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Improved method to raise polyclonal antibody using enhanced green fluorescent protein transgenic mice

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Recombinant fusion protein is widely used as an antigen to raise antibodies against the epitope of a target protein. However, the concomitant anticarrier antibody in resulting antiserum reduces the production of the desired antibody and brings about unwanted non-specific immune reactions. It is proposed that the carrier protein transgenic animal could be used to solve this problem. To validate this hypothesis, enhanced green fluorescent protein (EGFP) transgenic mice were produced. By immunizing the mice with fusion protein His6HAtag-EGFP, we showed that the antiserum from the transgenic mice had higher titer antibody against His6HA tag and lower titer antibody against EGFP compared with that from wild-type mice. Therefore, this report describes an improved method to raise high titer antipeptide polyclonal antibody using EGFP transgenic mice that could have application potential in antibody preparation.

Keywords   transgenic mice; polyclonal antibody; EGFP

Antibodies are widely used in life science research and clinical applications. In raising antibodies, antigen is often prepared by coupling the antigen peptide to a carrier protein, such as keyhole limpet hemocyanin [1–5] or bovine serum albumin [6,7]. In this case, the immunogenicity of the short target peptide is enhanced due to the enlargement of the antigen molecule, but it is a laborious process. Recombinant fusion proteins, using maltose-binding protein [8,9] and glutathione S-transferase (GST) [10–14] as the carrier proteins, have also been adopted to raise antibody. The fusion proteins are easy to acquire with recombinant DNA technology and purified by well-established affinity chromatography methods. However, the majority of antibody components in the antiserum generated by these methods are against the carrier proteins [15,16]. Therefore, the titer of the desired antibody is often low and a non-specific effect might exist. To avoid this problem, the synthetic high-density multiple antigenic peptide system has been applied to raise antipeptide antibody [17,18]. Although there is no anticarrier antibody in the antiserum, the use of the multiple antigenic peptide system is limited to very short peptides because of the space tendency of the branched multiple antigenic peptides [19].

It is well known that immune tolerance is established during development in animals [20], so the antibodies generated against endogenous proteins are abolished. The nature of this process implies that the production of anticarrier antibody could be reduced after immunization with the corresponding fusion protein, if the carrier protein was considered as an endogenous product through a transgenic approach.

To validate this hypothesis, we produced enhanced green fluorescent protein (EGFP) transgenic mice as immune animals and chose His6HAtag as the polypeptide epitope. After immunization with the fusion protein His6HAtag-EGFP, the antisera from immunized mice were characterized by the titer of anti-EGFP and anti-His6HA tag. Our data indicated that the immunized EGFP transgenic mice produced higher titer antibody against His6HA tag and lower titer antibody against the carrier protein EGFP compared with wild-type mice.

Materials and Methods

Generation of EGFP transgenic mice

To generate EGFP transgenic mice, plasmid pEGFP-N2 (BD Biosciences Clontech, Heidelberg, Germany) was digested with *Pvu*II, and the resulting 4.1 kb fragment was purified and injected into fertilized mouse eggs (C57Bl/6×CBA). The injected eggs were then implanted into oviducts of pseudo-pregnant mice for continued development. The offspring were screened for integration of the EGFP gene by polymerase chain reaction (PCR) analysis of their tail DNA. Mice were kept in the conventional animal facility of Shanghai Nan Fang Model Organism Research Center (Shanghai, China) in accordance with institutional guidelines. Transgenic mice were bred with C57Bl/6 mice and transmitted the EGFP gene for seven generations.

Construction of plasmids

Plasmid p6HHA-EGFP (kindly provided by Dr. GM Cheng of the Shanghai Institutes for Biological Sciences, Shanghai, China) is a T7 promoter-based vector that is used for expression of His6HAtag-EGFP in *Escherichia coli*. In this vector, the His6HA tag is fused in frame to the N-terminus of the EGFP coding sequence. The amino acid sequence of the His6HA tag is MRGSHHHHHHGMAS-MTGGQQMGRDLYDDDDKDRWGSTMSGYPYDVPD-YAGS (51 amino acids). Plasmid p6HHA-EGFP was digested with *Nco*I and *Sac*I to remove the EGFP coding sequence and replaced in frame with the GST coding sequence, amplified by PCR from plasmid pGEX-3x (Amersham Pharmacia Biotech, Uppsala, Sweden) using 5′-primer 5′-CCTACCATGGCCCCTATACTAGGTTAT-TG-3′ and 3′-primer 5′-GTAGGAGCTCGATGAATTCC-CGGGGAT-3′. The resulting plasmid was designated p6HHA-GST.

Plasmid pGEXA was constructed previously in our laboratory by adding multiple cloning sites between *Bam*HI and *Eco*RI sites of pGEX-3x. The DNA fragment encoding the EGFP protein was amplified by PCR from plasmid pEGFP-N1 (BD Biosciences Clontech) using 5′-primer 5′-GGTAGCGGCCGCATGGTGAGCAAGGGCGAGG-3′ and 3′-primer 5′-GTTTGAATTCTTTACTTGTACAGCT-CGTCC-3′. The PCR product was digested with *Not*I and *Eco*RI, then inserted into the *Not*I-*Eco*RI sites of pGEXA to construct plasmid pGST-EGFP.

The constructs was confirmed by DNA sequencing.

Expression and purification of proteins

Escherichia coli BL21(DE3) was transformed with the expression vectors p6HHA-EGFP, p6HHA-GST, and pGST-EGFP, then the transformed cells were grown in Luria-Bertani medium containing 100 μg/ml ampicillin. On reaching mid-log phase, the culture was induced with 0.8 mM isopropyl β-*D*-thiogalactopyranoside at 37 ℃ for 4 h. The cells were harvested by centrifugation and disrupted by sonication in phosphate-buffered saline (PBS) solution. The cytoplasmic extract was filtered and subjected to affinity chromatography. His6HAtag-EGFP and His6HAtag-GST were purified by a nickel affinity resin column (Anxin, Shanghai, China) and GST-EGFP was purified by a glutathione Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

Generation of polyclonal antibody

Mice were divided into two groups, EGFP transgenic mice and wild-type littermates. Each group contained five 8-week-old female mice. The antigen (His6HAtag-EGFP) was dissolved in PBS solution at a concentration of 160 μg/ml and emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, USA). At initial immunization, each mouse was injected subcutane-ously at several sites on the back with 40 μg immunogen in a total volume of 500 μl. Two and 4 weeks later, the mice received two booster injections intraperitoneally with the same amount of immunogen as the initial immunization except that it was emulsified with incomplete Freund's adjuvant (Sigma-Aldrich). After another 2 weeks, the mice were given an intravenous injection of 5 μg immunogen without adjuvant. Three days later, the mice were bled from the retro-orbital plexus to obtain antisera.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well polystyrene microtiter plates were coated with 100 μl/well purified protein solution at 1 μg/ml overnight at 4 ℃. Plates were blocked with 4% skim milk in PBS for 2 h at 37 ℃. After the plates were washed three times with PBS, each well was incubated with 100 μl diluted sera (dilution of 1:200, 1:400, 1:800, 1:1600, 1:3200, ..., 1:25,600) for 60 min at 37 ℃. Plates were washed four times with PBS-T (PBS and 0.1% Tween-20), and each well was incubated with 100 μl anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:7000 dilution; Sigma-Aldrich) for 60 min at 37 ℃. Plates were washed five times with PBS-T and 100 μl tetramethylbenzidin substrate solution was added to each well. After incubation at room temperature for 10 min, the reaction was stopped with the addition of 25 μl of 2 M H_2SO_4 into each well. The absorbances at 450 nm were measured with an ELISA

plate reader (Bio-Rad, Hercules, USA). The titer of antibody was defined as the serum dilution at which the absorbance at 450 nm was half of the maximal value.

Western blot analysis

Ten nanograms of purified GST-EGFP or His6HAtag-GST were electrophoresed in 15% sodium dodecyl sulfate-polyacrylamide gel. The samples were transferred to nitrocellulose membranes for 60 min at 100 V. The membranes were then rinsed with Tris-buffered saline Tween 20 (TBST) and blocked with 5% skim milk for 60 min. One membrane was incubated with antiserum (1:7000 dilution) from immunized transgenic mice and the other was incubated with antiserum (1:7000 dilution) from immunized wild-type mice in parallel for 60 min. Both membranes were then washed four times with TBST and incubated with goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:7000 dilution; Sigma-Aldrich) for 60 min and washed in TBST. Membranes were finally developed with enhanced chemiluminescence detection reagents (Sigma-Aldrich) and detected under the same conditions.

Results

Generation of EGFP transgenic mice

After screening of the offspring mice by PCR identification [**Fig. 1(A)**], the stable expression of EGFP in transgenic mice was confirmed by the detection of green fluorescence in live mice with ultraviolet excitation light. The high level of EGFP expression that was directed by a strong cytomegalovirus promoter could be detected in the transgenic newborns after the transgenic mice were bred with C57Bl/6 mice for seven generations [**Fig. 1(B)**]. Immunohistochemistry analysis with anti-EGFP antibody also showed the ubiquitous expression of EGFP in transgenic mice (data not shown).

Construction of plasmids, expression and purification of fusion proteins

In order to produce the antigen, the recombinant plasmid p6HHA-EGFP was transformed into *E. coli* BL21(DE3) to express fusion protein His6HAtag-EGFP, used to immunize both transgenic and wild-type mice. The purified proteins His6HAtag-GST and GST-EGFP were used for detection of the antibody titer against His6HAtag and EGFP [**Fig. 2(A)**]. The corresponding plasmids were named p6HHA-GST and pGST-EGFP, respectively.

The purified fusion proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [**Fig. 2(B)**]. The purity of the proteins was estimated to
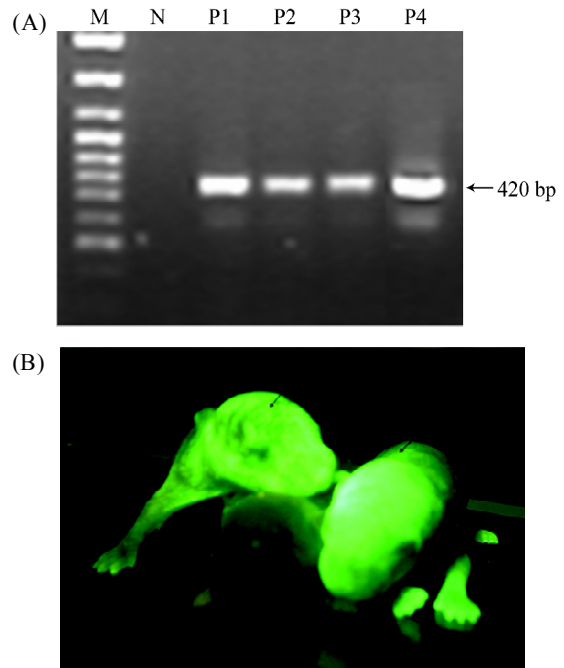

Fig. 1 Generation of enhanced green fluorescent protein (EGFP) transgenic mice with ubiquitous expression of EGFP in newborns (A) Genotyping of the transgenic mice by polymerase chain reaction identification. M, DNA marker; N, negative (wild-type) mice; P1–P4, positive transgenic mice 1–4. (B) The green fluorescence was visible in the live transgenic mice (as arrows indicate) when irradiated with excitation light with one wild-type mouse (middle) as the negative control. The photograph was taken under ultraviolet light.
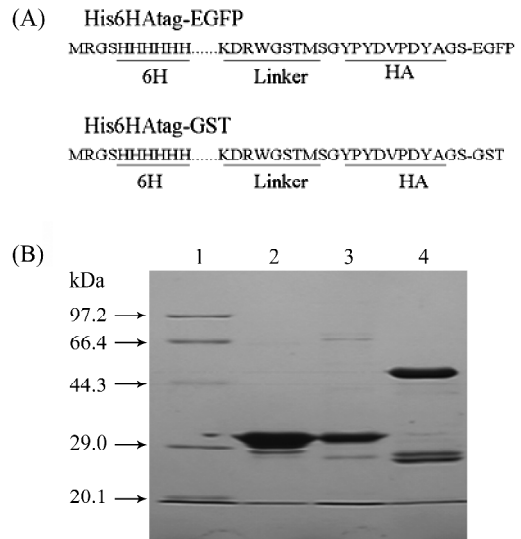

Fig. 2 Expression and purification of fusion proteins (A) Schematic illustration of two fusion proteins. (B) The purity of three purified proteins was shown by Coomassie Brilliant Blue staining using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 1, protein marker; 2, purified His6HAtag-enhanced green fluorescent protein (EGFP); 3, purified His6HAtag-glutathione S-transferase (GST); 4, purified GST-EGFP.

be approximately 90% for His6HAtag-EGFP and 85% for His6HAtag-GST.

EGFP transgenic mice generated higher titer polyclonal antibody against His6HAtag with a reduced titer of anti-EGFP

EGFP is the auto-antigen for EGFP transgenic mice, as pre-immune sera from EGFP transgenic mice did not give positive reactions to GST-EGFP (data not shown). After immunization with purified protein His6HAtag-EGFP, the antisera from EGFP transgenic mice and wild-type mice were titered against His6HAtag-GST and GST-EGFP, respectively, by ELISA. The results showed that the titer of the antiserum against His6HAtag-GST protein was raised to approximately 5300 for EGFP transgenic mice, and to approximately 2600 for wild-type mice [**Fig. 3(A)**]. As for the titer of antiserum against GST-EGFP protein, EGFP transgenic mice showed the average titer of 6500, whereas wild-type mice showed a titer of 15,000 [**Fig. 3(B)**]. The target/carrier ratio (the antiserum titer of anti-His6HAtag to that of anti-EGFP protein) of the transgenic mice was significantly higher than that of wild-type mice [**Fig. 3(C)**].

Furthermore, Western blot analysis showed that the antiserum from EGFP transgenic mice displayed a better detection sensitivity to His6HAtag-GST [**Fig. 4(A)**] and less sensitivity to GST-EGFP [**Fig. 4(B)**] than that from wild-type mice. These results suggested that by immunization of fusion protein His6HAtag-EGFP, the EGFP transgenic mice generated higher titer polyclonal antibody against His6HA tag with lower titer of anti-EGFP antibody.
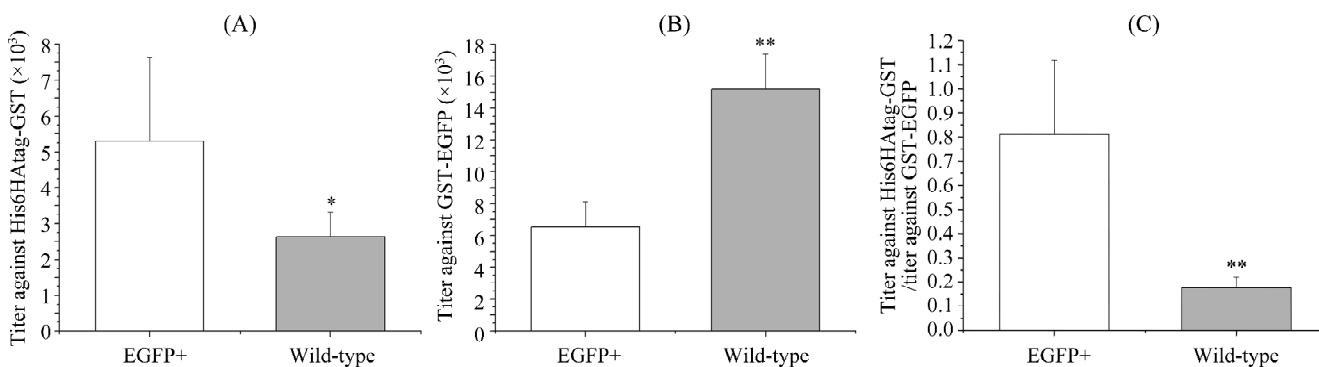

Fig. 3 Determination of antiserum titer by enzyme-linked immunosorbent assay   Comparison of the titer of antiserum against His6HAtag-glutathione S-transferase (GST) (A) and GST-enhanced green fluorescent protein (EGFP) (B) and the ratios (C) between EGFP transgenic mice (EGFP+) and wild-type mice. The antiserum titer was defined as the serum dilution at which the absorbance at 450 nm was half of the maximal value. Data are shown as the mean±SD for five mice analyzed (pooled into two groups). *$P<0.05$; **$P<0.01$.
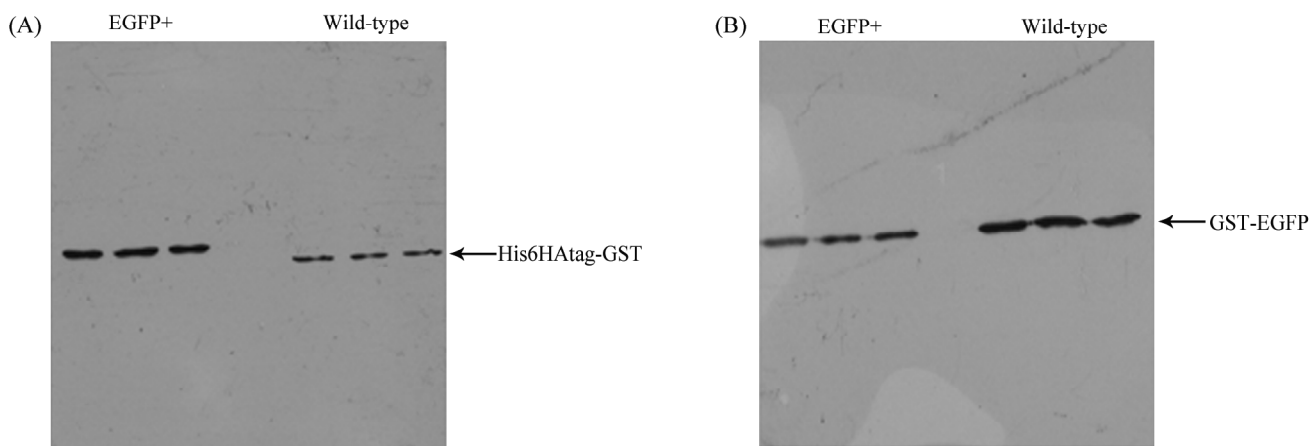

Fig. 4 Detection sensitivity of antiserum confirmed by Western blot analysis   The antiserum from enhanced green fluorescent protein (EGFP) transgenic mice was more sensitive to His6HAtag-glutathione S-transferase (GST) (A) and less sensitive to GST-EGFP (B) compared with that from wild-type mice. Left three lanes, incubated with the antiserum from EGFP transgenic mice (EGFP+); right three lanes, incubated with the antiserum from wild-type mice.

Discussion

In this study, we showed that EGFP transgenic mice could be used to raise high titer polyclonal antibodies against a foreign tag using EGFP fusion protein as the antigen. The antiserum from transgenic mice contained higher titer of antibody against the target peptide and lower titer of antibody against the carrier protein compared with that from wild-type mice. However, the titer of the antibody against the epitope, raised in the transgenic mice, was only twice that obtained in the wild-type mice, whereas the target/carrier ratio (the antiserum titer of antitarget peptide to that of anticarrier protein) of the antibody from transgenic mice was approximately 4-fold of that from wild-type mice [**Fig. 3(C)**]. EGFP could also be used as a protein fusion partner to monitor and optimize the production and purification of recombinant protein by the green fluorescence emission.

Significant titer against the carrier protein (EGFP) was nevertheless obtained in EGFP transgenic mice. This might be the result of using intensified immunization strategies that broke the immune tolerance. Another possible explanation is that EGFP transgenic mice produced antibodies against denatured EGFP protein during immunization. In addition, complete Freund's adjuvant might have partially denatured the immunogen.

The increase of the absolute titer of antibody against the tags in this study was limited and the improvement of the specificity of the antibody against the target tag requires substantiation with further evidence, however, the significant increase of the target:carrier ratio of the antibody titer in transgenic mice suggested that this method is valuable. This strategy might also be suitable for other fusion protein systems, such as GST and maltose-binding protein, if the corresponding transgenic mice are generated. Furthermore, this method is not limited to the field of polyclonal antibody preparation and might be more useful in monoclonal antibody development.

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