RESEARCH REPORT
SWINE HEALTH

Title: Novel DNA-vaccine for PRRSV with improved heterologous protection. **NPB project: #11-089**

Investigator: Alexander N. Zakhartchouk, Ph.D. and DVM

Institution: University of Saskatchewan

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Industry Summary

PRRS is recognized by the pig industry worldwide as one of the most important swine diseases, and it is estimated to cost the US Swine Industry $ 560 million per year. The disease is caused by a PRRS virus (PRRSV).

Vaccination against PRRS is the best way to protect animals and reduce the economic burden for producers. Now, modified live vaccines for PRRS are in use in USA and Canada. However, these vaccines cannot protect against all diverse PRRSV strains. In addition, there is a risk of reversion to virulence under farm conditions. Therefore, a new generation of vaccines with higher safety and protective efficacy is required to control PRRS. Also, the availability of a DIVA (differentiating infected from vaccinated animals) vaccine would be of significant value for infected herds monitoring and eventual eradication of the disease. Here, we suggest developing a novel vaccine candidate based on plasmid DNA. The DNA vaccine is safe, relatively inexpensive, easy to produce and can elicit cellular immune responses that necessary for heterologous protection. In addition, DNA vaccine has a DIVA potential. Therefore, the main goal of this project is to develop a new improved vaccine for PRRSV with increased protection against heterologous PRRSV strains.

The project has three objectives: (1) to construct plasmids expressing synthetic genes for GP5 and M; (2) to characterize the expression of GP5 and M in cell culture; (3) to test the immunogenicity and protective efficacy of the vaccine in pigs.

Under the first objective, synthetic PRRSV genes encoding major structural proteins GP5 and M were constructed and cloned into two plasmid vectors for expression in pig cells. The sequences of the genes were optimized to achieve maximal levels of the proteins expression. In addition, sequence encoding a novel intramolecular adjuvant (polyQ) was added to the M gene to increase cell-mediated immune responses against M. Under the second objective, expression of GP5, M and M-polyQ genes were studied after transfection of cultured swine cells with DNA of the plasmids. Unfortunately, we could not detect the protein expression using several immunological methods. Therefore, more work is needed to achieve abundant PRRSV antigens expression in swine cells. Under the third objective, group of pigs were vaccinated with the mixture of the plasmid DNAs. However, PRRSV-specific immune responses were not detected.

Alexander N. Zakhartchouk, Ph.D. and DVM
Research Scientist
Vaccine and Infectious Disease Organization – International Vaccine Center (VIDO-InterVac)
University of Saskatchewan
120 Veterinary Road
Saskatoon, SK, S7N 5E3 Canada
Phone: (306) 966-1570
Fax: (306) 966-7478
E.mail: alex.zak@usask.ca

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Scientific Abstract

Though a modified live attenuated vaccine is available against porcine reproductive and respiratory syndrome virus (PRRSV), its limitations in protection efficacy, safety and few others warrant the development of newer vaccines.

In this study, we have constructed a novel DNA vaccine that consists of two plasmids designed to express modified synthetic PRRSV genes for GP5 and M. To achieve the highest possible levels of gene expression in porcine cells, the gene sequences were codon-optimized using the GeneScript's OptimumGene™ computer program. In addition to codon-optimization, the GP5 gene has the following modifications. First, the decoy epitope (aa 27-33) in the GP5 sequence was mutated: the sequence VLANASN was changed to AVAGASA. Second, to eliminate glycan shielding, the aspargine triplet (AAC) in the GP5 gene sequence at positions N33 and N51 was replaced with the alanine triplet (GCC). Third, to facilitate the protein detection, the sequence encoding 15 amino acids HA tag was introduced at the 3'-end of the gene. The sequence of M gene was also codon-optimized, and the sequence encoding 8 amino acids FLAG tag was added to the 3'-end of the gene. Next, for gene expression, both GP5 and M genes were cloned under the control of the cytomegalovirus (CMV) promoter, intron and polyA signal. Furthermore, internal ribosome entry sequence (IRES) was introduced between the genes. Introduction of IRES allows expression of both genes from one mRNA, and this facilitates formation of GP5-M heterodimers.

Another construct was designed to increase M-specific cell-mediated immune responses. To stimulate aggregation of the M protein in cells, the M gene sequences were fused with polyglutamine (polyQ) domains, which are expected to enhance its antigenic potential. To this end, CAG/CAA repeats coding for 103 glutamine residues was added to the 3'-end of the M gene. To facilitate the M protein detection, the sequence encoding 8 amino acids FLAG tag was added to the 5'-end of the gene.

Two eukaryotic vectors were explored in order to express PRRSV antigens in pig cells: pMASIA and pCI-neo. All plasmids were sequenced and showed correct PRRSV gene sequences. However, several attempts to detect GP5 and M proteins in the transfected with the plasmids swine (ST) cells were unsuccessful despite using three different immunological methods: indirect immunofluorescence, Western blotting and immunoprecipitation. More work is needed to achieve abundant PRRSV antigens expression in swine cells.

Introduction

PRRS is recognized by the pig industry worldwide as one of the most important swine diseases. PRRS is estimated to cost the US industry approximately $ 560 million per year.

Early attempt to develop a vaccine based on expression of GP5 protein alone were only partially effective. Viral epitopes that are capable of inducing neutralizing antibodies (Abs) appear to reside on the M, GP2a, GP3, GP4 and GP5 proteins. Of these, neutralizing Abs to GP5 appear to be most relevant for protection since most of the neutralizing Abs found in infected animals are directed against GP5. A recent study using co-expression of GP5 and M showed that PRRSV-specific immune responses were significantly increased. Glycosylation of GP5 also plays an crucial role in escaping or minimizing virus-neutralizing Ab response by N-glycan-shielding mechanism. The glycosylation of GP5 diminishes the immunogenicity of the nearby enclosed neutralizing epitope, and critical for the induction or evasion of a neutralizing Abs. In addition, a decoy epitope (amino acids 27-30) was identified in GP5 that may interfere with the immune response to the main neutralizing epitope (amino acids 37-45). Furthermore, it has been demonstrated that level of GP5 expression can be increased by optimization of gene codon. Thus, all these studies indicate that, for vaccine development, GP5 should be optimized in its ability to induce neutralizing Abs by co-expression with other viral proteins, by reducing its glycosylation state, deleting decoy epitope and by optimization of its mRNA translation.

Another PRRSV protein, M, is a major structural protein which is the most conserved among viral strains. In the virion, the GP5 and M proteins interact to each other and form a disulphide-linked heterodimer. A significant T cell-proliferation and gamma interferon synthesis was demonstrated against M protein indicating that this protein may have a significant role in eliciting cellular immunity in PRRSV infection. Thus, for vaccine development, it is necessary to express the M protein.

Recently, adjuvant potential of aggregate-forming polyglutamine domains (polyQ) has been described. Fusion of the weakly immunogenic GFP antigen with long polyQ domain increased anti-GFP antibody and cell-mediated immune responses. Therefore, we proposed to fuse the PRRSV M protein with polyQ to enhance its immunogenicity.

Objectives:

1) To construct plasmids expressing synthetic genes for GP5 and M.
2) To characterize the expression of GP5 and M in cell culture.
3) To test the immunogenicity and protective efficacy of the vaccine in pigs.

Materials and Methods

Plasmids construction

Synthetic PRRSV GP5 and M genes were cloned into pUC57 vector by GeneScript. Next, these genes were sub-cloned into eukaryotic expression vectors, such as pMASIA and pCI-neo. Clones carrying the insert were selected using appropriate antibiotics, and the inserted gene sequence was verified by PCR and sequencing. Correct plasmids were propagated and purified using the QIAGEN midi prep DNA purification kit.

Transfection

ST cell monolayers seeded in 6-well plates, or Lab-Tek chamber slides were transfected with 5 µg plasmid DNA and 25 µl Lipofectin (Invitrogen) according to manufacturer's instructions.

Immunofluorescence

ST cells were plated on Lab-Tek chamber slides (Nunc) and transfected with plasmid DNA. At 48 h post transfection, the cells were fixed with absolute methanol for 10 min at -20^0C. The cells were washed with phosphate buffer saline (PBS) and incubated with monoclonal murine anti-FLAG antibody or monoclonal

murine anti-HA antibody and visualized with goat anti-mouse Cy2-conjugated IgG. The cells were examined using Axiovert 200M inverted microscope (Zeiss) and AxioVision software.

Western blot analysis

For Western blot, 5 µg of cell lysates were separated by 10% sodium-dodecyl sulphate (SDS) polyacrilamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 1% BSA fraction V and probed with anti-FLAG (SIGMA) and anti-HA (GenScript) monoclonal antibodies. The membranes were washed and exposed to goat anti-mouse IgG conjugated to alkaline phosphatase. The blot was developed using alkaline phosphatase color development kit (Bio-Rad).

Immunoprecipitation

ST cells in 6-well pales were transfected using Lipofectin method. At 48 h post transfection, the cells were incubated in methionine-cysteine-free medium for 1 h before being labeled with [^{35}S]methionine-cysteine (100 µCi/well). After 12 h of labeling, the cells were harvested. Proteins were immunoprecipitated from cell lysates with anti-FLAG monoclonal antibody or with anti-HA monoclonal antibody in RIPA buffer and analyzed by SDS-PAGE.

Swine study

Commercial crossbred weaned 3-week old piglets were used in this study and were maintained in the VIDO-InterVac BSL-2 facility under the supervision of a veterinarian. They were bled on arrival, and the sera were tested to confirm the absence of PRRSV-specific antibodies. All pigs were maintained and euthanized as per the protocol, approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Pigs in group A (n=8) were vaccinated with mixture of 300 µg pMASIA-GP5-IRES-M and pMASIA-MpolyQ DNA twice, three weeks apart, intramuscularly. Pigs in group B (n=8) were vaccinated with 300 µg of pMASIA DNA as a negative control. On day 56 post vaccination, all pigs were euthanized. Blood samples were collected on 21, 42, 48 and 56 d.p.v., and serum was used for analysis of PRRSV neutralization and ELISA antibody titers. In addition, on day 40 post vaccination, 10 ml of blood was collected from each animal for isolation of PBMCs and ELISPOT assay.

Serology

Serum samples, obtained as described above, were analyzed by ELISA using purified PRRSV as an antigen.

For virus neutralization test, all serum samples were heat inactivated (56^0C, 30 min) and serially diluted. Then, the serial dilutions of serum were mixed with the equal volumes containing 50 $TCID_{50}$ of PRRSV VR-2332. After 1 h incubation at 37^0C, the mixtures were transferred to MARC-145 cell monolayers in 96-well tissue culture plates. After incubation for 7 days, the cells were examined for CPE under a microscope. Endpoint titers were calculated as the reciprocal of the highest serum dilution that neutralized PRRSV infection in 50% of the wells.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

Unifilter 96-well plates (GE Healthcare) were coated overnight at 4°C with anti-porcine IFN-γ mAb (Thermo Scientific) at a concentration of 5 µg/ml. The next day, plates were washed with AIM V medium (Life Technologies) supplemented with 10% FBS and blocked with the same medium for 2 h at 37^0C. Peripheral blood mononuclear cells (PBMCs), isolated from each animal, were cultured in AIM V medium supplemented with 2% FBS at 5 X 10^5 cells per well in triplicate wells. The cells were infected with PRRSV VR-2332 at a multiplicity of infection (MOI) of 0.1. Control cells were left uninfected. After 20 h of incubation at 370C in a humidified atmosphere with 5% CO_2, the plates were washed five times with PBS/0.05% Tween-20 and incubated for 4 h at room temperature with rabbit anti-porcine IFN-γ polyclonal antibodies (Thermo Scientific) at a concentration of 2 µg/ml in PBS/0.05% Tween-20. After washing, 100 µl of 1:5000 diluted biotinylated goat anti-rabbit IgG (Life Technologies) solution was added in each well. Subsequently, the plates were incubated for 2 h at room temperature with AP-conjugated streptavidin (Jackson Immunoresearch) diluted 1:1000 in PBS. The bound antibodies were visualized by incubation with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium substrate (Sigma-Aldrich). The plates were washed in distilled water and air dried; spots were then counted using an inverted microscope.

Results

1) To construct plasmids expressing synthetic genes for GP5 and M.

Synthetic genes

The gene synthesis was ordered from GeneScript Inc. (Piscataway, USA). To achieve the highest possible levels of gene expression, the gene sequences were codon-optimized. The codon-optimization was performed using the GeneScript's OptimumGene™ computer program that considers a variety of critical factors involved in different stages of protein expression. In addition to codon-optimization, the GP5 gene has the following modifications. First, the decoy epitope (aa 27-30) in the GP5 sequence was mutated. Second, to eliminate glycan shielding, the aspargine triplet (AAC) in the GP5 gene sequence at positions N33, N44 and N51 was replaced with the alanine triplet (GCC). Third, to facilitate the protein detection, the sequence encoding 15 amino acids HA tag was introduced at the 3'-end of the gene. The gene was cloned into pUC57 vector using the *Eco*RV site.

The sequence of M gene was also codon-optimized, and the sequence encoding 8 amino acids FLAG tag was added to the 5'-end of the gene. To stimulate aggregation of the M protein in cells, CAG/CAA repeats coding for 103 glutamine residues were added to the 3'-end of the M gene. The gene was cloned into pUC57 vector using the *Eco*RI and *Stu*I sites.

The resulting plasmids were sequenced, and sequencing results were consistent with the target insert sequences.

Plasmids

Both synthetic GP5 and M genes were cloned into the pMASIA vector under the control of the cytomegalovirus (CMV) promoter, intron A and the bovine growth hormon polyA signal. Furthermore, internal ribosome entry sequence (IRES) was introduced between the genes. Introduction of IRES allows expression of both genes from one mRNA, and this will facilitate the formation of GP5-M heterodimers. The resulting plasmid was named pMASIA-GP5-IRES-M. The synthetic M gene containing glutamine residues tail was also cloned into the pMASIA vector to construct pMASIA-MpolyQ (Fig. 1).

Several other laboratories reported that the vector pCI (Promega) was suitable for abundant protein expression. Therefore, later we constructed two new plasmids: pCI-GP5-IRES-M and pCI-MpolyQ (Fig. 1).

2) To characterize the expression of GP5 and M in cell culture.

To detect the expression of PRRSV GP5 and M proteins by pMASIA-GP5-IRES-M and pMASIA-MpolyQ in transfected swine sells, we used three different methods: indirect immunofluorescence, Western blotting and immunoprecipitation. Since synthetic genes encode HA and FLAG tags, we used commercially available mouse monoclonal antibodies to detect these proteins.

Swine ST cells were transfected with DNA of the plasmids using Lipofectin (Invitrogen), fixed in ice-cold methanol and probed with anti-FLAG or anti-HA antibodies followed by staining with Cy2-conjugated goat anti-mouse secondary antibody. Cell nuclei were counter-stained with Hechst 33342 (Sigma-Aldrich), and images were taken under a fluorescent microscope (Fig. 2). No expression of the PRRSV proteins was detected in transfected cells.

Also, lysates of transfected and non-transfected ST cells were separated by SDS-PAGE under reducing conditions and analysed by Western blotting using anti-FLAG or anti-HA antibodies followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibody. Antibodies should recognize the 25 kDa and 20 kDa protein bands corresponding to modified GP5 and M proteins respectively. No such bands were detected (Fig. 3). To confirm that anti-FLAG and anti-HA antibodies are working, we loaded a positive cell lysate (Fig. 3, lane C). As expected, the antibodies recognized a 52 kDa protein containing FLAG and HA-tag.

In addition, immunoprecipitation experiment was carried out. To this end, ST cells were left un-transfected or transfected with pMASIA-GP5-IRES-M and pMASIA-MpolyQ. Next, the cells were metabolically labeled with [^{35}S]methionine-cysteine. Following labeling, the radiolabeled proteins were immunoprecipitated with anti-FLAG or anti-HA monoclonal antibodies and analyzed by SDS-PAGE under reducing conditions. The immunoprecipitation of the transfected cells did not reveal specific protein bands that may correspond to PRRSV GP5 or M (Fig. 4).

All these experiments were repeated using ST cells transfected with pCI-GP5-IRES-M and pCI-MpolyQ DNA with the same results (data not shown).

3) To test the immunogenicity and protective efficacy of the vaccine in pigs.

The humoral immune responses elicited by the DNA vaccine were examined by ELISA and serum neutralization assay. None of the vaccinated pigs showed PRRSV-specific neutralizing or ELISA antibody titers.

To determine the level of PRRSV-specific cellular immunity in vaccinated pigs, we measured the secretion of INF-γ by in vitro stimulated PBMCs using ELISPOT assay. After two vaccinations with the DNA vaccine, no INF-γ secreting cells were observed.

Discussion

Vaccination is the best method to protect pigs from PRRSV infection. The limited protection and other disadvantages of the currently available vaccines dictate the need to develop new vaccine candidates. Direct injection of plasmid DNA is an alternative strategy of vaccination and studies have shown that plasmids encoding PRRSV antigens can induce protective immune responses in pigs; however, the efficacy of DNA vaccines is considerably less than that of conventional vaccines. In this study, we had hoped to increase the efficacy of a DNA vaccine by creating more immunogenic synthetic genes encoding PRRSV antigens and using adjuvant potential of aggregate-forming polyglutamine domains.

Genes encoding PRRSV GP5 and M were synthetized by GeneScript. The proprietary OptimumGene™ codon optimization technology was used to comprehensively optimize DNA sequences to significantly increase

protein expression in swine cells. To facilitate protein expression identification, 8-amino acid tag (FLAG) was introduced at the N-terminus of the M protein and 15-amino acid tag (HA) at the C-terminus of the GP5 protein.

Two eukaryotic vectors were explored in order to express PRRSV antigens in pig cells: pMASIA and pCI-neo. Both these vectors are suitable for abundant protein expression in eukaryotic cells as has been demonstrated in our laboratory and several other laboratories. All plasmids were sequenced and showed correct PRRSV gene sequences. However, several attempts to detect GP5 and M proteins in the transfected with the plasmids swine (ST) cells were unsuccessful despite using three different immunological methods: indirect immunofluorescence, Western blotting and immunoprecipitation.

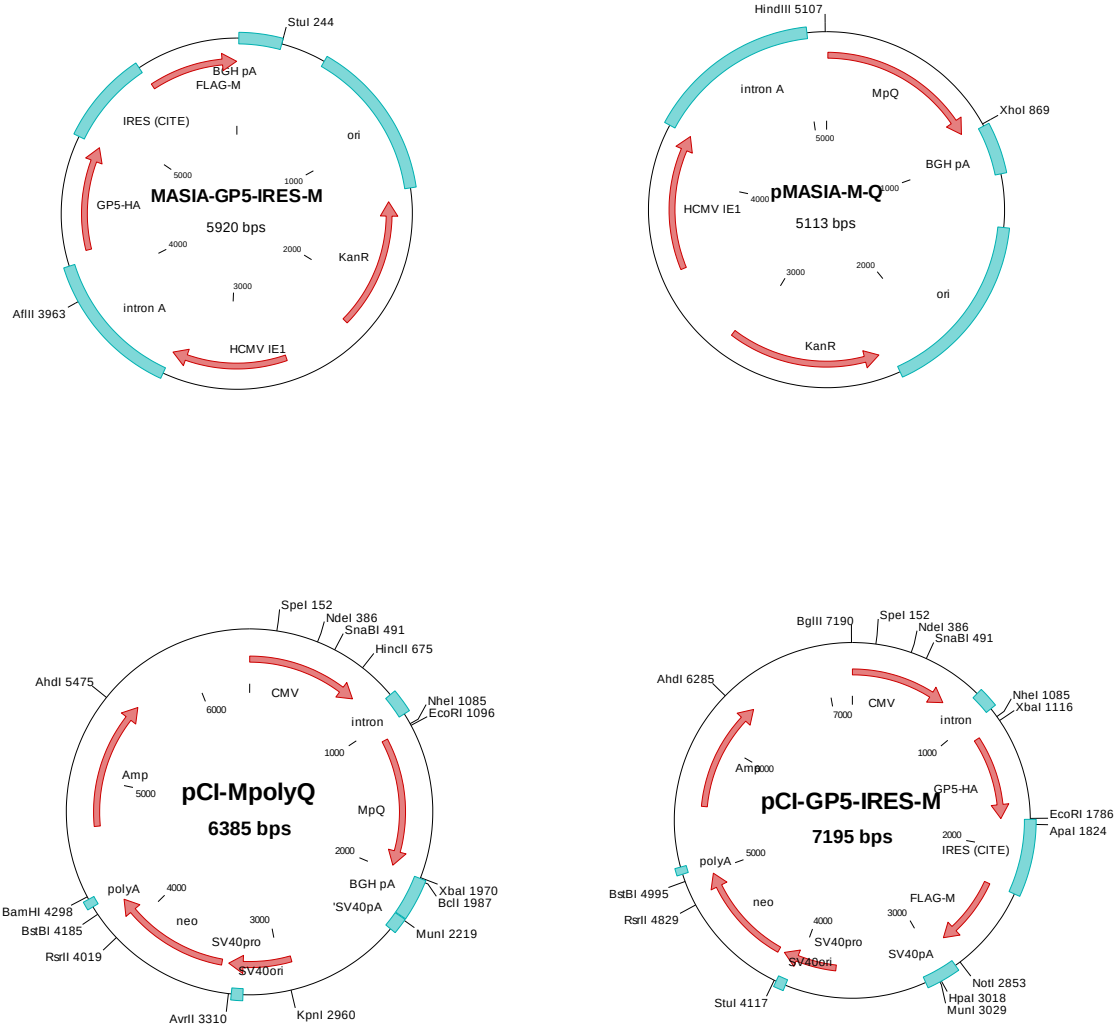

Fig. 1. Maps of the plasmids used in the study.

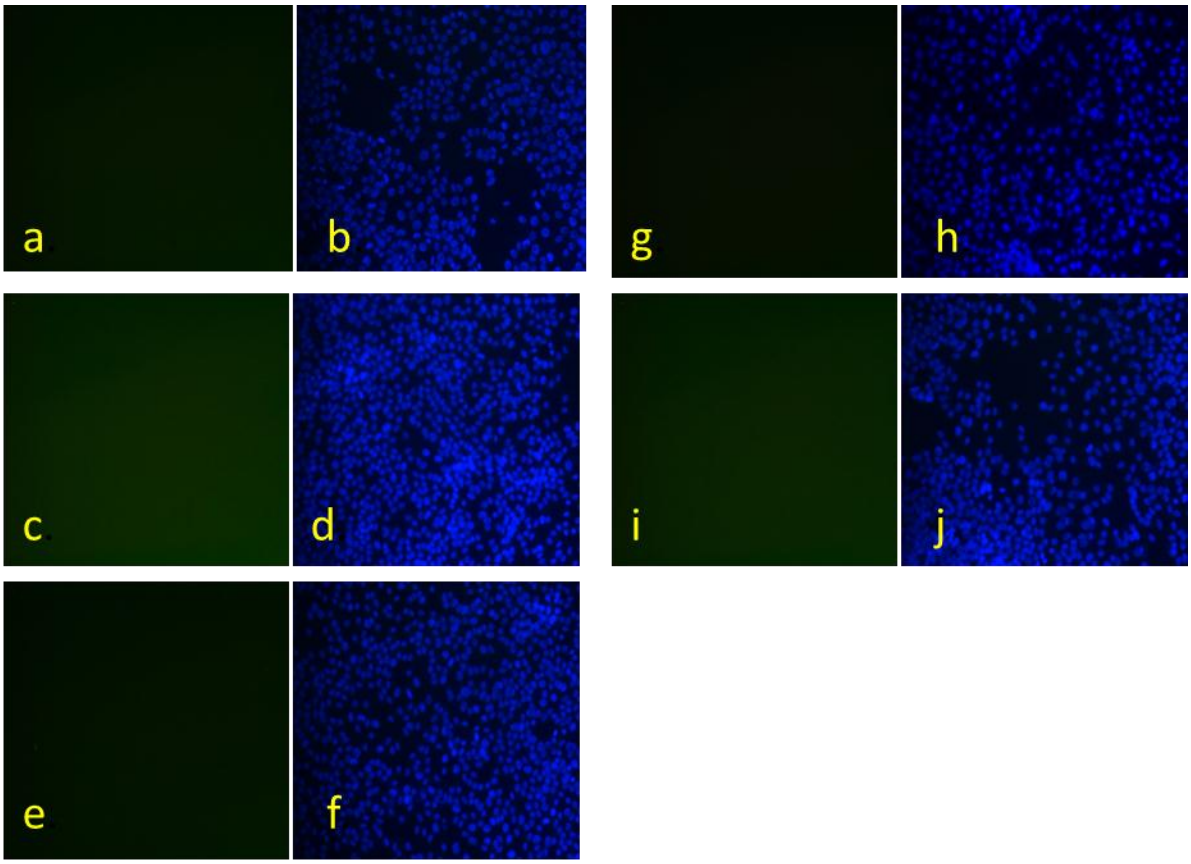

Fig. 2. Indirect immunofluorescence of ST cells transfected with pMASIA-GP5-IRES-M (**a** and **b**, **e** and **f**) or pMASIA-MpolyQ (**c** and **d**). Fixed cells were probed with monoclonal murine anti-FLAG antibody (**a**, **c**) or monoclonal murine anti-HA antibody (**e**) and visualized with goat anti-mouse Cy2-conjugated IgG. Un-transfected ST cells were also probed with anti-FLAG antibody (**g**) or with anti-HA antibody (**i**) and visualized with goat anti-mouse Cy2-conjugated IgG. Cell nuclei were stained with Hoechst 33342 (**b**, **d**, **f**, **h** and **j**).

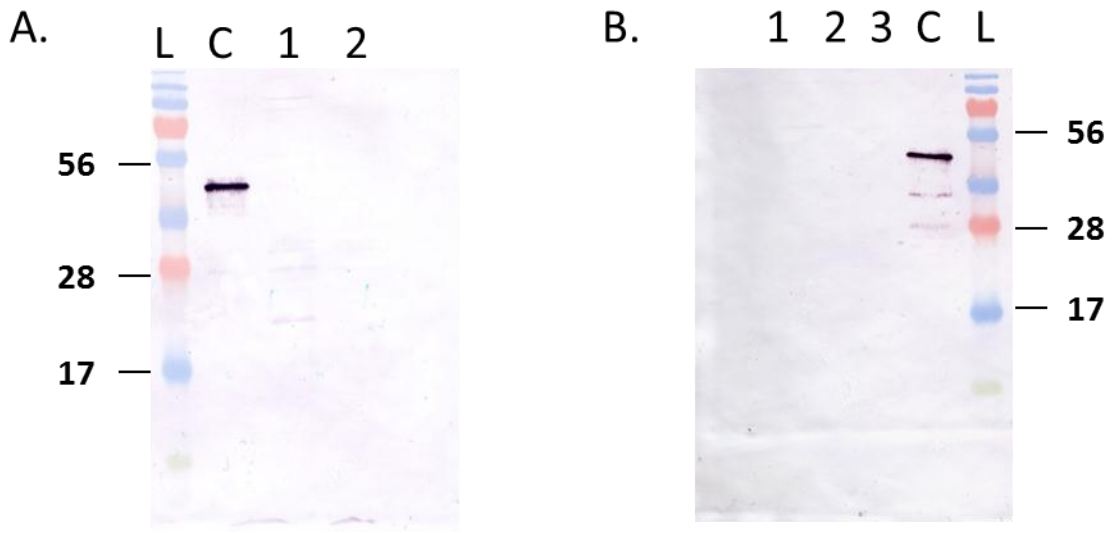

Fig. 3. Western blot analysis of proteins from the cell lysates of ST cells probed with anti-HA monoclonal antibody (**A**) or with anti-FLAG monoclonal antibody (**B**). Lane 1, untransfected cells; lane 2, transfected with pMASIA-GP5-IRES-M; lane 3, transfected with pMASIA-MpolyQ; lane C, Multiple Tag Cell Lysate (GenScript); lane L, PageRuler Prestained Protein Ladder Plus (Fermentas). Apparent molecular weight of proteins is in kDa.

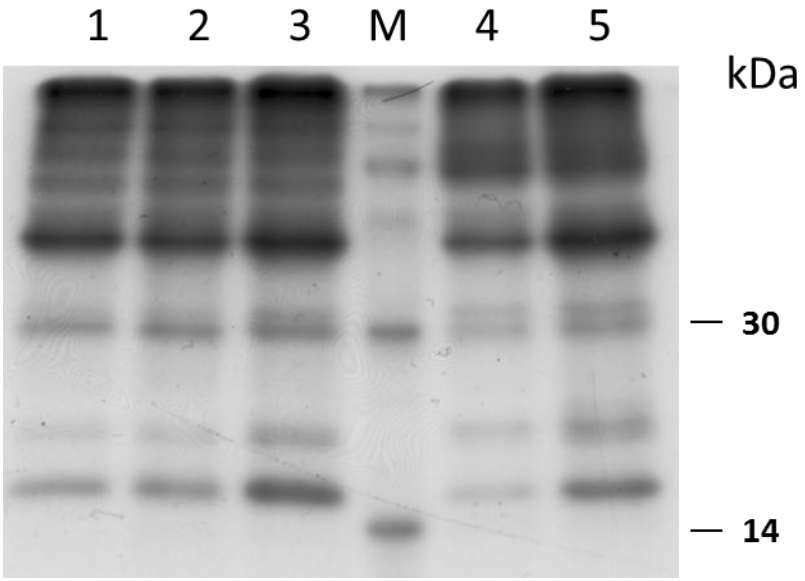

Fig. 4. Proteins from the lysates of radiolabeled ST cells, un-transfected (lane 1 and 4), transfected with pMASIA-MpolyQ (lane 2), pMASIA-GP5-IRES-M (lane 3 and 5) were immunoprecipitated with anti-FLAG monoclonal antibody (lanes 1 to 3) or anti-HA monoclonal antibody (lane 4 and 5). Lane M is a molecular weight marker containing [^{14}C] methylated proteins.