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Cloning and Expression of Transforming Growth Factor Beta 1 of Guanzhong Dairy Goat and its Effect on Proliferation of Goat Peripheral Blood Mononuclear Cells (PBMCs)

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Abstract: Transforming Growth Factor beta 1 (TGF-β1) is a pleiotropic cytokine that affects diverse biologic processes. In this study, the goat *TGF-β1* gene was cloned and sequenced with length of 1173 bp. The predicted peptide of goat TGF-β1 contained 390 amino acids and 29 amino acids signal peptide. Phylogenetic analysis of different species available in GenBank™ based on the complete amino acid sequences of TGF-β1 showed that the goat was clustered with sheep sistered to bovine. The TGF-β1 mature peptide including 112 amino acids was expressed with a fusion protein of approximately 32 kDa. The goat PBMCs were proliferative stimulated by recombinant TGF-β1 but the proliferation of PBMCs stimulated by ConA was inhibited under co-stimulation of recombinant TGF-β1. These results suggest that the goat recombinant TGF-β1 would play an important role in regulating the immune responses and sustaining the balance of organism physiological state.

Key words: TGF-β1, PBMCs, proliferation, dairy goat, physiological state, amino acid

INTRODUCTION

Transforming Growth Factor beta 1 (TGF-β1) is a pleiotropic cytokine with regulation role in cell proliferation, differentiation, survival and apoptosis that affects diverse biologic processes including development of autoimmune diseases and cancer and immune response to pathogenic agents (Bierie *et al*., 2009; Li *et al*., 2007). In the mouse Experimental Autoimmune Encephalomyelitis (EAE), a model of autoimmune disease, the risk of which was increased with the reduction of TGF-β1 that caused by decrease of the oral tolerance level to autoantigen (Chen *et al*., 1994). The TGF-β1 inducing Foxp3 expression regulation T (Treg) cells also have important functions in suppressing gastritis and other autoimmune diseases (Zhang *et al*., 2010; Nguyen *et al*., 2011). In the process of host immune to virus, bacteria and parasites, TGF-β1 is the key cytokine to regulate immune intensity of innate immnue and acquired immune via modulating lymphocytes activation inhibition and differentiation (Noah and Ruslan, 2009; Bonilla and Oettgen, 2010).

In the Peripheral Blood Mononuclear Cells (PBMCs) of orthotopic liver transplanted recipients with human cytomegalovirus infection, the expression of TGF-β1 mRNA level was high significantly increased compared with healthy group ($p<0.001$) (Zhang *et al*., 2009). The low amount of TGF-β1 could increase NO synthase level which effectively limited growth of malaria parasite in mammals, suggesting the protective role during parasite infection (Luckhart *et al*., 2008). Recently, an important T cell subset (Th17 cell) in the acquired immunity was discovered and has been widely studied in immune response in the autoimmune diseases and against pathogenic agents, especially in the development of inflammation (Niebuhr *et al*., 2011; Pappu *et al*., 2011). In the mouse nasal-associated lymphoid tissue with group A Streptococcus infection, the induction of TGF-β1 was associated with Th17 cellular immune response that provides sufficient protection against group A Streptococcus *in vivo* (Wang *et al*., 2010). TGF-β1 is also one of the indispensable cytokines in the generation of Th17 cell subset from naive $CD4^+$ T cells (Paul *et al*., 2006; Korn *et al*., 2009; Yoshimura *et al*., 2010). In the mouse, TGF-β1 together with DC-derived IL-6 were essential for differentiation of IL-17-producing T cells from naive $CD4^+$T *in vitro* and the effect could be amplified in the cytokines milieu of IL-1β and TNF-α (Veldhoen *et al*., 2006; Bettelli *et al*., 2006). In the human, the TGF-β1, IL-23, IL-1β and IL-6 co-provide the cytokines milieu for differentiation of human Th17 cells and TGF-β1 and IL-21 uniquely promote this effect accompanied with expression of the transcription factor RORC2 (Li *et al*., 2008; Volpe *et al*., 2008).

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TGF-β1 genes of human, murine, bovine, ovine, porcine have been cloned, expressed and their biological functions also have been preliminary studied (Derynck *et al*., 1985, 1986; Cheifetz *et al*., 1987; Van *et al*., 1987; Qian *et al*., 1990; Woodall *et al*., 1994). The TGF-β1 could be secreted by T, B, NK, PBMCs and endothelial cells *in vivo* or induced by mitogen (lipopolysaccharide or Concanavalin A) and cytokines milieu *in vitro*. The sequences of mature TGF-β1 peptides from different species displayed unusually high degree conservation. The product of this peptide is a 25 kDa homodimeric molcule (Suthanthiran *et al*., 2000; Li *et al*., 2007). The bone marrow osteoprogenitor was activated and matrix was synthesized by mice recombinant TGF-β1 in the old mice (Gazit *et al*., 1999). However, prior to the present study, there has no published report on cloning and biological activity of TGF-β1 in goat. The objectives of this study were to clone and express the mature TGF-β1 peptide gene of dairy goat and to study the proliferation effect of goat PBMC stimulated by this recombinant peptide alone or combined with mitogen Concanavalin A *in vitro*.

MATERIALS AND METHODS

Spleen lymphocyte cell culture, RNA isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) amplification: Spleens of Guanzhong dairy goats were collected from the slaughterhouse. Splenocytes were isolated using Lymphocytes Separation Medium (Shanghai, huajing), washed with phosphate-buffered saline (PBS, pH 7.2~7.4) containing 5% Fetal Bovine Serum (FBS) for two times and re-suspended in RPMI-1640 (100 mL of complete RPMI-1640 culture media containing 10 mL FBS, 1 mL of 0.1M Sodium pyruvate, 1 mL of 1M HEPES, 5×10^{-3} M β-Mercaptoethanol, 7.5% $NaHCO_3$ and 1 mL of L-Gln) at a concentration of 5×10^7 viable cells/mL. Then, Concanavalin A (ConA, Sigma) was added at a final concentration of 10 μg mL^{-1}. After 24 h incubation, the total RNA of lymphocytes was isolated from 1×10^8 cells using TRIZOL reagent (TaKaRa, DaLian, China) according to manufacturer's instruction. The mature peptide of *TGF-β1* gene was amplified with primers Shangu1 (5'-ATGCCGCCYTCGGGGCTGCGGCTGCTGC-3') and Shangd1 (5'-TCAGCTGCACTTGCAGG-AGCGCACGATC-3') using One Step RT-PCR kit (TaKaRa) according to manufacturer's instruction. RT-PCR reactions (25 μL) were performed in 1 μL of Primescript 1 Step Enzyme Mix, 0.5 μM of each primer, 12.5 μL of 2×1 step buffer and 2.5 μL of RNA sample in a thermocycler (Biometra) under the following conditions: after reverse transcriptase at 50°C for 30 min and initial denaturation at 94°C for 2 min then 94°C for 30 sec (denaturation), 56°C for 1.5 min (annealing), 72°C for 1 min (extension) for 30 cycles, followed by a final extension at 72°C for 10 min. Samples without RNA (no-RNA controls) was included in each amplification run and in no case was amplicon detected in the no-RNA. Each amplicon (5 μL) was examined by agarose gel electrophoresis to validate amplification efficiency.

Positive amplicons were selected, purified and sequenced using an ABI 377 automated DNA sequencer (using BigDye Terminator Chemistry) employing the same primers as used in the RT-PCR.

Sequence and phylogenetic analysis: For sequence and phylogenetic analysis, TGF-β1 amino acid sequences of *H. sapiens* (GenBank™ Accession No. NP_000651), *M. musculus* (NP_035707), *B. taurus* (NP_001159540), *O. aries* (NP_001009400), *S. scrofa* (NP_999180) in GenBank™ were considered. The predicted amino acid sequences of goat TGF-β1 were aligned with corresponding sequences of other animals in GenBank™ using program Clustal X 1.83 (Thompson *et al*., 1997). Pairwise comparisons were made of the level of sequence Differences (D) among different species using the equation (Chilton *et al*., 1995; Zhao *et al*., 2009):

$$D = 1-(M/L)$$

Where:

M = The number of alignment positions at which the two sequences have a base in common

L = The total number of alignment positions over which the two sequences are compared

The unrooted Neighbor-Joining (NJ) Method in MEGA Version 4.0 (Tamura *et al*., 2007) was used to construct genetic relationships, starting from a distance matrix based on the Kimura 2-parameter index (Kimura, 1980) with 1000 replicates of bootstrap analysis and values >50% reported.

Prokaryotic expression of goat-TGF beta 1: The gene region encoding mature TGF-β1 peptide was amplified with primers Shangu2 (5'-TAAGGATCCGCCCTGGA-CACCAACTACTGCTTCA-3') and Shangd2 (5'-TTACTCGAGTCAGCTGCACTTGCAGGAGCGCACGA TC-3'). PCR reactions (25 μL) were performed in 2 mM of $MgCl_2$, 2.5 mM of each primer, 2.5 μL of 10×ExTaq buffer, 0.2 mM of each dNTPs, 1.25 U of ExTaq DNA polymerase (TAKARA) and 1 μL of DNA sample in a thermocycler (Eppendorf) under the following conditions: after an initial denaturation at 94°C for 5 min then 94°C for 30 sec (denaturation), 50°C for 30 sec (annealing), 72°C for 1 min (extension) for 30 cycles followed by a final extension at

72°C for 10 min. These optimized cycling conditions for the specific and efficient amplification were obtained after varying annealing temperatures and Mg^{2+} concentration. Then purified PCR product was digested by $BamH^{-}$ and Xho^{-} enzymes and subcloned into pET32a plasmid (Novagen). Then, the positive recombinant was transformed into *Escherichia coli* BL21-condon plus (DE3)-RIL to be expressed under 0.5 mM IPTG (Promega) at 16°C for 14 h. The bacteria samples were collected 2 h interval post induction and analyzed by SDS-PAGE.

Purification of the recombinant goat-TGF-β1 fusion protein: Bacterial cells harvested from 200 mL of induced cultures were resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; pH 8 .0) and stored at -80°C. After frozen cells thawed at 37°C for 15 min, bacterial cells were disrupted by sonication for 20 min at power of 5. Supernatant was obtained with centrifugation at 4°C. The soluble $(His)_6$ tagged recombinant goat-TGF-β1 was purified from the supernatant by Nickel-Nitrilo-Triacetic (Ni-NTA) affinity chromatography using a HiTrap™ Chelating HP column (GE Healthcare). Recombinant protein was eluted using Phosphate Buffered Saline (PBS; pH 7.4) containing 40 or 500 mM imidazole then filtered with bag filter for 3 h in PBS and stored at -80°C.

Proliferation of peripheral blood mononuclear cells stimulated by ConA and TGF-β1 mature peptide: Goat Peripheral Blood Mononuclear Cells (PBMCs) were cultured in 200 μL of volume containing 1×10^6 viable cells/mL and stimulated with soluble TGF-β1 fusion protein (final concentration of 1, 10 or 25 ng mL^{-1}) alone or together with ConA (20 μg mL^{-1}). The proliferation of PBMCs were determined using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to procedures reported previously (Bounous *et al*., 1992; Keller *et al*., 2005) after 68 h cultured. Absorbance at 490 nm was measured using an model 680 microplate reader (Bio-Rad).

Statistical analysis: Mean, standard deviation and statistical significance were calculated using the GraphPad. Prism (Version 5.0) application Software. Statistical significance was determined using the non-parametric paired t-test. The differences were considered to be statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Molecular cloning and bioinformatic analyses of goat TGF-β1: The goat TGF-β1 was cloned and sequenced, with the length of 1068 bp which shared nucleotide identity of 98.47% with sheep TGF-β1 published in the GenBank™ (NM_001009400.1). The amino acid sequence of goat TGF-β1 was predicted with a 29 amino acids signal peptide and a 112 amino acids mature peptide, consistent with sheep TGF-β1. High degree of conservation was found in the mature peptide by alignment of the predicted amino acid sequences of goat TGF-β1 with that of *O. aries*, *B. taurus*, *H. sapiens* and *M. musculus* (Fig. 1) with amino acid identity 99.12, 99.12, 99.12 and 98.21%,

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  Goat MPPSGLRLLPLLLPLLWLLMLTPGRPVAGLSTCKTIDMELVKRKRIEAIRGQILSKLRLASPPSQGDVPPGPLPEAILALYNSTRDRVAGESAETEPEPE 100
 Sheep ............................................G....................................................... 100
Bovine .................................................................................................... 100
 Human ...................V......A.......................................E.........V.................P..... 100
 Mouse ...............P...V......A.......................................E.........V................DP..... 100
  Goat ADYYAKEVTRVLMVEYGNKIYDKMKSSSHSIYMFFNTSELREAVPEPVLLSRAELRLLRLKLKVEQHVELYQKYSNNSWRYLSNRLLAPSDSPEWLSFDV 200
 Sheep ....................................................DV.............................................. 200
Bovine ....................................................DV.............................................. 200
 Human ...............TH.E....F.Q.T........................................................................ 200
 Mouse ..............DRN.A..E.T.DI...........DI.......P.........Q...SS...................G....T.T.T........ 200
  Goat TGVVRQWLTHREEIEGFRLSAHCSCDSKDNTLQVDINGFSSGRRGDLATIHGMNRPFLLLMATPLERAQHLHSSRHRRALDTNYCFSSTEKNCCVRQLYI 300
 Sheep .................................................................................................... 300
Bovine .........R.......................................................................................... 300
 Human ........SRGG...............R...........TT..............................Q............................ 300
 Mouse ........NQGDG.Q...F...........K.H.E...I.PK....G..D.................................................. 300
  Goat DFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPGASAAPCCVPQALEPLPIVYYVGRKPKVEQLSNMIVRSCKCS           390
 Sheep ..........................................................................................           390
Bovine ..........................................................................................           390
 Human ..........................................................................................           390
 Mouse ....................................................S.....................................           390
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Fig. 1: Alignment of amino acid sequences of TGF-β1 in different species available in GenBank™

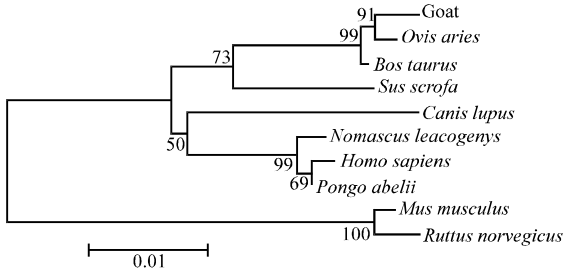

Fig. 2: Phylogenetic relationship of different species available in GenBank™ inferred by neighbor-joining analysis based on the complete amino acid sequences of TGF-β1. Bootstrap values (in percentage) >50% from 1,000 pseudo-replicates are shown. Scale bar indicates P distance

respectively. Phylogenetic analysis of different species available in GenBank™ based on the amino acids of TGF-β1 (Fig. 2) showed that the goat was clustered with *O. aries* sistered to *B. taurus*.

Expression of recombinant goat TGF-β1 mature peptide: The mature TGF-β1 peptide has been proved to be the main domain that plays the biologic functionsin cell proliferation, differentiation and survival (Brown *et al*., 1990; Li *et al*., 2007; Taylor, 2009; Yoshimura *et al*., 2010) and this peptide of some species (human and mouse) is available commercially (R and D systems). Therefore, in the present study, the mature peptide of goat TGF-β1 was expressed and its effect on proliferation of PBMCs was studied. The molecular weight of recombinant mature peptide was approximately 13 kDa and the goat TGF-β1 mature peptide was approximately 33 kDa (Fig. 3 and 4).

The proliferation effect of peripheral blood mononuclear cells by the goat TGF-β1 mature peptide: Three doses (1, 10 and 25 ng mL^{-1}) of recombinant protein were used to determine the effect on the proliferation of PBMCs. The proliferation of cells could be stimulated by three doses of recombinant mature TGF-β1 with statically significant at dose of 1 ng mL^{-1}. The Stimulation Index (SI) at the dose of 1 ng mL^{-1} was higher than that at doses of 10 and 25 ng mL^{-1} of recombinant TGF-β1 protein and the SI at dose of 10 ng mL^{-1} was higher than that at dose of 25 ng mL^{-1} but with no statically significant ($p>0.05$) (Fig. 5). However, the proliferation of PBMCs stimulated by three doses (1, 10 and 25 ng mL^{-1}) of recombinant protein together with ConA showed that the proliferation of cells by ConA was inhibited by different doses of recombinant protein but with no statically significant difference and the SI at dose of

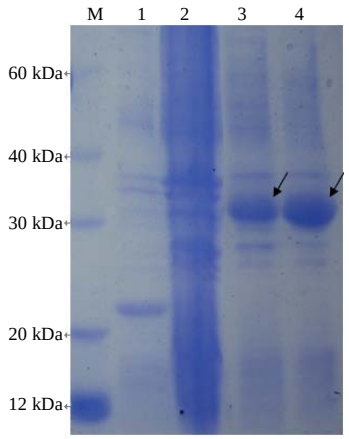

Fig. 3: The induction sample was analyzed by SDS-PAGE. Lane 1: *E. coli* BL21 (DE3) cells transformed with pET32a plasmid. Lane 2: Uninduced *E. coli* BL21 (DE3) cells transformed with TGF-β1-pET32a plasmid. Lane 3: The supernatant of *E. coli* BL21 (DE3) cell transformed with TGF-β1-pET32a plasmid expressing fusion protein. Lane 4: The sediment of *E. coli* BL21 (DE3) cells transformed with TGF-β1-pET32a plasmid expressing fusion protein. The TGF-β1 fusion protein was indicated by arrow

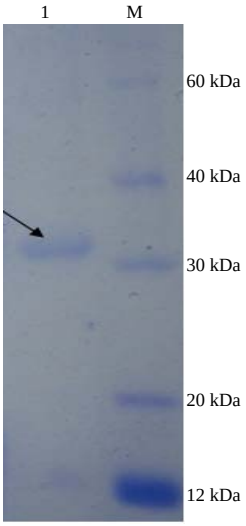

Fig. 4: The purified TGF-β1 fusion protein was analyzed by SDS-PAGE. The TGF-β1 fusion protein was indicated by arrow

25 ng mL^{-1} of recombinant c TGF-β1 together with ConA was higher that at doses of 1 and 10 ng mL^{-1} (Fig. 6).

In peripheral blood, the relative proportions of T and B cells account for about 75 and 10% of all lymphocytes

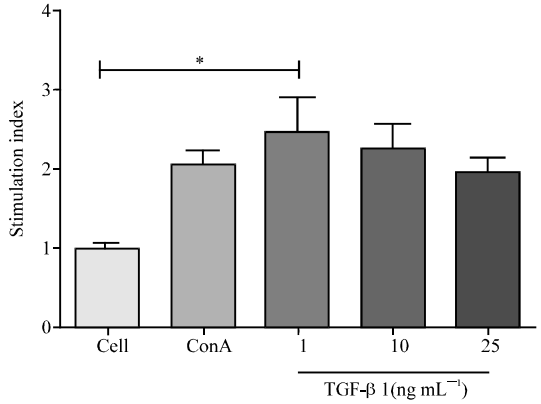

Fig. 5: The proliferation of PBMCs stimulated by different doses of goat recombinant TGF-β protein with ConA as control. Stimulation Index (SI) of PBMCs was calculated according to the equation, SI = (Stimulation group−Blank group)/(Negative group−Blank group). *represents $p<0.05$

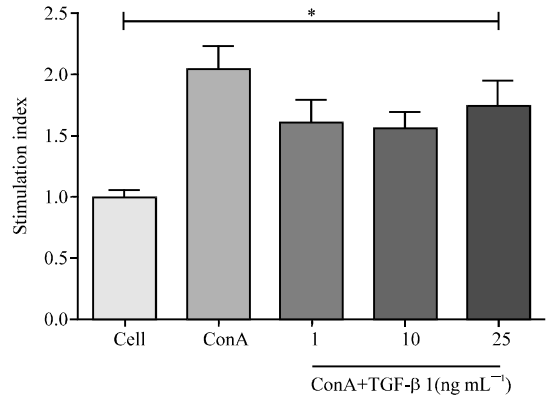

Fig. 6: The proliferation of PBMCs co-stimulated by ConA and different doses (1, 10 and 25 ng mL^{-1}) of goat recombinant TGF-β protein. Stimulation Index (SI) of PBMCs was calculated according to the equation, SI = (Stimulation group−Blank group)/(Negative group−Blank group). *represents $p<0.05$

that including naive T cells and effector T cells (Parslow, 2001). The PBMCs are treated as a good study model for pathogens infection, vaccine immunity and other processes of immune responses at cellular and molecular level (Symeonidou *et al*., 2010; Belisle *et al*., 2011). *In vivo*, the PBMCs microarray was analyzed after the Rhesus macaques, an important animal model for the study vaccines against HIV/AIDS were immunized with a DNA vaccine which was used to evaluate the level of cellular immunity (Belisle *et al*., 2011). *In vitro*, the antigen-specific IFN-γ secreting T cells in human PBMCs were induced by a clinical immunogenicity FPX1 in human PBMCs which elicited antigen specific immune response in 47% individual naive healthy donors (Wullner *et al*., 2010) and the inhibition of PBMCs proliferative response could be used to indicate the role of definite factor in the immune response.

Human PBMC proliferative response to *Leishmania infantum* promastigotes was inhibited by anti-IFN-γ mAb gamma123 which stressed the role of IFN-γ in the immune response to leishmaniasis (Zucca *et al*., 1995). The PWM-stimulated PBMCs was inhibited by TGF-β1 via suppression of IL-2 and IL-6 expression which suggested that TGF-β1 might suppress immune response (Reinhold *et al*., 1994). But TGF-β1 also positively regulates immune responses by promoting T cells survival and inducing IgA class switching in the B cells (Li *et al*., 2006).

In the present study, the proliferation of PBMCs stimulated by recombinant TGF-β1 was dose-dependent. The proliferative effect on the PBMCs of three doses was deteriorated following increasing the dose of recombinant TGF-β1 which might due to death of portion of actived T cells induced by TGF-β1 (Okamoto *et al*., 2005). The proliferation of PBMCs induced by TGF-β would be caused by differentiation and mitosis of different cell subsets. Xu and Silver have reported that polarized retinal antigen-specific memory/effector T cells were not fully inhibited by TGF-β1 (Xu *et al*., 2003). The memory/effector T cells in the PBMCs could be sustained and the proliferation of them could be enhanced by recombinant TGF-β1. In addition, IL-6 could be present in the PBMCs culture system secreted by actived T cells or B cells (Kishimoto *et al*., 1995). The nai ve $CD4^+$T in the PBMCs could be induced by IL-6 together with exogenous TGF-β1 to differentiate into Th17 cells (Kimura and Kishimoto, 2010). The TGF-β1 also might induce IgA isotype expression in the B cells and increase the numbers of precursors of IgA-producing cells (Min and Kim, 2003).

The PBMCs could be proliferated by ConA stimulation via promoting the mitogenesis of T cells that secreted IL-2 (Ravid *et al*., 1983). However, the proliferation of PBMCs was inhibited by co-stimulation of recombinant TGF-β1 and ConA. The possible explaination was that ConA induced $CD4^+CD25^-$T cells to differentiate into the $CD4^+CD25^+Foxp3^+$iTregs (Tregs) in the milieu of IL-2 and exogenous recombinant TGF-β1 (Thornton *et al*., 2004; Zheng, 2008) which suppressed the proliferation of the $CD4^+CD25^-$T cells in the PBMCs (Erhardt *et al*., 2007).

CONCLUSION

The goat TGF-β1 was cloned and sequenced with length of 1173 bp. The dose-dependent proliferation of goat PBMCs was found under stimulation of recombinant goat TGF-β1 but the proliferation of PBMCs stimulated by ConA was inhibited under co-stimulation of recombinant TGF-β1. These results would provide the basic data for studying recombinant TGF-β1 modulating host homeostasis during pathogens infection and autoimmune diseases.

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