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Molecular Identification and Expression Analysis of the *Interferon Beta* Gene in Sheep (*Ovis aries*)

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Abstract: Interferon beta (IFN-β) belongs to the group of inflammatory cytokines that are expressed in eukaryotic cells as an early response to viral infection. Sheep as an important livestock in the world, however, little is known about sheep Interferon beta (sIFN-β) and its role in immune responses. In this study, researchers amplified the cDNA of sIFN-β from sheep spleen by RT-PCR using primers according to the cattle IFN-β. The ORF of sIFN-β covers 561 bp encoding 186 amino acids. Sequence prediction shows that the amino acid of sIFN-β possess 22 aa signal peptide domain and two putative N-glycosylationlation sites. qPCR analysis indicated that sIFN-β mRNA was predominantly expressed in lymphoid node. The 36 kDa recombinant sIFN-β protein was efficiently expressed and purified using metal chelate affinity chromatography (Ni-NTA) then confirmed by SDS-PAGE and Western blotting. This study provides a deeper understanding of sIFN-β molecule structure and also contributes to further studies.

Key words: Sheep, interferon beta, tissue distribution, chelate, amino acid

INTRODUCTION

Type I IFN demonstrate a complex evolutionary history that has resulted in the divergence of at least eight distinct subfamilies: IFN-α, IFN-δ, IFN-ε, IFN-κ, IFN-ζ IFN-τ, IFN-ω and IFN-β (Krause and Pestka, 2005). Type I IFNs are the major contributors in innate immunity against viruses. IFN-β induces strong antiviral effects and is therefore an attractive agent to prevent or reduce the incidence of virus-mediated disease. Prophylactic IFN-β on respiratory epithelial cells infected with Rhinovirus (RV) show that IFN-β has not only a strong but also a long-lasting protective effect against RV infection (Gaajetaan *et al*., 2013). Early post exposure treatment with IFN-β significantly increased survival time of rhesus macaques infected with a lethal dose of Ebola virus. Early treatment with IFN-β also significantly increased survival time after Marburg virus infection (Smith *et al*., 2012).

However, IFN-β can also inhibit the growth of human malignant glioma and induces glioma cell apoptosis using the human *IFN-β* gene transfected into glioma cells. The possible mechanism of this procedure is induced S-phase cell cycle arrest in a p53-dependent manner by activating the ataxia telangiectasia mutated-dependent DNA damage pathway through both caspase-dependent apoptosis and necroptosis in cancer cells (Guo *et al*., 2012; Huang *et al*., 2012; Robinson *et al*., 2012). It was also shown that an aberrant activation of immune systems by high levels of type I IFNs contributes to the development of autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (Ronnblom and Alm, 2003).

Until now, 5 type I IFN genes IFN-α, IFN-ε, IFN-ω, OvIFN-δ and the new cytokine IFN-τ were reported to play an important role in maternal-fetal interactions during early pregnancy (Cochet *et al*., 2009; Leaman and Roberts, 1992; Lefevre and Boulay, 1993; Liu *et al*., 1996; Martal *et al*., 1998; Whaley *et al*., 1991). Sheep as an economically important animals of livestock farming, there is no study about the sequences of the *sIFN-β* gene, its phylogenetic classification and properties.

MATERIALS AND METHODS

Samples and RNA isolation: Samples of different tissues were collected from healthy female Chinese domestic sheep of about 6 months old and weighing 30-40 kg

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in breeding base of Yunnan Animal Science and Veterinary Institute, Yunnan, China. All animals used in this study have been approved by the scientific ethical committee of the Yunnan Animal Science and Veterinary Institute. About 7 tissues samples of spleen, heart, liver, lung, kidney, lymphoid node and muscle about 250 mg were disrupted and homogenized using a ribolyser and liquid nitrogen. Total RNAs was extracted using a TaKaRa kit and DNase treated using the DNase I kit (TaKaRa, Japan). Samples were eluted into 50 μL of nuclease free water. The final concentration of RNA was quantified using spectrophotometric OD260 measurements and purity was assessed by OD260/OD280 ratio. RNA integrity was electrophoretically verified on 1.2% agarose gels followed by ethidium bromide staining. RNAs were stored at -80°C until use.

PCR amplification of sIFN-β and analysis the tissue distribution: Primers for isolation sIFN-β were designed according the sequence of cattle which were closely related to sheep. PCR primers were designed out of the Opening Reading Frame (ORF) to get the full length of sIFN-β (Table 1). First-strand cDNA was synthesized using Reverse Transcriptase (Takara, Japan) according to the manufacturer's protocol. PCR conditions (30 cycles) were as follows: denature for 30 sec at 94°C, annealing for 30 sec at 55°C and extension for 1 min at 72°C. The PCR product was gel purified, cloned into the pMD19-T vector (Takara, Japan) and sequenced. The nucleotide sequence was submitted into Genbank. IFN-β mRNA expression was analyzed by relative quantitative two step RT-PCR. PCR amplification was then performed in triplicate on an ABI 7500 Fast (Applied Biosystems, USA) by SYBR Green Method with the cycling conditions (50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min). Data was normalized using (GAPDH).

Nucleotide sequence accession numbers and bioinformatics analyses: The new identified sequences used in this study have been deposited in the GenBank database: sheep, JX458084. Other sequences cited are as follows, goat, JX458085, cattle, NM_174350, pig, NM_001003923, horse, NM_001099400, cat, NM_001009297, dog, FJ194477, human, NM_002176, raccoon, EU916272, monkey, NM_010510, rat, NM_019127, mink, NM_001135795, chicken, NM_001024836. The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.Expasy.Org/) and the protein domain features of IFN-β were determined by using simple modular architecture research tool (http://smart.embl-heidelberg.de/). Sequence alignment was performed with the ClustalW and Mega 5.0.

Table 1: Primer sequence used in this study

Primers	Direction	Nucleotide sequence (5'-3')	Products (size)
IFN-β FW	Forward	atttcatcatgacctaccggtg	593 bp
IFN-β RV	Reverse	ctgacaggtcttcagtttcgg	
sIFN-β qFW	Forward	gcttctccactacggctctt	172 bp
sIFN-β qRV	Reverse	aactgctgcggctgctta	
GAPDH FW	Forward	aacctgccaagtatgatgag	119 bp
GAPDH RV	Reverse	agtgtcgctgttgaagtc	
rIFN-β FW	Forward	CGGGATCCatgacctaccggtgcct	578 bp
rIFN-β RV	Reverse	CCGCTCGAGtcagtttcggaggtaac	

Capital letters represent restriction endonuclease BamHI and XhoI and the protection nucleotides

Protein modeling: A three dimensional structures of the protein from its amino acid sequence were generated by Modeller. Easy Modeler, based on PerlTK uses a very simple and easy interface to implement the features of Modeller 9.7 (Tian *et al*., 2010). The interferon structure of sheep, human and cattle was modeled using Easy Modeller 2.0 and Swiss PDB viewer with 3V2W (Knapp *et al*., 2008). The initial model building and structural alignment was performed and the modeled protein was visualized using PyMol 1.5 (Seeliger and de Groot, 2010).

Expression of recombinant sIFN-β protein: The cDNA encoding the INF-β was amplified by PCR using primers rINF-β FW and rINF-β RV. The PCR product digested with BamHI and XhoI was subcloned into the pET32a expression vector (Clonetech, USA), forming a sequence encoding a fusion protein of sINF-β and an NH2-terminal His6-tag. This recombinant plasmid was sequenced and termed pET32a-sIFN-β. The constructed recombinant plasmid pET32a-sIFN-β was transformed into competent *Escherichia coli* BL21 (DE3) cells (Novagen, USA). The induction scheme after several tests was established as follows: final Isopropyl-b-Thiogalactopyranoside (IPTG) concentration, 0.2 mM; induction temperature, 30°C; total induction hours, 4 h and shaking speed 150 rpm. After induction, cells were harvested by centrifugation at 6000 g for 10 min at 4°C. The sIFN-β protein was collected by refrigerated centrifugation at 12000 g for 15 min at 4°C after sonication and was purified with Ni-NTA affinity chromatography (Qiagen, Germany).

Proteins from *E. coli* DE3 cell extracts were separated by SDS-PAGE and transferred to a PVDF membrane. Fusion protein was detected against anti-6His monoclonal. The signal was detected by ECL Plus reagent (Invitrogen, USA).

RESULTS AND DISCUSSION

Characterization of *sIFN-β* gene: sIFN-β was amplified using RNA extracted from sheep spleen tissue. The full length cDNA of sheep *IFN-β* gene with 561 bp was

obtained and deposited in GenBank (No. JX458084). It encodes a protein of 186 amino acids with 22 aa amino acids signal peptide domain and a 152 aa mature peptide. The molecular weight of sIFN-β is about 22.30 kDa with the calculated Isoelectric Point (PI) being 5.14.

Three cysteine (cys) sites were predicted which lies in Cys 17, 31 and 141. Cys residues are widely conserved with all IFN-β except rat and mouse. IFN-β contains one disulphide and one free cysteine that the disulphide bond was predicted to be between Cys 31 and 141. The putative N-glycosylation site (Asn-X-Ser/Thr) which exists in mouse and human is also found in sheep. The positions of substituted residues in the various subtypes are as indicated (Fig. 1). sIFN-β shows two unique potential N-glycosylation sites: one site conserved at position Asn 101 and another at Asn 131.

***sIFN-β* gene is closely related to goat and cattle *IFN-β* gene:** To clearly delineate the evolution of sIFN-β, researchers analyzed the phylogenetic relationship of the IFN-β of sheep, goat, cattle and other 11 species based on amino acid alignments. The analysis showed that sIFN-β clustered with goat and cattle IFN-β (Fig. 2). The relationship between sheep and cattle is much closer than between human and mouse. Multiple sequence alignment results showed that the sIFN-β of sheep and cattle share significant homology, 91.3% identity at the nucleotide level and 90.4% similarity at the protein level for the coding region.

Structure of the sIFN-β has typical characteristics of type I interferons: The predicted structure model of

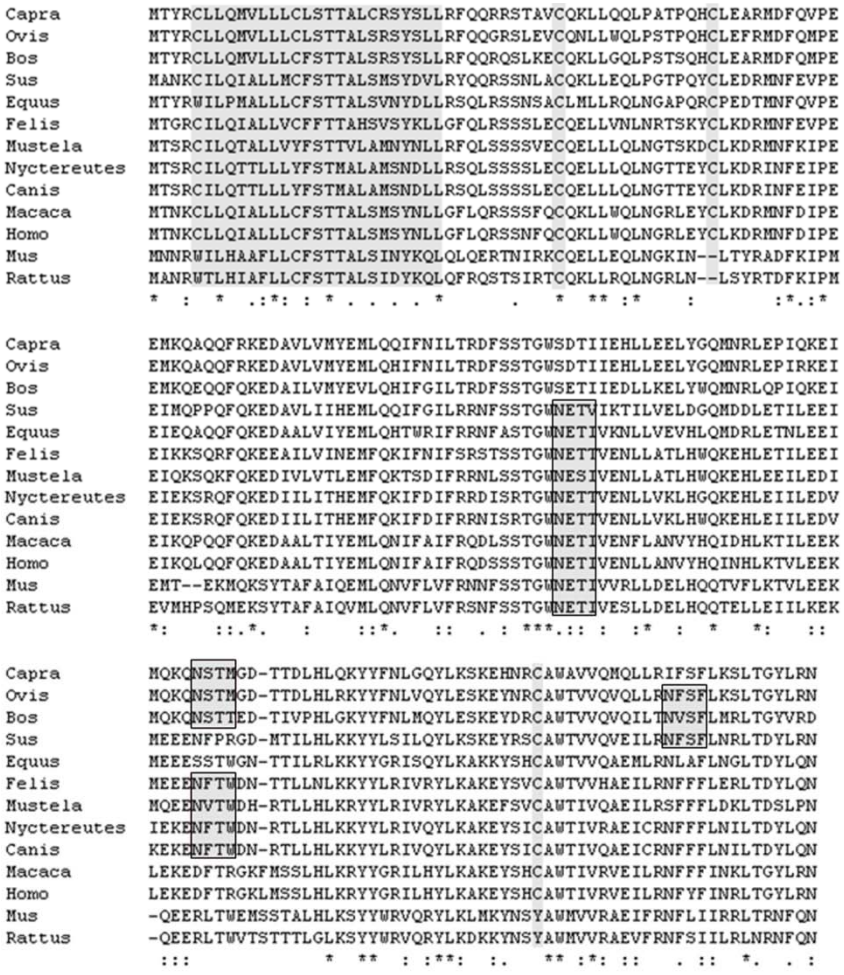

Fig. 1: Alignment of the deduced amino acid sequence of sINF-β. The putative signal peptide sequences predicted by SignalP and conserved cysteines are show in grey. Predicted glycosylation sites ate boxed. The identical amino acid residues are marked '*', conserved and semiconserved residues are depicted by ':' and '.,' respectively. Dashes indicate gaps introduced into the sequence to optimize the alignment

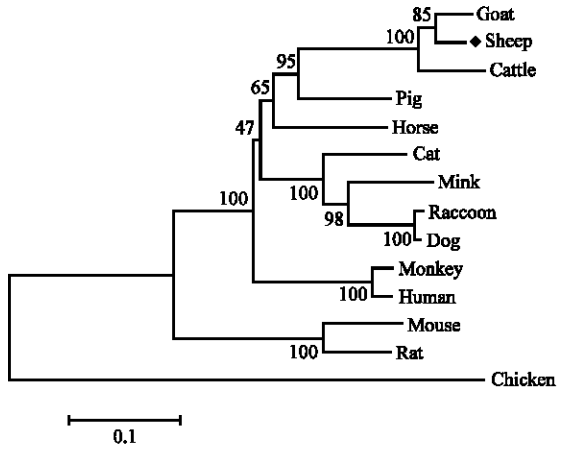

Fig. 2: Phylogenetic analysis based on amino acid alignments of sIFN-β with representative vertebrate species. Unrooted phylogenetic trees were constructed using the full length protein sequences with the neighbor-joining method within the MEGA5 package and bootstrapped 1000 times. The phylogenetic relationships among 13 placental mammals *IFN-β* genes and chicken *IFN-β* genes were retrieved from GenBank and their accession numbers

sheep and cattle was constructed according to human IFN-β (hIFN-β) shows that sIFN-β protein molecule roughly cylindrical in shape which belong to the family of long-chain helical cytokines. It has a characteristic barrel-shaped tertiary structure formed by the up-down arrangement of five α-helices, A (residues 36-34), B (residues 73-91), C (residues 102-125), D (residues 139-154) and E (residues 163-183). Helices A, B, C and E form a left-handed, type II four-helix bundle (Fig. 3a). Compared to human interferon model, structures of sIFN-β and cattle IFN-β (cIFN-β) are highly similar to their corresponding human proteins except the β-sheet.

By homology modeling both sheep and cIFN-β have putative tertiary structures very similar to each other (Fig. 3b). The only difference is β-sheet of hIFN-β was not same as cattle and sheep. These data suggest that the predicted structures of both human and sheep IFN-β have the general characteristics of IFN-β.

Tissue expression analysis of sIFN-β mRNA: The expression of sIFN-β in various tissues was analyzed by real-time qPCR and normalized to sheep GAPDH level. A dissociation curve shows a single peak at the melting temperature expected for that replication suggested specific amplification. As shown in Fig. 4, SYBR Green real-time qPCR analysis revealed that IFN-β was expressed in all tissues studied with highest mRNA level observed in lymphoid tissue.

Expression and purification of the sIFN-β protein: The sIFN-β protein was expressed with the pET32a plasmid. Most of the resulting protein was found in inclusion bodies. A predominant protein at 36 kDa was observed by SDS-PAGE analysis (Fig. 5, lane 2, 3) and there was almost no basal expression of sIFN-β without induction by IPTG (Fig. 5, lane 1) panel A. After one step purification of soluble fusion, the yield of eluted soluble proteins with purity no <90% was about 1 mg mL^{-1} (Fig. 5, lane 1) as assessed by the brad ford method. The expressed sIFN-β was confirmed by western blotting using an anti-His6-tag mouse antibody (Fig. 5, Lane 2) panel B.

The IFN-β are a family of cytokines with pleiotropic activities that include inhibition of viral replication and cell proliferation and activation of the immune system (Stark *et al*., 1998). Therefore, understanding the property of sIFN-β and expression the protein could lead to the development of novel therapeutic approaches to the treatment of sheep infection diseases.

In this study, researchers obtained the full length ORF of sIFN-β based on the most related species sequence. The results show that sIFN-β contained many conserved features such as share same signal peptide sequence and same large ORF when it was compared with other IFN-β. Sequence analysis shows that sheep has low homology with rat and chicken but it is higher with goat and cattle. These similarities of *IFN-β* genes reflect the origins of IFN-β and subsequent evolution. Since, all IFN-β act same function thus researchers conclude that they evolve from common ancestral genes and that the duplications that gave rise to the current subtypes. Phylogenetic analyses also indicate that sheep, goat and cattle is belong to same clade but form distinct clade with chicken.

The predicted structure and possible residues involved in the biological activity of the sIFN-β were identified based on the previous analysis of the residues involved in the biological activity of IFN-β. The results of aligning the primary structures of IFN-β enable us to predict which domain might impact the function of sIFN-β.

sIFN-β contains eight Asn residues with the NXS/T motif as N-linked glycosylation suggesting that sIFN is glycosylated. For most animals, the glycosylation site lies Asn 101 with consense NETT (Van-Pesch *et al*., 2004). Sheep have two predicted functional site which use NSTM and NFSF as common consense but this two site are not conserved in other mamals. However, the antiviral activity of fishes (zfIFN) has been demonstrated in

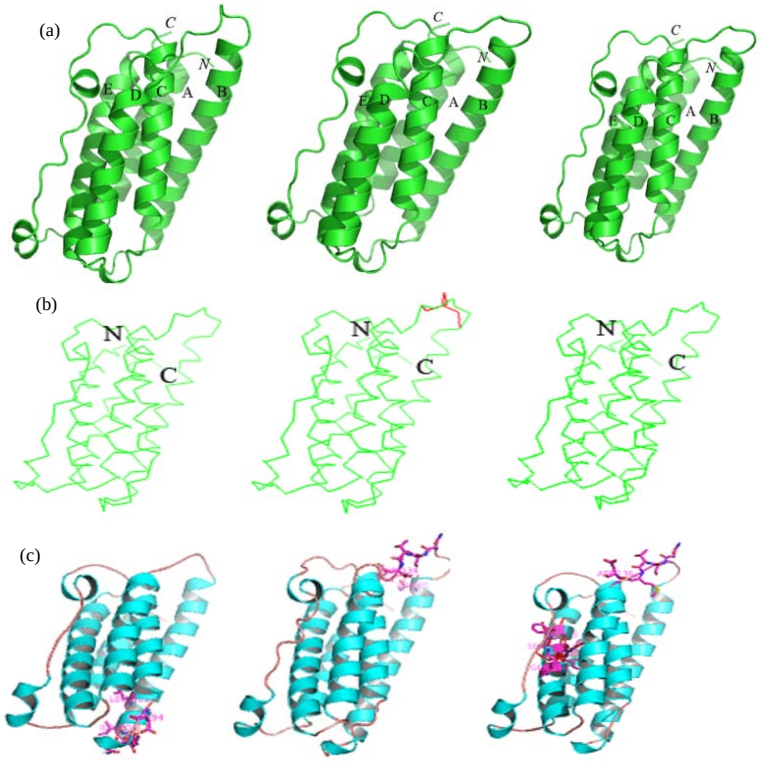

Fig. 3: The predicted structure of the sIFN-β: a) Backbones of sIFN-β (right) compared with cIFN-β (middle) and hIFN-β (left); b) Comparison of sIFN-β structures. The structure of sIFN-β (right) compared with cIFN-β (middle) and hIFN-β (left); c) Predicted glycosylation sites of sIFN-β (right) compared with cIFN-β (middle) and hIFN-β (left). The same structure was shown in green while the difference was shown as red. The N and C termini are labeled as well. Glycosylation sites (Asn) and the binding sites are shown in purple

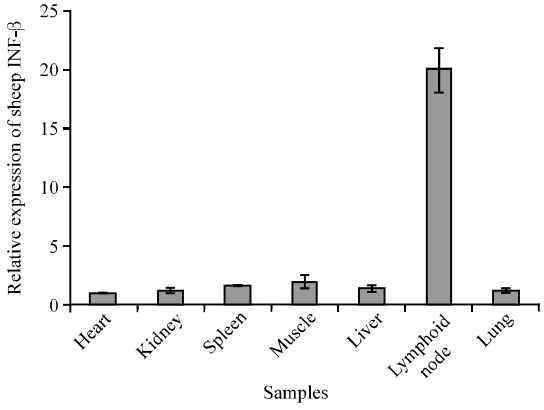

Fig. 4: sIFN-β expression levels in different tissues were analysised by qPCR. Mean mRNA levels (including standard deviations) in 7 tissues were analyzed. Data were $2^{-\Delta\Delta Ct}$ levels calculated relative to the tissue with lowest expression (heart) set to 1, normalized against GAPDH mRNA levels. Vertical bars represented the mean±SE

earlier report but glycosylation site does not exist thus it can be concluded that glycosylation does not appear to be necessary to play antiviral effect. Hence, glycosylation is unlikely to influence binding activity but might enhance IFN-β stability *in vivo*.

IFN-α/β are massively produced upon viral infection however, there is evidence for the constitutive expression of IFN-α/β, albeit at very low levels in the absence of viruses or other IFN inducers (De Maeyer-Guignard *et al*., 1988; Gresser, 1990). The IFN-α/β mRNAs can be detected in normally growing mouse embryonic fibroblasts, splenocytes and bone marrow cells by reverse transcriptase polymerase chain reaction analysis (Hata *et al*., 2001; Honda *et al*., 2004; Takayanagi *et al*., 2002). In the results show that sIFN-β mRNA expression can be detected in seven different sheep tissues, the highest expression level was found in lymphocyte node and no significant deviation was detected in other six tissues. These results are consistent with those low levels of IFNs are constitutively expressed in mouse and humans notably during embryogenesis (Samuel, 2001).

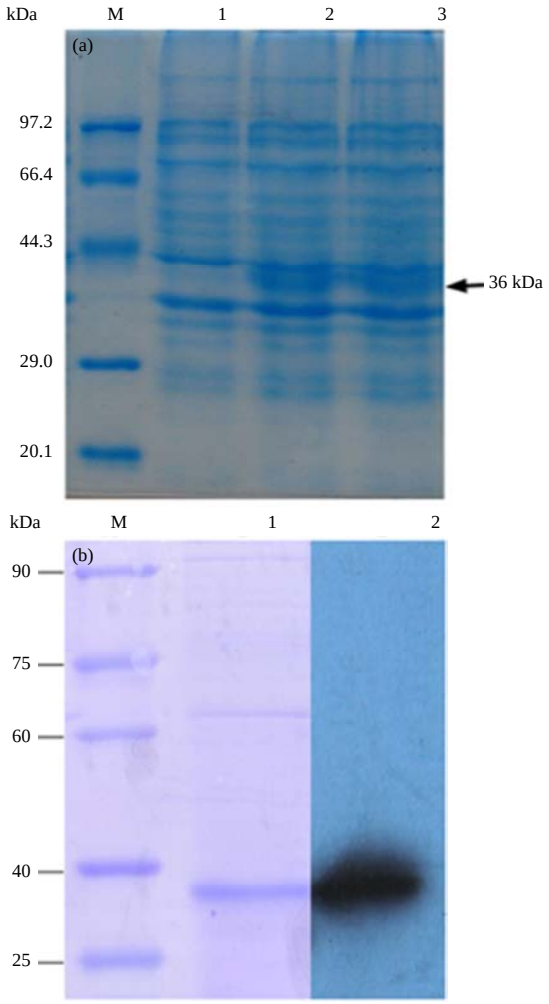

Fig. 5: SDS-PAGE analysis of sIFN-β protein expressed in *E. coli* BL21 (DE3); a) Lane 1: cell lysates of bacteria transformed with sIFN-β without IPTG induction. Lane 2, 3: cell lysates of bacteria transformed with sIFN-β under IPTG induction (Lane 2: 0.2 mM; Lane 3: 1 mM); b) Lane 1: sIFN-β protein purified by Ni-NTA affinity chromatography. Lane 2: Western blot analysis of sIFN-β using anti-His6-tag mouse antibody. The arrow indicates the location of sIFN-β protein. Low molecular weight marker is shown in the left lane

Considering the lymphocyte node expert important innate immune function in recognize pathogens thus it more easy to stimulated and response.

Low amounts of constitutively produced IFN might maintain immune cells in an activated state ready for a timely response to pathogens. However, virus also develop its owner specific mechanisms to circumvent early immune recognize. Avian H5N1 strains were attenuated for replication and provoked higher IFN secretion than wild virulent strains. Replication of avian viruses was significantly enhanced on interferon-deficient cells and exogenous IFN potently limited the growth of all viral strains (Matthaei *et al*., 2013; Palomares *et al*., 2013). Researchers also expressed the recombinant sIFN-β protein as a candidate drug to prevent and cure sheep disease especially the viral disease which can lead immune system impairment.

CONCLUSION

In this study, researchers cloned and analyzed the gene structure of sIFN-β. Furthermore, the protein of sIFN-β expressed in this study could be used to improve the immunity of goat and sheep against infectious diseases in the future.

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