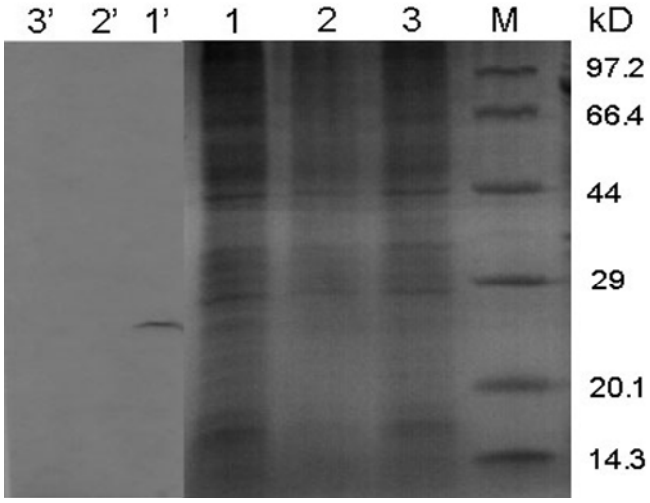

Figure 4. SDS-PAGE and Western blotting of BmN-hIFN-λ2. 1, transfected BmN cells with pIZT/V5-His-hIFN-λ2; 2, transfected BmN cells with pIZT/V5-His; 3, normal BmN cells; M, protein marker; 1', 2', 3', Western blotting, corresponding to lanes 1, 2 and 3.

observed under a microscope to visualize green fluorescence. Maximum transfection efficiency was achieved four weeks post-transfection and was approximately 80% (Figure 2). This study designated this stable transfected cell line BmN-IFN-λ2. The identity of this stable transformed cell line was identified by PCR on the genomic DNA from the cells (Figure 3). Expression of IFN-λ2 was confirmed by visualizing green fluorescence and Western blot with a band at approximately 26 kDa corresponding to IFN-λ2 (Figure 4). The expression level of hIFN-λ2 was approximately 8.14 ng in 4×10^5 cells (Table 1 and Figure 5). By contrast, the expression of hIFN-λ2 in normal BmN cells was not detected and the blank vector transfected cells.

Antiproliferative activity

The antiproliferative activity of hIFN-λ2 expressed in the BmN cells was estimated by inhibiting the growth of tumor cells. The results showed that IC_{50} of recombinant hIFN-λ2 on A549, HL60, BEL-7402 and M231 cells were approximately 3.21, 2.84, 6.29 and 9.32 ng/ml, respectively (Figure 6). And these results indicated that the inhibitory effect of recombinant hIFN-λ2 on HL60 was best in the four kinds of tumor cells.

DISCUSSION

Insect cell systems are widely used to produce proteins,

Table 1. The hIFN-λ2 activity of the transformed cell lines BmN-hIFN-λ2.

Sample	hIFN-λ2 standard (pg·mL^{-1})					OpIE-hIFN-λ2 cell line (cell·mL^{-1})	Normal cell (cell·mL^{-1})	CK
	1000	500	250	125	62.5	4 × 10^5	4×10^5	
OD_{450}	0.290	0.250	0.230	0.221	0.217	0.862	0. 017	0. 013

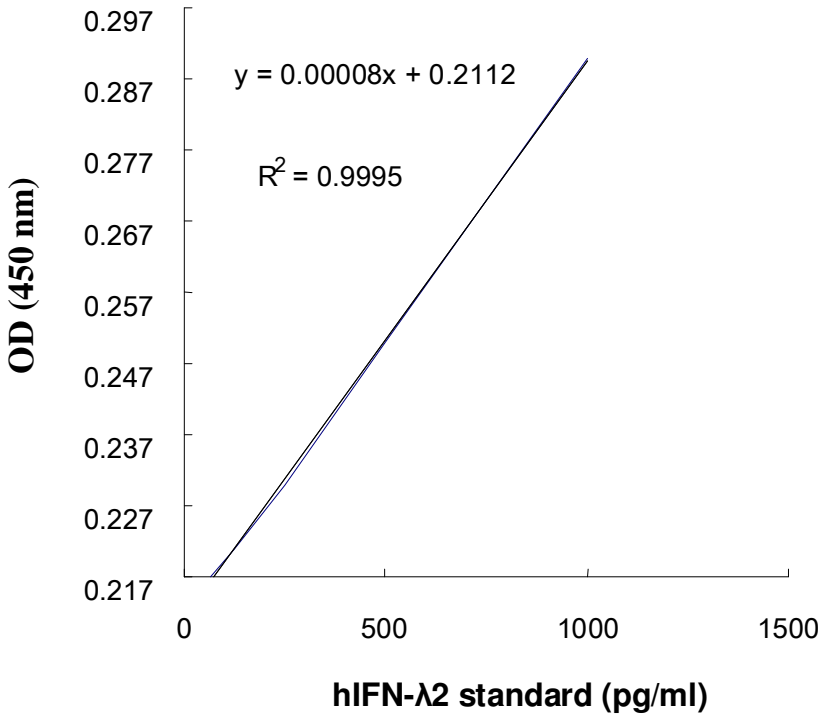

Figure 5. The calibrator curve of hIFN-λ2 tested by ELISA.

because insect cells have a similar pattern and capacity of co-translational and post-translational modifications as mammalian cells, such as glycosylation, phosphorylation and protein processing. There are two usually employed strategies, one is to use an insect expression vector and the other is to use transposable elements, for example PiggyBac. PiggyBac is a class II movable component, first isolated from a *Trichoplusia ni* cell line. The functions of the PiggyBac transposon have been previously identified and developed into an important insect embryo transformation system (Handler et al., 1998).

In the present study, hIFN-λ2 expression was investigated using a new and highly efficient insect expression vector, pIZT/V5-His in silkworm cells *in vitro*. The pIZT/V5-His vector was developed by invitrogen for stable expression of exogenous genes in Sf-9 insect cells. This vector employed the IE-2 promoter from Orgyia pseudotsugata nucleopolyhedrovirus (OpNPV) to control exogenous gene expression. Moreover, with the assistance of the Zeocin resistance marker, it is easy to screen and determine the integration of the exogenous gene into the host cell genome (Zhou et al., 2007; Wilson et al., 2007; Balu et al., 2005; Guo et al., 2000).

In this study, the biological effects of hIFN-λ2 on tumor cell growth were also investigated and demonstrated that hIFN-λ2 had specific antitumor activity *in vitro*. The MTT assay results indicated that hIFN-λ2 obviously inhibited the growth of the A549, HL60, M321 and BEL-7402 cell lines. HIFN-λ2 displayed variable IC_{50} on different tumor cells, this divergence may be related to the number of hIFN-λ2 receptors, as well as the type of the tumor cells. Previous research indicates that hIFN-λ displays selectivity against tumor cells. HIFN-λ has antiviral and antiproliferative activities and these activities may be dependent on the expression of IFN receptors (Langer et al., 2004; Weissmann and Weber, 1999; Meager et al., 2005; Uzé and Monneron, 2007).

In this study, a stable transformed BmN cell to express hIFN-λ2 was successfully constructed. Meanwhile, it has been shown that the blank vector transfected cells had no

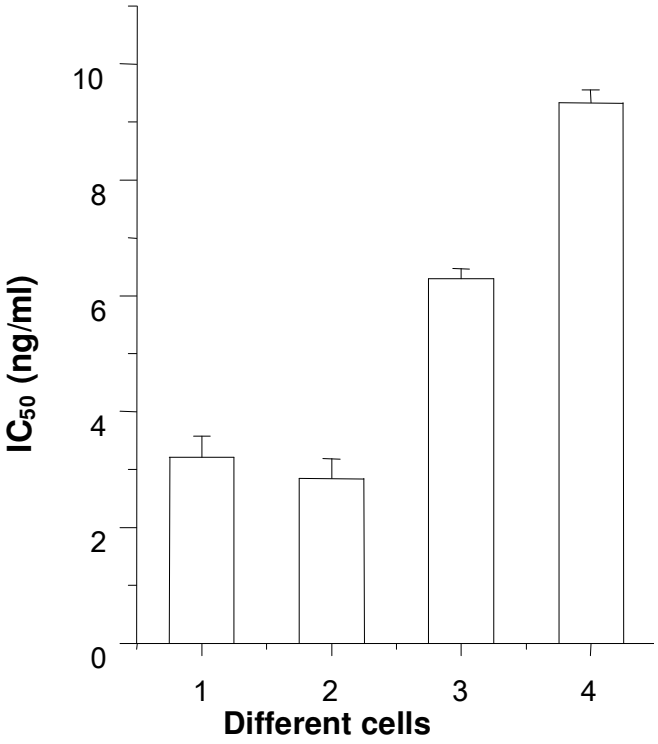

Figure 6. Antiproferative activity of BmN-hIFN-λ2. Lane 1, the inhibitory concentration (50%) of IFN-λ2 on growth of A549; Lane 2, the inhibitory concentration (50%) of IFN-λ2 on growth of HL60; Lane 3, the inhibitory concentration (50%) of IFN-λ2 on growth of BEL7402; Lane 4, the inhibitory concentration (50%) of IFN-λ2 on growth of M231; n = 3. Data are mean ± SD.

anti-proliferative activity; by contrast, the transfected cell could produce functional antitumor hIFN-λ2. Previous research has demonstrated that IFN-λ may hopefully reduce the severe side effects associated with type I IFN treatments (Ma et al., 2006; Sterlund et al., 2005; Meager et al., 2002). These results presented here have laid a solid foundation for further study of hIFN-λ anti-tumor mechanisms, as well as the therapeutic potential of hIFN-λ.

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