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(71) **Applicant** *(for US only)*: **CHEN, Chung-Chin** [US/US]; Bacon & Thomas, PLLC, 625 Slaters Lane, 4th Flr., Alexandria, Virginia 22314 (US).

(72) **Inventor; and**
(71) **Applicant: LIAO, Chao-Wei**; 18F.-1, No. 135, Ciyun Rd., East Dist., Hsinchu City, 300 (TW).

(72) **Inventor: LIAO, Ting-Kai**; 18F.-1, No. 135, Ciyun Rd., East Dist., Hsinchu City, 300 (TW).

(74) **Agent: URCIA, Benjamin E.** et al.; Bacon & Thomas, PLLC, 625 Slaters Lane, 4th Flr., Alexandria, Virginia 22314 (US).

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(54) **Title:** FUSION POLYPEPTIDE FOR IMMUNO-ENHANCEMENT AND METHOD FOR ENHANCING STIMULATION OF IMMUNE RESPONSE USING THE SAME

(57) **Abstract:** A fusion polypeptide is disclosed, which includes: (a) a mucosa targeting polypeptide; (b) a translocating peptide for translocation; and (c) an antigenic epitope. In addition, a method for enhancing a stimulation of an immune response using the aforementioned fusion polypeptide is also disclosed.

Fusion polypeptide for immuno-enhancement and method for enhancing stimulation of immune response using the same

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of filing date of U. S. Provisional Application Serial Number 62/412,896, filed October 26, 2016 under 35 USC § 119(e)(1).

BACKGROUND

1. Field

The present disclosure relates to a fusion polypeptide, a composition comprising the same and a method for enhancing a stimulation of an immune response using the same.

2. Description of Related Art

Over 35 years, the antigenic peptides are critical factors in mobilizing the immune system against foreign invaders and cancers. Most of the highly successful treatments, including vaccines, have been made empirically, with little immunological insight. Until 1986, it was never considered that peptides could have an essential role in the control of the specificity of immune responses in conjunction with molecules—MHC class I and II—best known for their involvement in transplant rejection. However, new knowledge about the chemistry and pharmacological properties of antigenic peptides as well as of the molecular biology of antigen processing, presentation, and recognition by immune cells, has now enabled a more rational approach to vaccine and immune enhancer design. Harnessing the immune system to treat disease or cancer therapy will be facilitated by a greater understanding of the origins and roles of antigenic peptides in immunity

The roles of antigenic peptides in both the innate and adaptive immune systems are these key players can be used for therapy against infectious disease, cancer and auto-immune conditions. Innate immunity is the first line shotgun approach of defense against infections and is exemplified by antimicrobial peptides, also known as host defense peptides (HDPs). Adaptive immunity relies on the capacity of immune cells to distinguish between the body's own antigens and unwanted invaders and tumor cells. The antigen peptide belonging to the type of adaptive immunity, there is a path of the molecule from initial generation by proteolytic processing to its presentation to immune cells by major histocompatibilty complex (MHC) molecules in immune system. Therefore, key players are MHC-I and MHC-II that form a noncovalent complex with antigen peptides and present these peptides in the context of antigen-presenting cells to T cells of the immune system

Although the antigenic peptides are known to be an effective manner for stimulating immune response, many researches and studies still focus on finding novel antigenic peptides with improved effect. Therefore, an object of the present disclosure is to find an adjuvant, which can effectively transport to immune target organs for adaptive immunity route to achieve the purpose of enhancing the stimulation of the immune response.

SUMMARY

The present disclosure provides a fusion polypeptide, which can be used as an enhancer for stimulating the immune response.

The present disclosure also provides a composition for enhancing a stimulation of an immune response, which comprises the aforesaid fusion polypeptide. In addition, the present disclosure further provides a method with the aforesaid fusion polypeptide.

The fusion polypeptide of the present disclosure comprises: (a) a mucosa

targeting polypeptide; (b) a first translocating peptide for translocation; and (c) a first antigenic epitope.

The mucosa targeting polypeptide is a binding epitope facilitating the binding of the fusion polypeptide to a receptor in a subject in need. The first translocating peptide for translocation is used as a carrier which is able to facilitate cytosolic localization and antigen presentation. The first antigenic epitope can enhance the immune-modulating activity. When the fusion polypeptide of the present disclosure comprises the aforesaid three components, the fusion polypeptide can be effectively delivered into a subject in need and enhance immune response to endogenously process target antigens.

Furthermore, another fusion polypeptide of the present disclosure comprises: (b) a first translocating peptide for translocation; and (c) a first antigenic epitope selected from SEQ ID NOs: 5 and 6. (E622, E713).

The first translocating peptide for translocation is used as a carrier which is able to facilitate cytosolic localization and antigen presentation. The first antigenic epitope is a modified antigenic epitope, which can enhance the immune-modulating activity. Especially, the sequence shown by SEQ ID NOs: 5 and 6 (i.e. E622 and E713) is modified and different from the known polypeptides of viral proteins of E6 and E7, which can further enhance the immune-modulating activity. When the fusion polypeptide of the present disclosure comprises the aforesaid two components, the fusion polypeptide can be effectively delivered into a subject in need and enhance immune response to endogenously process target antigens.

In addition, the present disclosure also provides a composition for enhancing a stimulation of an immune response, comprising: a vaccine and any one of the aforesaid fusion polypeptide. Herein, the vaccine may comprise: a second translocation peptide for translocation and a second antigenic epitope. The second translocation peptide is similar to the first translocation peptide, and the second antigenic epitope is similar to the second antigenic epitope.

Thus, the descriptions about the second translocation peptide and the second antigenic epitope are not repeated again. Furthermore, in the present disclosure, the first antigenic epitope and the second antigenic epitope has to be compatible to each other. For example, both the first antigenic epitope and the second antigenic epitope are HPV antigenic epitopes; but the first antigenic epitope and the second antigenic epitope is not necessarily the same. Or, both the first antigenic epitope and the second antigenic epitope are Myostatin epitopes; but the first antigenic epitope and the second antigenic epitope is not necessarily the same. Or, both the first antigenic epitope and the second antigenic epitope are PRRSV antigenic epitopes; but the first antigenic epitope and the second antigenic epitope is not necessarily the same.

Moreover, the present disclosure also provides the method for enhancing a stimulation of an immune response, comprising: administering the aforesaid composition to a subject in need. Herein, the subject can be mammalian, such as human, pig, etc.

In one embodiment of the present disclosure, the antigenic epitope can be an HPV antigenic epitope, a Myostatin epitope, or a PRRSV antigenic epitope.. In another embodiment of the present disclosure, the composition is orally administered to the subject in need.

Other objects, advantages, and novel features of the disclosure will become more apparent from the following detailed description.

EMBODIMENT

Without intent to limit the scope of the invention, exemplary instruments, apparatus, methods and their related results according to the embodiments of the present invention are given below. Note that titles or subtitles may be used in the examples for convenience of a reader, which in no way should limit the scope of the invention. Moreover, certain theories are proposed and disclosed

herein; however, in no way they, whether they are right or wrong, should limit the scope of the invention so long as the invention is practiced according to the invention without regard for any particular theory scheme of action.

The present disclosure is related to a platform for generating some immune-modulating chimeric polypeptides evolved in local mucosa systems, including the type-I and type-II mucosa immune system that contains a mucosa targeting polypeptide which can be a M-cell or an epithelial cell targeting domain, a translocating peptide for translocation which can be from pseudomonas exotoxin, and a antigenic epitope which can be a Th1 antigenic epitope.

We survey the immune responses against the antigenic epitopes of chimeric polypeptides. Especially, the immune responses can be elicited when the fusion polypeptide of the present disclosure is administered through orally administration. The fusion polypeptide of the present disclosure evolves the mucosal immune system in the intestine. Hereinafter, there are three groups of PE-based fusion antigens to demonstration the present disclosure that their specific immunities can be enhanced.

1. Mucosa targeting polypeptide construct

The significant advances have been made in the identification of M-cell-specific surface markers (Kim SH et al., *J Immunol.* 2010 Nov 15;185(10):5787-95). In the oral route, it should comprise the M-cell or epithelial cell ligand for mucosal targeting (Azizi et al., *PLoS Pathog.* 2010 Nov 11;6(11); K J Syrjänen, *J Clin Pathol.* 2002 Oct; 55(10): 721–728; Roy C. et al., GI Motility online (2006) doi:10.1038/gimo15). We have explored efficient recombinant production of several bio-polypeptides by exploiting the natural peptides as a fusion partner polypeptide.

The following embodiments use three kind peptides of binding epitopes as examples of the M-cell target peptides in type-I mucosa immune system. Also, one kind peptide of binding epitope is as an example of the epithelial cell

targeting peptide in type-II mucosa immune system (Azizi et al., *PLoS Pathog.* 2010 Nov 11;6(11); K J Syrjänen, *J Clin Pathol.* 2002 Oct; 55(10): 721–728; Roy C. et al., *GI Motility online* (2006) doi:10.1038/gimo15; Mulder DJ et al., *Dig Dis Sci.* 2012 Mar;57(3):630-42). The M-cell target peptides used in the following examples are shown in Table 1. The epithelial cell targeting peptide is HPV L2-200, as shown in Table 2) for epithelial targeting ligand (Mulder DJ et al., *Dig Dis Sci.* 2012 Mar;57(3):630-42).

Table 1. The M-cell targeting polypeptides in type-I mucosa immune system

SEQ ID NO.	Name of peptide	Amino acid codon
01	CO-1	SFHQ<u>LPAR</u>S<u>PAPL</u>Q
02	DQ-2	S<u>SFH</u>LFHH<u>LPARAPL</u>APSE<u>LQ</u>P
03	RV-3	STP<u>FH</u>P<u>LPAR</u>KPL<u>PLQ</u>P

Table 2. The intestine epithelial cell targeting peptide

SEQ ID NO.	Name of peptide	Amino acid codon
04	L2-200 (from HPV L2 protein)	<u>*EF*</u>HM<u>*VD*</u>GMSIRAKRRKRASATQLYKTC KQAGTCPPDIIPKVEGKTIAEQILQYGSM GVFFGGLGIGTGSGTGGRTGYIPLGTRPP TATDTLAPVRPPLTVDPVGPSDPSIVSLVE ETSFIDAGAPTSVPSIPPDVSGFSITTSTDT TPAILDINNNTVTTVTTHNNPTFTDPSVL QPPTPAETGGHFTLSSSTISTHNYEEIPMD TKDEL<u>*LE*</u>

*: EF, VD and LE shown in italics are the restriction enzyme sites, EcoR1 and Xho1, which are added for subcloning purpose.

1-1. Construction of M-cell targeting polypeptide

The CO-1 peptide core sequence, SFHQ<u>LPAR</u>S<u>PAPL</u>Q (SEQ ID NO. 01), is chosen as described in Kim SH et al., *J Immunol.* 2010 Nov 15;185(10):5787-95. The DQ-2 and RV-3 amino acid code sequence are

searched by Basic Local Alignment Search Tool (BLAST) to find the regions to mimic CO-1 sequence. The program compares protein sequences to sequence databases and calculates the statistical significance. From the amino acid codon sequence, we can find two CO-1-like peptides, DQ-2 (SEQ ID NO. 02): SFHLFHHLPARAPLAPL in *Daedalea quercina* Fr. (Polyporus-like Fugus), and RV-3 (SEQ ID NO. 03): STPFHPLPARKPLP in *Dasypus novemcinctus*.

To design and generate a DNA codon sequence without DNA template, the codon substitutions without altering the original amino acid sequence of the selected peptide segment was made for avoiding spurious restriction sites and for optimal expression in E. coli. Restriction site linkers were added at the ends of the peptide segment-encoding DNA sequence, EcoR1 and Xho1 at 5'end and 3'end, for insertion and sub-cloning into vector plasmids. The DNA fragment was generated by PCR and multiple primers extension. The PCR DNAs, as shown in Table 3, encoded above amino acid sequences were respectively subcloned in to E coli vector plasmids by recombinant technique.

All synthesized and/or subcloned nucleotide fragments were analyzed by restriction enzyme cutting and electrophoresis to check if they were of the expected sizes and right cutting sites. Once a sample had been obtained, DNA sequences were produced automatically by machine and the result displayed on computer. Sequence analysis performed to identify the sequence of nucleotides in a nucleic acid, or amino acids in a polypeptide.

Table 3. The DNA sequence encoded M-cell target peptide epitopes

SEQ ID NO.	Name of DNA	DNA codon
05	CO-1	<u>GAATTC</u>AGCAGCTTTCATCTGTTCCACCATCTGCCAGCGCGTGCGCCATTAGCGCCTTCTGAATTACAGCCC<u>CTCGAG</u>

06	DQ-2	GAATTCAGCAGCTTTCATCTGTTCCACCATCTGCCAGCGCGTGCGCCATTAGCGCCTTCTGAATTACAGCCCCTCGAG
07	RV-3	GAATTCTCTACTCCTTTCCACCCATTGCCTGCCCGCAAACCATTGCCTCTGGTGCCCCTCGAG

1-2. Construction of intestine epithelial targeting polypeptide

L2-200 DNA fragment encoding the N-terminal domain (aa 1 to 200; "L2-200") of HPV16 L2 protein was synthesized by multi-step PCR using primer pairs listed in Table 6. The primer pairs 1-10 generated DNA fragments of size 82 bp, 147 bp, 219 bp, 285 bp, 354 bp, 420 bp, 492 bp, 552 bp, and 613 bp, respectively. The final DNA product was digested by restriction enzymes Mun I and Sal I to isolate a 630 bp large DNA fragment, which was then subcloned into an EcoRI and Xho I digested vector.

Table 4

Target peptide	No. of primer pairs	Fw.* primer	Nucleotide sequence of the forward primer	SEQ ID NO.	Rv.** primer	Nucleotide sequence of the reverse primer	SEQ ID NO.
L2-200	Pair 1	F1	gttgacccggttggtccgtccgacccgtccatcgtttccctggttgaa	55	R1	aggtcggagcaccagcgtcgatgaaggaggtttcttcaaccagggaaac	56
	Pair 2	F2	gacaccctggctccggttcgtccgccgctgaccgttgacccggttggt	57	R2	accggaaacgtccggcgggatggacggaacggaggtcggagcaccagc	58
	Pair 3	F3	tacatcccgctgggtacccgtccgccgaccgctaccgacaccctggctccg	59	R3	agccggggtagtgtcggtggaggtggtgatggagaaaccggaaacgtccgg	60

	Pair 4	F4	ggtaccggttccggtactggcggtcgtaccggttacatcccgctgggt	61	R4	tacggtagtaacggtgttgttgttgatgtccaggatagccggggtagtgtc	62
	Pair 5	F5	ggttctatgggtgtttcttcggcggtctgggcatcggtaccggttccggt	63	R5	cgggtcggtgaaggtcgggttgttgtgagtggttacggtagtaacggt	64
	Pair 6	F6	ggtaaaaccatcgctgaacagatcctgcaatacggttctatgggtgtt	65	R6	ggtttcagccggggtcggcggttgcagaacggacgggtcggtgaaggt	66
	Pair 7	F7	ggtacctgcccgccggacatcatcccgaaagttgaaggtaaaaccatcgct	67	R7	ggagatggtggaagaggacagggtgaagtgaccaccggtttcagccggggt	68
	Pair 8	F8	acccagctgtacaaaacctgcaaacaggctggtacctgcccgccg	69	R8	gtccatcgggatttcttcgtagttgtgggtggagatggtggaaga	70
	Pair 9	F9	ccgtgctaaacgtcgtaaacgtgcttccgctacccagctgtacaaaa	71	R9	attatttttctcgagcagttcgtctttggtgtccatcgggatttcttc	72
	Pair 10	F10	cccgaattccatatggtcgacggtatgtccatccgtgctaaacgtcgt	73	R10	attatttttctcgagcagttcgtctttggtgtccatcgggatttcttc	74

*Fw: Forward.
**Rv: Reversed.

Table 5. The DNA sequence encoded L2-200 target polypeptide

SEQ ID NO.	Name of DNA	DNA codon

08	L2-200 (from HPV L2 protein)	GAATTCCATATGGTCGACGGTATGTCCATCCGTGCTA AACGTCGTAAACGTGCTTCCGCTACCCAGCTGTACA AAACCTGCAAACAGGCTGGTACCTGCCCGCCGGAC ATCATCCCGAAAGTTGAAGGTAAAACCATCGCTGAA CAGATCCTGCAATACGGTTCTATGGGTGTTTTCTTCG GCGGTCTGGGCATCGGTACCGGTTCCGGTACTGGCG GTCGTACCGGTTACATCCCGCTGGGTACCCGTCCGC CGACCGCTACCGACACCCTGGCTCCGGTTCGTCCGC CGCTGACCGTTGACCCGGTTGGTCCGTCCGACCCGT CCATCGTTTCCCTGGTTGAAGAAACCTCCTTCATCG ACGCTGGTGCTCCGACCTCCGTTCCGTCCATCCCGC CGGACGTTTCCGGTTTCTCCATCACCACCTCCACCG ACACTACCCCGGCTATCCTGGACATCAACAACAACA CCGTTACTACCGTAACCACTCACAACAACCCGACCT TCACCGACCCGTCCGTTCTGCAACCGCCGACCCCGG CTGAAACCGGTGGTCACTTCACCCTGTCCTCTTCCA CCATCTCCACCCACAACTACGAAGAAATCCCGATGG ACACCAAAGACGAACTGCTCGAG

As shown in Tables 1 and 2, the mucosa targeting polypeptide used in the fusion polypeptide of the present disclosure may be an M-cell targeting polypeptide (such as CO1, DQ2 and RV3) or an intestine epithelial targeting polypeptide (such as L2-200). The polypeptides CO1, DQ2, RV3 and L2-200 respectively have an amino acid sequence represented by SEQ ID NOs: 1 to 4.

In the present disclosure, the antigenic epitope comprised in the fusion polypeptide of the present disclosure is a Th1 antigenic epitope. Examples of the Th1 antigenic epitope can be an HPV antigenic epitope, a Myostatin epitope, or a PRRSV antigenic epitope. Hereinafter, the syntheses of the HPV antigenic epitope, the Myostatin epitope and the PRRSV antigenic epitope are described in detail.

2. The HPV Th1 epitopes and its modified polypeptides of viral proteins of E6 and E7

In the present disclosure, the HPV antigenic epitope can be an E7 peptide sequence or an E6 peptide sequence of human papillomavirus type 16.

Sarkar A.K. et al (2005) suggest that cellular immune responses specific

to the E6 and E7 peptides have a role in the protective immunity against HPV-associated CIN (Sarkar AK et al., *Gynecol Oncol.* 2005 Dec;99 (3 Suppl 1):S251-61). A fused peptide E601, SEQ ID NO. 9: EFVDQLLRREVFCGFRDLVYDFAFSDLKLPQLCTELKLPQLCTELLE was introduced since the peptide Q15L (QLLRREVYDFAFRDL) and V10C (VYDFAFRDLC) of HPV-16 E6 had a good CMI response.

A fused peptide E701, SEQ ID NO. 11: EFVDQAEPDQAEPDRARAHYNIRARAHYNILRAHYNIVIFRAHYNIVIFLE was synthesized, according to Tindle R.W et al in 1995 (Tindle R.W et al., *Clin Exp Immunol.* 1995; 101:265-271). They report that an ISCAR (= Immunostimulatory Carrier) with a BT5 peptide of HPV16 E7 which contains the linear B epitope 44QAEPD48, the Th epitope 48DRAHYNI54, and the overlapping CTL epitope 49RAHYNIVTF57, has a good therapeutic effect.

The E622 and E713 were modified from E601 and E701-polypeptide sequences according to the proteasome cleavage site prediction software (http://www.imtech.res.in/raghava/pcleavage/): an SVM based method for prediction of constitutive proteasome and immunoproteasome cleavage sites in antigenic sequences for Th1 pathway. The polypeptides sequence of E601, E622, E701 and E713 are shown as Table 6.

Table 6: The Th1 epitopes and its modified polypeptides of viral proteins of E6 and E7

SEQ ID NO.	Name of peptide	Amino acid codon
09	E601	*EFVD*QLLRREVFCGFRDLVYDFAFSDLKLPQLCTELKLPQLCTEL*LE*
10	E622	*EFVD*KDELREVYNFAFLLVLRREVYDKDELLLLLEDRQLLRREVFCGFRDLLEDRVYDFAFSDLKLPQLCTELKLPQLCTELKDELKDELVLLL*LE*

11	E701	*EFVD*QAEPDQAEPDRARAHYNIRARAHYNILRAHYNIVIFRAHYNIVIF*LE*
12	E713	*EFVD*QAEPDQAEPDRDELVLRARAHYNIRARAHYNILEDRLLVLRAHYNIVIFRAHYNIVIFKDELLV*LE*

*: EF, VD and LE shown in italics are the restriction enzyme sites, EcoR1 and Xho1, which are added for subcloning purpose.

The DNA encoded of the E601, E622, E701 and E713 was designed. Codon substitutions without altering the original amino acid sequence of the selected peptide segment were made for avoiding spurious restriction sites and for optimal expression in E. coli. Restriction site linkers were added at the ends of the peptide segment-encoding DNA sequence, EcoR1, Sal1 and Xho1 at 5'end and 3'end, for insertion and sub-cloning into a vector plasmid. The target DNA sequence of E601, E622, E701 and E713 were generated by PCR (polymerase chain reaction) and multiple primers extension, the DNA condones are shown as Table 7. The DNA fragments of the modified nucleic acid sequence encoding target chimeric polypeptides were synthesized by PCR with multiple primers (US 8,372,407). Non-DNA template PCR was performed. After the first run PCR, 0.01-1 μl of the DNA product were used as a DNA template for the second run PCR, in which the second primer pair was added together with dNTPs, reagents and Pfu polymerase. The remaining primer pairs were sequentially added in this manner at the subsequent runs of PCR until the target peptide-encoding DNA fragments were respectively synthesized (US 8,372,407).

Table 7: The DNA codon sequences of various HPV polypeptides

SEQ ID NO.	Name of DNA	DNA sequence

13	E601	*<u>GAATTC</u>***<u>GTCGAC</u>**CAACTGTTGCGTCGTGAAGTTTTCTGTGGCTTTCGTGATCTGGTCTATGACTTCGCCTTTAGTGATTTGAAGCTGCCACAATTGTGTACGGAACTGAAACTGCCTCAACTGTGTACAGAACTGAAGGATGAGCTG***<u>CTCGAG</u>***
14	E622	*<u>GAATTC</u>***<u>GTCGAC</u>**AAAGATGAACTGCGTGAGGTGTATAACTTTGCGTTCCTGTTAGTGTTACGCCGTGAGGTTTATGACAAGGACGAGTTGTTACTGCTGTTAGAAGATCGCCAACTGTTGCGTCGTGAAGTTTTCTGTGGCTTTCGTGATCTGTTAGAAGACCGCGTCTATGACTTCGCCTTTAGTGATTTGAAGCTGCCACAATTGTGTACGGAACTGAAACTGCCTCAACTGTGTACAGAACTGAAGGATGAGCTGAAAGATGAATTAGTGCTGTTATTG***<u>CTCGAG</u>***
15	E701	*<u>GAATTC</u>***<u>GTCGAC</u>**CAGGCGGAACCAGATCAAGCGGAACCTGACCGTGCCCGCGCACATTATAACATTCGCGCACGTGCACACTATAATCTGGAGGCGCATTATAACATTGTCATCTTCCGCGCACATTATAACATCGTCATTTTC***<u>CTCGAG</u>***
16	E713	*<u>GAATTC</u>***<u>GTCGAC</u>**CAGGCGGAACCAGATCAAGCGGAACCTGACCGTGACGAGCTGGTGTTACGCGCCCGCGCACATTATAACATTCGCGCACGTGCACACTATAATCTGGAGGATCGTTTACTGGTCTTGCGTGCGCATTATAACATTGTCATCTTCCGCGCACATTATAACATCGTCATTTTCAAAGATGAGTTGCTGGTT***<u>CTCGAG</u>***

3. The polypeptides of Myostatin epitopes

Myostatin (also known as growth differentiation factor 8, abbreviated GDF-8) is a myokine, a protein produced and released by myocytes that acts on muscle cells' autocrine function to inhibit myogenesis: muscle cell growth and differentiation. In humans, it is encoded by the MSTN gene. Myostatin is a secreted growth differentiation factor that is a member of the TGF beta protein family. Animals either lacking myostatin or treated with substances that block the activity of myostatin have significantly more muscle mass. Furthermore, individuals who have mutations in both copies of the myostatin gene have significantly more muscle mass and are stronger than normal. Studies into myostatin shows that myostatin may

have therapeutic application in treating muscle wasting diseases such as muscular dystrophy (Hamrick MK, *IBMS BoneKEy*. 2010 January;7(1):8-17).

3-1. M14 and M27 construct

M14 peptide epitope was selected a partial fragment of myostatin binding domain; DFGLDCDEHSTESRCCRYPLTVDFEAFGWDWIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQANPRGSAGPCCTPTLMSPINMLYFNGKEQIIYGKIPAMVVDRCGCS, which has been patented in US20020127234. The M14 peptide core sequence, FLQKYPHTHLVHQA, was selected. Codon substitutions without altering the original amino acid sequence of the selected peptide segment were made for avoiding spurious restriction sites and for optimal expression in E. coli. Restriction site linkers were added at the ends of the peptide segment-encoding DNA sequence, EcoR1- Sal1 and Xho1 at 5'end and 3'end, for insertion and sub-cloning into a vector plasmid. The DNA sequence encoded the amino acid sequence of EFVDFLQKYPHTHLVHQALE was 5'-GAATTCGTCGAC**GTGTTTTTACAAAAATATCCTCATACGCACCTGGTCCATCAGGCG**CTCGAC-3'. The DNA fragment was generated by PCR and multiple primers extension. Furthermore, the polypeptide with four repeated core sequence, EFVD**VFLQKYPHTHLVHQA**LD**VFLQKYPHTHLVHQA**LD**VFLQKYPHTHLVHQA**LD**VFLQKYPHTHLVHQA**LE (SEQ CO NO. 17), was generated with repeated insertion by recombinant technique.

M27 was modified polypeptide to present a Th1 epitope characteristics according to the proteasome cleavage site prediction software (http://www.imtech.res.in/raghava/pcleavage/): an SVM based method for prediction of constitutive proteasome and immunoproteasome cleavage sites in antigenic sequences. There are two polypeptide fragments: **HTHLVHQA**

peptide and E**FLQKYPH** peptide are fused and modified to create the M27 chimeric polypeptide (no. 3). The HTHLVHQA peptide is searched by Basic Local Alignment Search Tool (BLAST) to find regions of similarity between biological sequences. The program compares protein sequences to sequence databases and calculates the statistical significance. Its amino acid codon sequence can be found in the CHLNCDRAFT_134428 protein of Chlorella variabilis (sequence ID: ref XP_005847307). The amino acid codon sequence of EFLQKYPH peptide can be found in the ROOT PRIMORDIUM DEFECTIVE 1 isoform X4 of Cicer arietinum (sequence ID: ref XP_012571622.1). The two fusion peptide genes are respectively subcloned in to E coli vector plasmids and then fused together by recombinant technique. The modified DNA fragment encoded of LEP-HTHLVHQA-NVLLALQLLLEDREF, which added one Xho1 restriction enzyme site at 5'-end and a DNA linker or bridge encoded with NVLLALQLLLEDREF peptide at 3'-end, is generated by primer extension and PCR running method. This DNA linker encoded NVLLALQLL peptide is created by an SVM based method for proteasome cleavage prediction. LEDR signal also is added into fusion peptide in order to enhance Th1 immune response when the peptide is sorted nearby ER location. The DNA fragment encoded of LE**PHTHLVHQA**NVLLALQLLLEDREF peptide is generated by PCR technique. The DNA fragment encoded of **HTHLVHQA** is inserted into the site of Xho1 and EcoR1 of E. coli plasmid vector. The DNA fragment encoded of E**FLQKYPHV**D with two restriction enzyme sites, EcoR1 and Sal1, is generated by PCR technique. The DNA fragment encoded of the peptide of LE**PHTHLVHQA**NVLLALQLLLEDREFV**FLQKYPH**VD is fused the DNA fragment encoded of **HTHLVHQA** and the DNA fragment encoded of E**FLQKYPHV**D by recombinant technique. Furthermore, a DNA fragment with eight repeated insertion was created.

As shown in Table 8, a core of fused epitope:

EFVFLQKYPHVE**PHTHLVHQAN**V was generated, and the amino acid sequence is similar to the **EFVFLQKYPHTHLVHQAN** of Myostatin domain in US20020127234. Finally, the polypeptides sequence of M14 (SEQ ID NO. 17) and M27 (SEQ ID NO. 18) are shown as Table 8. The DNA coded M14 and M27 are shown as Table 9.

Table 8. The modified polypeptides of Myo14 and Myo27

SEQ ID NO.	Name of peptide	Amino acid codon
17	M14	*EFVD*VFLQKYPHTHLVHQALDVFLQKYPHTHLVHQALDVFLQKYPHTHLVHQALDVFLQKYPHTHLVHQA*LE*
18	M27	*EF*L*LE*PHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPHVEPHTHLVHQANVLLALQLLLEDREFVFLQKYPH*VD*

*: VD, EF and LE shown in italics are the restriction enzyme sites, EcoR1 and Xho1, which are added for subcloning purpose.

Table 9. The DNA code sequences of Myo14 and Myo27

SEQ ID NO.	Name of DNA	DNA code sequence
19	M14	*GAATTC***GTCGAC**GTGTTTTTACAAAAATATCCTCATACGCACCTGGTCCATCAGGCGCTCGACGTGTTTTTACAAAAATATCCTCATACGCACCTGGTCCATCAGGCGCTCGACGTGTTTTTACAAAAATATCCTCATACGCACCTGGTCCATCAGGCGCTCGACGTGTTTTTACAAAAATATCCTCATACGCACCTGGTCCATCAGGCG***CTCGAG***

20	M27	*<u>GAATTC</u>*CTC***<u>CTCGAG</u>***CCACATACGCACTTAGTGCAT CAAGCGAACGTTTTGCTGGCACTGCAATTATTATTA GAAGATCGTGAATTTGTCTTCTTGCAAAAATATCCA CACGTCGAGCCACATACGCACTTAGTGCATCAAGC GAACGTTTTGCTGGCACTGCAATTATTATTAGAAGA TCGTGAATTTGTCTTCTTGCAAAAATATCCACACGT CGAGCCACATACGCACTTAGTGCATCAAGCGAACG TTTTGCTGGCACTGCAATTATTATTAGAAGATCGTG AATTTGTCTTCTTGCAAAAATATCCACACGTCGAGC CACATACGCACTTAGTGCATCAAGCGAACGTTTTG CTGGCACTGCAATTATTATTAGAAGATCGTGAATTT GTCTTCTTGCAAAAATATCCACACGTCGAGCCACAT ACGCACTTAGTGCATCAAGCGAACGTTTTGCTGGC ACTGCAATTATTATTAGAAGATCGTGAATTTGTCTT CTTGCAAAAATATCCACACGTCGAGCCACATACGC ACTTAGTGCATCAAGCGAACGTTTTGCTGGCACTG CAATTATTATTAGAAGATCGTGAATTTGTCTTCTTGC AAAAATATCCACACGTCGAGCCACATACGCACTTA GTGCATCAAGCGAACGTTTTGCTGGCACTGCAATT ATTATTAGAAGATCGTGAATTTGTCTTCTTGCAAAA ATATCCACACGTCGAGCCACATACGCACTTAGTGCA TCAAGCGAACGTTTTGCTGGCACTGCAATTATTATT AGAAGATCGTGAATTTGTCTTCTTGCAAAAATATCC ACAC**<u>GTCGAC</u>**

4. The fusion polypeptides of PRRSV epitope

Porcine reproductive and respiratory syndrome virus (PRRSV) causes chronic, economically devastating disease in pigs. Frequent mutations in the viral genome result in viruses with immune escape mutants. Irrespective of regular vaccination, control of PRRSV remains a challenge to swine farmers. At present, enhancing the early immunological mechanisms in PRRSV-infected pigs can improve preventive or therapeutic purpose (O. J. Lopez et al., *Clin Vaccine Immunol.* 2007 Mar; 14(3): 269–275). Recently, we try to develop an immune enhancer for oral administration. Three immunomodulating polypeptides (GP317, GP417 and GP437) from PRRSV GP3 and GP4 epitopes are selected.

4-1. GP317, GP417 and GP437 construct

GP317, GP417 and GP437 DNA fragment encoding the virus neutralization domain of PRRSV GP3 and GP4 proteins, as shown in Table 10, were synthesized by multi-step PCR using primer pairs. Codon substitutions without altering the original amino acid sequence of the selected peptide segment were made for avoiding spurious restriction sites and for optimal expression in E. coli. Restriction site linkers were added at the ends of the peptide segment-encoding DNA sequence, EcoR1 and Xho1 at 5'end and 3'end, for insertion and sub-cloning into a vector plasmid. The final DNA products, as shown in Table 11, were digested by restriction enzymes EcoR1 and Xho1 to isolate DNA fragments, which were then subcloned into an EcoRI and Xho I digested vector. Once a sample had been obtained, DNA sequences were produced automatically by machine and the result displayed on computer. Sequence analysis performed to identify the sequence of nucleotides in a nucleic acid, or amino acids in a polypeptide.

Table 10. The immunomodulating polypeptide-enhancers

SEQ ID NO.	Name of peptide	Amino acid codon
21	GP317	*EF*VSFSTGGSQNWTVERLLQAEFCSTSQAARQRLETGRNCSTGQAARQRLEPGRNLVLCLTSQAAQQRLEPGGNCQTSQAAHQRLEPGRNCRTSQAASQRLEPGRNCRTSQAAHQRLEPGRNCSTRQAAQQRLEPGRNLLCPTSQAAHQRRLEPGRNCSTSQAAYQRLEPGRNCPTSRAARQRLEPGRNLLCSTSQAALQRLEPGRNLCPTSQAAKQRLEPGRNLVVCLTSQAARQRLEPGRNCSTSQAASQRLEPGRNCPTSQAARQRLEPGRNVLLLCLTSQAAHQRLEPGRN*LE*
22	GP417	*EF*GVSAAQEKISFGLLGVPTAQETTSIREVLEVSTAQENSPFMLGASATEEKTSLRLGASTTQETSFGKCLRPHGVSAAQGTTPFRGVSTTQENTSFGRVPTAQENVSFGLHGVPAAQKTNSFGGVPTAQENISFKEVSATQREIPFRCLRPHGVSTAQETPFRGVSTAQETIPFRGVSATHENISFGCLRPHGVSAAQESIPIRLGASAAQENTSFRGTPAAQEKIP*LE*

23	GP437	*EF*LGVSAAQERIPIREVSADKEVSAEKKEISFGVSTA QGNISFGLGVSTAQEAIPFLALGVSTAQETIPFGLLG VSTAQGIISFGGVSTAQENISFGGVSTAQETISFGLLG VSTAQENISFGCLRTHEVSAAQEKISFGGVSEAQKIS FGVSAAGVSAAQEEIPFGCLRPHGLPAAQEKTSFGG VSAAQEKTSFGGVSAAQEEFSFGCLRPHRVSAAQE KISFEVSALEVSAAQEKISFGVSAALGVSAAQEKNS FGCLRPHGVSAAQEKTSFGGVSAAQKKISFG*LE*

*: VD, EF and LE shown in italics are the restriction enzyme sites, EcoR1 and Xho1, which are added for subcloning purpose.

Table 11. The DNA sequence encoded the immunomodulating polypeptide-enhancers

SEQ ID NO.	Name of peptide	Amino acid codon
24	GP317	<u>GGAATTC</u>GTGAGCTTTAGCACGGGTGGCAGCCAG AACTGGACGGTGGAACGTCTGCTGCAAGCCGAGT TCTGTAGTACTTCTCAGGCGGCGCGCCAGCGTCTG GAACCAGGGCGTAATTGTTCTACAGGCCAGGCCGC ACGTCAACGTTTAGAGCCAGGTCGCAATTTAGTTT TGTGTCTGACGAGCCAGGCCGCACAGCAGCGCTT GGAACCAGGCGGTAACTGTCAAACTTCTCAAGCG GCCCATCAACGCCTGGAACCAGGTCGCAACTGTCG CACTAGCCAAGCCGCCAGCCAACGTTTAGAGCCA GGCCGCAACTGTCGCACGAGTCAGGCGGCGCACC AACGTCTGGAACCAGGCCGTAATTGTAGTACGCGC CAAGCAGCCCAGCAACGCTTAGAACCAGGGCGCA ACCTGTTATGTCCAACTTCTCAGGCGGCCCATCAA CGCCGCTTAGAACCAGGGCGTAATTGTAGCACGTC TCAAGCAGCATATCAACGTCTGGAACCAGGCCGCA ACTGTCCAACTTCTCGTGCGGCACGCCAGCGCTTA GAACCAGGTCGTAATTTATTATGTTCTACTAGCCAA GCCGCATTACAGCGTTTAGAGCCAGGGCGTAACCT GTGTCCAACTAGCCAAGCAGCAAAACAACGCCTG GAGCCAGGTCGTAATTTAGTGGTCTGTTTAACGAG CCAAGCGGCGCGTCAACGCTTAGAACCAGGTCGC AATTGTTCTACTAGCCAAGCGGCCAGTCAACGTTT AGAACCAGGGCGCAACTGTCCAACGAGCCAAGCG GCGCGCCAACGTTTAGAGCCAGGGCGCAACGTTTT ATTGTTGTGTCTGACGAGTCAAGCCGCCCATCAAC GTCTGGAACCAGGTCGCAAT<u>CTCGAG</u>

25	GP417	<u>GAATTC</u>GGCGTGAGCGCGGCCCAGGAAAAGATCA GTTTCGGCCTGTTAGGTGTGCCAACGGCCCAAGAG ACTACAAGTATTCGCGAGGTTTTGGAAGTCAGTAC TGCACAAGAAAACAGTCCATTTATGTTAGGCGCGA GTGCCACGGAGGAAAAAACGTCTTTGCGCCTGGG GGCAAGCACAACGCAGGAGACGAGTTTTGGCAAG TGTTTACGTCCACATGGGGTTTCTGCAGCCCAAGG GACGACTCCATTTCGCGGTGTCAGTACAACGCAAG AAAACACGAGTTTTGGTCGTGTCCCAACGGCACA AGAGAACGTGTCTTTTGGCCTGCATGGTGTTCCAG CAGCGCAAAAGACGAACAGCTTCGGTGGCGTTCC AACGGCACAAGAAAACATTAGTTTTAAGGAGGTTA GTGCCACGCAACGTGAAATCCCATTCCGTTGTTTA CGCCCACACGGGGTTAGCACAGCCCAGGAGACTC CATTTCGCGGGGTGAGTACTGCCCAGGAGACGATC CCATTCCGTGGGGTTTCTGCAACGCATGAAAACAT CAGTTTTGGGTGTTTGCGTCCACATGGTGTCAGCG CCGCACAGGAATCTATTCCAATCCGTCTGGGCGCG AGCGCAGCCCAAGAGAATACCAGTTTTCGCGGGA CACCAGCGGCACAGGAGAAAATCCCATTGGAACT CGAG

26	GP437	GAATTCCTGGGCGTGAGCGCAGCCCAAGAGCGCA TCCCAATTCGCGAGGTGAGCGCCGACAAAGAGGT GAGTGCCGAGAAGAAAGAGATCTCTTTCGGGGTG AGCACCGCGCAGGGTAATATCAGTTTTGGTTTGGG CGTCAGCACCGCACAGGAGGCAATTCCATTCTTGG CACTGGGGGTCAGTACCGCCCAGGAAACTATTCCA TTTGGCTTGCTGGGGGTTAGCACTGCACAAGGTAT CATTAGTTTCGGCGGGGTCTCTACTGCGCAGGAGA ATATCAGCTTTGGCGGGGTTAGTACTGCGCAAGAG ACCATTAGTTTTGGTTTGCTGGGCGTTTCTACCGCC CAGGAGAATATTAGCTTTGGTTGTTTACGCACTCAT GAAGTTAGTGCCGCACAAGAGAAAATTAGCTTCGG CGGCGTTAGTGAAGCGCAAGAGAAGATTAGTTTCG GGGTCTCTGCAGCAGGCGTCAGCGCCGCCCAAGA GGAGATTCCATTTGGGTGTCTGCGCCCACACGGCC TGCCAGCGGCGCAGGAGAAAACCAGCTTCGGCGG CGTTAGTGCCGCCCAGGAAAAGACCTCTTTCGGTG GTGTCAGCGCAGCACAAGAAGAGTTCTCTTTTGGT TGTTTGCGCCCACATCGTGTTAGTGCCGCACAGGA AAAGATCAGCTTTGAAGTTAGCGCGCTGGAAGTCA GTGCCGCGCAAGAGAAGATTAGTTTTGGCGTTAGC GCGGCATTGGGTGTCAGCGCAGCACAAGAAAAGA ACTCTTTCGGTTGTTTACGCCCACACGGTGTTAGC GCCGCGCAAGAGAAAACCAGCTTCGGGGGTGTTA GTGCCGCACAAAAAAAGATCAGCTTTGGGCTCGA G

As shown in Tables 6, 8 and 10, the antigenic epitope used in the fusion polypeptide of the present disclosure may be E622, E713, Myo27, Myo14, GP317, GP417 or GP437, which respectively has a sequence shown by SEQ ID NOs: 10, 12, 17, 18, 21, 22 or 23.

In the present disclosure, a translocating peptide is comprised in the fusion polypeptide of the present disclosure as a carrier. The translocating peptide can be from pseudomonas exotoxin. In one aspect of the present disclosure, the translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III. Hereinafter, the synthesis of the translocating peptide is described in detail.

***5. Pseudomonas* exotoxin (PE) toxoid carrier constructs for delivered**

peptide antigens in the intracellular processing system

Pseudomonas exotoxin A (PE) polypeptide contains domains Ia, II, Ib and III. It has a total of 613 amino acids, the full length PE-DNA fragment was published (Liao CW et al., *Applied Microbiology and Biotechnology*, July 1995, Volume 43, Issue 3, pp 498–507) and the subcloned strains are stored in Liao Lab. To investigate whether the C-terminal deletions of the PE polypeptide fragment would impact the immunogenicity of PE-fused Th1 epitopes, A series of DNA fragments encoding various lengths of *Pseudomonas* exotoxin A polypeptide were synthesized using primer pairs listed in Table 12: PE-425, PE-407, PE-49. The term "PE-*N*" stands for a PE fragment consisting of aa 1-N, in which N is an integer. For example, "PE-425" is a PE fragment consisting of aa 1-425, and "PE-407" is a PE consisting of aa 1-407.

5-1. Example 1: pPE49 plasmid

PE49 target gene is cloned in pET15b plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Plasmid pPE49 was constructed as follows. A 159bp DNA fragment comprising a nucleotide sequence encoding PE aa 1-49 was synthesized by PCR using the primer pair PE-F1 and PE-R1, as shown in Table 12. The 165bp PCR product was digested by Xho1 I and Nde I to isolate a 159bp fragment. It was then subcloned into the 5.9 kb large DNA fragment which was cut from pET15b with Xho1 I and Nde I to generate plasmid pPE-49 (6079bp).

5-2. Example 2: pPE407 plasmid

PE407 target gene is cloned in pET15b plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Plasmid pPE407 was constructed as follows. A 1224bp DNA fragment comprising a nucleotide sequence encoding PE aa 1-407 was synthesized by PCR using the primer pair PE-F1 and PE-R2 as shown in Table 12. The 1238bp

PCR product was digested by Xho1 I and Nde I to isolate a 1224bp fragment. It was then subcloned into the 5.9 kb large DNA fragment which was cut from pET15b with Xho1 I and Nde I to generate plasmid pPE-407 (7159bp).

5-3. Example 3: pPE425 plasmid

PE425 target gene is cloned in pET15b plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Plasmid pPE425 was constructed as follows. A 1299bp DNA fragment comprising a nucleotide sequence encoding PE aa 1-425 was synthesized by PCR using the primer pair PE-F1 and PE-R3 as shown in Table 12. The 1299bp PCR product was digested by Xho1 I and Nde I to isolate a 1293bp fragment. It was then subcloned into the 5.9 kb large DNA fragment which was cut from pET15b with Xho1 I and Nde I to generate plasmid pPE-425 (7213bp).

The nucleotide and amino acid sequences of the above PE fragments are poly-His epitopes, flanked by linkers MGSSHHHHHH and LEHHHHHHZ at 5'- and 3'- (or N- and C-) ends, respectively.

Table 12

Target PE	Fw.* primer	Nucleotide sequence of the forward primer	SEQ ID No.	Rv.** primer	Nucleotide sequence of the reverse primer	SEQ ID No.
PE49	PE-F1	ccccatatggccgaagaagc t	75	PE-R1	tttctcgagttgaattccatgga gtagttcatcactccctggccg ttgg	76
PE407	PE-F2	ccccatatggccgaagaagc t	77	PE-R2	tttctcgaggaattcgacgtcg ccgccgtcgccgaggaactc cg	78
PE425	PE-F3	ccccatatggccgaagaagc t	79	PE-R3	tttctcgaggaattccgcctgg agcagccgctccaccg	80

*Fw: Forward.
**Rv: Reversed.

The target DNA fragment of PE-fused antigenic epitopes can be inserted into Xho1 restriction enzyme site of pPE407 and pPE425.

6. Plasmid constructions for the Mucosal targeting fused polypeptides of immune enhancer

6-1. Plasmid vector construction for subcloning

PE49-3, plasmid construction for insertion of the DNA fragment of M-cells target peptide epitopes. The plasmid is original from the pPE49 plasmid. It was modified by PCR and recombinant manipulation. The primers and sequence modification were shown in Tables 13 and 14.

Table 13

Target PE	Fw.* primer	Nucleotide sequence of the forward primer	SEQ ID No.	Rv.** primer	Nucleotide sequence of the reverse primer	SEQ ID No.
PE49-3	PE-F4	tataccatggccgaacaattggtg gacctc	81	PE-R4	tttctcgaggaattcttcc atgcagtagtgcagcac gccc	82

*Fw: Forward.
**Rv: Reversed.

Table 14. The difference of N-terminal amino acid sequence and DNA sequences and its restriction sites between pPE49 and pPE49-3

The N-terminal amino acid sequence of P49: MGSSHHHHHHSSGLVPRGSA<u>M</u>AE*EAF*DLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHY<u>SMEFLEHM</u> The N-terminal amino acid sequence of P49-3: <u>M</u>AE*QLV*DLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHY<u>*C*MEFLEHM</u>

The 5'-end pP49 DNA sequence behind T7 promotor site direction: ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCC Nde1 GCGCGGCAGCG**CCATGG**CCGAAGAAGCTTTCGACCTCTGGAACGAA TGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAG CCGCATGAGCGTCGACCCGGCCATCGCCGACACCAACGGCCAGGG Nco1 EcoR1 Xho1 Nde1 CGTGCTGCACTACT**CCATG***GAATTC***CTCGAG***CATATG*GCCGAAG HindIII **AAGCTT**TCGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGA Sal1 CCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGC**GTCGAC**
The 5'-end pP49-3 DNA sequence behind T7promotor site direction: Nco1 Mfe1 TTTTGTTTAACTTTAAGAAGGAGATATA**CCATGG**CCGAA**CAATTG**GT GGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAG GACGGCGTGCGTTCCAGCCGCATGAGCGTCGACCCGGCCATCGCCG EcoR1 ACACCAACGGCCAGGGCGTGCTGCACTACTGCATG**GAATTC** Xho1 Nde1 HindIII CTCGAG*CATATG*GCCGAAG**AAGCTT**TCGACCTCTGGAACGAATGCGC CAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCA TGAGC**GTCGAG**CACCACCACCACCACCACTGA

6-2. Plasmid constructions for the M-cell targeting polypeptides

Three DNA fragments (CO1, DQ2, RV3) of M-cell ligand were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE49-3/pET plasmids so that the DNA fragment encode the M-cell ligand was inserted down-stream of T7 promoter. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance. The partial DNA sequences of the insertion portions of pP49-3-CO1, pPE49-3-DQ2, and pPE49-3-RV3 plasmids are shown in Table 15.

Table 15

Plasmid name	SEQ ID NO.	DNA sequence

pP49-3-CO1	27	GTTTAACTTTAAGAAGGAGATATACCATGGCCGAACAATTGGTGGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGACCCGGCCATCGCCGACACCAACGGCCAGGGCGTGCTGCACTACTGCATGGAATTCTCTTTTCATCAGCTGCCAGCGCGTTCTCCAGCCCCACTGCAGCTCGAGCATATGGCCGAAGAAGCTTTCGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAAC
pP49-3-DQ2	28	GTTTAACTTTAAGAAGGAGATATACCATGGCCGAACAATTGGTGGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGACCCGGCCATCGCCGACACCAACGGCCAGGGCGTGCTGCACTACTGCATGGAATTCAGCAGCTTTCATCTGTTCCACCATCTGCCAGCGCGTGCGCCATTAGCGCCTTCTGAATTACAGCCCCTCGAGCATATGGCCGAAGAAGCTTTCGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAAC
pP49-3-RV3	29	GTTTAACTTTAAGAAGGAGATATACCATGGCCGAACAATTGGTGGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGACCCGGCCATCGCCGACACCAACGGCCAGGGCGTGCTGCACTACTGCATGGAATTCTCTACTCCTTTCCACCCATTGCCTGCCCGCAAACCATTGCCTCTGGTGCCCCTCGAGCATATGGCCGAAGAAGCTTTCGACCTCTGGAACGAATGCGCCAAAGCCTGCGTGCTCGACCTCAAGGACGGCGTGCGTTCCAGCCGCATGAGCGTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAAC

6-3. Plasmid construction of the epithelial cell targeting polypeptides

L2-200 of targeting ligand DNA was ligated into EcoR1 and XhoI

restriction enzymes digested pPE49-3/pET plasmid. Plasmid containing insert was respectively transformed into E. coli and clones selected for by ampicillin resistance. The partial DNA sequences of the insertion portion of pL2-200 plasmid are shown in Table 16.

Table 16

Plasmid name	SEQ ID NO.	DNA sequence
pP49-3-L2-200	30	GTTTAACTTTAAGAAGGAGA*TATA*CCATGGC CGAACAATTGGTGGACCTCTGGAACGAATG CGCCAAAGCCTGCGTGCTCGACCTCAAGGA CGGCGTGCGTTCCAGCCGCATGAGCGTCGA CCCGGCCATCGCCGACACCAACGGCCAGG GCGTGCTGCACTACTGCATGGAATTC*CATAT* *G*GTCGAC**GGTATGTCCATCCGTGCTAAAC** **GTCGTAAACGTGCTTCCGCTACCCAGCT** **GTACAAAACCTGCAAACAGGCTGGTACC** **TGCCCGCCGGACATCATCCCGAAAGTTG** **AAGGTAAAACCATCGCTGAACAGATCCT** **GCAATACGGTTCTATGGGTGTTTTCTTCG** **GCGGTCTGGGCATCGGTACCGGTTCCGG** **TACTGGCGGTCGTACCGGTTACATCCCG** **CTGGGTACCCGTCCGCCGACCGCTACCG** **ACACCCTGGCTCCGGTTCGTCCGCCGCT** **GACCGTTGACCCGGTTGGTCCGTCCGAC** **CCGTCCATCGTTTCCCTGGTTGAAGAAA** **CCTCCTTCATCGACGCTGGTGCTCCGAC** **CTCCGTTCCGTCCATCCCGCCGGACGTT** **TCCGGTTTCTCCATCACCACCTCCACCG** **ACACTACCCCGGCTATCCTGGACATCAAC** **AACAACACCGTTACTACCGTAACCACTCA** **CAACAACCCGACCTTCACCGACCCGTCC** **GTTCTGCAACCGCCGACCCCGGCTGAAA** **CCGGTGGTCACTTCACCCTGTCCTCTTC** **CACCATCTCCACCCACAACTACGAAGAA** **ATCCCGATGGACACCAAAGACGAACTG**C TCGAG*CATATG*GCCGAAG*AAGCTT*TCGACCT CTGGAACGAATGCGCCAAAGCCTGCGTGCT CGACCTCAAGGACGGCGTGCGTTCCAGCCG CATGAGCGTCGAGCACCACCACCACCACCA **CTGA**GATCCGGCTGCTAAC

6-4. Preparation of the expressed Mucosa targeting fused polypeptides

The chimeric polypeptides had expression in *E. coli*, BL21(DE3) system. Codon substitutions without altering the original amino acid sequence of the selected peptide segment were made for avoiding spurious restriction sites and for optimal expression in *E. coli*. Restriction site linkers were added at the ends of the peptide segment-encoding DNA sequence for insertion into the expression vector.

7. Plasmid constructions and preparations of the recombinant PE-based fused antigens and immune enhancers

7-1. PE-based chimeric antigens construct

Two DNA fragments (E601; SEQ ID NO. 13, and E701; SEQ ID NO. 15) of HPV Th1-antigenic epitopes were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE425 plasmids (PE toxoid vector derivative plasmids) so that the fusion protein was added at the PE(ΔIII) fragment C-terminal. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance.

Two DNA fragment (M14; SEQ ID NO. 19, and M27; SEQ ID NO. 20) of the Myostatin epitope was ligated into EcoR1 and XhoI restriction enzymes digested pPE407 plasmids.

Three DNA fragments (GP317; SEQ ID NO. 24, GP417; SEQ ID NO 25. and GP437; SEQ ID NO. 26) of PRRSV epitopes were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE425 plasmids (pET and PE toxoid vector derivative plasmids) so that the fusion protein was added at the PE(ΔIII) fragment C-terminal. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance.

Table 17. The examples of the PE-based recombinant chimeric antigens

Groups	SEQ IO NO.	Name of PE-based chimeric antigen

HPV E601, E701	31	PE-E601
	32	PE-E701
M14, M27	33 34	PE-M14 PE-M27
GP317 GP417 GP437	35	PE-GP317
	36	PE-GP417
	37	PE-GP437

SEQ ID NO.	Name of peptide	Amino acid codon
31	PE-E601	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDVSFSTRGTQNWTVERLLQA**EF**QL LRREVFCGFRDLVYDFAFSDLKLPQLCTELK LPQLCTELKDEL**LE**HHHHHH*

32	PE-E701	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDVSFSTRGTQNWTVERLLQA**EF**Q AEPDQAEPDRARAHYNIRARAHYNLEAHY NIVIFRAHYNIVIF**LE**HHHHHH*
33	PE-M14	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDV**EF**VFLQKYPHTHLVHQALDVFL QKYPHTHLVHQALDVFLQKYPHTHLVHQA LDVFLQKYPHTHLVHQA**LE**HHHHHH*

34	PE-M27	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDV**EF**LLEPHTHLVHQANVLLALQL LLEDREFVFLQKYPHVEPHTHLVHQANVLL ALQLLLEDREFVFLQKYPHVEPHTHLVHQA NVLLALQLLLEDREFVFLQKYPHVEPHTHL VHQANVLLALQLLLEDREFVFLQKYPHVEP HTHLVHQANVLLALQLLLEDREFVFLQKYP HVEPHTHLVHQANVLLALQLLLEDREFVFL QKYPHVEPHTHLVHQANVLLALQLLLEDRE FVFLQKYPHVEPHTHLVHQANVLLALQLLL EDREFVFLQKYPHV**E**HHHHHH*

35	PE-GP317	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDVSFSTRGTQNWTVERLLQA**EF**VS FSTGGSQNWTVERLLQAEFCSTSQAARQRL ETGRNCSTGQAARQRLEPGRNLVLCLTSQA AQQRLEPGGNCQTSQAAHQRLEPGRNCRTS QAASQRLEPGRNCRTSQAAHQRLEPGRNCS TRQAAQQRLEPGRNLLCPTSQAAHQRRLEP GRNCSTSQAAYQRLEPGRNCPTSRAARQRL EPGRNLLCSTSQAALQRLEPGRNLCPTSQA AKQRLEPGRNLVVCLTSQAARQRLEPGRNC STSQAASQRLEPGRNCPTSQAARQRLEPGR NVLLLCLTSQAAHQRLEPGRN**LE**HHHHHH*

36	PE-GP417	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDVSFSTRGTQNWTVERLLQA**EFG** VSAAQEKISFGLLGVPTAQETTSIREVLEVST AQENSPFMLGASATEEKTSLRLGASTTQETS FGKCLRPHGVSAAQGTTPFRGVSTTQENTS FGRVPTAQENVSFGLHGVPAAQKTNSFGGV PTAQENISFKEVSATQREIPFRCLRPHGVSTA QETPFRGVSTAQETIPFRGVSATHENISFGCL RPHGVSAAQESIPIRLGASAAQENTSFRGTP AAQEKIP**LE**HHHHHH*

37	PE-GP437	MGSSHHHHHHSSGLVPRGSHMAEEAFDLW NECAKACVLDLKDGVRSSRMSVDPAIADTN GQGVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGSWS LNWLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHESNE MQPTLAISHAGVSVVMAQTQPRREKRWSE WASGKVLCLLDPLDGVYNYLAQQRCNLDD TWEGKIYRVLAGNPAKHDLDIKPTVISHRLH FPEGGSLAALTAHQACHLPLETFTRHRQPRG WEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLAL TLAAAESERFVRQGTGNDEAGAANADVVS LTCPVAAGECAGPADSGDALLERNYPTGAE FLGDGGDVSFSTRGTQNWTVERLLQA**EF**LG VSAAQERIPIREVSADKEVSAEKKEISFGVST AQGNISFGLGVSTAQEAIPFLALGVSTAQETI PFGLLGVSTAQGIISFGGVSTAQENISFGGVS TAQETISFGLLGVSTAQENISFGCLRTHEVSA AQEKISFGGVSEAQKISFGVSAAGVSAAQEE IPFGCLRPHGLPAAQEKTSFGGVSAAQEKTS FGGVSAAQEEFSFGCLRPHRVSAAQEKISFE VSALEVSAAQEKISFGVSAALGVSAAQEKN SFGCLRPHGVSAAQEKTSFGGVSAAQKKISF G**LE**HHHHHH*

7-2. PE-based HPV Fusion Polypeptides

To develop a PE-based HPV fusion protein as a vaccine, a reverse genetic engineering method was employed to construct a highly efficient, viral fusion protein expression vector. Through injection administration, the PE-based HPV vaccine can deliver the viral polypeptides into cells including antigen presenting cells (APCs) to elicit a strong immune response (Liao; Cancer research 2005). Thus, the strategy for developing a PE-based fusion polypeptide was also to fuse the C-terminus of the PE fragment with a viral protein Th1 epitope such as E601 and E701 polypeptides from HPV type 16 as shown in Table 6. However, any other types of HPV (e.g., type 18, type 35 and any other HPV types) can also be used as the HPV antigenic epitope.

Four DNA fragments (E601, E622, E701, and E713) of HPV antigens Th1-epitope were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE425 plasmids (pET23a and PE toxoid vector derivative plasmids) so that the fusion protein was added at the PE(ΔIII) fragment C-terminal. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance.

All synthesized and/or subcloning nucleotide fragments were analyzed by restriction enzyme cutting and electrophoresis to check if they were of the expected sizes and right cutting sites. Once a sample had been obtained, DNA sequences were produced automatically by machine and the result displayed on computer. Sequence analysis performed to identify the sequence of nucleotides in a nucleic acid, or amino acids in a polypeptide.

7-3. PE-based Myostatin epitopes fused polypeptides construct

The strategy of developing a PE-based fusion protein was to fuse the C-terminus of the PE fragment with a myostatin protein binding epitope (M14) to elicit anti-Myostatin antibodies for blocking the activity of myostatin.

Two DNA fragments (M27, M14) of Myostatin binding epitope were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE407 plasmids (pET23a and PE toxoid vector derivative plasmids) so that the fusion protein was added at the PE(ΔIII) fragment C-terminal. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance. Once a sample had been obtained, DNA sequences were produced automatically by machine and the result displayed on computer. Sequence analysis performed to identify the sequence of nucleotides in a nucleic acid, or amino acids in a polypeptide.

7-4. PE-based GP3 and GP4 fused polypeptides construct

The strategy of developing a PE-based fusion protein was to fuse the C-terminus of the PE fragment with virus serum neutralization epitopes (GP3 and GP4) to elicit antibodies against PRRSV infection.

The DNA fragments (GP317, GP417, and GP437) were respectively ligated into EcoR1 and XhoI restriction enzymes digested pPE425 plasmids (pET23a and PE toxoid vector derivative plasmids) so that the fusion protein was added at the PE(ΔIII) fragment C-terminal. Plasmids containing inserts were respectively transformed into E. coli and clones selected for by ampicillin resistance. Once a sample had been obtained, DNA sequences were produced automatically by machine and the result displayed on computer. Sequence analysis performed to identify the sequence of nucleotides in a nucleic acid, or amino acids in a polypeptide.

8. Plasmid construction of the PE-based mucosal targeting fused peptides

All the constructed plasmids, as shown in Table 18, are belonged to the fusion biogenic polypeptides, which can serve an immune enhance functions through the mucosal targeting and Th1 immuno-proteasome processing.

The larger DNA fragment containing DQ2 epitope was cleaved from pP49-3-DQ2 plasmid with HindIII and Pst1, followed by respectively ligating into various DNA fragments, which containing PE and Th1 epitope plasmids, such as pPE-E713, pPE-E622, pPE-M14, pPE-M37, pPE-GP317, pPE-GP417 and pPE-GP437. Those new plasmids containing DQ2 epitope were cleaved with Mfe1 and HindIII and then exchanged other two DNA fragments (P493-CO1, and RV3). The larger DNA fragment containing HPV epithelial cell targeting epitope was cleaved from pP49-3-L2-200 plasmid with HindIII and Pst1, followed by ligating with HindIII and Pst1 restriction enzymes digested pPE-E713 or pPE-E622 plasmid which containing PE with HPV Th1 epitope.

Table 18. The examples of the fusion biogenic polypeptides for immune enhance functions

Group	Name of immune enhancer
HPV E6 and E7 Th1-epiopes: E622, E713	CO1-PE-E622, CO1-PE-E713
	DQ2-PE-E622, DQ2-PE-E713

	RV3-PE-E622, RV3-PE-E713
	L2-200-PE-E662, L2-200-PE-E713
Myostatin-like polypeptide: M27, M14	CO1-PE-M27, CO1-PE-M14
	DQ2-PE-M27, DQ2-PE-M14
	RV3-PE-M27, RV3-PE-M14
PRRSV VN-epitopes: GP417, GP437, GP317	DQ2-PE-GP417, DQ2-PE-GP437 DQ2-PE-GP317

SEQ ID NO.	Name of peptide	amino acid codon
38	CO1-PE-E622	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSFHQL PARSPAPLQLEHMAEEAFDLWNECAKAC VLDLKDGVRSSRMSVDPAIADTNGQGVL HYSMVLEGGNDALKLAIDNALSITSDGLT IRLEGGVEPNKPVRYSYTRQARGSWSLN WLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHES NEMQPTLAISHAGVSVVMAQTQPRREKR WSEWASGKVLCLLDPLDGVYNYLAQQR CNLDDTWEGKIYRVLAGNPAKHDLDIKP TVISHRLHFPEGGSLAALTAHQACHLPLE TFTRHRQPRGWEQLEQCGYPVQRLVALY LAARLSWNQVDQVIRNALASPGSGGDLG EAIREQPEQARLALTLAAAESERFVRQGT GNDEAGAANADVVSLTCPVAAGECAGPA DSGDALLERNYPTGAEFLGDGGDVSFST RGTQNWTVERLLQA*EFVD*KDEL**REVYN** **FAFLLVLRREVYDK**DELLLLLEDR**QLLR** **REVFCGFRDL**LEDR**VYDFAFSDLKLPQ** **LCTELKLPQLCTEL**KDELKDELVLLL*LE* HHHHHH*

39	DQ2-PE-E622	MAEQLVDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYCMEFSSFHLFHHLPARAPLAPSELQPLEHMAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQARGSWSLNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFFVRAHESNEMQPTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQA*EFVD*KDEL**REVYNFAFLLVLRREVYDK**DELLLLLEDR**QLLRREVFCGFRDL**LEDR**VYDFAFSDLKLPQLCTELKLPQLCTEL**KDELKDELVLLL*LE*HHHHHH*
40	RV3-PE-E622	MAEQLVDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYCMEFSTPFHPLPARKPLPLVPLEHMAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQARGSWSLNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFFVRAHESNEMQPTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQA*EFVD*KDEL**REVYNFAFLLVLRREVYDK**DELLLLLEDR**QLLRREVFCGFRDL**LEDR**VYDFAFSDLKLPQLCTELKLPQLCTEL**KDELKDELVLLL*LE*HHHHHH*

41	L2-200-PE-E662	**MAEQLVDLWNECAKACVLDLKDGVRS** **SRMSVDPAIADTNGQGVLHYCM*EF***HM* ***VD**GMSIRAKRRKRASATQLYKTCKQAGT CPPDIIPKVEGKTIAEQILQYGSMGVFFGG LGIGTGSGTGGRTGYIPLGTRPPTATDTLA PVRPPLTVDPVGPSDPSIVSLVEETSFIDAG APTSVPSIPPDVSGFSITTSTDTTPAILDINN NTVTTVTTHNNPTFTDPSVLQPPTPAETG GHFTLSSSTISTHNYEEIPMDTKDELLEH MAEEAFDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYSMVLEGGN DALKLAIDNALSITSDGLTIRLEGGVEPNK PVRYSYTRQARGSWSLNWLVPIGHEKPS NIKVFIHELNAGNQLSHMSPIYTIEMGDE LLAKLARDATFFVRAHESNEMQPTLAISH AGVSVVMAQTQPRREKRWSEWASGKVL CLLDPLDGVYNYLAQQRCNLDDTWEGK IYRVLAGNPAKHDLDIKPTVISHRLHFPEG GSLAALTAHQACHLPLETFTRHRQPRGW EQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARL ALTLAAAESERFVRQGTGNDEAGAANA DVVSLTCPVAAGECAGPADSGDALLERN YPTGAEFLGDGGDVSFSTRGTQNWTVER LLQA*EFVD*KDEL**REVYNFAFLLVLRREV** **YDK**DELLLLLEDR**QLLRREVFCGFRDL** LEDR**VYDFAFSDLKLPQLCTELKLPQL** **CTEL**KDELKDELVLLL*LE*HHHHHH*

42	CO1-PE-E713	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSFHQL PARSPAPLQLEHMAEEAFDLWNECAKAC VLDLKDGVRSSRMSVDPAIADTNGQGVL HYSMVLEGGNDALKLAIDNALSITSDGLT IRLEGGVEPNKPVRYSYTRQARGSWSLN WLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHES NEMQPTLAISHAGVSVVMAQTQPRREKR WSEWASGKVLCLLDPLDGVYNYLAQQR CNLDDTWEGKIYRVLAGNPAKHDLDIKP TVISHRLHFPEGGSLAALTAHQACHLPLE TFTRHRQPRGWEQLEQCGYPVQRLVALY LAARLSWNQVDQVIRNALASPGSGGDLG EAIREQPEQARLALTLAAAESERFVRQGT GNDEAGAANADVVSLTCPVAAGECAGPA DSGDALLERNYPTGAEFLGDGGDVSFST RGTQNWTVERLLQA*EFVD***QAEPDQAEP** **DRDELVLRARAHYNIRARAHYNIL**EDR LLVL**RAHYNIVIFRAHYNIVIF**KDELLV*L* *E*HHHHHH*
43	DQ2-PE-E713	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSSFHL FHHLPARAPLAPSELQPLEHMAEEAFDL WNECAKACVLDLKDGVRSSRMSVDPAI ADTNGQGVLHYSMVLEGGNDALKLAID NALSITSDGLTIRLEGGVEPNKPVRYSYTR QARGSWSLNWLVPIGHEKPSNIKVFIHEL NAGNQLSHMSPIYTIEMGDELLAKLARD ATFFVRAHESNEMQPTLAISHAGVSVVM AQTQPRREKRWSEWASGKVLCLLDPLDG VYNYLAQQRCNLDDTWEGKIYRVLAGN PAKHDLDIKPTVISHRLHFPEGGSLAALTA HQACHLPLETFTRHRQPRGWEQLEQCGY PVQRLVALYLAARLSWNQVDQVIRNALA SPGSGGDLGEAIREQPEQARLALTLAAAE SERFVRQGTGNDEAGAANADVVSLTCPV AAGECAGPADSGDALLERNYPTGAEFLG DGGDVSFSTRGTQNWTVERLLQA*EFVD***Q** **AEPDQAEPDRDELVLRARAHYNIRARA** **HYNIL**EDRLLVL**RAHYNIVIFRAHYNIVI** **F**KDELLV*LE*HHHHHH*

44	RV3-PE-E713	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSTPFH PLPARKPLPLVPLEHMAEEAFDLWNECA KACVLDLKDGVRSSRMSVDPAIADTNGQ GVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGS WSLNWLVPIGHEKPSNIKVFIHELNAGNQ LSHMSPIYTIEMGDELLAKLARDATFFVR AHESNEMQPTLAISHAGVSVVMAQTQPR REKRWSEWASGKVLCLLDPLDGVYNYL AQQRCNLDDTWEGKIYRVLAGNPAKHD LDIKPTVISHRLHFPEGGSLAALTAHQAC HLPLETFTRHRQPRGWEQLEQCGYPVQR LVALYLAARLSWNQVDQVIRNALASPGS GGDLGEAIREQPEQARLALTLAAAESERF VRQGTGNDEAGAANADVVSLTCPVAAG ECAGPADSGDALLERNYPTGAEFLGDGG DVSFSTRGTQNWTVERLLQA*EFVD***QAEP** **DQAEPDRDELVLRARAHYNIRARAHY** **NIL**EDRLLVL**RAHYNIVIFRAHYNIVIF**K DELLV*LE*HHHHHH*

45	L2-200-PE-E713	MAEQLVDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYCMEFHMVDGMSIRAKRRKRASATQLYKTCKQAGTCPPDIIPKVEGKTIAEQILQYGSMGVFFGGLGIGTGSGTGGRTGYIPLGTRPPTATDTLAPVRPPLTVDPVGPSDPSIVSLVEETSFIDAGAPTSVPSIPPDVSGFSITTSTDTTPAILDINNNTVTTVTTHNNPTFTDPSVLQPPTPAETGGHFTLSSSTISTHNYEEIPMDTKDELLEHMAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNGQGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQARGSWSLNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFFVRAHESNEMQPTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQA*EFVD***QAEPDQAEPDRDELVLRARAHYNIRARAHYNIL**EDRLLVL**RAHYNIVIFRAHYNIVIF**KDELLV*LE*HHHHHH*

46	CO1-PE-M14	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSFHQL PARSPAPLQLEHMAEEAFDLWNECAKAC VLDLKDGVRSSRMSVDPAIADTNGQGVL HYSMVLEGGNDALKLAIDNALSITSDGLT IRLEGGVEPNKPVRYSYTRQARGSWSLN WLVPIGHEKPSNIKVFIHELNAGNQLSHM SPIYTIEMGDELLAKLARDATFFVRAHES NEMQPTLAISHAGVSVVMAQTQPRREKR WSEWASGKVLCLLDPLDGVYNYLAQQR CNLDDTWEGKIYRVLAGNPAKHDLDIKP TVISHRLHFPEGGSLAALTAHQACHLPLE TFTRHRQPRGWEQLEQCGYPVQRLVALY LAARLSWNQVDQVIRNALASPGSGGDLG EAIREQPEQARLALTLAAAESERFVRQGT GNDEAGAANADVVSLTCPVAAGECAGPA DSGDALLERNYPTGAEFLGDGGDV***EFVD*** **VFLQKYPHTHLVHQAL**D**VFLQKYPHT** **HLVHQAL**D**VFLQKYPHTHLVHQAL**DV **FLQKYPHTHLVHQA***LE*HHHHHH*
47	DQ2-PE-M14	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSSFHL FHHLPARAPLAPSELQPLEHMAEEAFDL WNECAKACVLDLKDGVRSSRMSVDPAI ADTNGQGVLHYSMVLEGGNDALKLAID NALSITSDGLTIRLEGGVEPNKPVRYSYTR QARGSWSLNWLVPIGHEKPSNIKVFIHEL NAGNQLSHMSPIYTIEMGDELLAKLARD ATFFVRAHESNEMQPTLAISHAGVSVVM AQTQPRREKRWSEWASGKVLCLLDPLDG VYNYLAQQRCNLDDTWEGKIYRVLAGN PAKHDLDIKPTVISHRLHFPEGGSLAALTA HQACHLPLETFTRHRQPRGWEQLEQCGY PVQRLVALYLAARLSWNQVDQVIRNALA SPGSGGDLGEAIREQPEQARLALTLAAAE SERFVRQGTGNDEAGAANADVVSLTCPV AAGECAGPADSGDALLERNYPTGAEFLG DGGDV*EFVD***VFLQKYPHTHLVHQALD** **VFLQKYPHTHLVHQALDVFLQKYPHT** **HLVHQALDVFLQKYPHTHLVHQA***LE*H HHHHH*

48	RV3-PE-M14	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSTPFH PLPARKPLPLVPLEHMAEEAFDLWNECA KACVLDLKDGVRSSRMSVDPAIADTNGQ GVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGS WSLNWLVPIGHEKPSNIKVFIHELNAGNQ LSHMSPIYTIEMGDELLAKLARDATFFVR AHESNEMQPTLAISHAGVSVVMAQTQPR REKRWSEWASGKVLCLLDPLDGVYNYL AQQRCNLDDTWEGKIYRVLAGNPAKHD LDIKPTVISHRLHFPEGGSLAALTAHQAC HLPLETFTRHRQPRGWEQLEQCGYPVQR LVALYLAARLSWNQVDQVIRNALASPGS GGDLGEAIREQPEQARLALTLAAAESERF VRQGTGNDEAGAANADVVSLTCPVAAG ECAGPADSGDALLERNYPTGAEFLGDGG DV*EFVD***VFLQKYPHTHLVHQALDVFLQ** **KYPHTHLVHQALDVFLQKYPHTHLVH** **QALDVFLQKYPHTHLVHQA***LE*HHHHH H*

49	CO1-PE-M27	**MAEQLVDLWNECAKACVLDLKDGVRS** **SRMSVDPAIADTNGQGVLHYCMEFSFH** **QLPARSPAPLQLEHMAEEA**FDLWNECA KACVLDLKDGVRSSRMSVDPAIADTNGQ GVLHYSMVLEGGNDALKLAIDNALSITS DGLTIRLEGGVEPNKPVRYSYTRQARGS WSLNWLVPIGHEKPSNIKVFIHELNAGNQ LSHMSPIYTIEMGDELLAKLARDATFFVR AHESNEMQPTLAISHAGVSVVMAQTQPR REKRWSEWASGKVLCLLDPLDGVYNYL AQQRCNLDDTWEGKIYRVLAGNPAKHD LDIKPTVISHRLHFPEGGSLAALTAHQAC HLPLETFTRHRQPRGWEQLEQCGYPVQR LVALYLAARLSWNQVDQVIRNALASPGS GGDLGEAIREQPEQARLALTLAAAESERF VRQGTGNDEAGAANADVVSLTCPVAAG ECAGPADSGDALLERNYPTGAEFLGDGG DV*EF*LLEPHTHLVHQANVLLALQLLLED REFVFLQKYPHVEPHTHLVHQANVLLAL QLLLEDREFVFLQKYPHVEPHTHLVHQA NVLLALQLLLEDREFVFLQKYPHVEPHT HLVHQANVLLALQLLLEDREFVFLQKYP HVEPHTHLVHQANVLLALQLLLEDREFV FLQKYPHVEPHTHLVHQANVLLALQLLL EDREFVFLQKYPHVEPHTHLVHQANVLL ALQLLLEDREFVFLQKYPHVEPHTHLVH QANVLLALQLLLEDREFVFLQKYPHV*E*H HHHHH*

50	DQ2-PE-M27	**MAEQLVDLWNECAKACVLDLKDGVRS SRMSVDPAIADTNGQGVLHYCMEFSSF HLFHHLPARAPLAPSELQPLEHMAEEA** FDLWNECAKACVLDLKDGVRSSRMSVD PAIADTNGQGVLHYSMVLEGGNDALKL AIDNALSITSDGLTIRLEGGVEPNKPVRYS YTRQARGSWSLNWLVPIGHEKPSNIKVFI HELNAGNQLSHMSPIYTIEMGDELLAKL ARDATFFVRAHESNEMQPTLAISHAGVS VVMAQTQPRREKRWSEWASGKVLCLLD PLDGVYNYLAQQRCNLDDTWEGKIYRV LAGNPAKHDLDIKPTVISHRLHFPEGGSL AALTAHQACHLPLETFTRHRQPRGWEQL EQCGYPVQRLVALYLAARLSWNQVDQVI RNALASPGSGGDLGEAIREQPEQARLALT LAAAESERFVRQGTGNDEAGAANADVV SLTCPVAAGECAGPADSGDALLERNYPTG AEFLGDGGDV*EF*LLEPHTHLVHQANVLL ALQLLLEDREFVFLQKYPHVEPHTHLVH QANVLLALQLLLEDREFVFLQKYPHVEP HTHLVHQANVLLALQLLLEDREFVFLQK YPHVEPHTHLVHQANVLLALQLLLEDRE FVFLQKYPHVEPHTHLVHQANVLLALQL LLEDREFVFLQKYPHVEPHTHLVHQANV LLALQLLLEDREFVFLQKYPHVEPHTHLV HQANVLLALQLLLEDREFVFLQKYPHVE PHTHLVHQANVLLALQLLLEDREFVFLQ KYPHV*E*HHHHHH*

51	RV3-PE-M27	**MAEQLVDLWNECAKACVLDLKDGVRS** **SRMSVDPAIADTNGQGVLHYCMEFSTP** **FHPLPARKPLPLVPLEHMAEEA**FDLWN ECAKACVLDLKDGVRSSRMSVDPAIADT NGQGVLHYSMVLEGGNDALKLAIDNAL SITSDGLTIRLEGGVEPNKPVRYSYTRQA RGSWSLNWLVPIGHEKPSNIKVFIHELNA GNQLSHMSPIYTIEMGDELLAKLARDATF FVRAHESNEMQPTLAISHAGVSVVMAQT QPRREKRWSEWASGKVLCLLDPLDGVY NYLAQQRCNLDDTWEGKIYRVLAGNPA KHDLDIKPTVISHRLHFPEGGSLAALTAH QACHLPLETFTRHRQPRGWEQLEQCGYP VQRLVALYLAARLSWNQVDQVIRNALAS PGSGGDLGEAIREQPEQARLALTLAAAES ERFVRQGTGNDEAGAANADVVSLTCPVA AGECAGPADSGDALLERNYPTGAEFLGD GGDV*EF*LLEPHTHLVHQANVLLALQLLL EDREFVFLQKYPHVEPHTHLVHQANVLL ALQLLLEDREFVFLQKYPHVEPHTHLVH QANVLLALQLLLEDREFVFLQKYPHVEP HTHLVHQANVLLALQLLLEDREFVFLQK YPHVEPHTHLVHQANVLLALQLLLEDRE FVFLQKYPHVEPHTHLVHQANVLLALQL LLEDREFVFLQKYPHVEPHTHLVHQANV LLALQLLLEDREFVFLQKYPHVEPHTHLV HQANVLLALQLLLEDREFVFLQKYPHV*E* HHHHHH*

52	DQ2-PE-GP317	**MAEQLVDLWNECAKACVLDLKDGVRS** **SRMSVDPAIADTNGQGVLHYCMEFSSF** **HLFHHLPARAPLAPSELQPLEHMAEEA** FDLWNECAKACVLDLKDGVRSSRMSVD PAIADTNGQGVLHYSMVLEGGNDALKL AIDNALSITSDGLTIRLEGGVEPNKPVRYS YTRQARGSWSLNWLVPIGHEKPSNIKVFI HELNAGNQLSHMSPIYTIEMGDELLAKL ARDATFFVRAHESNEMQPTLAISHAGVS VVMAQTQPRREKRWSEWASGKVLCLLD PLDGVYNYLAQQRCNLDDTWEGKIYRV LAGNPAKHDLDIKPTVISHRLHFPEGGSL AALTAHQACHLPLETFTRHRQPRGWEQL EQCGYPVQRLVALYLAARLSWNQVDQVI RNALASPGSGGDLGEAIREQPEQARLALT LAAAESERFVRQGTGNDEAGAANADVV SLTCPVAAGECAGPADSGDALLERNYPTG AEFLGDGGDVSFSTRGTQNWTVERLLQA *EF*VSFSTGGSQNWTVERLLQAEFCSTSQ AARQRLETGRNCSTGQAARQRLEPGRNL VLCLTSQAAQQRLEPGGNCQTSQAAHQR LEPGRNCRTSQAASQRLEPGRNCRTSQA AHQRLEPGRNCSTRQAAQQRLEPGRNLL CPTSQAAHQRRLEPGRNCSTSQAAYQRL EPGRNCPTSRAARQRLEPGRNLLCSTSQA ALQRLEPGRNLCPTSQAAKQRLEPGRNL VVCLTSQAARQRLEPGRNCSTSQAASQR LEPGRNCPTSQAARQRLEPGRNVLLLCLT SQAAHQRLEPGRN*LE*HHHHHH*

53	DQ2-PE-GP417	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSSFHL FHHLPARAPLAPSELQPLEHMAEEAFDL WNECAKACVLDLKDGVRSSRMSVDPAI ADTNGQGVLHYSMVLEGGNDALKLAID NALSITSDGLTIRLEGGVEPNKPVRYSYTR QARGSWSLNWLVPIGHEKPSNIKVFIHEL NAGNQLSHMSPIYTIEMGDELLAKLARD ATFFVRAHESNEMQPTLAISHAGVSVVM AQTQPRREKRWSEWASGKVLCLLDPLDG VYNYLAQQRCNLDDTWEGKIYRVLAGN PAKHDLDIKPTVISHRLHFPEGGSLAALTA HQACHLPLETFTRHRQPRGWEQLEQCGY PVQRLVALYLAARLSWNQVDQVIRNALA SPGSGGDLGEAIREQPEQARLALTLAAAE SERFVRQGTGNDEAGAANADVVSLTCPV AAGECAGPADSGDALLERNYPTGAEFLG DGGDVSFSTRGTQNWTVERLLQA*EF*GVS AAQEKISFGLLGVPTAQETTSIREVLEVST AQENSPFMLGASATEEKTSLRLGASTTQE TSFGKCLRPHGVSAAQGTTPFRGVSTTQE NTSFGRVPTAQENVSFGLHGVPAAQKTN SFGGVPTAQENISFKEVSATQREIPFRCLR PHGVSTAQETPFRGVSTAQETIPFRGVSAT HENISFGCLRPHGVSAAQESIPIRLGASAA QENTSFRGTPAAQEKIP*LE*HHHHHH*

54	DQ2-PE-GP437	MAEQLVDLWNECAKACVLDLKDGVRSS RMSVDPAIADTNGQGVLHYCMEFSSFHL FHHLPARAPLAPSELQPLEHMAEEAFDL WNECAKACVLDLKDGVRSSRMSVDPAI ADTNGQGVLHYSMVLEGGNDALKLAID NALSITSDGLTIRLEGGVEPNKPVRYSYTR QARGSWSLNWLVPIGHEKPSNIKVFIHEL NAGNQLSHMSPIYTIEMGDELLAKLARD ATFFVRAHESNEMQPTLAISHAGVSVVM AQTQPRREKRWSEWASGKVLCLLDPLDG VYNYLAQQRCNLDDTWEGKIYRVLAGN PAKHDLDIKPTVISHRLHFPEGGSLAALTA HQACHLPLETFTRHRQPRGWEQLEQCGY PVQRLVALYLAARLSWNQVDQVIRNALA SPGSGGDLGEAIREQPEQARLALTLAAAE SERFVRQGTGNDEAGAANADVVSLTCPV AAGECAGPADSGDALLERNYPTGAEFLG DGGDVSFSTRGTQNWTVERLLQA*EF*LGV SAAQERIPIREVSADKEVSAEKKEISFGVS TAQGNISFGLGVSTAQEAIPFLALGVSTAQ ETIPFGLLGVSTAQGIISFGGVSTAQENISF GGVSTAQETISFGLLGVSTAQENISFGCLR THEVSAAQEKISFGGVSEAQKISFGVSAA GVSAAQEEIPFGCLRPHGLPAAQEKTSFG GVSAAQEKTSFGGVSAAQEEFSFGCLRP HRVSAAQEKISFEVSALEVSAAQEKISFG VSAALGVSAAQEKNSFGCLRPHGVSAAQ EKTSFGGVSAAQKKISFG*LE*HHHHHH*

Among the fusion polypeptides shown in Table 18, the mucosa targeting polypeptide is located at an N-terminal of the fusion polypeptide, the antigenic epitope is located at a C-terminal of the fusion polypeptide, and the translocation peptide is located between the mucosa targeting polypeptide and the antigenic epitope. However, the present disclosure is not limited thereto. In other embodiments of the present disclosure, the translocating peptide is located at an N-terminal of the fusion polypeptide, the antigenic epitope is located at a C-terminal of the fusion polypeptide, and the mucosa targeting polypeptide is located between the translocating peptide and the antigenic

epitope.

9. Fusion polypeptide expression and purification

The plasmids, under the control of T7 promoter, were transformed into E. coli BL21 (DE3) for expression. After the induced expression of the recombinant polypeptides, the inclusion bodies in the lysates were recovered; inclusion granules were harvested from the insoluble fraction by differential centrifugation (1,450 x g, 10 min). The pellet was then homogenized in TNE buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 50 mM NaCl) containing 100 mM PMSF and 1 mg/ml DOC, and a further 1,450 x g supernatant was collected. After three harvests, the pellets were collected from the combined supernatants by centrifugation (27,000 x g, 20 min). Urea soluble contaminants were removed by re-suspending in three washes of 0.1 M Tris-HCl, pH 8.5 containing 1 M urea followed by centrifugation (27,000 x g, 20 min). The final granule preparation was solubilized in 20 volumes of 8 M Urea in TNE, with gentle stirring for overnight at room temperature. The proteins were then purified by S200 gel filtration chromatography in denatured and reduced condition (10 mM DTT) with 6 M urea in TNE buffer. Protein elution fractions were renatured by dialysis against TNE buffers containing from 4 M to 0 M urea in a Pellicon device (Millipore, Billerica, MA, USA).

The clones were grown up from 2 ml of glycerol storage stocks by inoculation into 500 ml flask containing 200 ml of LB with 500 μg/ml Ampicillin. The flasks were shaken at 150 rpm and 37°C, until the cultures had an OD_{600} of 1.0±0.3. Aliquots of 50ml were inoculated in each one of eight sterilized 3000 ml flasks containing 1250 ml LB fortified with 500 μg/ml of Ampicillin and 50 ml 10% glucose, incubated in a 37°C rotating incubator and shaken at 150 rpm for 2-3 hours. IPTG was then added to a final concentration of 50 ppm, and the culture was incubated at 37°C with shaking for another 2 hours to complete the protein induction. The chimeric peptides were quantified by densitometry of Coomassie Blue stained SDS-PAGE. 0.03±0.003 mg of

chimeric polypeptides was used for high-dose administration, and 0.01±0.0001 mg was used for low-dose administration in the mice animal test. For each 10 liters of bacterial culture about 300-400 mg of polypeptides was obtained, which was sufficient for 3000-9000 administrations.

10. Preparation of microspheres of chimeric antigens and immune enhancers for oral administration

The recombinant polypeptides of chimeric antigens and immune enhancer preparations

The polypeptides production and sample preparation performed in a class 100 laminar flow. Each recombinant polypeptide solution was equivalent to 300 mg protein content. It was transferred in 3L volumetric flask and then added 70-80ml of 8M urea with powder carriers as described in Table 19.

Table 19 presents the microsphere formulations. Microsphere powders were prepared with a co-spray drying procedure using a Model L-8 device (Ohkawara Kakohki) as described in Liao et al. (2001). The spray drying process was set based on several manufacturing factors: atomizer speed at 30,000 rpm, feeding rate at 1 mL/min, hot air inlet temperature of 50°C, exhaust temperature of 35°C, and cyclone pressure of ~110 to 120 mm H_2O. Microspheres were stored at 4°C (Liao et al., 2001).

Table 19. The formulation of microsphere powder preparation

300 mg recombinant polypeptide solution in 8M urea	80 ml
Sterile water	1920 ml
HPMC-AS	10 gram
Ethyl-cellulose N20	35 gram
Talc	50 gram

Sodium alginate	5 gram

To test the stability of microsphere formulation, the size and surface structure of microspheres and an in vitro protein-release study were analyzed after the formulations were stored at various temperatures for at least 3 months. The dissolution test of the enteric-coated microspheres was conducted according to the pH-changing method of USP-XXII A (United States Pharmacopoeia, 1990). Microspheres with a total weight of 3 g were placed in a jacketed beaker containing 500 mL of 0.02 N HCl solution (pH 1.5) at 37°C and rotated at 100 rpm. 5 mL of 0.2 M tri-sodium phosphate buffer were added to adjust the pH to 7 for further dissolution experiments, and each test was performed in triplicate. During dissolution testing, 1.5 mL of the samples were taken out at 30-min intervals, which were replaced by 1.5 mL of buffer solution to maintain the volume in the dissolution vessel. Samples were centrifuged at 15,000 g for 5 min, and the supernatants were aspirated and stored at -20°C. Finally, the quantity of protein released was determined by using a Coomassie protein assay.

11. Experiment: Oral administration of M-cell ligand chimeric polypeptides enhancing the mice antibody titers against specific antigens

11-1. To demonstrate that immune enhancement effect of the fusion biogenic polypeptides, vaccination program in mouse tests through orally administration of recombinant antigens

Male ICR mice were obtained at age 4 weeks, quarantined for 1 week before the study, and maintained throughout the study on libitum with pelleted food and water. Growth curve analysis were marked at 4th weeks and weighed twice a week up to 10 weeks. Males and females were separated at 4 weeks and littermates of the same sex placed in the same cage in order to keep the same environment for the different treatment during the rest of the experiment. The

animals were assigned randomly to groups, which received combinations of oral dosing fusion antigen with bio-peptide enhancers as shown in Table 20. For each treatment, at least four mice were analyzed. Weights were plotted on growth curves and Anova statistical analysis using Stat View software was performed.

Used for oral immunization of PE-based vaccines and/or mucosal targeting bio-peptide enhancers were 2 different dosing schedules (3 mg low dose and 6 mg high dose of recombinant antigen formulation, i.e., equal to 10 mcg and 20 mcg protein concentrations). Mice were orally administered with one dose / one time/per week and 4 times with this microsphere solution using a blunt-tipped feeding needle inserted into their stomachs. The positive control group was immunized by subcutaneous injection with 0.25 mL PE-based vaccine that included 20 mcg (H dose) protein antigens at 2-week intervals for a total of four times immunizations.

Two weeks after each immunization, three mice were exsanguinated and blood was collected by puncturing their retroorbital plexuses. From the results in serum and intestinal specific antibodies titers against the fusion antigen was examined. Serum was obtained by coagulation at 4°C for 12 h followed by centrifugation. Intestine lavage samples done at necropsy were collected by instilling 1 mL of washing buffer (PBS containing 100g/mL soybean trypsin inhibitor, 50mM EDTA, 1mM PMSF, 0.5% gelatin, and 0.05% NaN_3) into the intestine. The lavage was collected and stored at -20°C, and lung samples after necropsy were gathered by homogenizing one-half of the lung with 0.5 mL of washing buffer. Finally, the supernatant of a lung-homogenized sample was collected and stored at -20°C following centrifugation. Blood samples were taken and the serum assayed in an ELISA for the titer of anti-E7 or anti-E6 or anti-M14 or anti-GP3 or anti-GP4 specific antibodies using serial ten-fold dilutions. The specific IgA or IgG or IgG1 or IgG2a antibody titer was detected after the second round of immunization. The PRRS virus neutralization assay

was examined with PE-GP417, 437 &317 by oral and injection vaccine groups.

The very high dose (VH) (100 mcg protein) and high-dose injections (H) (20 mcg protein) induced similar titers after the third round immunization and reached a plateau after the fourth round. The low dose of injection induced a lower titer, but was still detectable at a 1:3250 dilution after the fourth round immunization.

11-2. Experimental tables:

Table 20. The vaccination program and examination of immune enhancement effect of the fusion biogenic polypeptides in mouse tests

Table 20-1. Experiment No.1

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
1. Blank	0 dose	-	0 dose
2. PE-E6	L dose	PE-E622	L dose
3. PE-E6	L dose	DQ2-PE-E622	L dose
4. PE-E601	L dose	-	0 dose
5. PE-E6	L dose	-	0 dose
6. Positive control through subcutaneous injection PE-E6* or PE-E106 (4 times injection with H dose)			
PE-E6 or PE-E106 monovalent vaccine	H dose (20 mcg total protein /dose)		

*: PE-E6 vaccine published in Cheng WF et al., *PLoS One*. 2013 Sep 13;8(9):e71216.

Table 20-2. Experiment No.2

Vaccine groups one dose / 1 time/per week and 4 times orally	Immune enhancer L dose/ 1 time/per week and 4 times orally

7. Blank	0 dose	-	0 dose
8. PE-E601	2 L-doses	-	0 dose
9. PE-E601	L dose	L2-200-PE-E622	L dose
10. PE-E601	L dose	CO1-PE-E622	L dose
11. PE-E601	L dose	DQ2-PE-E622	L dose
12. PE-E601	L dose	RV3-PE-E6222	L dose

Table 20-3. Experiment No.3

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
13. Blank	0 dose	-	0 dose
14. PE-E601	2 L-doses	-	-
15. PE-E601	L dose	CO1-PE-E601	L dose
16. PE-E601	L dose	DQ2-PE-E601	L dose
17. PE-E601	L dose	RV3-PE-E601	L dose
18. PE-E601	L dose	L2-200-PE-E601	-

Table 20-4. Experiment No.4

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
19. Blank	-	-	-
20. PE-E7	L dose	PE425-E713	L dose
21. PE-E7	L dose	DQ2-PE425-E713	L dose
22. PE-E701	L dose	-	-
23. PE-E7	L dose	-	-
24. Positive control through subcutaneous injection PE-E701 (4 times injection with H dose)			
PE-E7 monovalent vaccine H dose (20 mcg total protein /dose)			

Table 20-5. Experiment No.5

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
25. Blank	-	-	0 dose
26. PE-E701	L dose	PE-E713	L dose
27. PE-E701	L dose	L2-200-PE-E713	L dose
28. PE-E701	L dose	CO1-PE-E713	L dose
29. PE-E701	L dose	DQ2-PE-E713	L dose
30. PE-E701	L dose	RV3-PE-E713	L dose

Table 20-6. Experiment No.6

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
31. PE-M14	2 L-doses	-	-
32. PE-M14	L dose	CO1-PE-M14	L dose
33. PE-M14	L dose	DQ2-PE-M14	L dose
34. PE-M14	L dose	RV3-PE-M14	L dose
35. PE-M14	L dose	PE-M27	L dose
36. PE-M14	L dose	CO1-PEM27	L dose
37. PE-M14	L dose	DQ2-PE-M27	L dose
38. PE-M14	L dose	RV3-PE-M27	L dose
39. Blank	L dose	-	0 dose

Table 20-7. Experiment No.7

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
40. Blank	-	-	-

41. PE-GP417	L dose	CO1-PE-GP417	L dose
42. PE-GP417	L dose	DQ2-PE-GP417	L dose
43. PE-GP417	L dose	RV3-PE-GP417	L dose
44. PE-GP417	2 L-doses	-	-
45. PE-GP437	L dose	CO1-PE-GP437	L dose
46. PE-GP437	L dose	DQ2-PE-GP437	L dose
47. PE-GP437	L dose	RV3-PE-GP437	L dose
48. PE-GP437	2 L-doses	-	-
49. Positive control through subcutaneous injection PE-GP417&437 (4 times injection with H dose)			
PE-GP417&437 bivalent vaccine		H dose (20 mcg total protein /dose)	

Table 20-8. Experiment No.8

Vaccine groups one dose / 1 time/per week and 4 times orally		Immune enhancer L dose/ 1 time/per week and 4 times orally	
50. Blank	-	-	-
51. PE-GP317	L dose	CO1-PE-GP317	L dose
52. PE-GP317	L dose	DQ2-PE-GP317	L dose
53. PE-GP317	L dose	RV3-PE-GP317	L dose
54. PE-GP317	2 L-doses	-	-

11-3. Oral immunization experiment in pig model : Oral administration of M-cell ligand chimeric polypeptides enhancing the swine antibodies titer against specific antigens

In the pig model, each 24 piglets were selected respectively from six sows source in a healthy pig farming. Three piglets from each sow were assigned and marked with ear-number randomly to the vaccinated and control groups as listed in Table 21. The oral vaccine group totally contained nine piglets, which received orally L dose (3 mg) or H dose (6 mg) suspended in a

2% acetate solution at the ages 4, 6, 7, 9, 12, 14, 16, 18, 21, 24, 26 and 28-day. The intramuscular administration group contained another three piglets that received a VH dose (100 mcg for each antigen) of PE-GPs trivalent with two times injection. These were immunized at ages 14 and 28-day by intramuscular injection with 1 mL of vaccine that included 0.5 mL of formalin-inactive broth (300 mcg total protein of GP417, 437, and 317 antigens) and 0.5 mL aluminum gel. These last 3 piglets served as controls. After accomplishment of the oral and injection administration program, all the experiment piglets, age 29-30-day, were moved and kept in groups of ten in straw-bedded pens 3.8 m × 4.5 m (17 m^2). Air temperature and humidity were recorded twice a day throughout the testing period. Animals were fed the same complete feed mixture.

Table 21. The vaccination program and examination of immune enhancement effect of the mucosal targeting polypeptides in piglet tests

Vaccine groups (N=3) L dose / 3 time/per week and 12 times orally/ 4 weeks		Immune enhancer (N=3) L dose/ 3 time/per week and 12 times orally/ 4 weeks	
Blank	0 dose	-	0 dose
PE-M14	L dose	DQ2- PE-M14	L dose
PE-M14	L dose	DQ2- PE-M27	L dose
PE-M14	H dose	-	-
PE-GP417, 437, 317	L dose	DQ2-PE-GP417	L dose
PE-GP417, 437, 317	L dose,	additional with PE-GP417	L dose
PE-GP417, 437, 317	H dose	-	-
Positive control through intramuscular injection (N=3) twice at ages 14 and 28-day			
PE-GP417, 437, 317 trivalent vaccine		VH dose (300mcg total protein /dose)	

The observation and monitored for grow performance and health was preceded by a 2-day period during which the piglets were adapted to the new environment. The data was subjected to analysis of variance (ANOVA) to obtain the effect of post-weaning age on weight gain and linear measurements.

Two weeks after finishing immunization program, piglet blood was collected by puncturing their retroorbital plexuses. From the results in serum and intestinal specific antibodies titers against the fusion antigen was examined. Serum was obtained by coagulation at 4°C for 12 h followed by centrifugation. Blood samples were taken and the serum assayed in an ELISA for the titer of anti-Myo14 or anti-GP317 or anti-GP417&437 specific antibodies using serial 2.5-fold dilutions. The specific IgA or IgG or IgG1 or IgG2a antibody titer was detected after the second round of immunization. The PRRS virus neutralization assay was examined PE-GP417, 437 &317 oral and injection vaccine groups.

11-4. Measurement of antibody responses by ELISA

Polystyrene microdilution plates (Costar) were coated with antigens. The plates were coated with 100 μl per well with this antigen at a concentration of 2 μg/mL protein in coating buffer (carbonate-bicarbonate buffer at pH 9.6). Binding antibody titers induced the six proteins (E7, E6, Myo14, Myo27, GP3, GP4) were analyzed using ELISA tests. Following overnight adsorption each protein antigens at 4°C, in-directed ELISA was performed as described (Liao 2003). Horse radish peroxidase (HRP)-conjugated secondary antibodies (goat anti-Mouse IgG, IgA, IgG2, IgG1) were diluted at the optimal concentration (x1000 fold) in blocking buffer immediately before use. Note that a positive reference sample with a high antibody titer should be used to determine the titers of antibodies. This reference sample was made into aliquots and preserved in the lyophilized state. The serum sample was diluted 500x, 2500x, 12500x, and 62500x, respectively. The intestinal and lung samples were diluted 10-fold times for routine work. To interpret the results accurately, ELISA (E)

value was determined by the following formula: E value = D x (S-N)/(P-N), where the OD value of injection vaccine sample = positive control serum or lavage, N = negative control serum or lavage, S = test sample, and D = sample dilution fold.

11-5. PRRSV serum neutralization test by indirect immunofluorescence

PRRS virus neutralization test by indirect immunofluorescence assay. All filtered oral fluid and serum samples (0.2-μm filter) used in the NA assay were treated with UV light (254 nm) at a distance of two inches from the samples in plates for 45 min and were then heat inactivated for 30 min at 56°C. Each test sample was 2-fold serially diluted (1:2 to 1:128) in serum-free DMEM (100 μl per well) and incubated with an equal volume of 100 TCID50 of one of the PRRSV strains (PRRSV TC-01) for 1 h at 37°C. After incubation, 100 μl of the supernatant was transferred into a 96-well microtiter plate containing a confluent monolayer of Alveolar macrophage 3D4/31 (ATCC CRL-2844; Alveolar macrophage; immortalized with SV40 large T antigen); each sample was run in duplicate. After 1 h of incubation, 100 μl of DMEM containing 2% horse serum and an antibiotic-antimycotic mixture was added, and the plate was incubated for 48 h at 37°C in a CO_2 incubator. Cells were fixed using an acetone/Milli-Q water (8:2) mixture for 10 min at room temperature (~20°C), and plates were dried completely before being immunostained as described previously. Cells were treated with anti-PRRSV nucleocapsid protein-specific monoclonal antibody (SDOW17) (Rural Technologies, Inc., SD) (1:5,000) for 2 h at 37°C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG(H+L) (Invitrogen, CA) secondary antibody (1:3,000). The plate was examined under a fluorescence microscope after mounting with glycerol–phosphate-buffered saline (PBS) (6:4). The virus-neutralizing antibody (NA) titer was determined to be the reciprocal dilution ratio of the sample at which >90% inhibition in the PRRSV-induced immunofluorescence was observed.

12. Result of experiments

12-1. The efficacy of fusion polypeptides as immune enhancers on the PE-based E6 oral vaccine

The high-risk human papillomavirus E6 (hrHPV E6) protein has been widely studied due to its implication in the process of malignant transformation of human cells. HPV 16 E6 oncoprotein could affect the IL-18 induced IFN-γ production in human PBMCs to elucidate the possible immune escape mechanisms of HPV infected cervical lesion including cervical cancer. The E6 oncoprotein of HPV-16 and HPV-18 inhibit immune response by interacting with host IRF3 and TYK2 (Li S et al., *Oncogene*. 1999 Oct 14;18(42):5727-37; Masaud Shah et al., *Scientific Reports* **5**, Article number: 13446 (2015); Cheng WF et al., *PLoS One*. 2013 Sep 13;8(9):e71216.; Cho YS et al., *FEBS Lett*. 2001 Jul 20;501(2-3):139-45). In our previous research, the E6-specific immune responses generated by the PE-E6 vaccine were weaker than the E7-specific immune responses of the PE-E7 through injection mice model. In this disclosure, we evaluated the efficacy of PE-E6 oral vaccine on immune response through oral administration in mice model. In the experiments, we demonstrated that the mucosal targeting epitopes, including DQ2 and RV3 had something enhancement activity of E6-specific immune response from fallowing data analysis.

In Table 22, oral administrations of PE-E6 or PE-E601 demonstrated weak immune response against E6 or E601 antigen according to the mice serum antibodies activity data of the group no. 4 and 5 by ELISA test. However, the serum IgA and IgG antibodies activity of PE-E6 vaccine with additional PE-E622 or DQ2-PE-E622 immune enhancer, group no. 2 and 3, could be enhanced. From the E6-specific IgG activity in serum dilution 500-fold samples, the data of the group No.3 was significantly higher than other oral vaccine groups without enhancer additional ($p < 0.05$).

Table 22. Serum levels of IgG and IgA against HPV-16 E6 in the mice

groups by ELISA test

Group no. (n=3)	Serum anti-E6 IgG activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
1	Blank	-	0.35	0.10	0.27	0.03	0.22	0.02
2	PE-E6	PE-E622	0.70	0.11	0.45	0.06	0.19	0.06
3	PE-E6	DQ2-PE-E622	0.77	0.08	0.59	0.08	0.25	0.08
4	PE-E601	-	0.39	0.05	0.22	0.05	0.18	0.05
5	PE-E6	-	0.55	0.14	0.34	0.10	0.22	0.10
6	Inject (H dose)	-	0.90	0.05	0.71	0.07	0.35	0.07

Group no. (n=3)	Serum anti-E6 IgA activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
1	Blank	-	0.15	0.08	0.18	0.03	0.18	0.03
2	PE-E6	PE-E622	0.40	0.14	0.30	0.11	0.25	0.06
3	PE-E6	DQ2-PE-E622	0.43	0.10	0.33	0.08	0.24	0.08
4	PE-E601	-	0.27	0.05	0.20	0.05	0.18	0.05
5	PE-E6	-	0.38	0.14	0.26	0.10	0.19	0.10
6	Inject (H dose)	-	0.45	0.05	0.37	0.07	0.22	0.07

In the table 23, E6 or E601-specific serum IgG1 and IgG2a responses were assayed by ELISA, 2-3 weeks after oral administration of vaccine and/or additional immune enhancers. From the data of the group No.3, oral administration of PE-E6 vaccine with additional DQ2-PE-E622 immune enhancer could elicit a good Th1 pathway according to the high ratio of

IgG2a/IgG1. The IgG2a/IgG1 ratio was 1.4-1.5 in the group no.3 (PE-E6 oral vaccine with additional DQ2-PE-E622 enhancer). It was significantly higher than other groups without enhancer additional ($p<0.05$). The IgG2a/IgG1 ratio of the E6-specific antibodies in group no.2 (PE-E6 oral vaccine with additional PE-E622 enhancer) was 1.13, that was not strong significantly compared with other groups which without enhancer additional ($p> 0.05$).

Table 23. Serum activity of IgGs anti-E6 or anti-E601 and its IgG2a/IgG1 ratio

Group no. (n=3)	Anti-E6 activity in ELISA-Test		IgG1		IgG2		IgG2a/IgG1
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	
1	Blank	-	0.17	0.10	0.09	0.03	
2	PE-E6	PE-E622	0.36	0.11	0.45	0.06	1.24
3	PE-E6	DQ2-PE-E622	0.42	0.08	0.59	0.08	1.41
4	PE-E601	-	0.19	0.05	0.18	0.05	0.96
5	PE-E6	-	0.38	0.14	0.24	0.10	0.63
6	Inject (H dose)	-	0.48	0.05	0.42	0.07	0.08

Group no. (n=3)	Anti-E601 activity in ELISA-Test		IgG1		IgG2		IgG2a/IgG1
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	
1	Blank	-	0.15	0.08	0.18	0.03	
2	PE-E6	PE-E622	0.40	0.14	0.45	0.11	1.13
3	PE-E6	DQ2-PE-E622	0.43	0.10	0.65	0.08	1.51
4	PE-E601	-	0.29	0.05	0.27	0.05	0.94
5	PE-E6	-	0.32	0.14	0.24	0.10	0.75

6	Inject (H dose)	-	0.33	0.05	0.22	0.07	0.67

In Table 24, five groups of mice were oral administration of PE-E601 vaccine additional with various enhancers including PE-E601, L2-L200-PE-E622, CO1-PE-E622, DQ2-PE -E622 and RV3-PE-E622, respectively.

The immune responses of those groups of mice against E601 antigen were examined according to mice serum IgG1, IgG2a antibodies activity by ELISA test. Through the IgG2a/IgG1 ratio data, the Th1 or Th2 immune pathway could be prospected. The IgG2a/IgG1 ratio was 1.56 or 1.46 in the group no.11 or 12 (PE-E601 oral vaccine with additional DQ2-PE-E622 or RV3-PE-E622 enhancer), but those of the group 8 or 9 or10 (PE- E601 oral vaccine additional with PE-E601 or L2-200-PE-E622 or CO1-PE-E622 enhancer) was lower than 1.05. According to the high ratio of E601-specific IgGs and IgG2a/IgG1 data in mice experiments, the DQ2-PE-E622 and RV3-PE-E622 enhancers could strongly elicit a good Th1 immunity when PE-based E601 oral vaccine administration with additional the enhancers. The Th1 immunity enhancing efficacy of DQ2-PE-E622 and RV3-PE-E622 were significantly different from other groups ($P<0.05$).

Table 24

Group no. (n=3)	Anti-E601 activity in ELISA-Test		IgG1		IgG2		IgG2a/IgG1
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	
7	Blank	-	0.15	0.08	0.18	0.03	
8	PE-E601 2 L-doses		0.33	0.04	0.28	0.07	0.83
9	PE-E601	L2-200-PE-E622	0.40	0.03	0.38	0.03	0.94
10	PE-E601	CO1-PE-E622	0.42	0.03	0.44	0.03	1.04

11	PE-E601	DQ2-PE-E622	0.29	0.04	0.45	0.05	1.56
12	PE-E601	RV3-PE-E6222	0.31	0.03	0.45	0.04	1.46

In Table 25, five groups of mice are oral administration of PE-E601 vaccine additional with various enhancers including PE-E601, L2-200-PE-E601, CO1-PE-E601, DQ2-PE-E601 and RV3-PE-E601, respectively.

The immune response of those groups of mice against E601 antigen examined according to mice serum IgG1, IgG2a antibodies activity by ELISA test. Through the IgG2a/IgG1 ratio data, the Th1 or Th2 pathway could be prospected. The IgG2a/IgG1 ratio is 1.24 or 1.28 in the group no.16 or 17 (PE-E601 oral vaccine additional with DQ2-PE-E622 or RV3-PE-E622 enhancer), but those of the group 14 or 15 or18 (PE-E601 oral vaccine additional with PE-E601 or CO1-PE-E622 or L2-200-PE-E622) was lower than 1.05. According to the high ratio of IgG2a/IgG1 data in mice experiments, the DQ2-PE-E622 and RV3-PE-E622 enhancers can elicit a good Th1 pathway when PE-based E016 oral vaccine administration additional with the enhancers ($p<0.05$).

Table 25

Group no. (n=3)	Anti-E601 activity in ELISA-Test		IgG1		IgG2		IgG2a/IgG1
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	
13	Blank	-	0.15	0.08	0.18	0.03	
14	PE-E601 2 L-doses		0.33	0.05	0.28	0.07	0.83
15	PE-E601	CO1-PE-E601	0.34	0.05	0.34	0.03	1.00
16	PE-E601	DQ2-PE-E601	0.35	0.05	0.44	0.03	1.24
17	PE-E601	RV3-PE-E601	0.35	0.04	0.45	0.05	1.28

18	PE-E601	L2-200-PE-E601	0.33	0.05	0.31	0.04	0.94

12-2. The effect of fusion biogenic polypeptides enhancers on the PE-based E7 oral vaccine

Our previous studies indicated that a based E7 fusion protein vaccine enhanced MHC class I and II presentation of E7, leading to dramatic increases in the number of E7-specific CD8+ and CD4+ T-cell precursors and markedly raised titers of E7-specific antibodies. These results indicated that retrograde-fusion protein via the delivery domains of exotoxins with an antigen greatly enhances in vivo antigen-specific immunologic responses and represents a novel strategy to improve cancer injection vaccine potency (Ebrahimpoor S et al., *Iran J Allergy Asthma Immunol*. 2013 Aug 28;12(4):361-7.). In the present disclosure, we try to evaluate the efficacy of PE-E7 oral vaccine on immune response of mice test through oral administration. We performed a study on the oral administration experiments of PE-E7 in mice immunization test that we got similar results that were found in PE-E6 study. Orally administrations of PE-E7 or PE-E701 demonstrated weak immune response against E7 or E701 antigen. In the present disclosure, we demonstrated that the mucosal targeting epitopes, including DQ2 and RV3 had something enhancement activity of E6-specific antibodies from fallowing data analysis.

In Table 26, oral administrations of PE-E7 or PE-E701 demonstrated weak immune response against E7 or E701 antigen according to the mice serum antibodies activity data of the group no. 22 and 23 by ELISA test.

Table 26

Group no. (n=3)	Serum anti-E7 IgG activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
19	Blank	-	0.30	0.10	0.24	0.03	0.18	0.04

20	PE-E7	PE-E713	0.72	0.15	0.43	0.10	0.30	0.07
21	PE-E7	DQ2-PE-E713	0.93	0.05	0.55	0.06	0.39	0.05
22	PE-E701	-	0.44	0.07	0.22	0.06	0.19	0.05
23	PE-E7	-	0.68	0.14	0.34	0.10	0.22	0.10
24	Inject (H dose)	-	1.50	0.15	0.75	0.07	0.48	0.07

Group no. (n=3)	Serum anti-E7 IgA activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
19	Blank	-	0.21	0.08	0.18	0.03	0.18	0.03
20	PE-E7	PE-E713	0.50	0.16	0.30	0.10	0.25	0.06
21	PE-E7	DQ2- PE-E713	0.60	0.12	0.33	0.05	0.24	0.08
22	PE-E701	-	0.21	0.15	0.20	0.07	0.18	0.05
23	PE-E7	-	0.43	0.20	0.26	0.07	0.19	0.10
24	Inject (H dose)	-	0.60	0.15	0.37	0.07	0.22	0.07

However, the serum IgA and IgG antibodies activity of PE-E7 vaccine group with additional PE-E622 or DQ2-PE-E622, group no. 20 and 21, could be enhanced. From the E7-specific IgG activity in serum dilution 200-fold samples, the data of the group No.21 was significantly higher than other oral vaccine groups without enhancer additional ($p< 0.05$).

In Table 27, five groups of mice were oral administration of PE-E701 vaccine additional with various enhancers including PE-E713, L2-L200-PE-E713, CO1-PE-E713, DQ2-PE-E713 and RV3-PE-E713, respectively.

Table 27

Group no. (n=3)	Serum anti-E701 IgGs activity in ELISA-Test		IgG1		IgG2		IgG2a/IgG1
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	
25	Blank	-	0.18	0.09	0.19	0.05	
26	PE-E701	PE-E713	0.36	0.06	0.37	0.10	1.03
27	PE-E701	L2-200-PE-E713	0.42	0.06	0.65	0.05	1.55
28	PE-E701	CO1-PE-E713	0.47	0.06	0.63	0.07	1.34
29	PE-E701	DQ2-PE-E713	0.47	0.08	0.67	0.09	1.43
30	PE-E701	RV3-PE-E713	0.48	0.06	0.69	0.05	1.44

The immune responses of those groups of mice against E701 antigen were examined according to mice serum IgG1, IgG2a antibodies activity by ELISA test. Through the IgG2a/IgG1 ratios data, the Th1 or Th2 immune pathway could be prospected. The IgG2a/IgG1 ratios were 1.55, 1.34, 1.43 and 1.44 in the group no.27, no. 28, no. 29 and no.30 which were PE-E701 oral vaccine with additional L2-200-PE-713, CO1-PE-E713, DQ2-PE-E713, RV3-PE-E713 enhancers, respectively. The IgG2a/IgG1 ratios of group 26 (PE-E701 oral vaccine additional with PE-E713 enhancer) was lower than 1.05. According to the high activity of E701-specific IgG2a and high IgG2a/IgG1 ratios data, the L2-200-PE-713, CO1-PE-E713, DQ2-PE -E622 and RV3-PE -E622 enhancers could strongly elicit a good Th1 immunity when PE-based E713 oral vaccine administration with additional these enhancers in mice immunization model. The immunity enhancing efficacy of E701-specific IgG2a showed that L2-200-PE-713, CO1-PE-E713, DQ2-PE-E713 and RV3-PE-E713 groups had significantly different from PE-E713 enhancer groups ($P<0.05$).

12-3. The efficacy of fusion polypeptides as immune enhancers on the PE-

based myostatin oral vaccine

Recombinant myostatin can induce immune responses to myostatin by oral route, resulting in increasing body weight in mice. It is an important step towards transforming cells into edible vaccine to improve meat production in farm animals and combat muscle-waste genetic diseases in human (Zhang T et al., *BMC Biotechnol.* 2012 Dec 19;12:97; Aravind S et al., *J Virol Methods.* 2012 Nov;185(2):234-8).

We have established a PE-based myostatin fused oral vaccine for animal use. There were several fusion biogenic polypeptide enhancers were developed for PE-based myostatin fused oral vaccine formulation. In the present disclosure, we demonstrated that the mucosal targeting epitopes, including CO1, DQ2 and RV3 had something enhancement activity of myostatin epitope-specific antibodies from fallowing data analysis.

In Tables 28 and 29, the M14-specific serum IgG and IgA activities of ELISA in serial serum dilutions (1:200-1:1250) were examined in various serum samples.

Table 28

Group no. (n=3)	Serum anti-M14 IgG activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
31	PE-M14 2 L-doses		0.56	0.20	0.51	0.07	0.34	0.04
32	PE-M14	CO1-PE-M14	0.80	0.23	0.78	0.16	0.41	0.04
33	PE-M14	DQ2-PE-M14	0.81	0.16	0.66	0.06	0.44	0.04
34	PE-M14	RV3-PE-M14	0.95	0.24	0.74	0.17	0.36	0.06
39	Blank	-	0.30	0.10	0.27	0.03	0.22	0.02

Table 29

Group no. (n=3)	Serum anti-M14 IgA activity in ELISA-Test		dilx 200		dilx 500		dilx 1250	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev
35	PE-M14	PE-M27	0.58	0.24	0.45	0.01	0.29	0.09
36	PE-M14	CO1-PE-M27	0.75	0.12	0.69	0.16	0.35	0.05
37	PE-M14	DQ2-PE-M27	0.75	0.06	0.64	0.07	0.33	0.03
38	PE-M14	RV3-PE-M27	0.79	0.13	0.66	0.16	0.29	0.05
39	Blank	-	0.27	0.02	0.25	0.03	0.23	0.03

According to serial dilutions ELISA data of the group no. 31 (PE-M14 vaccine group), it demonstrated that oral administration of PE-M14 vaccine elicited a good M14-specific IgG and IgA serum titers in the 1:200 to 1:1250 fold serum dilutions. Furthermore, the serum IgA and IgG antibodies activities of the groups of PE-M14 vaccine with additional CO1-PE-M14, DQ2-PE-M14 and RV3-PE-M14, CO1-PE-M27, DQ2-PE-M27 and RV3-PE-M27, corresponded to the group no. 32, 33 34, 36, 37 and 38 could be extremely enhanced. From the M14-specific IgG activity in serum dilution 500-fold samples, the data of the group No.32, No. 33 and No.34 were significantly higher than other oral vaccine groups ($p < 0.05$).

In Table 30, the data demonstrated that PE-based M14 fused oral vaccines could be enhanced by the mucosal targeting ligands, CO1, DQ2 and RV3. The serum specific-M14 antibodies activities (with serum 500 dilutions-ELISA) of IgG and IgA of the groups no. 32, 33 and 34, which treated with additional CO1-PE-M14, DQ2-PE-M14 and RV3-PE-M14 enhancer respectively, were slightly higher than that of activities of the group No.31 ($p > 0.1$). Furthermore, the activities of groups no. 36, 37, 38 presented very high level than that of groups no. 31 or no. 35, without Mucosal targeting ligands

additional. Specifically, the IgG and IgA levels of group no. 37 and 38 were significantly higher ($p<0.05$) when compared with the control group (such as group no. 31 or 35).

Table 30

Group no. (n=3)	Serum anti-M14 IgA activity in ELISA-Test		Value of OD405 by ELISA (the level of IgG antibody)		Value of OD405 by ELISA (the level of IgA antibody)	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev
31	PE-M14 2 L-doses		0.48	0.07	0.40	0.05
32	PE-M14	CO1-PE-M14	0.62	0.10	0.53	0.10
33	PE-M14	DQ2-PE-M14	0.60	0.16	0.55	0.08
34	PE-M14	RV3-PE-M14	0.65	0.13	0.57	0.05
35	PE-M14	PE-M27	0.51	0.07	0.45	0.08
36	PE-M14	CO1-PE-M27	0.78	0.22	0.69	0.16
37	PE-M14	DQ2-PE-M27	0.68	0.05	0.64	0.07
38	PE-M14	RV3-PE-M27	0.75	0.10	0.66	0.06
39	Blank	-	0.30	0.10	0.27	0.1

12-4. The efficacy of fusion polypeptides as immune enhancers on the PE-based PRRS GP3-GP4 epitopes oral vaccine.

Porcine reproductive and respiratory syndrome (PRRS) causes devastating economic losses due to late-term reproductive failure and severe pneumonia in neonatal pigs. PRRS disease is a high-consequence animal disease with current vaccines providing limited protection from infection due to the high degree of genetic variation of field PRRS virus. Serum neutralizing

antibodies (NAs) considered being an important correlate of protective immunity against PRRSV. The role that NA have in protection against infection with PRRSV had been demonstrated by Lopez et al (2007). His results identified certain threshold of serum virus neutralization (SVN) titer (≥1:8) at which the dissemination of PRRSV in the serum of a young pig would be blocked, as well as a higher threshold (≥1:32) that could imply complete protection of the animal from PRRSV infection. We had been developed a PE-based PRRSV subunit vaccine. However, the subunit vaccine has to be improved about SVN titer eliciting against PRRS disease control. In the present disclosure, the fallowing experimental result could show that the efficacy of fusion polypeptides as oral immune enhancers could elicit SVN.

In Table 31, the data demonstrated that PE-based GPs fused oral vaccines could be enhanced by the mucosal targeting ligands, CO1, DQ2 and RV3.

According to serial dilutions ELISA data of the group no. 44 and 48 (PE-GP417 and PE-GP437 vaccine groups), it showed that oral administration of PE-GP417 and PE-GP437 elicited a good GP417 and GP437-specific IgG serum titers in the 1:200 to 1:1250 fold serum dilutions. The serum IgG antibodies activities of PE-GP417 or PE-GP437 vaccine with additional CO1-PE-GP417 or CO1-PE-GP437, DQ2-PE-GP417 or DQ2-PE-GP437 and RV3-PE-GP417 or RV3-PE-GP437 immune enhancers, corresponded to the group no. 41 or 45, 42 or 46 and 43 or 47, could increase the antibodies activity. From the GP417-specific IgG activity in serum dilution 500-fold samples, the data of the group No.41, No. 42 and No.43 were significantly higher than other oral vaccine groups ($p < 0.02$). From the GP-437-specific IgG activity in that dilution fold samples of the group No. 46 and 47, it showed good immune enhancing effect, but No. 45 group was not. From the result, the mucosal targeting epitopes including CO1, DQ2 and RV3 had good enhancement activity in GP417 epitope-specific antibodies, and DQ2 and RV3 had good enhancement activity in the GP437 antibodies.

Table 31

Group no. (n=3)	Serum anti-GP417 & 437 IgG activity in ELISA-Test		dilx 200		dilx 500		dilx 1250		dilx 3125	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
40	Blank	-	0.31	0.10	0.23	0.08	0.22	0.05	0.27	0.08
41	PE-GP417	CO1-PE-GP417	0.60	0.11	0.49	0.06	0.28	0.06	0.22	0.06
42	PE-GP417	DQ2-PE-GP417	0.77	0.08	0.59	0.08	0.39	0.08	0.22	0.03
43	PE-GP417	RV3-PE-GP417	0.69	0.11	0.52	0.05	0.38	0.05	0.25	0.05
44	PE-GP417 2 L-doses		0.52	0.13	0.34	0.10	0.25	0.11	0.28	0.05
45	PE-GP437	CO1-PE-GP437	0.64	0.11	0.51	0.11	0.26	0.08	0.24	0.05
46	PE-GP437	DQ2-PE-GP437	0.85	0.12	0.63	0.08	0.44	0.09	0.32	0.03
47	PE-GP437	RV3-PE-GP437	0.75	0.10	0.54	0.10	0.38	0.10	0.28	0.05
48	PE-GP437 2 L-doses		0.58	0.14	0.34	0.10	0.22	0.10	0.25	0.06
49	Inject (H dose)	-	1.50	0.20	0.10	0.30	0.58	0.20	0.25	0.06

Table 32

Group no. (n=3)	Serum anti-GP417 & 437 IgA activity in ELISA-Test		dilx 200		dilx 500		dilx 1250		dilx 3125	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
40	Blank	-	0.28	0.10	0.27	0.03	0.21	0.08	0.21	0.03

41	PE-GP417	CO1-PE-GP417	0.65	0.12	0.38	0.08	0.29	0.05	0.24	0.04
42	PE-GP417	DQ2-PE-GP417	0.70	0.10	0.45	0.05	0.28	0.05	0.26	0.10
43	PE-GP417	RV3-PE-GP417	0.60	0.12	0.38	0.06	0.27	0.06	0.28	0.10
44	PE-GP417 2 L-dose		0.50	0.11	0.31	0.13	0.25	0.10	0.22	0.10
45	PE-GP437	CO1-PE-GP437	0.59	0.15	0.39	0.09	0.28	0.03	0.22	0.06
46	PE-GP437	DQ2-PE-GP437	0.81	0.11	0.45	0.10	0.31	0.05	0.27	0.07
47	PE-GP437	RV3-PE-GP437	0.72	0.14	0.48	0.11	0.34	0.05	0.27	0.05
48	PE-GP437 2 L-doses		0.53	0.10	0.29	0.13	0.26	0.05	0.22	0.09
49	Inject (H dose)	-	0.80	0.20	0.50	0.15	0.30	0.08	0.23	0.04

From the results shown in Table 32, the GP417 and GP437-specific IgA activity in PE-GP417 and PE-GP437 vaccine were slightly enhanced by the mucosal targeting epitopes, including CO1, DQ2 and RV3 (p>0.02).

In Tables 33 and 34 , the data showed that serum virus-neutralization (VN) antibodies has increased when PE-based GP417&437 and GP317 fused oral vaccines additional with the mucosal targeting ligands, CO1, DQ2 and RV3.

Table 33

Serum titers of the oral vaccine immunization in the experiment group in mice model

Group no. (n=3)	Group treatment		IgG-ELISA titers (S/P) coating antigen GP417 & 437	PRRSV TC-01 in Alveolar macrophage 3D4/31
	Oral vaccine	Enhancer		VN titers
40	Blank	-	0	0
41	PE-GP417	CO1-PE-GP417	149	16
42	PE-GP417	DQ2-PE-GP417	207	32
43	PE-GP417	RV3-PE-GP417	167	16
44	PE-GP417 2 L-doses		63	8
45	PE-GP437	CO1-PE-GP437	161	16
46	PE-GP437	DQ2-PE-GP437	230	32
47	PE-GP437	RV3-PE-GP437	178	16
48	PE-GP437 2 L-doses		64	8
49	Inject (H dose)	-	500	64

Table 34

Group no. (n=3)	Serum anti-GP317 IgG activity in ELISA-Test		dilx 200		dilx 500	
	Vaccine	Enhancer	Average	Stdev	Average	Stdev
50	Blank	-	0.28	0.10	0.22	0.08
51	PE-GP317	CO1-PE-GP317	0.50	0.10	0.35	0.06
52	PE-GP317	DQ2-PE-GP317	0.62	0.10	0.51	0.07
53	PE-GP317	RV3-PE-GP317	0.58	0.10	0.44	0.07
54	PE-GP317 2 L-doses		0.52	0.10	0.46	0.11
55	Inject (H dose)	-	0.88	0.12	0.68	0.10

Group no. (n=3)	Serum anti-GP317 IgG activity in ELISA-Test		IgG-ELISA titers (S/P) GP317	PRRSV TC-01 in Alveolar
	Oral vaccine	Enhancer		VN titers
50	Blank	-	0	0
51	PE-GP317	CO1-PE-GP317	141	8
52	PE-GP317	DQ2-PE-GP317	315	32
53	PE-GP317	RV3-PE-GP317	239	8
54	PE-GP317 2 L-doses		261	8
55	Inject (H dose)	-	500	32

From the results shown in Tables 31 and 32, only the group of No. 42, 46, 52 had the highest level of VN titers. The oral administration of PE-GP317, PE-GP414 and PE-GP437 vaccine without the immune enhancer, such as the group no. 44, 48 and 54, all showed a lower VN titers and only 8 score. However, the DQ2 fused polypeptide, which proposed a good mucosal targeting ligand, demonstrated a good immune enhancing efficacy when it added into vaccine groups such as the group No. 42, 46 and 52.

13. Pigs experiment: Oral administration of M-cell ligand chimeric polypeptides enhancing the swine antibody titer against specific antigen

The vaccination program and examination of immune enhancement effect of the fusion biogenic polypeptides in swine tests is shown in Table 21. In pig oral vaccine experiment of PE-M14 H-dose group without enhancer, there were no interaction effects of age x dietary program on growth performance from weaning to 10 weeks of age. The PE-M14 H-dose group did not affect growth performance from weaning to 10 weeks of age. It had no effect ($P>0.05$) on pig weight at 10 weeks of age. However, preweaning ADG (0.172 kg/day for $\leq$ 0.104 kg birth weight to 0.27 kg/day for $\geq$ 1.99 kg birth weight), weaning weight (5.26 kg to 8.85 kg), weaning BCS (2.69 to 2.93), and

preweaning mortality (24.2% to 4.6%) were improved for pigs of heavier birth weight categories.

Over the entire 5-wk postweaning phase, PE-M14 H-dose oral vaccine additional with DQ2-PE-M14 or DQ2-PE-M27 enhancer group, the piglets had a 23-24% higher weight gain ($P < 0.05$) and showed more play behavior (4.0 ± 0.3 vs. 2.8 ± 0.3 freq/h, $P < 0.05$) than that of placebo group.

Table 35

Vaccine group (N=3) L dose/ 3 times/ per week and 12 times orally/ 4 weeks		Immune enhancer (N=3) L dose/ 3 times/ per week and 12 times orally/ 4 weeks		IgG-ELISA titers (S/P) coating antigens: GP417, GP437 and GP 317	PRRSV TC-01 in Alveolar macrophage 3D4/31 VN titers
Blank	0 dose	-	0 dose	1	0
PE-GP417, 437, 317	L dose	DQ2-PE-GP417	L dose	320	32
PE-GP417, 437, 317 L dose and PE-GP417 L dose				160	16
PE-GP417, 437, 317	H dose	-	-	160	8
Positive control through intramuscular injection (N=3)				640	128

From the data shown in Table 33, the IgG-ELISA and VN titers in oral vaccine groups were lower than that of injection groups. VN titers could increase the score when the oral vaccine additional with DQ2-PE-GP417. It showed that DQ2 fused polypeptide, which proposed a good mucosal targeting ligand, demonstrated a good immune enhancer for PE-based PRRSV-NT oral vaccine.

In conclusion, when the vaccine is used with a fusion polypeptide of the present disclosure, good immune response can be achieved when the

composition is orally administered. Especially, when the fusion polypeptide of the present disclosure comprises a mucosa targeting polypeptide, the immune response is significantly increased.

Although the present disclosure has been explained in relation to its embodiment, it is to be understood that many other possible modifications and variations can be made without departing from the spirit and scope of the disclosure as hereinafter claimed.

What is claimed is:

1. A fusion polypeptide, comprising:

(a) a mucosa targeting polypeptide;

(b) a first translocating peptide for translocation; and

5 (c) a first antigenic epitope.

2. The fusion polypeptide of claim 1, wherein the mucosa targeting polypeptide is located at an N-terminal of the fusion polypeptide, the first antigenic epitope is located at a C-terminal of the fusion polypeptide, and the first translocation peptide is located between the mucosa targeting polypeptide 10 and the first antigenic epitope.

3. The fusion polypeptide of claim 1, wherein the mucosa targeting polypeptide is an M-cell targeting polypeptide or an intestine epithelial targeting polypeptide.

4. The fusion polypeptide of claim 1, wherein the mucosa targeting 15 polypeptide comprises an amino acid sequence selected from SEQ ID NOs: 1 to 4.

5. The fusion polypeptide of claim 1, wherein the first translocating peptide is from pseudomonas exotoxin.

6. The fusion polypeptide of claim 1, wherein the first translocating 20 peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III.

7. The fusion polypeptide of claim 1, wherein the first antigenic epitope is a Th1 antigenic epitope.

8. The fusion polypeptide of claim 1, wherein the first antigenic 25 epitope is an HPV antigenic epitope, a Myostatin epitope, or a PRRSV antigenic epitope.

9. The fusion polypeptide of claim 8, wherein the HPV antigenic epitope is an E7 peptide sequence or an E6 peptide sequence of human papillomavirus type 16.

10. The fusion polypeptide of claim 1, wherein the first antigenic epitope is selected from SEQ ID NOs: 10, 12, 17, 18, 21, 22 and 23.

11. A method for enhancing a stimulation of an immune response, comprising:

5 administering a composition to a subject in need, wherein the composition comprises a vaccine and a fusion polypeptide, and the fusion polypeptide comprises:

(a) a mucosa targeting polypeptide;

(b) a first translocating peptide for translocation; and

10 (c) a first antigenic epitope.

12. The method of claim 11, wherein the mucosa targeting polypeptide is located at an N-terminal of the fusion polypeptide, the first antigenic epitope is located at a C-terminal of the fusion polypeptide, and the first translocation peptide is located between the mucosa targeting polypeptide and the first 15 antigenic epitope.

13. The method of claim 11, wherein the mucosa targeting polypeptide is a M-cell targeting polypeptide or an intestine epithelial targeting polypeptide.

14. The method of claim 11, wherein the mucosa targeting polypeptide comprises an amino acid sequence selected from SEQ ID NOs: 1 to 4.

20 15. The method of claim 11, wherein the first translocating peptide is from pseudomonas exotoxin.

16. The method of claim 11, wherein the first translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III.

17. The method of claim 11, wherein the first antigenic epitope is a Th1 25 antigenic epitope.

18. The method of claim 11, wherein the first antigenic epitope is an HPV antigenic epitope, a Myostatin epitope, or a PRRSV antigenic epitope.

19. The method of claim 18, wherein the HPV antigenic epitope is an E7 peptide sequence or an E6 peptide sequence of human papillomavirus type

16.

20. The method of claim 11, wherein the first antigenic epitope is selected from SEQ ID NOs: 10, 12, 17, 18, 21, 22 and 23.

21. The method of claim 11, wherein the composition is orally
5 administered to the subject in need.

22. The method of claim 11, wherein the vaccine comprises: a second translocation peptide for translocation and a second antigenic epitope.

23. The method of claim 22, wherein the second translocating peptide is from pseudomonas exotoxin.

10 24. The method of claim 22, wherein the second translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III.

25. The method of claim 22, wherein the second antigenic epitope is a Th1 antigenic epitope.

26. The method of claim 22, wherein the second antigenic epitope is an
15 HPV antigenic epitope, a Myostatin epitope, or a PRRSV antigenic epitope.

27. The method of claim 11, wherein the vaccine is an anti-HPV vaccine, an anti-Myostatin vaccine or an anti-PRRSV vaccine.

28. A fusion polypeptide, comprising:

(b) a first translocating peptide for translocation; and

20 (c) a first antigenic epitope selected from SEQ ID NOs: 10 and 12.

29. The fusion polypeptide of claim 28, wherein the first translocating peptide is from pseudomonas exotoxin.

30. The fusion polypeptide of claim 28, wherein the first translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain
25 III.

31. A method for treating or preventing HPV, comprising:

administering a composition to a subject in need, wherein the composition comprises an anti-HPV vaccine and a fusion polypeptide, and the fusion polypeptide comprises:

(b) a first translocating peptide for translocation; and

(c) a first antigenic epitope selected from SEQ ID NOs: 10 and 12.

32. The method of claim 31, wherein the first translocating peptide is from pseudomonas exotoxin.

5 33. The method of claim 31, wherein the first translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III.

34. The method of claim 31, wherein the composition is orally administered to the subject in need.

35. The method of claim 31, wherein the vaccine comprises: a second 10 translocation peptide for translocation and a HPV antigenic epitope.

36. The method of claim 35, wherein the second translocating peptide is from pseudomonas exotoxin.

37. The method of claim 35, wherein the second translocating peptide comprises a pseudomonas exotoxin A fragment deleted of only domain III.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/58419

A. CLASSIFICATION OF SUBJECT MATTER
IPC - A61K 39/12, 39/104, 38/16, 38/10, 47/66; A61P 31/20; C07K 14/025, 14/21 (2018.01)
CPC - A61K 39/12, 39/104, 38/10, 38/16, 38/162, 47/66, 47/646; C07K 14/025, 14/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHENG, W. et al. Fusion Protein Vaccines Targeting Two Tumor Antigens Generate Synergistic Anti-Tumor Effects. PLoS ONE. September 2013, Vol. 8, e71216, pages 1-8, doi:10.1371/journal.pone.0071216; figures 1A, 3, 5; abstract; page 2, first column, third-fourth paragraphs; page 2, second column, third-fourth paragraphs; page 3, first column, second paragraph- second column, first paragraph; page 6, first column, second paragraph; page 6, second column, first-second paragraphs; page 7, second column, first-second paragraphs.	1, 3, 5-9, 11, 13, 15-19, 22-27
Y	KIM, S. et al. The M Cell-Targeting Ligand Promotes Antigen Delivery And Induces Antigen-Specific Immune Responses In Mucosal Vaccination. The Journal of Immunology. 15 October 2010, Vol. 185, pages 5787-5795, doi: 10.4049/jimmunol.0903184; abstract; page 5788, second column, fourth and sixth paragraphs; page 5789, first column, second paragraph; pages 5791, second column, second paragraph.	1, 3, 5-9, 11, 13, 15-19, 22-27
A	Lo, D. et al. M Cell Targeting By A Claudin 4 Targeting Peptide Can Enhance Mucosal IgA Responses. BMC Biotechnology. 2012, Vol. 12, pages 1-9, doi:10.1186/1472-6750-12-7; figure 3; abstract; page 3, first column, third paragraph; page 4, first column, second paragraph.	2, 12
A	Lin, K. et al. Carboxyl-terminal Fusion Of E7 Into Flagellin Shifts TLR5 Activation To NLRC4/NAIP5 Activation And Induces TLR5-independent Anti-tumor Immunity. Scientific Reports. 11 April 2016, Vol. 6, pages 1-10, DOI: 10.1038/srep24199; figure 1A; abstract; page 2, third-fourth paragraphs.	2, 12
A	KR 20120096323 A (INDUSTRIAL COOPERATION FOUNDATION CHONBUK NATIONAL UNIVERSITY) 30 August 2012; abstract; page 7, second column, third paragraph; page 8, second column, fourth paragraph; page 10, second column, second paragraph.	4, 14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 2018 (05.02.2018)	20 FEB 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/58419

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:
      - ☒ in the form of an Annex C/ST.25 text file.
      - ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/58419

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
-***-Please See Supplemental Page-***-

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No. PCT/US17/58419

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KR 20160030056 A (SEOUL NATIONAL UNIVERSITY R&DB FOUNDATION) 16 March 2016; abstract; page 5, second column, seventh paragraph; page 6, second column, first paragraph; page 8, second column, fourth paragraph.	4, 14
A	WO 2009/073133 A1 (VAXINATE CORPORATION) 11 June 2009; abstract; page 3, lines 1-4; page 7, lines 26-28; page 29, lines 1-8; page 46, lines 6-12; page 50, line 31- page 51, line 12.	10, 20, 28-37
A	US 2006/0154238 A1 (HU, Y.) 13 July 2006; paragraph [0065].	10, 20, 28-37
A	CHEN, S. et al. De-oncogenic HPV E6/E7 Vaccine Gets Enhanced Antigenicity And Promotes Tumoricidal Synergy With Cisplatin. Act Biochim Biophys Sin. 14 November 2013, Vol. 46, pages 6-14, DOI: 10.1093/abbs/gmt121; figure 1; abstract; page 8, first column, first paragraph.	31-37

-***-Continued from Box No. III: Observations Where Unity of Invention is Lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-37, SEQ ID NO: 1 (mucosa targeting polypeptide) and SEQ ID NO: 10 (first antigenic epitope) are directed toward a fusion polypeptide, comprising: (a) a mucosa targeting polypeptide; (b) a first translocating peptide for translocation.

The fusion peptide will be searched to the extent that it encompasses SEQ ID NO: 1 (mucosa targeting polypeptide) and SEQ ID NO: 10 (first antigenic epitope). Applicant is invited to elect additional mucosa targeting and/or antigenic epitope polypeptide sequence(s), with specified SEQ ID NO: for each, to be searched. Additional mucosa targeting and/or antigenic epitope polypeptide sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-3, 4 (in-part), 5-9, 10 (in-part), 11-13, 14 (in-part), 15-19, 20 (in-part), 21-27, 28 (in-part), 29 (in-part), 30 (in-part), 31 (in-part), 32 (in-part), 33 (in-part), 34 (in-part), 35 (in-part), 36 (in-part) and 37 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (mucosa targeting polypeptide) and SEQ ID NO: 10 (first antigenic epitope). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be fusion polypeptide comprising SEQ ID NO: 2 (mucosa targeting polypeptide); SEQ ID NO: 12 (first antigenic epitope).

No technical features are shared between the antibody sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a fusion polypeptide, comprising: (a) a mucosa targeting polypeptide; (b) a first translocating peptide for translocation; and (c) a first antigenic epitope; a method for enhancing a stimulation of an immune response, comprising: administering a composition to a subject in need, wherein the composition comprises a vaccine and a fusion polypeptide, and the fusion polypeptide comprises: (a) a mucosa targeting polypeptide; (b) a first translocating peptide for translocation; and (c) a first antigenic epitope; a fusion polypeptide, comprising: (b) a first translocating peptide for translocation; and (c) a first antigenic epitope; a method for treating or preventing HPV, comprising: administering a composition to a subject in need, wherein the composition comprises an anti-HPV vaccine and a fusion polypeptide, and the fusion polypeptide comprises:
(b) a first translocating peptide for translocation; and (c) a first antigenic epitope.

However, these shared technical features are previously shared by the publication entitled 'Fusion Protein Vaccines Targeting Two Tumor Antigens Generate Synergistic Anti-Tumor Effects' by Cheng, et al. (hereinafter 'Cheng') in view of US 7,485,321 B2 to O'Mahony et al. (hereinafter 'O'Mahony').

Cheng disclose a fusion polypeptide (fusion protein; abstract), comprising: (a) a first polypeptide (PE(delta III); abstract; figure 1A); (b) a first translocating peptide for translocation (KDEL3 translocating peptide for translocation; figure 1A; page 6, second column, first paragraph); and (c) a first antigenic epitope (E6 epitope (first antigenic epitope); abstract; figure 3); a method for enhancing a stimulation of an immune response (a method for generating more potent immune responses (enhancing a stimulation of an immune response; page 6, first column, second paragraph; figure 5), comprising: administering a composition to a subject in need (administered to mice for tumor protection (subject in need); page 3, first column, second paragraph), wherein the composition comprises a vaccine and a fusion polypeptide (wherein the composition comprises a vaccine and a fusion polypeptide; page 3, first column, third paragraph), and the fusion polypeptide comprises (abstract): (a) a first polypeptide (PE(delta III); abstract; figure 1A); (b) a first translocating peptide for translocation (KDEL3 translocating peptide for translocation; figure 1A; page 6, second column, first paragraph); and (c) a first antigenic epitope (E6 epitope (first antigenic epitope); abstract; figure 3); a fusion polypeptide (abstract), comprising: (b) a first translocating peptide for translocation (KDEL3 translocating peptide for translocation; figure 1A; page 6, second column, first paragraph); and (c) a first antigenic epitope (E6 epitope (first antigenic epitope); abstract; figure 3); a method for treating or preventing HPV (page 7, second column, first paragraph), comprising: administering a composition to a subject in need (administered to mice for tumor protection (subject in need); page 3, first column, second paragraph), wherein the composition comprises an anti-HPV vaccine and a fusion polypeptide (vaccine comprises PE(ΔIII)/E6 (composition comprises an anti-HPV vaccine and a fusion polypeptide); page 3, first column, third paragraph; page 7, second column, first paragraph), and the fusion polypeptide (abstract) comprises: (b) a first translocating peptide for translocation (KDEL3 translocating peptide for translocation; figure 1A; page 6, second column, first paragraph); and (c) a first antigenic epitope (E6 epitope (first antigenic epitope); abstract; figure 3).

Cheng does not disclose a mucosa targeting polypeptide.

O'Mahony discloses a mucosa targeting peptide (abstract; column 8, lines 55-59).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of Cheng, to include a mucosa targeting polypeptide, as previously disclosed by O'Mahony, in order to provide a superior method for transporting an immune enhancing vaccine across mucosa to effectively treat or prevent HPV infection.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Cheng and O'Mahony references, unity of invention is lacking.