A Dissertation
for the Degree of Master of Science

Identification of porcine Toll-like receptor2 (TLR2) targeting peptide ligands using cell-based phage display combined with Illumina sequencing

일루미나 시퀀싱과 세포 기반 파지 디스플레이를 통한 돼지 TLR2 표적 펩타이드의 동정

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Summary

Toll-like receptors (TLRs) are the representative PRRs and play an important role in innate and adaptive immunity. Among various types of TLRs, TLR2 could recognize a wide range of bacterial and fungal components and induce innate and adaptive immune responses. In particular, it is known that TLR2-targeting formulations in vaccines showed adjuvants properties and could effectively induce humoral and cellular immune responses.

In this study, porcine TLR2-targeting peptide ligands were identified using cell-based phage display combined with high-throughput sequencing. Porcine TLR2-overexpressing cell line was constructed using lentiviral vector and established cell line was confirmed through flow cytometry, western blot and ligand binding assay. Cell-based phage display was performed on the constructed cell line and two types of biopanning were proceeded : subtractive and non-subtractive biopanning. After 3 rounds of biopanning, eluted phage from each round was sequenced using Illumina platform and three candidate peptides were identified: NAGHLSQ, VPSKPGL, RANLDGQ.

In the binding assay, NAGHLSQ showed the highest binding affinity to the constructed cell line and binding of NAGHLSQ to TLR2 overexpessing cell line was gradually decreased in response to increasing concentration of TLR2 ligand. Thus, NAGHLSQ peptide could be used in porcine vaccines as TLR 2 targeting formulation.

Keywords: TLR, Lentivirus, Cell-based phage display, Illumina sequencing

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Contents

Summary ····· Ⅰ
Contents ····· Ⅱ
List of Tables and Figures ····· Ⅴ
List of Abbreviations ····· Ⅶ

Ⅰ. Introduction ····· 1

Ⅱ. Review of Literature ····· 3
1. Toll-like receptors (TLRs) and their signalling ····· 3
1) Types of TLRs and their ligands ····· 3
2) TLR signalling ····· 3
2. Targeting TLR2 for vaccine development ····· 6
1) TLR2 ligands and TLR2 signalling ····· 6
2) TLR2 expression on specific cells and tissues ····· 8
3) Immunomodulation by TLR2-targeting formulations ····· 11
(1) Antigen ····· 11
(2) Lymphocyte activity ····· 12
(3) Mucosal immune responses ····· 14
3. Phage display ····· 19
1) General information about phage display ····· 19
2) Types of phage library ····· 21
(1) Peptide library ····· 21
(2) Antibody library ····· 22
3) Types of screening ····· 23
(1) *in vitro* screening ····· 23
(2) *in vivo* screening ····· 24

Ⅲ. Materials and Methods
1. Cell culture ····· 25

2. Construction of porcine TLR2-overexpressing cell line ····· 26
1) Confirmation of VSVG envelope tropism on IPEC-J2 cells ····· 26
2) Plasmid construction ····· 26
3) Preparation of porcine TLR2-expressing lentiviruses ····· 27
4) Lentiviral transduction on IPEC-J2 cells ····· 27
3. Validation of constructed cell line ····· 29
1) Flow cytometric analysis ····· 29
2) Western blot ····· 30
3) Ligand binding assay ····· 30
4. Cell-based phage display ····· 31
1) Phage display peptide library ····· 31
2) Biopanning *in vitro* ····· *31*
(1) *in vitro* phage binding assay ····· 31
(2) Phage titration ····· 34
(3) Phage amplification ····· 34
5. Illumina sequencing ····· 36
1) Isolation of phage DNA ····· 36
2) Preparation of sequencing templates ····· 36
(1) PCR amplification of phage DNA ····· 37
(2) Construction of sequencing library ····· 37
3) Processing of sequencing data ····· 38
6. Characterization of porcine TLR2 targeting peptides ··· 39
1) Peptide binding assay ····· 39
2) Competition assay ····· 39
7. Statistical analysis ····· 40

Ⅳ. Results and Discussion ····· 41
1. Construction of porcine TLR2-overexpressing cell line ···
····· 41

2. Validation of constructed cell line ····· 43
3. Cell-based phage display ····· 46
4. Illumina sequencing ····· 48
1) Characterization of naive library ····· 48
2) Characterization of enrichment process ····· 49
3) Identification of false positives ····· 53
5. Characterization of porcine TLR2 targeting peptide ····· 55
1) Peptide binding assay ····· 55
2) Competition assay ····· 57

Ⅴ. Conclusion and Further Aspects ····· 59

Ⅵ. Literature Cited ····· 61

Ⅶ. Summary in Korean ····· 78

List of Tables and Figures

<Table>

Table 1. TLR2 ligands ····· 7
Table 2. TLR2 expression in livestock ····· 10
Table 3. Primers used for amplification of porcine TLR2 ····· 27
Table 4. Barcoded primers used for amplification of phage ssDNA ····· 37
Table 5. Number of total sequences and unique sequences along 3 rounds of selection in two types of biopanning ····· 50
Table 6. Candidate peptides ····· 55

<Figure>

Figure 1. Scheme of TLR types and TLR signalling pathway ····· 5
Figure 2. Cellular signal pathway of TLR2 ····· 7
Figure 3. Immunomodulation effects of targeting TLR2 in vaccine ····· 18
Figure 4. The structure and genome of M13 bacteriophage ····· 20
Figure 5. Phage display peptide library from NEB (USA) ····· 21
Figure 6. Construction of ScFvs phage libraries ····· 22
Figure 7. The typical procedures of in vitro biopannnig ····· 24
Figure 8. The procedure of establishment of porcine TLR2-over expressing cell line ····· 28
Figure 9. The procedure of cell-based phage display in this study ····· 33
Figure 10. Confirmation of tropism of VSVG envelope on

IPEC-J2 cells using GFP-carrying lentiviruses ····· 42
Figure 11. Detection of GFP fused porcine TLR2 expression on lentiviral transduced IPEC-J2 cells using fluorescence microscopy ····· 42
Figure 12. Comparative analysis of transduction or transfection efficiency in IPEC-J2 cells using flow cytometry ····· 44
Figure 13. Western blot analysis of lentiviral transduced IPEC-J2 cells ····· 44
Figure 14. Co-localization of porcine TLR2 and its ligand (zymosan) ····· 45
Figure 15. A progressive enrichment of phage during 3 rounds of biopanning ····· 47
Figure 16. Preparation of DNA templates for Illumina sequencing. ····· 51
Figure 17. Analysis of amino acid distribution of Ph.D.C7C-50 library ····· 51
Figure 18. Progressive decrease in the number of unique sequences along 3 rounds of biopanning ····· 52
Figure 19. Counts of most abundant peptides in two types of biopanning ····· 52
Figure 20. Percentage of overlap of most abundant peptides ····· 53
Figure 21. Counts of sequences recorded in SAROTUP and PepBank along 3 rounds of biopanning ····· 54
Figure 22. Binding assay of Rhodamine-conjugated synthetic peptides ····· 56
Figure 23. Competition binding assay of Rhodamine-conjugated synthetic peptides and TLR2 lignads (zymosan) ····· 58

List of Abbreviations

BSA : Bovine serum albumin

DMSO : Dimethyl sulfoxide

FACS : Fluorescence-activated cell sorting

GALT: Gut-associated lymphoid tissue

GFP : Green fluorescent protein

HEK 293 : Human embryonic kidney cell line

HPLC : High-performance liquid chromatography

IPEC-J2 : Intestinal porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet

IPTG : Isopropyl-β-D-thiogalactoside

M cell : Membranous cells

MHC : Major histocompatibility complex

NK cell : Natural killer cell

PAMP : Pathogen-associated molecular pattern

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PEG : Polyethylene glycol

PFA : Paraformaldehyde

PRR : Pathogen recognition receptor

Tet : Tetracyclin

VSVG : Vesicular stomatitis virus glycoprotein

X-gal : 5-Bromo-4-Chloro-3-Indolyl -D-Galactopyranoside

Ⅰ. Introduction

TLRs are transmembrane type I glycoproteins and consist of three domains : extracellular domain, which is involved in the recognition of TLR ligands; transmembrane domain and intracellular parts that have toll/IL-1 receptor (TIR) domain for signal transduction (Basto *et al*., 2014). TLR is one of the pathogen recognition receptors (PRRs) that recognize various components of microorganisms. 13 members of the TLRs have been identified in mammals (Basto *et al*., 2014). Different TLRs could be classified according to ligands, signalling pathway and their cellular localization. When TLRs are activated by their ligands, cascades of intracellular signalling could induce inflammation and involved in both innate and adaptive immunity (Koropatnick, *et al*., 2004).

In particular, TLR2 could be targeted for therapeutic and vaccine applications (Basto *et a*l., 2014). TLR2 dimerizes with another TLRs such as TLR1 and TLR6, thus TLR2 can recognize the largest range of bacterial and fungal component (Gibson *et al*., 2010). It has been reported that TLR2-targeting formulations such as synthetic TLR2 ligands and expression of TLR2 ligands fused with antigen exert immunomodulation effects in vaccine. For example, TLR2-targeting formulations modulate migration of antigen presenting cells (APCs), mediate internalization, processing and presentation of antigen, induce $CD4^+$ T cell polarization and $CD8^+$ T cell cytotoxicity, increase NK cells activity, involved in the generation and longevity of antibody secreting cells (ASC) and induce mucosal immunity

(Basto *et al.*, 2014). Therefore, targeting TLR2 in vaccine could be effective strategies to enhance immune responses elicited after vaccinization.

In this study, porcine TLR2-targeting peptide ligands were identified using cell-based peptide phage display combined with Illumina sequencing. Peptide phage display is one of the most powerful tools to identify targeting moiety against specific biological targets, using enormously diverse peptide phage libraries. Cell-based phage display is to use whole native cells or target gene transfected cells, thus maintaining the native conformation of receptor with normal post-translational modifications (Watters *et al.*, 1997).

In traditional phage display, Sanger sequencing is used for phage sequencing but there are many disadvantages such as false positives, time-costing (1Mb/day) and random-clone picking problems.('t Hoen *et al.*, 2012). In this situation, high-throughput sequencing such as Illumina platform could overcome the limitations of traditional phage sequencing. It is possible to read large number of DNA sequence simultaneously, rapidly characterize and quantify the enrichment process during biopanning. Also, the highly enriched clones could be identified without several selection rounds. (A. C.'t Hoen *et al.*, 2012).

The aim of this study is to identify porcine TLR2-targeting peptides and *in vitro* cell-based phage display was performed on TLR2-overexpressing cell line. Porcine TLR2-targeting formulations could be applicable in not only vaccines but also developing targeting therapeutics.

Ⅱ. Review of Literature

1. Toll-like receptors (TLRs) and their signalling

1) Types of TLRs and their ligands

Toll-like receptors (TLRs) are representative pattern recognition receptors (PRRs), which recognize various microbial molecules referred as pathogen-associated molecular patterns (PAMPs). It is known that they recognize pathogens or microbes regardless of their pathogenicity (Koropatnick, *et al*., 2004). TLRs can be classified into two groups according to their cellular location. TLRs which recognize fungal and bacterial components are expressed on the cell surface, while TLRs that recognize viral components are localized into intracellular compartment (Hennessy *et al*., 2010). Therefore, different TLRs can be targeted by different types of ligands. There are three groups of TLR ligands: proteins, lipid-based molecules and nucleic acids (Kanzler *et al*., 2007). For example, TLR2 recognizes Gram-positive bacterial and fungal components and TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria. TLR5 binds bacterial flagellin and TLR7, TLR8 binds ssRNA of virus and TLR9 recognizes unmethylated CpG islands and the synthetic unmethylated CpG motifs. (Hennessy *et al*., 2010).

2) TLR signalling

When TLRs are stimulated by their ligands, TLR signalling can induce innate immune responses like inflammation and also can be involved in adaptive immunity (Lavelle *et al*., 2010). After

ligand binding, Toll-interleukin 1 resistance (TIR) domains on each TLR leads to a conformational change and recruits adaptor proteins (O'Neill *et al.*, 2013). It is known that there are some differences among TLRs in adaptor usage. TLR2, TLR5, TLR7 and TLR9 stimulate MyD88 dependent-pathway, which use myeloid differentiation primary-response protein 88 (MyD88) and MyD88-adaptor-like protein (MAL) as a adaptor protein. On the contrary, TLR3 stimulate MyD88 independent pathway, which use TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) as a adaptor protein (Gay *et el.*, 2014). TLR4 specially moves from the cell surface to the endosomes, switching signalling from MyD88 to TRIF. These adaptor proteins stimulates downstream signalling pathways. MyD88 and MAL activate Intereukin 1 receptor-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), leading to the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinase and production of pro-inflammatory cytokines. TRIF and TRAM activate TRAFs and the transcription factor interferon-regulatory factors 3 (IRF3), producing Type I interferons (O'Neill *et al.*, 2013). The ability of TLRs to induce immune response makes them as attractive therapeutic targets. The scheme of TLR types and TLR signalling pathway is illustrated in Figure 1.

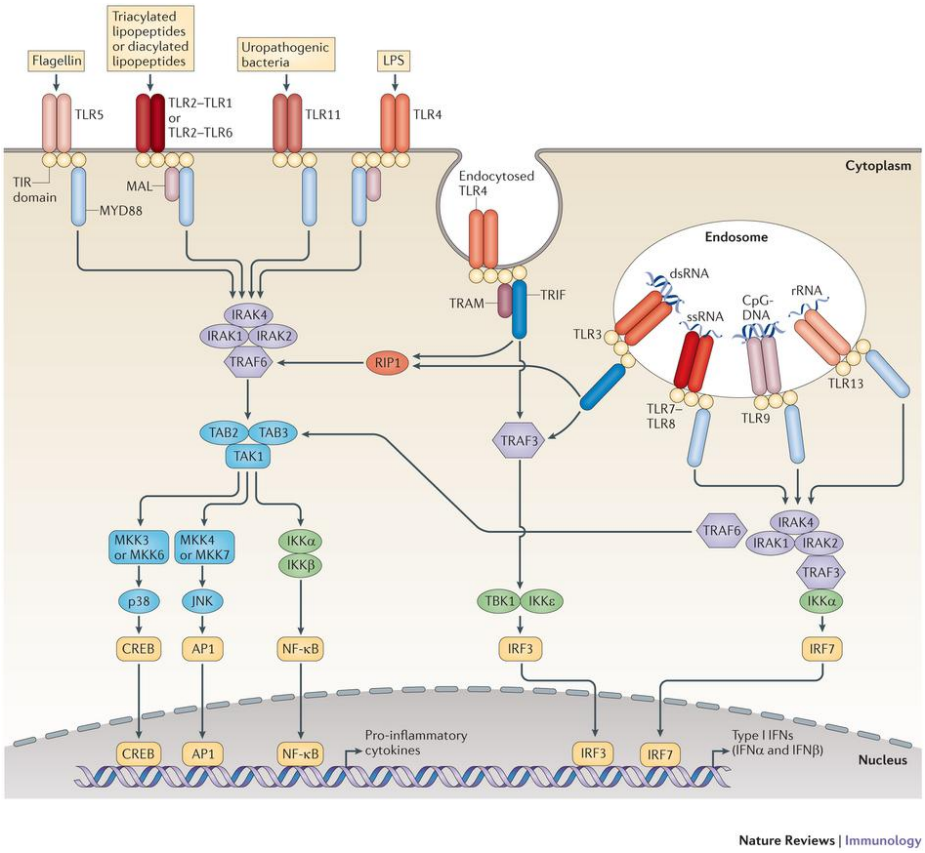


Figure 1. Sheme of TLR types and TLR signalling pathway. (O'Neill *et al.*, 2013).

2. Targeting TLR2 for vaccine development

1) TLR2 ligands and TLR2 signalling

In particular, TLR2 is one of the cell surface TLRs and mostly expressed on immune cells such as macrophage, dendritic cells and monocytes. It widely recognizes bacterial and fungal components than any other TLRs (Gibson *et al.*, 2010). It mostly binds the components of Gram-positive bacteria. TLR2 binds lipoteichoic acid (LTA) and heterodimerizes with TLR1 or TLR6. recognizing triacylated lipopeptides and diacylated lipopeptides, respectively. It is known that this dimerization of TLRs allows the receptors to recognize a wide range of microbial components (Basto *et al.*, 2014). TLR2 signaling is MyD88 dependent pathway as described in the previous chapter. MyD88 and MAL, which are the recruited adaptor proteins, activate IRAKs and TRAFs, inducing various cytokines such as IL-2, IL-6, IL-12 and TNF-a. TLR2 ligands is shown in Table 1 and cellular signal pathway of TLR2 is illustrated in Figure 2.

Table 1. TLR2 ligands

Origin	Component	Species	Usage
Bacterial	Diacyl lipopeptides	Mycoplasma	TLR2/TLR6
	Triacyl lipopeptides	Bacteria	TLR2/TLR1
	Peptidoglycans	Gram-positive bacteria	TLR
	Lipoteichoic acid	Gram-positive bacteria	TLR2/TLR6
	Glycolipids	*Treponema maltophilum*	TLR2
Fungal	Zymosan	*Saccharomyces cerevisiae*	TLR2/TLR6
	Phospholipomannan	*Candida alibicans*	TLR2

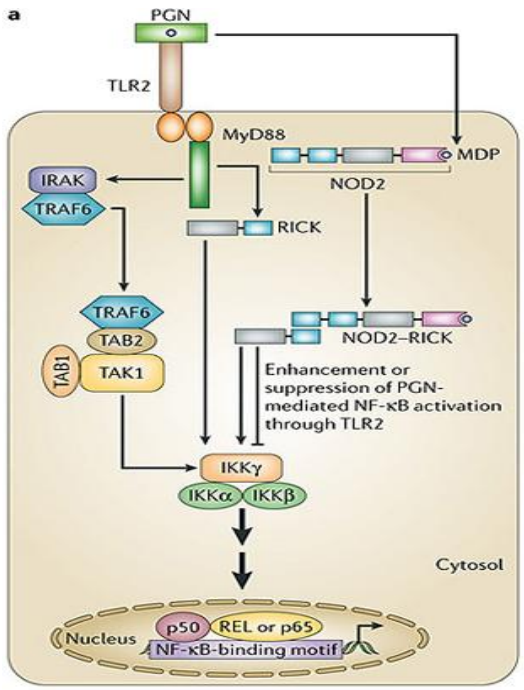


Figure 2. Cellular signal pathway of TLR2 (Strober *et al*., 2006).

2) TLR2 expression on specific cells and tissue

In human and mouse, it has been reported that TLR2 is expressed mostly on antigen presenting cells (APCs) such as monocytes, macrophages and dendritic cells (DCs) including CD8α^+, CD8α^-, interstitial and Langerhans DCs in humans and plasmacytoid DCs in the mouse (Pulendran *et al.*, 2004). Also, granulocyte, some epithelial cells and lymphocytes including B cells, $CD4^+$, $CD8^+$ T cells, regulatory T cells (T_{reg}), γδ T cells and NK cells express TLR2 (Gibson *et al.*, 2010).

In addition, recent researches elucidate TLR2 expression on livestock. In the bovine, monocytes and monocyte-derived macrophages showed a high expression level, alveolar macrophages, monocyte-derived DCs showed an intermediate expression and $CD172a^+$, $CD172a^-$ DC subsets of afferent lymph showed a weak expression (Kwong *et al.*, 2011). No expression was detected on $CD21^+$ B cells (Werling *et al.*, 2006). In case of swine, TLR2 is expressed on T cells, B cells, macrophages, monocytes, granulocytes but not on peripheral blood lymphocytes. TLR2 was also expressed in non-immune tissue such as heart, thymus, skeletal muscle and epithelial cells in lung, jejunum, kidney and liver (Alvarez *et al.*, 2008). The most distinct feature of swine is that TLR2 is expressed in gut-associated lymphoid tissue (GALT) (Tohno *et al.*, 2005). TLR2 is highly expressed in the mesenteric lymph nodes and Peyer's patches than spleen and also detected in membranous cells (M cells). Because GALT has defense mechanism against mucosal pathogens, it seems that targeting TLR2 could effectively induce mucosal immune responses against mucosal diseases. Chicken TLR2 was highly

expressed in heart, liver, muscle, gizzard, spleen, cecal tonsil, and bursa. Also, heterophils, macrophages, monocytes, B cells and T cells express TLR2 (Philbin *et al*., 2005). Expression of TLR2 in domestic animals is shown in Table 2.

Table 2. TLR2 expression in livestock (Basto *et al.*, 2014).

Species	Cells and tissues	Method	Expression level
Human and Mouse	Antigen presenting cells: macrophage monocytes, and DCs(CD8α^+, CD8α^-, interstitial and Langerhans DCs in humans and plasmacytoid DCs in the mouse Lymphocytes: B cells, $CD4^+$, $CD8^+$ T cells, regulatory T cells, γδ T cells and NK cells Granulocytes: neutrophils, basophils some epithelial cells		
Bovine	Monocytes	RT-PCR/FC	strong
	Monocyte derived-macrophages	RT-PCR/FC	
	Alveolar macrophages	RT-PCR/FC	Intermediate
	Monocyte derived-DCs	RT-PCR/FC	
	$CD172^+$ DCs	RT-PCR/FC	Weak
	$CD172^-$ DCs	RT-PCR/FC	
	$CD21^+$ B cells	RT-PCR	No signal
Swine	Mesenteric lymph nodes and Peyer's patches	RT-PCR/FC	Higher than spleen
	Heart, thymus, lung, kidney, skeletal muscle, small intestine	RT-PCR	Lower than spleen
	M cells	IHC,FC	
	T and B cells	FC	
	Monocytes, macrophage, and granulocytes	FC	
	Epithelial cells lining body entries (Lung, jejunum, kidney, liver)	IHC	
Chicken	Heart, liver, gizzard, muscle	RT-PCR	Strong
	Spleen, caecal tonsil, bursa, liver	RT-PCR	Strong
	Heterophils, monocytes, macrophages, B cells, T cells	RT-PCR	

* RT-PCR: Reverse transcription-PCR; FC: Flow cytometry; WB: Western blot
IHC: Immunohistochemistry

2) Immunomodulation by Formulations Targeting TLR2

(1) Antigen

① Antigen internalization

Antigen internalization is mediated by TLR2 at the inflammatory sites. Increasing internalization of antigen leads to decrease in the motility of mature DCs, which process and present antigen (Datta *et al.*, 2003). Also, treatment of anti-TLR2 monoclonal antibody internalized the ligand into endosomes and presented through MHC class II (Schjetne *et al.*, 2003).

② Antigen processing and presentation

To induce adaptive immune responses, antigen must be processed in APCs and presented through MHC class. There are growing evidences that TLR activation leads to phagosome maturation, promoting antigen presentation by APCs. It is reported that inducible maturation of phagosome is occurred only in the presence of TLR signalling (Blander *et al.*, 2008; Blander *et al.*, 2004; Blander *et al.*, 2006). Moreover, TLR signalling also regulate MHC class II loading of antigen (De Gassart *et al.*, 2008). Conjugation of antigen and TLR ligands increased cellular and humoral immune responses compared to mixed formulations, because TLR activation was occurred in the same phagosomes that contain the antigen (Khan *et al.*, 2007; Zeng *et al.*, 2002). Also, activation of TLR2 signalling with TLR2 ligands upregulate MHC class II and co-stimulatory molecules (Zeng *et al.*, 2002; Chen *et al.*, 2009).

③ Cross-presentation of antigen

Increased antigen internalization and increase in TAP and proteasomal activity could induce increased cross-presentation of antigens by DCs (Gil Torregrosa *et al.*, 2004; West *et al.*, 2004). MyD88-dependent signalling pathway of TLR2 dislocate TAP to the early endosomes, increasing cross-presentation of exogenous antigens like PAMPs (Burgdorf *et al.*, 2008). It was suggested that cross-presentation of antigen by TLR2 signalling is increased by the interaction of lipopeptides with the plasma membrane lipids, entering in the MHC class I processing pathway (Martinon *et al.*, 1992).

(2) Lymphocyte activity

① Modulation of APCs migration

There are some reports that TLR2 was proved to play an important role in leukocyte migration to inflammation site (Caproni *et al.*, 2012). Pam_3CSK_4, the synthetic triacyl lipopeptide, was effective in early induction of $CD11b^+$ blood cells (Caproni *et al.*, 2012). Moreover, TLR2 ligands increase the time for contact betwwen DCs and antigen at the inflammatory sites (West *et al.*, 2008).

② $CD4^+$ T cell polarization

There are some controversial issues related to the relationship between TLR2 and $CD4^+$ T cells. Some researchers insisted that TLR2 activation induce Th2 responses (Dillon *et al.*, 2004; Kiura *et al.*, 2006). TLR2 activation induces ERK1/2 signalling, which decrease IL-12 production and increase IL-10 production.

However, other authors insisted that TLR2 stimulation induce Th1 responses (Brightbill *et al*., 1999; Cote-Sierra *et al*., 2002; Chua *et al*., 2008; Ghielmetti *et al.*, 2005). Unlike other TLRs, TLR2 ligands could induce IFN-γ production in mouse. (Imanishi *et al.*, 2007). Also, synthetic lipopeptides or bacterial lipoproteins induce Th1/T_{reg} differentiation and inhibit Th2 responses (Revets *et al*., 2005; Akdis *et al.*, 2003; Lombardi *et al*., 2008).

These different observations were obtained from different TLR2 ligands and distinct models. However, it is evident that TLR2 plays an important roles in both effector and regulatory immune mechanisms.

③ $CD8^+$ T cell cytotoxicity

It is reported that monoacylated lipopeptides induce $CD8^+$ T cell responses (Andrieu, *et al*., 2000; Zhang *et al*., 2005). Another researchers observed that TLR2 ligands conjugated antigen enhance virus specific memory $CD8^+$ CTL and this phenomenon was hardly observed in $TLR2^{-/-}$ and $MyD88^{-/-}$ mice (Zhang *et al*., 2009). In addition, when ovalbumin was inoculated with TLR2 ligands, it induced $CD8^+$ responses (Prajeeth *et al.*, 2010).

④ Induction of NK cells activity

NK cell activation is effective in defending against virus and bacteria (Kim *et al*., 2012; Lindgren *et al*., 2011; Marcenaro *et al.*, 2008; Martinez *et al*., 2010). The activation of NK cells by various TLR2 agonists has been reported and effectiveness is different among different TLR2 ligands (Eriksson *et al.,* 2006;

Sawaki *et al.*, 2007). Injection of Pam_2CSK_4 around NK sensitive tumor cells inhibit tumor growth (Sawahata *et al.*, 2011). In addition, TLR2 agonists could induce antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells (Kiura *et al.*, 2006).

⑤ Generation of antibody-secreting cell (ASC)

Induction of antibody response is important in evaluating effectiveness of vaccine. It has been reported that TLR2 plays an important role in the longevity of antibody secreting cells (ASC). In addition, TLR2-mediated CD40 signalling could promote differentiation of B cells into ASC (Komegae *et al.*, 2013; Boeglin *et al.*, 2011).

(3) Mucosal immune responses

Many diseases of humans and livestock are caused by pathogens entering into mucosal surfaces. In this context, mucosal vaccine has several advantages (Hase *et al.*, 2009). Mucosal vaccine could induce antigen specific IgA responses at the site of infection and also at the distant mucosal sites from the immunization site (Park *et al.*, 2014). In addition, unlike conventional injection vaccines, mucosal vaccine induce not only mucosal immune responses but also systemic immune responses (Lycke *et al.*, 2012). It is reported that TLR2 targeting formulations has been proved to induce strong mucosal and systemic immune responses such as production of mucosal IgA and serum IgG (Lee *et al.*, 2011; Jackson *et al.*, 2004; Zhang *et al.*, 2009; Nardelli *et al.*, 1994; Bricknell *et al.*, 2005).

In particular, mucosal vaccine has several advantages especially

in livestock. In case of domestic animals, most pathogens access through the mucosal surface such as Porcine epidemic diarrhea(PED) and Foot-and-mouse disease (FMD) in pigs, causing a huge amount of economic loss. In addition, unlike mucosal vaccine, conventional injection vaccine can only stimulate systemic immune responses and cause stress to domestic animals. Finally mucosal vaccination gives passive immunity of IgA to young domestic animals like calfs and piglets during colostrum (Park *et al.*, 2014).

① Gut imprinting

DCs can modulate immune responses in a tissue-specific manner (Mora *et al.*, 2008). In particular, DCs from GALT referred as gut-associated DCs exhibit gut-specific imprinting properties, including the capacity to generate gut-tropic lymphocytes. They induce expression in a high level of gut-specific receptors such as integrin $\alpha_4\beta_7$, chemokine receptor CCR9 on B cells and T cells (Mora *et al.*, 2003; Mora *et al.*, 2006). The ability of gut-associated DC to induce gut-tropic lymphocytes is explained by their ability to metabolize vitamin A (retinol) into all-trans retinoic acid (RA) because RA is required to imprint gut-homing lymphocytes (Coombes *et al.*, 2007). It is reported that gut-associated DCs have high expression levels of retinal dehydrogenases (RALDH), which is the key enzymes for RA biosynthesis. In recent study, it is known that unlike other TLRs, MyD88-dependent TLR2/1 signals, can educate extra-intestinal DCs with gut-specific features (Wang *et al.*, 2011). In $MyD88^{-/-}$ mice, low levels of RALDH was expressed

in gut- associated DCs and their ability to induce gut-homing T cells was significantly impaired. On the contrary, RALDH production gut-homing of lymphocytes was induced after treatment of extra-intestinal DC with a TLR1/2 agonist through a mechanism. Considering that an immune responses at distal site from the immunization site would be more freely modulated, the ability of TLR2 signalling to educate extra-intestinal DCs with gut-homing properties could enhance the mucosal immune responses elicited by vaccines.

② Targeting porcine M cells

M cell is located in follicle associated epithelium (FAE) of peyer's patch in GALT. M cell lacks microvili on their apical surface and has a basolateral pocket containing lymphocytes or dendritic cells (Frey *et al.*, 1996). It is known that M cell uptakes and delivers antigen from the gut lumen to dendritic cells across mucosa epithelia via transcytosis. After transported antigen is uptake by DCs, antigen is presented to T cells in peyer's patch and B cells in germinal center are activated. Then, IgA^+ B cells and T cells migrate to lamina propria through mesentric lymph node, then IgA^+ B cells mature into plasma cells, producing IgA. Also, antigen driven DCs migrate to the mesentric lymph node, distributing of antigen in other secondary lymphoid organ through blood circulation (Abreu *et al.*, 2010). Therefore M cell is the beginning point of mucosal immune reponses and an ideal target for mucosal vaccine delivery. Targeting M cells could increase delivery efficiency of vaccines and enhance not only mucosal immune responses but also systemic immune responses elicited

by vaccinization (Park *et al.*, 2014).

It is reported that TLR2 is expressed on the M cells in adult swine (Tohno *et al.*, 2005). Porcine TLR2 in the mesenteric lymph nodes and peyer's patches was expressed 15- and 9- fold higher than that of spleen on mRNA level. In flow cytometric analysis, cytokeratin 18^{+}/$TLR2^{+}$ cells was approximately 40%, considering M cell comprise very small population on intestinal epithelial cells. Immunohistochemistry analysis confirmed that TLR2 is not only expressed in the cytoplasm but also in the apical membrane of M cell, suggesting that porcine TLR2 could be involved in ligand-specific endocytosis in M cells. Immunomodulation effects by targeting TLR2 in vaccines are shown in Figure 3.

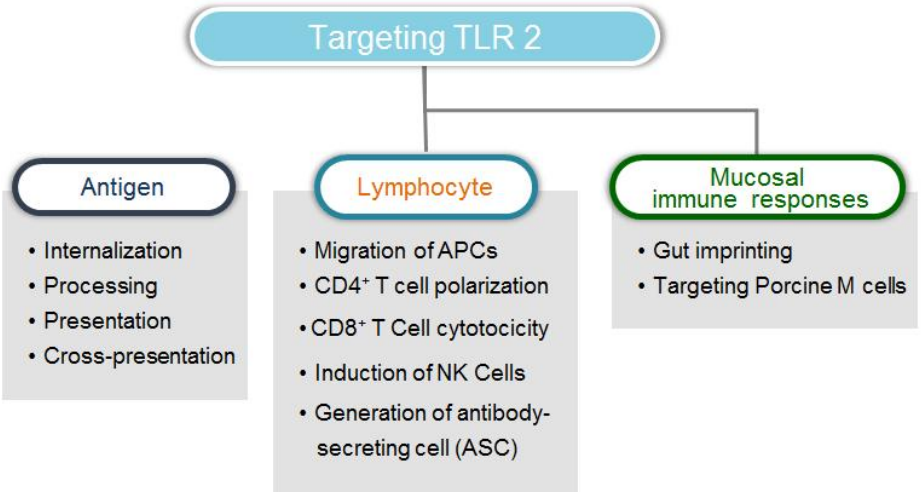


Figure 3. Immunomodulation effects of targeting TLR2 in vaccine

3. Phage display

1) General information about phage display

Phage display was first developed in 1985 by G.P. Smith and used as a powerful tools for identification of targeting ligands such as peptide ligands and antibody fragments (Smith *et al.*, 1985). The principle of phage display is the display of random peptide or antibody on the surface of coat protein on filamentous phage. From this population, a selection is proceeded against desired target (Pande *et al.*, 2010).

Phage display has several advantages than other display methods such as simplicity, speed and cost effectiveness. The most significant advantages of this technique is generation of the diverse screening libraries (Hamzeh-Mivehroud *et al.*, 2013). Phage display has been used as a powerful tools in drug discovery, proteomics, enzymology. To be specific. it was used in identification of receptor agonists, epitope mapping, antibody engineering, studying protein - protein interactions and developing organs, tissue of tumor targeting drugs (Molek *et al.*, 2011).

Filamentous bacteriophage used in phage display infects F plasmid-containing gram-negative bacteria, such as *E.coli*. M13 phage, f1 phage and fd phage are one of the filamentous bacteriophage (Qi *et al.*, 2012). Because filamentous bacteriophage is lysogenic, not lytic, phage infected host cells release new phage without any bacterial lysis. Filamentous bacteriophage has ssDNA and the genome consists of 11 genes : capsid proteins (pIII, pVI, pVII, pVIII and pIX; DNA replication related proteins (pII, pV and pX); and assembly related proteins (pI, pIV and

pXI) (Bratkovič *et al.*, 2010). The most frequently used coat proteins for the phage display are pIII and pVIII, known as the minor and major coat proteins, respectively (Hamzeh-Mivehroud *et al.*, 2013). The displayed peptide is expressed at the N-terminus of pIII protein and only short peptides can be displayed at the N-terminus of pVIII protein (Molek *et al.*, 2011). The structure and genome of M13 bacteriophage is shown in Figure 4.

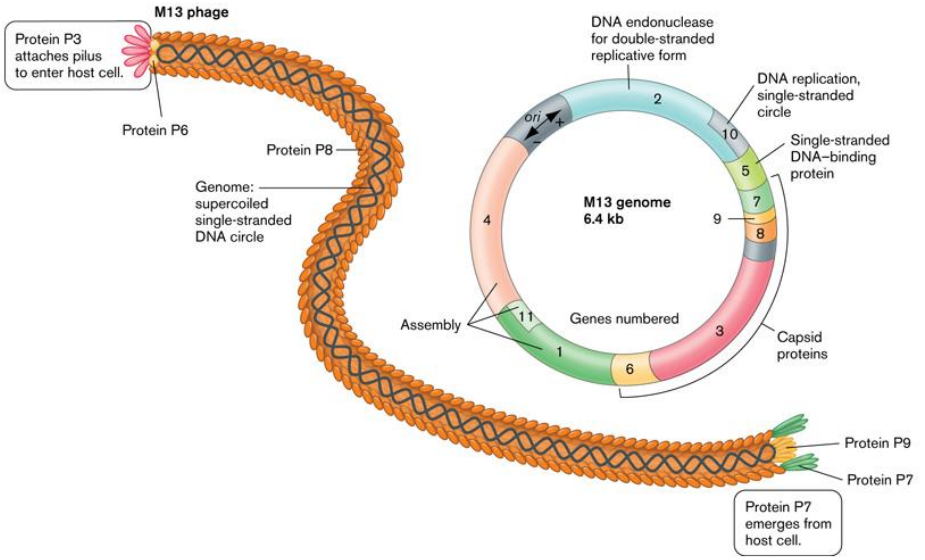


Figure 4. The structure and genome of M13 bacteriophage (Microbiology, W. W. Norton & Company).

2) Types of phage library

(1) Peptide library

Among various targeting ligands, peptide ligands have several advantages. Peptide ligands can be synthesized by chemical methods on a large scale and can be easily conjugated with peptide drug, antigen and carriers. Also, Peptide ligands could transported into cells through receptor-mediated endocytosis (Park *et al.*, 2014). In peptide phage display, degenerate oligonucleotides such as $(NNK)_n$ (N: 1:1 mixture of A,T,G and C, K: 1:1 mixture of G and T) codon are inserted into the phage genome and approximately 10^{10} different peptides can be displayed (Hamzeh-Mivehroud *et al.*, 2013).

Because peptides have many advantages over proteins such as costs, stability, efficiency of organ and tissue penetration and immunogenicity, peptides have been used as therapeutic drugs. Several peptide drugs have been developed by phage display. Commercial phage peptide library is illustrated in Figure 5.

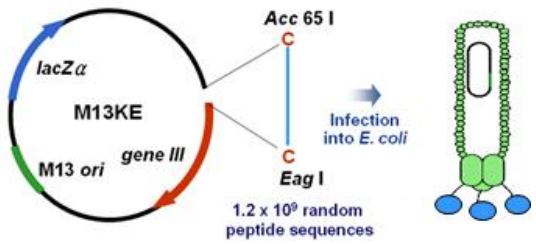


Figure 5. Phage display peptide library from NEB (USA)

(2) Antibody library

The antibody molecule consist of heavy (H) and light (L) chains, containing variable (V) and constant (C) domains. The various modified antibody fragments can be displayed on the phage (Hamzeh-Mivehroud *et al.*, 2013). Single-chain fragment variable(ScFv) is one of the engineered antibody fragments and consist of only one chain of V_H and V_L linkged by a short linker (Tang *et al.*, 1996). To make antibody fragment libraries, cDNA of V_H and V_L gene from B cells are prepared and inserted into the genome of phage coat protein. (Zhu *et al.*, 2009). Advantage of antibody phage display over peptide phage display is high specificity. Using antibody phage display, monoclonal antibodies against biological target such as TNF-α were developed for last several decades and have been approved for use in the clinical applications (Hoogenboom *et al.*, 1998). Construction of ScFvs phage libraries is shown in Figure 6.

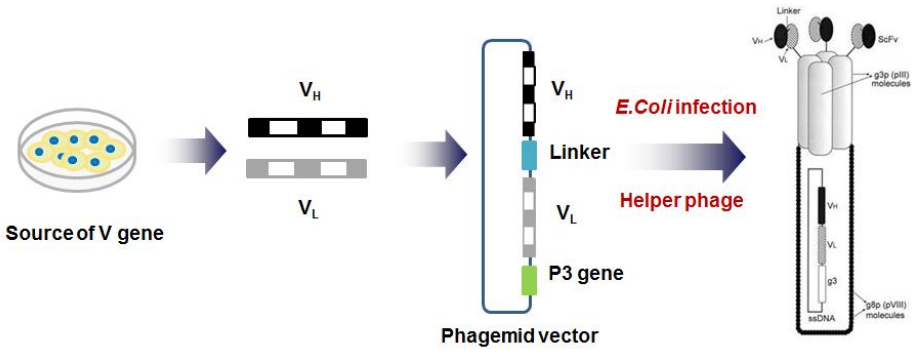


Figure 6. Construction of ScFvs phage libraries

3) Types of screening

(1) *in vitro* screening

Traditional *in vitro* screening using phage display involves the following steps: target immobilization, phage binding, washing, phage elution and repetition of selection rounds (Azzazy *et al.*, 2002). First, purified target is immobilized on plates and the phage library is added for binding to target. Then, the unbound phage is removed by washing and target-bound phage is eluted by changing pH or competing with ligands. To identify the phage population that have much higher affinity against target, the eluted phage is amplified in *E.coli* and these procedures are repeated for 3 or 4 times. Finally, eluted phage is sequenced.

In some cases, *in vitro* screening can be performed on whole cell, which referred as cell-based phage display (Hamzeh-Mivehroud *et al.*, 2012; Heitner *et al.*, 2001). In particular, cell-based phage display could be used for identification of membrane targeting ligands (Molek *et al.*, 2011). Because it is difficult to purify the membrane receptor in a soluble form and maintain the natural conformation. Therefore using native cells or target gene transfected cells could maintain the native conformation of receptor with normal post-translational modification. However, because non-target molecules are expressed on cells, there is a possibility that non-target binding occurs and false positives could be selected. To overcome this problem, subtraction is usually performed in cell-based phage display (Hamzeh-Mivehroud *et al.*, 2012). The typical biopanning of *in vitro* screening is shown in Figure 7.

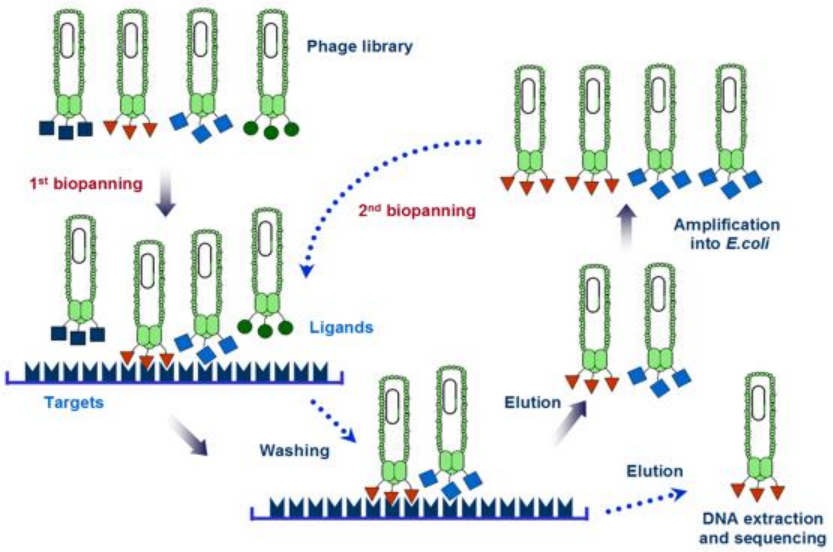


Figure 7. The typical procedures of *in vitro* biopannnig (Kang *et al*., 2008)

(2) *in vivo* screening

in vivo biopanning could be used in identification specific tissue or organ-targeting ligands. Firstly, phage library is injected in the circulation. This allows phage to circulate a period of time. Then, the organs are dissected and tissue or organ-bound phage is recovered and sequenced. *in vivo* selection has several advantages: first, peptides are identified after testing functionally and overcoming degradation. Secondly, unspecific phage is removed from circulation. Finally, it could identify receptor expressed selectively on endothelium of the specific tissue (Kang *et al*., 2008). There receptors could be used as molecular targets for the diagnostics and targeting therapies.

Ⅱ. Materials and Methods

1. Cell culture

IPEC-J2, non-transformed porcine jejunum epithelial cell line was generously provided by Min-Jeong Gu (Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Korea) and maintained in Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 medium mixture supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% insulin-transferrin-selenium-X (ITS-X) (Gibco, USA) and antibiotics (penicillin/streptomycin). HEK 293 LTV cell line was kindly gifted by Kwang-Hwan Choi (Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Korea) and cultured according to the manufacturer's instructions (Cell Biolabs, USA). IPEC-J2 and HEK 293 LTV cells were grown at 37 ℃ in an atmosphere of 5 % CO_2.

2. Construction of porcine TLR2-overexpressing cell line

1) Confirmation of VSVG envelope tropism on IPEC-J2 cells

Lentiviruses that express eGFP (Kwang Hwan *et al.*, 2013) was provided from Kwang-Hwan Choi (Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Korea). IPEC-J2 cells were treated with lentiviruses in the presence of 8 μg/mL polybrene (sigma). After 48h, cells were dissociated into single cells using 0.25% trypsin and fixed with 4% PFA. The fixative was removed by three washes with PBS and cells were analyzed using flow cytometry (FACS Aria).

2) Plasmid construction

Porcine TLR2 gene was amplified from pUNO1-pTLR02 (Invivogen, USA) by PCR with the designed primers (Table 3). The primers include restriction enzyme sites and additional sequences were inserted to remove stop codon in TLR2 sequence and to fit in the eGFP frame for the C-terminal eGFP-tagged TLR2 expression. PCR was initiated with a 4-min denaturation at 95 ℃, followed by 5 cycles of 30 s at 95 ℃, 30 s at 43 ℃, 2-min at 72 ℃ and 30 cycles of 30 s at 95 ℃, 30 s at 62 ℃ and 2-min at 72 ℃. PCR product was inserted into a lentiviral transfer plasmid pWPXL (Addgene, USA) by Pme/Mlu sites to generate the pWPXL-pTLR2-eGFP.

Table 3. Primers used for amplification of porcine TLR2

Primer	Sequence	Restriction enzyme site
1F	AGCTTTGTTTAAACGCCACCATGCCATGTGCTTT	Pme I
1R	ACATGCATGCTCACAGGCTGAGTT	Sph I
2F	ACATGCATGCCTGGCCCTTCCTAC	Sph I
2R	CGCGACGCGTAAGGACTTGATCGCAG	Mlu I

3) Preparation of porcine TLR2-expressing lentiviruses

For the production of lentiviruses, three plasmids were used: lentiviral expression plasmid, pWPXL; packaging plasmid, PsPAX2 (Addgene, USA); and envelope plasmid, pLP/VSVG (Invitrogen, USA). Preparation of porcine TLR2-exprerssing lentiviruses was performed according to the standard protocol. Briefly, three hours before transfection, the cell medium was replaced with 25 μM chloroquine-containing medium. Then, 26.5 μg of pWPXL-pTLR2-eGFP, 9.2μg of PsPAX2 and 5μg of pLP/VSVG were transfected into 80% confluent HEK 293 LTV cells using calcium phosphate precipitation method. After 12h of transfection, transfected cells were treated with 15% glycerol solution and incubated for another 24 h. Culture supernatants were harvested every 12 h and filtered using 0.45-μm pore filters (Satorius, Korea). To precipitate lentiviruses, PEG 10000 and NaCl was added to the lentiviral supernatant and concentrated by centrifugation at 15,000×g for 2 h at 4°C. The virus pellet was dissolved in DMEM and stored at - 70°C.

4) Lentiviral transduction on IPEC-J2 cells

IPEC-J2 cells were seeded at density of 1×10^5 and grown to be

60% confluent in 35mm culture dish before transduction. Cells were treated with lentiviruses in the presence of 8 μg/mL polybrene (Sigma). 48h after transduction, media was replaced with fresh media and transgene expression was observed by fluorescence microscopy. The scheme of construction of porcine TLR2-overexpressing cell line is shown in Figure 8.

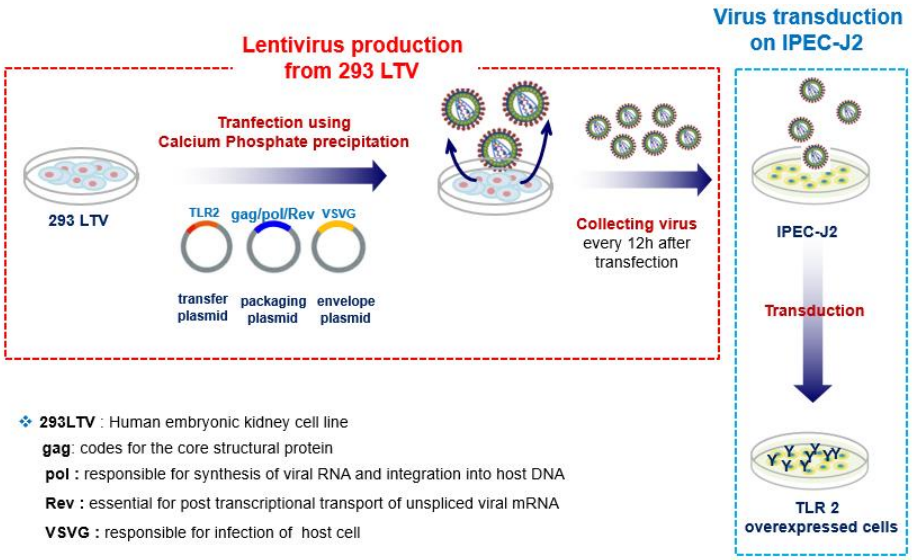


Figure 8. The procedure of establishment of porcine TLR2 overexpressing cell line

3. Validation of constructed cell line

1) Flow cytometric analysis

To evaluate lentiviral transduction efficiency, eGFP expression level of transduced IPEC-J2 cells was analyzed compared to IPEC-J2 cells transfected by Lipofectamine 3000 (Invitrogen, USA); the conventional transfection reagent. Cells were detached using 0.25% trypsin and fixed with 4% PFA. Fixed cells was analyzed using flow cytometry (FACS Aria).

2) Western blot

IPEC-J 2 cells were transduced by pTLR2-eGFP carrying lentiviruses or carrying only GFP. Cells were washed with ice cold PBS and lysed by radioimmunoprecipitation assay RIPA buffer (Sigma, USA) with protease inhibitor and phosphate inhibitor (Roche, Germany). Lysate was transferred into a new tube and protein concentration was measured by Bradford assay. Equivalent amounts of protein mixed with 5X sample buffer(10% SDS, 5% β-2-mercaptoethanol, 20% glycerol, 0.5M Tris-HCl, 0.05% Bromophenol blue) were boiled for 5 min at 95℃. Samples were loaded and electrophoretically separated on SDS-PAGE gels (12%). Protein was transferred to the nitrocellulose membrane on 13 V for 80 min. Membranes were blocked with 5% skim milk and 0.05% Tween-20 in Tris-buffered saline (TBS) for 1h at room temperature and incubated overnight at 4℃ with rabbit anti β-actin IgG (Santa Cruz Biotechnology, USA)(1:2000). The membranes were then washed three times with 0.05% Tween-20

in TBS and incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, USA) (1:20000) for 1 h at room temperature. After washing, membranes were reacted with ECL detection reagents (Santa Cruz Biotechnology, USA) and analyzed by luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). The membrane was then stripped with stripping buffer (Thermo Scientific, USA) and re-probed for GFP using HRP conjugated rabbit anti GFP polyclonal antibody (Santa Cruz Biotechnology, USA)(1:500).

3) Ligand binding assay

IPEC-J2 cells were seeded and grown to be 60% confluent in 35 mm confocal glass bottom dishes (SPL, Korea). Cells were treated with pTLR2-eGFP carrying lentiviruses in the presence of 8 μg/mL polybrene (Sigma) and incubated for 48h. 10 μg/mL of Texas Red- labeled Zymosan A from *S. Cerevisiae* (Molecular probes, USA) was treated for 1h at 37℃ and washed three times with PBS. Cells were then fixed in 4% PFA and analyzed using confocal microscopy (Leica TCS SP8X).

4. Cell-based phage display

1) Phage display peptide library

The Ph.D.-C7C^{TM} Phage Display Peptide Library Kit (New England Biolabs, USA) was used in this study. This system is based on a recombinant filamentous M13 bacteriophage where random peptide 7-mers are expressed at the N-terminal of minor coat protein(pⅢ). This peptide library features randomized sequence flanked by a pair of cystein residues, which results in constrained 7-residue disulfide loop to enhance binding efficiency compared to the same sequence expressed in a liner form. The peptides are followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pⅢ sequence. The library has 1.2 × 10^9 diversity and amplified once to yield ~200 copies of each sequence in 10 μl of the supplied phage.

2) Biopanning *in vitro*

(1) *in vitro* phage binding assay

① Biopanning without subtractive round

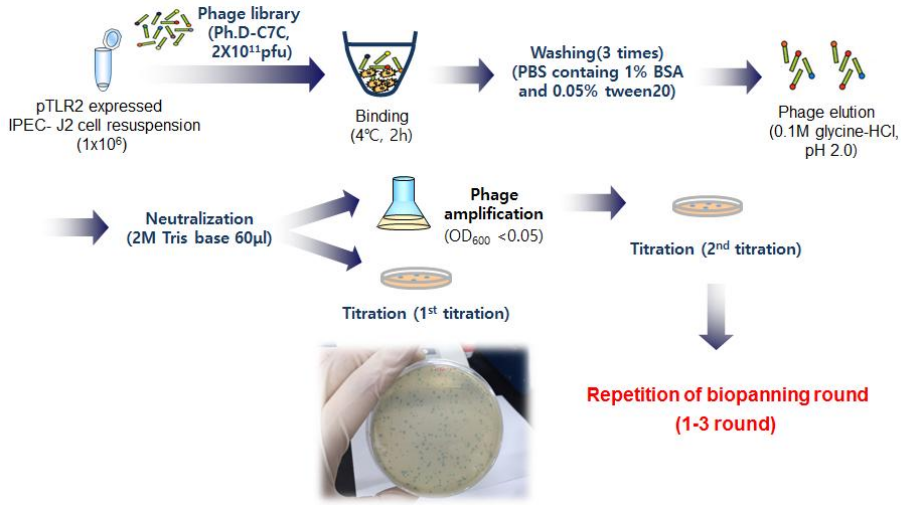
After transduction with pTLR2-eGFP carrying lentiviruses, IPEC-J2 cells are harvested with ice cold PBS (without Ca^{2+} and Mg^{2+}) containing 5mM EDTA. Harvested cells were then washed with DMEM containing 1% BSA and the cell number was counted. 1× 10^6 cells were prepared in DMEM containing 1% BSA and 2× 10^{11} pfu of phage library (10 μl of the phage library) were added to cell suspensions. Cells were incubated for 2h on ice to prevent endocytosis of any bound phage. The cell suspensions were centrifuged at 3000rpm for 3 min at 4℃ and

supernatant was discarded. In order to remove unspecific phage, cell pellet was washed three times with 500 μl of PBS that contains 1% BSA and 0.05% Tween 20 by gently pipetting. At the end of the final washing, 1 ml of 0.1M glycine-HCl (pH 2.0) was added to cell pellet to elute cell-bound phage. After several vortexing and 3 min of incubation, the tube was centrifuged at 13000 rpm for 3 min at 4℃. Supernatant that contains eluted phage was transferred into a new 1.5 ml tube and neutralized with 60 μl of 2M Tris base. Above steps refers to as '1^{st} round of biopanning'.

② Biopanning with subtractive round

Experimental procedures of biopanning with subtractive round were same as above biopanning without subtractive round except for one process. At the 1^{st} round of biopanning, 2×10^{11} pfu of phage were added to 1×10^{6} naive IPEC-J2 cells for 30 min on ice, centrifuged at 2700rpm for 4 min at 4℃ and cell pellets which contains cell-bound phage was discarded to remove naive IPEC-J2 cell-bound phage. Supernatant was then added to 1×10^{6} lentiviral transduced IPEC-J2 cells and positive selection was performed. The procedure of cell-based phage display is shown in Figure 9.

(a)



(b)

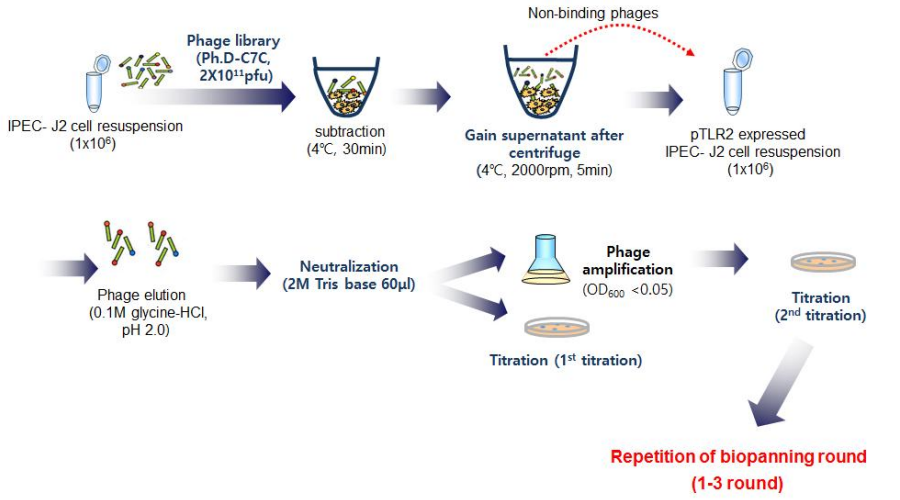


Figure 9. The procedure of cell-based phage display in this study. (a): Biopanning without subtrative round, (b): Biopanning with subtrative round

(2) Phage titration

To determine the number of phage in the eluate sample(or in the amplified sample), phage titer was measured according to the manufacturer's instructions (NEB, USA). Before titration, a single colony of ER2738 (NEB, USA) was inoculated in 5 ml of LB/Tet broth and incubated until it reached mid-log phase (OD_{600} ~0.5). For the titration of phage, the phage solutions were ten-fold serially diluted in LB medium. Suggested dilution ranges were 10^{1}~10^{4} for eluates and 10^{8}~10^{11} for amplified phage. 10 μl of each dilution was added to 190 μl of ER2738 culture and 100 μl of infected cells were dispensed into 45℃ melting Agarose Top, voltexed quickly, and quickly poured onto pre-warmed LB/Tet/IPTG/X-gal plates. After overnight incubation, phage was titrated by counting blue plaques and number of phage plaques was multiplied by the dilution factor for the plate. Every titration was performed three times.

(3) Phage amplification

Succeeding round of biopanning should be proceeded with equal pfu of phage library (2×10^{11}). Thus, eluate from each round should be amplified for the next round of biopanning. Phage amplification was proceeded according to the manufacturer's instructions (NEB, USA). Before phage amplification, a single colony of ER2738 was inoculated in 5 ml of LB/Tet broth and incubated until it reached mid-log phase (OD_{600} ~0.5). The phage eluate was added into the 20 ml ER2738 culture and incubated for 4.5h at 37℃ with vigorous shaking. The culture was transferred to a 50 ml centrifuge tube and centrifuged for 10 min

at 10,000 rpm, 4℃. The supernatant was transferred to a fresh tube and re-spin to remove the rest of *E.coli*. The upper 80% of the supernatant was taken to a new tube, mixed with 1/6 volume of PEG/NaCl and incubated overnight at 4℃ to precipitate phage. For the recovery of amplified phage, PEG/NaCl precipitate was centrifuged for 15 min at 10,000 rpm, 4℃ and supernatant was discarded. The phage pellet was suspended in 1 ml of TBS and the suspension was transferred to a new 1.5 ml tube and centrifuged for 2 min at 14,000 rpm, 4℃. The supernatnat was moved to a 2 ml tube, re-precipitated with 1/6 volume of PEG/NaCl, and incubated on ice for 60 min. After then, samples were centrifuged for 10 min at 14,000 rpm, 4℃ to get precipitated phage pellet. The supernatant was discarded and pellet was suspended in 200 μl of TBS and centrifuged for 1 min at 14,000 rpm, 4℃. The supernatant was transferred to a new 1.5 ml tube. The amplified phage was quantified by titration and $2x10^{11}$ pfu of amplified phage was used for the next round of biopanning.

5. Illumina sequencing

1) Isolation of phage DNA

There were seven samples used for Illumina sequencing: naive library(2×10^{11}pfu), phage eluates from 1, 2 and 3 round of biopanning with subtractive round and without subtractive round.

Isolation of phage ssDNA was performed using NaI/EtoH precipitation method (Matochko *et al.*, 2012). Briefly, 500 µl of phage solution was mixed with 200 µl of PEG/NaCl solution, quickly inverted and incubated for 2h on ice. The precipitate was centrifuged for 15 min at 14000rpm, 4℃ and the supernatant was discarded. The phage pellet was dissolved in 63 µl of NaI solution and 156 µl of 100% ethanol was added, incubated for 2h on ice to precipitate DNA. Centrifugation for 15 min at 14000 rpm, 4℃ yielded DNA pellet and pellet was washed with 200 µl of ice cold 70% ethanol. After centrifugation and drying, the DNA pellet was further purified using phenol - chloroform extraction. The pellet was resuspended in 400 µl of RNAse free water and equivalent amount of phenol - chloroform (1:1 v/v) was added, centrifuged for 1min at 14,000 rpm. The aqueous layer was transferred into a new tube and additional equivalent amount of phenol - chloroform was repeatedly added. 400 µl of final aqueous layer was treated with 40 µl of 3M sodium acetate, 800 µl of 100% ethanol and 2 µl of glycogen and incubated for 2h at -20℃ to precipitate DNA. After centrifuging and washing pellet, the pellet was resuspended in RNAse free water.

2) Preparation of sequencing templates

(1) PCR amplification of phage DNA

50 ng of isolated phage ssDNA was amplified using sample specific barcode primers (Table 4) flanking the variable region at the N-terminus of pⅢ gene of M13 phage. PCR reaction(50 μl) contained 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 1.5U Ex-taq polymerase and 10 pmole of forward and reverse barcode primer (all from TAKARA, Japan). 35 cycles (10 s at 98 °C, 20 s at 58 °C, 30 s at 72 °C) were performed. PCR products were electrophoresed on 2.5 % agarose gel.

Table 4. Barcoded primes used for amplification of phage ssDNA

Primer	Sequence
1	TGCAATCAGCTGTATTCTCACTCTGCT
2	GCATTGATCGACTATTCTCACTCTGCT
3	TGCACAGGCTATTATTCTCACTCTGCT
4	ATGCCTCTGAAGTATTCTCACTCTGCT
5	GCATGCTTACGATATTCTCACTCTGCT
6	CATGACTTGACGTATTCTCACTCTGCT
7	ATGCTACGAGTCTATTCTCACTCTGCT

(2) Construction of sequencing library

PCR products were purified from a 2.5% agarose gel using QIAquick Gel Extraction Kit (QIAGEN, Germany). The concentration of DNA was measured using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, USA). 40 ng of each samples were pooled together in 100 μl volume. Sequencing library containing Illumina-compatible sequencing adaptor was constructed by PCR using specific primers and sequencing was performed using Illumina Hiseq 2000 (Macrogen, Korea).

3) Processing of sequencing data

The sequence reads, which are exactly matched with the two nucleotide sequences consisting of the flanking region of phage display variable region (upstream: 5'-TATTCTCACTCTGCTTGT-3' and downstream 5'-TGCGGTGGAGGT-3'), were retained for the further process. The processed reads were sorted into each sample by barcode sequences. Nucleotide sequences of phage display variable regions for each sample were extracted and translated into amino acids using a conventional codon table, and the variable regions containing the stop codon were discarded. Then, the phage display variable regions encoding each unique peptide sequence was counted for each sample. The relative abundance of each peptide was calculated as the fraction of total reads in the library that encoded the peptide.

6. Characterization of porcine TLR2 targeting peptide

1) Peptide binding assay

Four peptides were synthesized to a purity of >95% by HPLC (PEPTRON, Korea): three candidate peptides (ACNAGHLSQCG, ACRANLDGQCG, ACVPSKPGLCG) and control peptide (ACGLHPAFQCG). These peptides include consensus motif (CX_7C: 9 mers, a single intra disulfide bond), Rhodamine B conjugated as fluorescence label and alanine and glycine residues at the terminal of the peptide to increase stability of the synthetic peptides. Each peptide was solubilized to a concentration of 20 mg/ml in DMSO.

IPEC-J2 cells and lentiviral transduced IPEC-J2 cells were incubated with 2 nM, 20 nM, 200 nM, 2 μM of each peptide. Cells then washed three times with PBS for 3min and fixed in 4 % PFA. The fluorescence was measured using FACS Aria.

2) Competition assay

IPEC-J2 cells and transduced IPEC-J2 cells were pre-incubated with 2 μM of the each peptide for 30 min at 4℃, and 0, 1, 2, 5, 10 μg of Zymosan A from *S. cerevisiae* (Thermo Scientific, USA) was added. Cells then washed three times with PBS for 3min and fixed in 4 % PFA. The fluorescence was measured using FACS Aria.

7. Statistical analysis

Results were expressed as the mean and standard deviation (SD). Statistical significance was determined through t-test using GraphPad PRISM software (GraphPad Software, Inc.) All statistical significance is denoted by *P < 0.05, **P < 0.01, and *** P < 0.001.

Ⅲ. Results and Discussion

1. Construction of porcine TLR2-overexpressing cell line

The major problem of cell-based selection in phage display is the expression non-target receptors. Viral vector as porcine TLR2 gene delivery system has some advantages in this study. Lentivirus can stably express TLR2 gene and maximize the expression of TLR2 gene because of its high gene delivery efficiency, thus decreasing the possibility of selecting non-target receptor binder. In this study, porcine TLR2-expressing IPEC J2 cells were established to identify porcine TLR2 targeting peptide ligands using lentiviruses that carry TLR2-eGFP fusion protein.

Before producing porcine TLR2 gene carrying lentiviruses, VSVG envelope tropism on IPEC-J2 cells was investigated through flow cytometric analysis. Compared to negative control, IPEC-J2 cells that transduced by VSVG-pseudotyped GFP carrying lentiviruses showed strong GFP signal (Figure 10). As a results, it was confirmed that VSVG envelope can efficiently infect IPEC-J2 cells.

In this study, GFP was fused to the C-terminus of porcine TLR2 gene, because there is signal peptides in the N-terminal region of TLR2. Also, GFP should be distinctly localized at the plasma membrane because GFP could bind to specific phage library. Fluorescence microscopic data showed that GFP gene is mostly expressed at plasma membrane on IPEC-J2 cells transduced by porcine TLR2-carrying lentiviruses (Figure 11).

(a) (b)

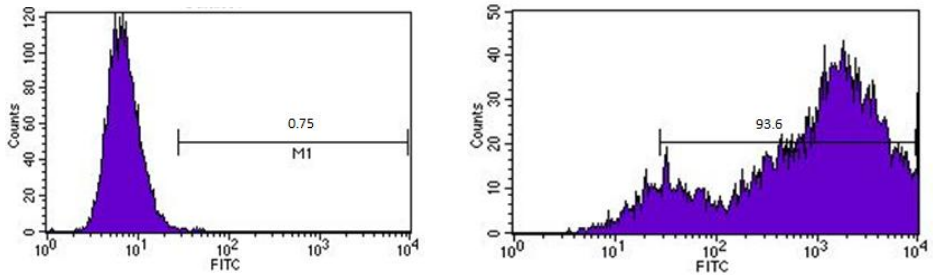


Figure 10. Confirmation of tropism of VSVG envelope on IPEC-J2 cells using GFP-carrying lentiviruses. (a): Non-treated IPEC-J2 cells. (b): IPEC-J2 cells treated with GFP-carrying lentiviruses.

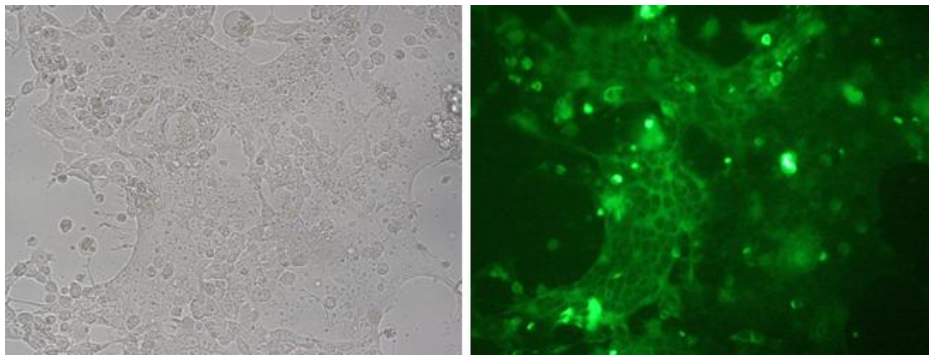


Figure 11. Detection of GFP fused porcine TLR2 expression on lentiviral transduced IPEC-J2 cells using fluorescence microscopy. IPEC-J2 cells were treated with pTLR2-eGFP carrying lentiviruses and incubated for 48 hours in the presence of 8μg/ml polybrene.

2. Validation of constructed cell line

Flow cytometric analysis was performed to measure lentiviral transduction efficiency on IPEC-J2 cells (Figure 12). Compared to negative control, lentiviral transduced IPEC-J2 cells showed approximately 83% transduction efficiency, which is much higher than that of conventional liposome mediated-transfection (47%)

To verify the molecular weight of GFP fused porcine TLR2 protein, IPEC-J2 cells that transduced by lentiviruses carrying pTLR2-eGFP or carrying only GFP were analyzed through western blotting (Figure 13). In case of the cells transduced by only GFP carrying lentiviruses, eGFP band (27 kDa) was detected. On the other hand, cells that transduced by porcine TLR2-eGFP carrying lentiviruses showed eGFP fused porcine TLR2 band, which has an expected molecular weight (115 kDa)

Because TLR2 is a transmembrane receptor, it is necessary to confirm whether the expressed porcine TLR2 maintain its normal function. In this context, TLR2 ligand binding assay was performed using Texas Red-labeled Zymosan as a TLR2 ligand. (Figure 14). Zymosan is the cell wall components from *S.cerevisiae* and act as a TLR2 agonist. As expected, unstimulated cells express GFP signal at plasma membrane. However, after 1h of ligand stimulation, receptor internalized and the signal of GFP and Texas Red was co-localized, suggesting that transduced porcine TLR2 maintain its normal function.

(a) (b) (c)

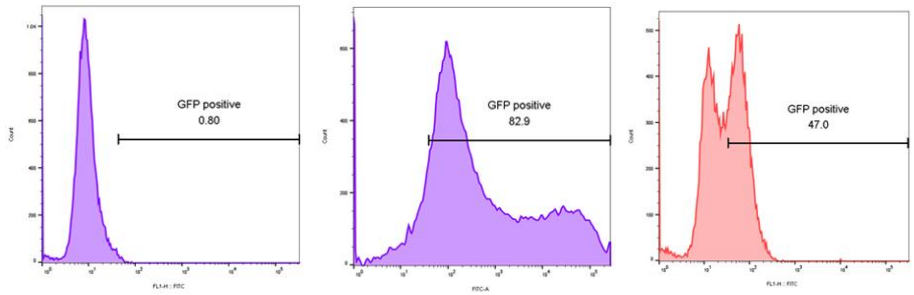


Figure 12. Comparative analysis of transduction or transfection efficiency in IPEC-J2 cells using flow cytometry. (a): non-treated IPEC-J2 cells. (b): IPEC-J2 cells treated with pTLR2-eGFP carrying lentiviruses. (c) IPEC-J2 cells transfected with equivalent amount of porcine TLR2 DNA using Lipofectamine 2000.

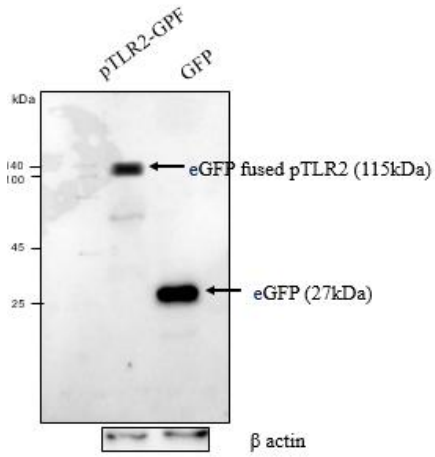


Figure 13. Western blot analysis of lentiviral transduced IPEC-J2 cells. First lane is protein marker, second lane is IPEC-J2 cells transduced with pTLR2-eGFP carrying lentiviruses and third lane is IPEC-J2 cells transduced with only eGFP-carrying lentiviruses. 20 μg of each sample was loaded and incubated with anti-GFP antibody.

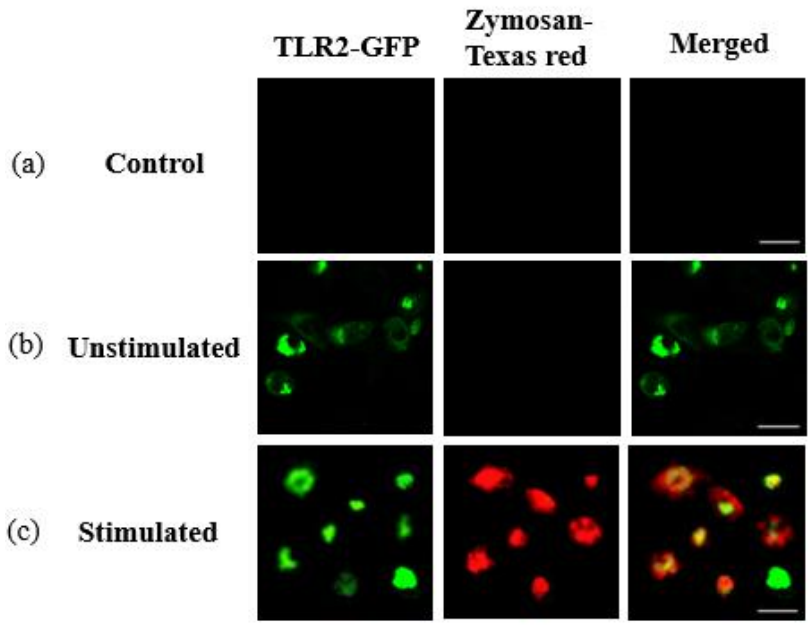


Figure 14. Co-localization of porcine TLR2 and its ligand (zymosan). (a): non-treated IPEC-J2 cells (b): IPEC-J2 cells transduced with pTLR-eGFP carrying lentiviruses, but not stimulated by zymosan (c): IPEC-J2 cells treated with 10 μg/mL of Texas Red conjugated zymosan after transduced with pTLR-eGFP carrying lentiviruses Green signals represents GFP and red signal represents zymosan signal. Scale bar represents 100 μm.

3. Cell-based phage display

In this study, porcine TLR2 binding assay were performed using *in vitro* cell-based phage display. As TLR2 is a transmembrane receptor, it is difficult to purify and maintain natural conformation or receptor. In this context, cell-based phage display could help maintain the native conformation of receptor with normal post-translational modification. However, cell-based phage display has a significant problem that non-target receptor could affect the phage binding. To overcome the disadvantages of cell-based phage display, two types of biopanning were performed: biopanning without subtractive round and with subtractive round. Negative selection against naive IPEC-J2 cells could decrease non-target receptor binders but it may lose TLR2 binders because IPEC-J2 cells express endogenous TLR2. Thus, biopanning rounds with subtraction and without subtraction were compared each other to identify true binders of porcine TLR2.

Phage titration data shows the progressive enrichment of specific phage population along the 3 rounds in both two types of biopanning (Figure 15). In case of subtractive biopanning, phage titer increased approximately 8-fold in 3 round compared to the first round of biopanning. Similarly, 6-fold increase of phage titer was shown in non-subtractive biopanning. Also, higher phage titer was observed in non-subtractive biopanning than subtractive biopanning, suggesting some phage population was removed through negative selection. Enrichment of phage titer along the round of biopanning could be significant evidence of selection pressure that specific phage population was steadily increased.

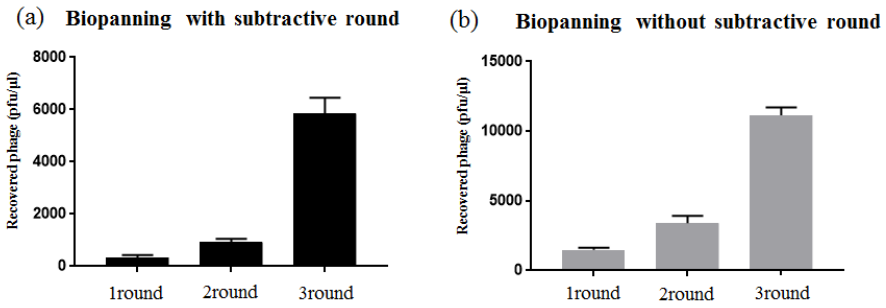


Figure 15. A progressive enrichment of phage during 3 rounds of biopanning. (a): titration data of eluted phage in biopanning with subtractive round (b): titration data of eluted phage in biopanning without subtractive round. All values represent the means ± SD (n=3).

4. Illumina sequencing

1) Characterization of naive library

The ssDNA of phage was isolated and sequencing templates were prepared (Figure 16). Before analyzing sequencing results of phage libraries selected against porcine TLR2, naive library (2×10^{11}pfu) was analyzed by Illumina sequencing to investigate the quality of library and to verify sequencing quality. Phage library of 2×10^{11} pfu theoretically contains approximately 200 copies of 1.2×10^{9} random peptides, but the entire library could not be analyzed because of the limited sequencing depth.

Figure 17a is the theoretical amino acid composition for Ph.D.-C7C naive library based on the random $(NNK)_7$ oligo-nucleotide library. However, the manufacturer (NEB, USA) provides actual amino acid distribution of naive library which is somewhat different from theoretical composition after sequencing by Ion torrent sequencer (Figure 17b).

We obtained more than 20 million unique sequences of naive library and analyzed amino acid distribution. The sequencing results of naive library are shown in Figure 17c,d. The observed amino acid distribution was almost same as actual composition provided by the manufacturers, suggesting that sequencing results are reliable. Proline, threonine, histidine and asparagine was overrepresented and valine, arginine, cystein, glutamine was underrepresented among 20 amino acids in the naive library. Especially, arginine and cystein was the most under-represented (-3.14%, -2.44% respectively), threonine and asparagine was the most overrepresented amino acids (+3.45%, +1.82% respectively).

2) Characterization of enrichment process

Phage libraries eluted after one, two and three round of biopanning were sequenced. The number of unique sequences decreased along the round in both two types of biopanning, but non-subtractive biopanning showed more diverse sequence than that of subtractive biopanning (Figure 18). In case of subtractive biopanning, the number of unique sequences decreased from 158,375 to 28,745 along 3 rounds, while subtractive biopanning showed decrease from 305,411 to 56,812. (Table 5). It means that the overall diversity of phage population decreased and selection occurred after biopanning rounds.

Also, enrichment profiles of the most abundant peptides was analyzed (Figure 19). The false positives identified through SAROTUP and Pepbank were excluded. The details of identifying false positives will be discussed later. In subtractive biopanning, NAGHLSQ was the most abundant peptides during 3 rounds of biopanning. NAGHLSQ appeared 8,546 times in round 1, 1,016,557 times in round 2 and 7,268,173 times in round 3, increasing by 850-fold from round 1 to round 3. In case of non-subtractive biopanning RANLDGQ showed the highest binding affinity. RANLDGQ appeared 4,896 times in round 1, 226,754 times in round 2 and 6,156,589 times in round 3, increasing by 1257-fold from round 1 to round 3. Interestingly, there were common sequences from both types of biopanning. VPSKPGL was the third rank in subtractive biopanning and second rank in non-subtractive biopanning. Illumina sequencing results suggest that the frequency of the most abundant peptides gradually increased along the round of biopanning. Additionally, the percentage of overlap among the

most enriched peptides was high in both types of biopanning (Figure 20).

In case of subtractive biopanning, the overlapped percentage of the top 10 peptides was 60% between rounds 1 and 3, 80% between round 2 and 3. In non-subtractive biopanning, approximately 70% of top 10 peptides between round 1 and 3, 80% between round 2 and 3 was overlapped. It means that high affinity peptides against porcine TLR2 was already identified after the first round of biopanning because of sequencing depth of Illumina. The similar effects of high-throughput sequencing in phage display has been already proved in many researches (A. C.'t Hoen *et al.*, 2012).

Table 5. Number of total sequences and unique sequences along 3 rounds of selection in two types of biopanning

Round	Number of sequences	Number of unique sequences
1 (with subtraction)	11,028,969	158,375
1 (without subtraction)	21,606,549	305,411
2 (with subtraction)	12,004,478	82,351
2 (without subtraction)	16,416,249	193,264
3 (with subtraction)	15,386,519	28,745
3 (without subtraction)	13,130,332	56,812
naive library	33,853,550	20,539,490

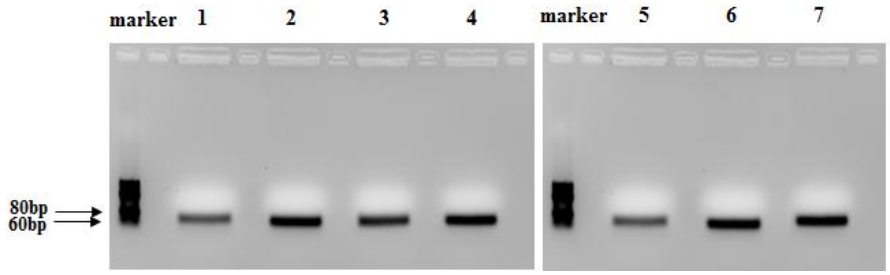


Figure 16. Preparation of DNA templates for Illumina sequencing. Phage ssDNA was isolated and amplified using sample specific barcode primers flanking the variable region at the N-terminus of pⅢ gene of M13 phage. PCR products were electrophoresed on 2.5 % agarose gel. Lane 1: Naïve library; Lane 2: phage eluate from 1R biopanning with subtraction; Lane 3: phage eluate from 1R biopanning without subtraction ;Lane 4: phage eluate from 2R biopanning with subtraction; Lane 5: phage eluate from 2R biopanning without subtraction; Lane 6: phage eluate from 3R biopanning with subtraction; Lane 8: phage eluate from 3R biopanning without subtraction

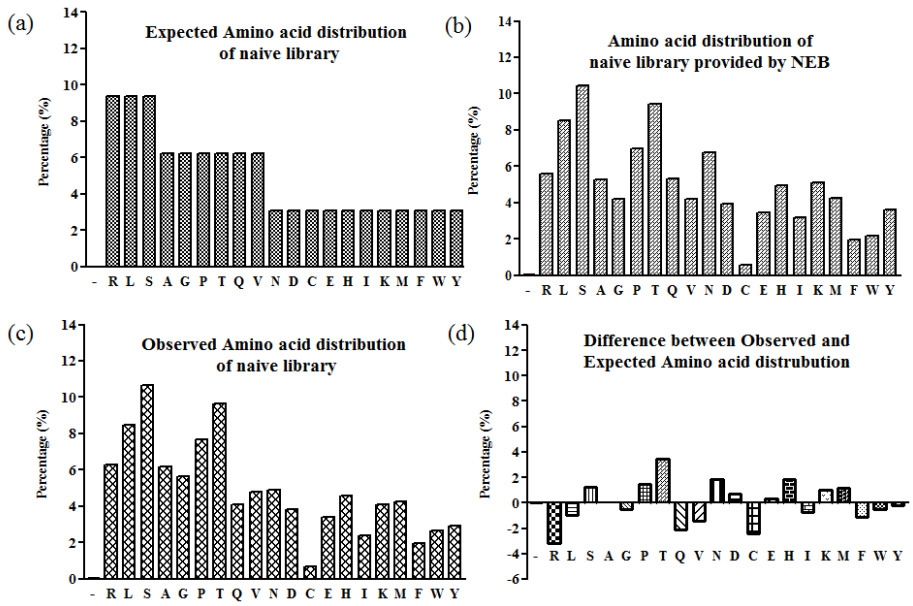


Figure 17. Analysis of amino acid distribution of Ph.D.C7C- library. Average amino acid composition (%) of all sequenced unique peptides is indicated. (a): Theoretical amino acid composition based on the $(NNK)_7$ inserts (b): amino acid composition provided by manufacturer (c): observed amino acid distribution after Illumina sequencing (d): Difference between the observed and the theoretical composition
- : stop codon.

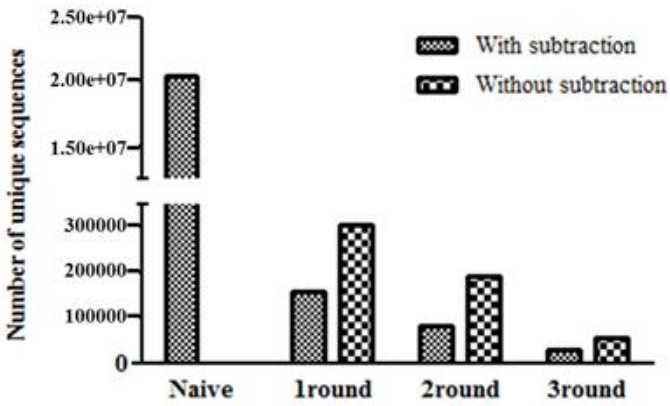


Figure 18. Progressive decrease in the number of unique sequences along 3 rounds of biopanning

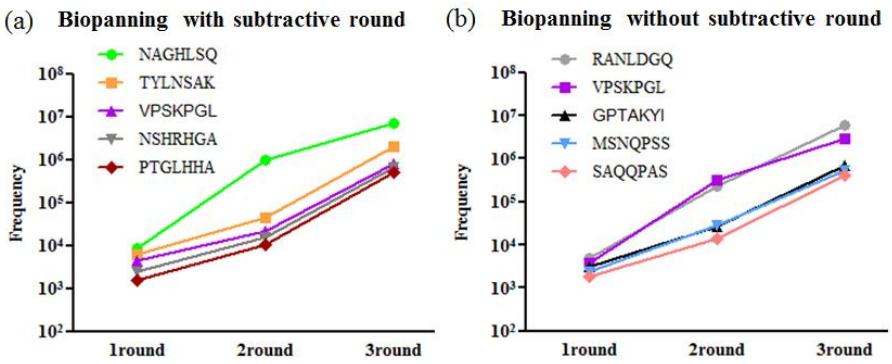


Figure 19. Counts of most abundant peptides in two types of biopanning. False positives were excluded.

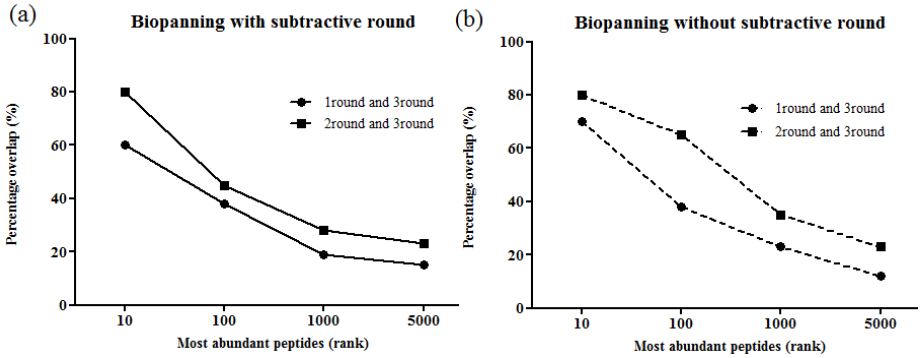


Figure 20. Percentage of overlap of most abundant peptides. Obtained sequences from 1 round and 2 round are compared with those of 3 round and overlapped sequences are indicated as percentage.

3) Identification of false positives

It is known that false positives occur in phage display for two reasons. One is target-unrelated sequences that bind to unrelated materials during biopanning such as metal ion, plastic, albumin. The other is propagation advantages that occur in phage amplification step. Specific phage grows faster than the other phage and phage that has these propagation advantages is greatly accelerated along the round of biopanning. For example, HAIYPRH peptide in the ph.D.-7 library has been identified as a representative false positives that grows faster in *E.coli* (Vodnik *et al.*, 2011) and found 41,257 times after one round of biopanning and 237,565 times after three rounds of biopanning (A. C.'t Hoen *et al.*, 2012).

In this study, two web-based program was used to identify false positives: SAROTUP for identification of non-target related sequences and sequences that have propagation

advantages and PepBank searching for sequences already identified in other researches. Three rounds of selection caused steadily increase in target-unrelated peptides (Figure 21). In both case of subtractive biopanning and non-subtractive rounds of biopanning, approximately 3-fold increase in false positives was observed between round 1 and 3. These false positives could not be easily identified in traditional sequencing of phage display.

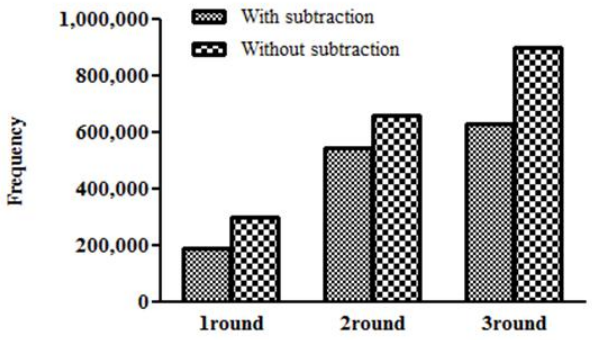


Figure 21. Counts of sequences recorded in SAROTUP and PepBank along 3 rounds of biopanning.

5. Characterization of porcine TLR2 targeting peptide

We selected three candidate peptides (NAGHLSQ, RANLDGQ, VPSKPGL) as porcine TLR2 targeting peptides (Table 6). All three peptides were not identified as false positives through SAROTUP and PepBank. NAGHLSQ and RANLDGQ were the most enriched peptides in subtractive biopanning and non-subtractive biopanning, respectively. They showed progressive enrichment along three rounds of selection. VPSKPGL is the common enriched peptide in both two types of biopanning. Candidate peptides were chemically synthesized by Peptron (Korea). Also, control peptide (CGLHPAFQC) was prepared. CGLHPAFQC is a transdermal tissue targeting peptide identified in our laboratory (Lee *et al.*, 2011).

Table 6. Candidate peptides

Group	Peptide sequence	Counts in 1R	Counts in 2R	Counts in 3R	SARO TUP	Pep Bank
Subtractive biopanning	NAGHLSQ	8,546	1,016,557	7,268,172	No	No
Non-subtractive biopanning	RANLDGQ	4,896	226,754	6,156,589	No	No
Subtractive and non-subtractive biopanning	VPSKPGL	4,326 3,852	22,146 327,346	809,923 2,926,354	No	No

1) Peptide binding assay

Rhodamine B conjugated synthesized peptides were treated to naive IPEC-J2 cells and lentiviral transduced IPEC-J2 cells. Control peptide did not show any significant binding affinity to

two types of cells. In case of naive IPEC-J2 cells, RANLDGQ, which was the most abundant peptides in non-subtractive biopanning showed the highest binding affinity and binding signal became more stronger in response to increasing concentrations of peptides. VPSKPGL, the common enriched sequence in both two types of biopanning, showed moderate binding affinity to naive IPEC-J2 cells. On the contrary, NAGHLSQ, which was the most enriched peptides in subtractive biopanning showed second lowest binding affinity to naive IPEC-J2 cells and binding signal did not significantly increased in response to peptide concentrations.

On lentiviral transduced IPEC-J2 cells, no binding affinity was observed in control peptide. NAGHLSQ showed the highest binding affinity and the binding signals became stronger according to increasing concentrations of peptides. It is completely different from the results of naive IPEC-J2 cells binding assay. RANLDGQ showed the second highest binding affinity. Binding affinity of VPSKPGL was similar with that of naive IPEC-J2 cell binding assay.

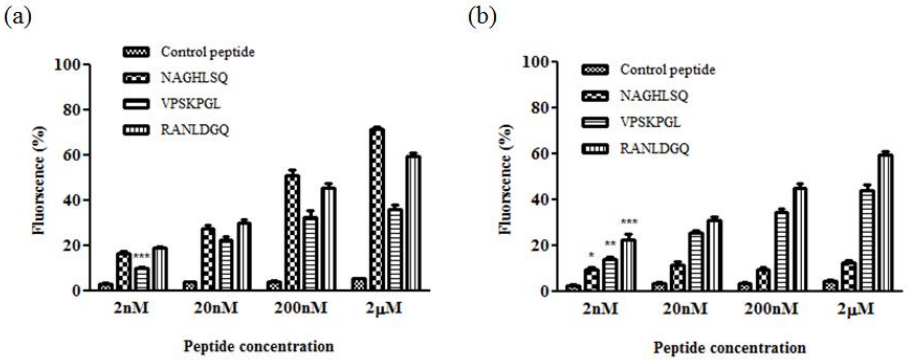


Figure 22. Binding assay of Rhodamine-conjugated synthetic peptides. (a): IPEC-J2 cells transduced with GFP fused porcine TLR2-carrying lentiviruses are treated with different concentrations of candidate peptides and control peptide. Fluorescence from Rhodamine-conjugated synthetic peptides was measured. (b): Naive IPEC-J2 cells are treated with different concentrations of candidate peptides and control peptide. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA

2) Competition assay

To confirm whether the binding is mediated by porcine TLR2, ligand competition assay was performed using zymosan as TLR2 native ligand. If the binding of peptides is mediated by TLR2, peptides would compete with the zymosan. IPEC-J2 cells and lentiviral transduced IPEC-J2 cells were pre-incubated with synthesized peptides and treated with different concentrations of zymosan.

In case of transduced IPEC-J2 cells, fluorescence signal of NAGHLSQ decrease from 77% to 7% in response to increasing concentrations of zymosan, suggesting that NAGHLSQ binds to transduced IPEC-J2 cells through TLR2 binding. RANLDGQ did not respond to increasing concentration of zymosan, suggesting that it binds to transduced IPEC-J2 cells but the binding is not mediated by TLR2. Unlike RANLDGQ, VPSKPGL showed small response to increasing concentration of zymosan.

On naive IPEC-J2 cells, NAGHLSQ showed no considerable binding affinity, while it showed the highest binding affinity to transduced IPEC-J2 cells. RANLDGQ, did not responded to increasing concentrations of zymosan, which is similar with the results of transduced IPEC-J2 cells. It means that RANLDGQ

binds non-TLR2 target. On the other hand, VPSKPGL showed moderate binding affinity to naive IPEC-J2 cells and slightly respond to increasing concentrations of zymosan. It suggests that VPSKPGL could bind both TLR2 and non-TLR2 target but the binding affinity is much lower than that of NAGHLSQ.

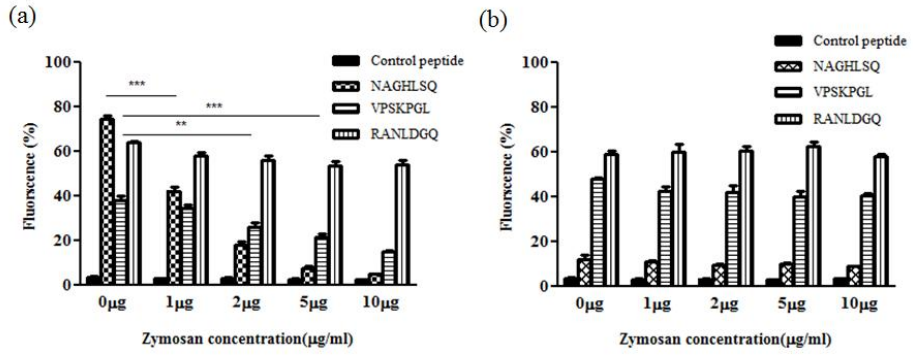


Figure 23. Competition binding assay of Rhodamine-conjugated synthetic peptides and TLR2 ligands (zymosan). (a): IPEC-J2 cells transduced with GFP fused porcine TLR2-carrying lentiviruses are treated with 10 nM of synthetic peptides and different concentrations of zymosan are incubated for 1 hour at 4℃. (b): Naive IPEC-J2 cells are treated with 10 nM of synthetic peptides and different concentrations of zymosan are incubated for 1 hour at 4℃. a*$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA

Ⅴ. Conclusion and Further Aspects

In this study, we performed *in vitro* cell-based phage display combined with Illumina sequencing. Porcine TLR2-overexpressing IPEC-J2 cell line was established using lentiviral vector. Transduction efficiency of lentivirus was 83% and expressed porcine TLR2 has an expected size. Also, porcine TLR2 was co-localized with TLR2 ligands, suggesting that porcine TLR2 maintain its normal function.

Two types of biopanning and 3 rounds of selection was performed on TLR2-overexpressing IPEC-J2 cells. A progressive enrichment of phage population was observed along three rounds of biopanning and Illumina sequencing results showed increase in the number of unique sequences in both types of biopanning and enrichment of the most abundant peptides. NAGHLSQ and RANLDGQ was the most enriched peptides in subtractive biopanning and non-subtractive biopanning, respectively. VPSKPGL was the commom peptides enriched in both types of biopanning. In addition, overlapped percentages of peptides between round 1, 2 and round 3 was high enough that non-repetitive selection could identify high-affinity peptides. False positives such as non-target related binders and propagation advantages were identified using web-based tools: SAROTUP and PepBank. As the biopanning round proceeded, false positives also increased.

In peptide binding assay, NAGHLSQ showed the highest binding affinity to TLR2-overexpressing IPEC-J2 cells among three

candidate peptides, but lower binding ability to naive TLR2. Also, in competition assay, binding affinity of NAGHLSQ was disrupted in response to increasing concentrations of TLR2 ligands, but no response in naive IPEC-J2 cells. Considering that TLR2 expression level is too low to detect in protein level on IPEC-J2, NAGHLSQ distinctly binds to IPEC-J2 cells through TLR2.

Therefore, NAGHLSQ could be used for immunogenic formulations in porcine vaccines. For example, NAGHLSQ could be conjugated with antigen and delivered into nanoparticles or expressed as fusion protein with antigen protein. Porcine TLR2-targeting NAGHLSQ would show adjuvants properties and effectively induce cellular and humoral immune responses. Moreover, NAGHLSQ could be used in not only vaccine design but also developing targeting therapeutics.

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Ⅶ. Summary in Korean

TLR은 대표적인 패턴인식 수용체로서 다양한 미생물 성분을 인식하여 선천성 및 후천성 면역반응에 있어 중요한 역할을 수행한다. 그 중에서도 TLR2의 경우 세포 표면에 발현되어 있으며 다른 TLR과 비교하였을 때 가장 다양한 미생물 성분을 인지하는 것으로 알려져 있다. 따라서 TLR2를 표적하는 전략은 표적형 약물 개발, 세포 치료제 등 다양한 분야에서 활용되어 왔다. 특히 TLR2 표적 제형은 백신 분야에서 널리 이용되어 왔는데 백신에 의한 세포성 및 체액성 면역반응을 증진시키는 것으로 알려져 왔다. 특히 돼지의 경우 면역 세포 이외에도 장내 점막면역기구 중의 하나인 peyer's patch와 외부 항원을 포집하여 점막 면역반응을 개시하는 M세포에서 TLR2가 매우 높은 수준으로 발현되어 있는데, 따라서 돼지 TLR2 표적 작용기를 동정하는 것은 구제역(FMD), 돼지 유행성 설사(PED) 등 점막을 통해 침투하는 질병을 효과적으로 방어할 수 있는 점막 백신의 백신 전달 효율 증가 및 백신 항원에 의한 면역반응 증진 효과를 가져올 것으로 기대된다.

본 연구에서는 돼지에 있어 백신 효과 증진을 위해 세포 기반 파지 디스플레이와 차세대 시퀀싱 기법인 일루미나 시퀀싱을 이용하여 돼지 TLR2 표적 펩타이를 동정하였다. 먼저, 렌티 바이러스를 이용하여 돼지 장 상피 세포주에 돼지 GFP 유전자가 융합된 TLR2 유전자를 도입함으로써 돼지 TLR2 과발현 세포주를 구축하였다. 구축된 세포주를 유세포 분석, 웨스턴 블랏, 리간드 결합 실험을 통해 검증한 결과 생성된 렌티 바이러스는 약 83%의 유전자 전달 효율을 가지며 GFP가 융합된 TLR2 단백질이 예상되는 사이즈에서 검출이 되는 것을 관찰하였다. 또한 리간드와 GFP 형광이 중첩되는 것을 확인함으로써 도입된 TLR2가 수용체로서 정상적으로 기능하는 것을 확인하였다.

이렇게 구축된 돼지 TLR2 과발현 세포주를 이용하여 세포 기반 파지 디스플레이를 수행하였다. 바이오패닝의 경우 subtraction을 진행한 경우와 진행하지 않은 경우로 나누어서 수행하였다. naive library와 각 라운드별로 용출된 파지를 일루미나 시퀀싱을 진행한 결과 바이오패닝 라운드가 진행될수록 펩타이드의 다양성은 감소하며 가장 높은 빈도수로 나타나는 펩타이드의 비율이 증가하는 것을 관찰하였다. 또한 라운드가 진행됨에 따라 false-positive의 빈도와 비율 또한 증가하는 것을 확인함으로써 기존의 시퀀싱 방법과 비교하였을 때 차세대 시퀀싱을 접목한 파지 디스플레이의 강점을 증명하였다. 최종적으로 후보 펩타이드 3종 (NAGHLSQ, RANLDGQ, VPSKPGL) 을 선정하였는데 NAGHLSQ의 경우 subtraction을 진행한 바이오패닝에서 가장 높은 빈도수를 보인 펩타이드, RANLDGQ은 subtraction을 진행하지 않은 바이오패닝에서 가장 높은 빈도수를 보인 펩타이드, 그리고 VPSKPGL의 경우 두 타입의 바이오패닝에서 공통적으로 높게 나타난 펩타이드이다.

후보 펩타이드와 대조군 펩타이드를 각각 합성한 후 검증 실험을 진행하였다. 먼저 NAGHLSQ의 경우 펩타이드 결합 실험에서 TLR2 과발현 세포주에 가장 높은 결합력을 보였지만 과발현 전의 세포주에서는 낮은 결합력을 보였다. 리간드 경쟁 실험에서는 TLR2 과발현 세포주에서 TLR2 리간드의 농도에 의존적으로 결합 능력이 감소하는 것을 관찰함으로써 NAGHLSQ가 TLR2에 높은 친화력으로 결합하는 것을 검증하였다. 두 번째로 RANLDGQ의 경우 TLR2 과발현 세포주와 과발현 전 세포주에서 모두 높은 결합력을 보였지만 리간드 경쟁 실험에서 TLR2 리간드에 반응하지 않음을 통해 RANLDGQ은 TLR2가 아닌 다른 물질과의 결합을 통해 세포에 결합할 것임을 추측할 수 있었다. 마지막으로 VPSKPGL의 경우 과발현 세포주와 과발현 전 세포주에서 중간 정도의 결합력을 보였지만 리간드 경쟁 실험에서는 RANLDGQ에 비해 TLR2 리간드에 반응하

는 것을 확인함으로써 TLR2에 낮은 친화력으로 결합함을 확인하였다.

본 연구를 통해 동정된 돼지 TLR2 표적 펩타이드는 점막 백신 항원을 담지한 나노파티클 전달체 표면에 수식되거나 백신 항원 단백질과 함께 발현되어 전달됨으로써 돼지 점막 백신의 전달 효율 및 백신 항원에 의한 면역반응 증진을 유도할 것으로 기대된다. 이 외에도 TLR2 표적 작용기로서 특정 조직, 질병 표적 약물 개발 분야에서도 널리 활용될 수 있을 것으로 생각된다.