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Production and Characterization of Polyclonal and Monoclonal Abs Against the RNA-Binding Protein QKI

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Abstract RNA-binding protein QKI, a member of the Signal Transduction and Activation of RNA family, is found to be essential in the blood vessel development and postnatal myelination in central nervous system (Woo et al., *Oncogene* 28:1176–1186, 2009; Lu et al., *Nucleic Acids Res* 31(15):4616–4624, 2003; Bohnsack et al., *Genesis* 44(2):93–104, 2006). However, its wide expression pattern suggests other fundamental roles in vivo (Kondo et al., *Mamm Genome* 10(7):662–669, 1999). To facilitate the understanding of QKI function in various systems, we prepared the polyclonal and monoclonal antibodies against QKI. To obtain the antigen, recombinant His-tagged QKI was expressed in *Escherichia coli* and highly purified by Ni^{2+}-chelated column combined with hydrophobic and ion exchange methods. Following three types of immunizations with different adjuvants, including Freund's, PAGE gel, and nitrocellulose membrane, only the antiserum produced with Freund's adjuvant is effective for Western blot detection. Several McAb clones are able to recognize both endogenous and over-expressed QKI with high affinity in Western blot and immunofluorescence. The specificity of Ab was validated as weakening, and no specific signals were observed in cells with QKI knocking down. Immunohistochemistry analysis further showed positive staining of QKI in kidney where QKI mRNA was abundantly expressed, ensuring the wide applications of the QKI Abs in the ongoing mechanistic studies.

Keywords QKI · Prokaryotic expression · Immunization · Protein purification · Antibody

Jie Zhang, Bo Huang, and Fang Yu contributed equally to this work and should be considered as co-first authors.

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Introduction

The expression profile of a protein in different tissues or cells provides valuable clues for functional characterization. Therefore, production of specific antibodies against the protein is a prerequisite step. Antigen preparation, subsequent antibody production, and specificity characterization are generally required.

QKI is an RNA-binding protein harboring a highly conserved KH domain [5]. As a member of the "Signal Transduction and Activation of RNA metabolism" ("STAR") family, it plays an important role in embryonic development, particularly in blood vessel system formation and postnatal myelination in central nervous system (CNS). There are three major isoforms of QKI produced by alternative splicing of QKI transcripts [6]. These isoforms (QKI-5, QKI-6, and QKI-7) differ in their 3′ untranslated regions at mRNA levels and carboxyl 8-30-amino-acid sequence correspondingly [7]. Among them, QKI-5 is a nuclear isoform possessing a nuclear localization signal and displayed a more wide expression pattern [8, 9]. It may exist as a homodimer or heterodimers with two other cytoplasmic isoforms, like QKI-6 or QKI-7, and dynamically shuttles between nucleus and cytoplasm [10, 11]. As an RNA-binding protein, QKI exerted its posttranscriptional regulation on certain specific target mRNAs, affecting the alternative splicing, localization, mRNA stability, or translational efficiency [8, 12]. According to the consensus bipartite *cis*-elements recognized by QKI, more than 1,430 putative direct mRNA targets regulated by QKI were predicted, implicating its ubiquitous role [13]. The lethal phenotype in QKI knock-out mice and ethylnitrosourea (ENU)-induced homozygous point mutant QKI mice emphasized its importance in early development [14–16]. Although it is of great significance to study the function of QKI, the fact that there are no commercially available antibodies against QKI, especially for histochemistry and immunofluroscene, is currently the biggest hurdle for in vivo study. Thus, obtaining a reliable antibody (Ab) is indispensable for exploring the critical role of QKI in vivo.

Here, the amino terminus of QKI-7 fused with 6× histidine was expressed and purified [17]. Since different isotypes of QKI share more than 90% homology in their amino acid sequence [18], their antigenic epitopes are largely shared. Immunization with the above purified antigen was supposed to generate the antibody applicable for all isoforms. As expected, the rabbit polyclonal antiserum and several clones of McAb were successfully prepared, and subsequently the specificity of Ab was determined.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The expression vector pET-28a carrying the human 6× His fused QKI-7 was kindly provided by Prof. Yue Feng (Department of Pharmocology, Emory University). *Escherichia coli* strain BL21 (DE3) was purchased from Novagen. The plasmid was transformed into the expression host BL21 (DE3). Transformant was picked and inoculated in 5 ml Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin and incubated under 37 °C with agitation overnight. The overnight culture was then used to inoculate 500 ml LB medium and incubated for 10 h. The cell pellets were then harvested by centrifugation and used to inoculate in 2× YT medium supplemented with 100 μg/ml ampicillin in a 30-l fermentor for 9 h. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a concentration of 0.5 mM to induce expression for an additional 4 h.

Expression and Purification of Recombinant QKI-7 for Antigen Production

The induced culture medium was centrifuged at 4,000 rpm for 25 min; cell pellets were lysed by overnight stirring with lysis buffer containing 20 mM Tris–HCl pH=10, 3 mol/l beta-mercaptoethanol and 6 M urea. The lysate was then subjected to ultrasonication for five episodes. Each episode consists of 90 cycles of 9 s with 15 s interval. The final product was centrifuged at 12,000×*g* for 30 min, and the supernatant was harvested and filtered. The filtered supernatant was then applied to IMAC (Sepharose Fast Flow, GE healthcare) column chelated with Ni^{2+} and equilibrated with solution A (20 mM Tris–HCl pH=8.0, 150 mM NaCl, 6 M urea), with a flow rate of 8 ml/min. The column was then washed with solution A to baseline level. Bound protein was washed down with solution B (20 mM Tris–HCl pH=8.0, 150 mM NaCl, 6 M urea, 200 mM imidazole). The fraction corresponding to the protein peak was collected and further applied to a hydrophobic column (source 30RPC GE healthcare). The column was pretreated with solution C (20 mM Tris–HCl pH=8.0, 1 ml/l mercaptoethanol, 5 M urea, 500 ml/l isopropanol) and washed with solution D (20 mM Tris–HCl pH=8.0, 1 ml/l mercaptoethanol, 5 M urea) to baseline level. The corresponding protein fractions were termed peak1, peak2, and peak3, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that target protein resided in peak2. Peak2 was then subjected to strong ion exchange chromatography (QKI-7, GE healthcare). For strong ion exchange chromatography, the column was washed by solution E (20 mM Tris–HCl pH=8.0, 1 ml/l mercaptoethanol, 5 M urea, 1 M NaCl) and then equilibrated with solution D. The fractions containing the protein peaks were collected and termed peakA, peakB, and peakC. SDS-PAGE analysis showed that the target protein resides in peakB, with the purity of above 90% determined by gel scanning. The final product was dialyzed against solution F with more than ten volumes higher than the samples (20 mM Tris–HCl pH=8.0, NaCl 150 mM) for 24 h then aliquoted and frozen at −20 °C.

Preparation of Rabbit Antiserum

Six New Zealand rabbits were randomly divided into three groups. Each group was immunized with 200 μg purified recombinant *QKI-7* in combination with Freund's adjuvant (Gibco BRL), polyacrylamide gel, or nitrocellulose membrane, respectively, as the adjuvant. First boost immunization was carried out 30 days after the first immunization. Two weeks later, a secondary boost was carried out. For the primary immunization and the first boost 1, 200 μg recombinant protein was mixed with equal volume of Freund incomplete adjuvant and injected subcutaneously at sub-axillary and groin regions, respectively. For the secondary boost, 200 μg recombinant protein was injected into the ear vein. The protocols followed in two other groups were the same as above mentioned except the type of adjuvant used. One milliliter blood sample was obtained for the titer test 2 weeks later after the secondary boost.

The whole blood antiserum was obtained by intra-cardiac canaling.

Preparation of Monoclonal Antibodies

Female BALB/c mice (6–8 weeks old) were immunized three times with 50 μg QKI, each mixed with Freund's complete (Sigma, St. Louis, MO) by subcutaneous injection at 3-week intervals. Seven days after the last immunization, the serum titers taken from mice caudal vein were determined by indirect enzyme-linked immunosorbent assay (ELISA). The mouse with the highest serum titer was boosted with 50 μg antigen by intra-peritoneal

injection. Three days later, the boosted mouse spleen cells were fused with SP2/0 myeloma cells in the presence of polyethylene glycol/dimethylsulfoxide solution (Sigma). The hybrids that reacted positively to QKI were selected by indirect ELISA and excluded by His Ag. The selected hybrids were sub-cloned three times using the limiting dilution method. Monoclonal antibodies were produced either from supernatants of the hybridoma cell culture or from ascites. The isotype of MAb was determined by using a commercially available mouse MAb isotyping kit (HyCult Biotechnology, Uden, The Netherlands), following the manufacturer's protocol.

Enzyme-Linked Immunosorbent Assay

Indirect ELISA was used to screen serum and hybridoma supernatants against QKI. Briefly, 96-well polystyrene plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4 °C with 1 mg per well of QKI diluted in coating buffer (0.05 M carbonate/bicarbonate buffer [pH 9.5]). After washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20, the serially diluted serum or supernatants were added and incubated at 37 °C for 1 h, then washed and incubated with 100 μl horseradish peroxidase conjugated goat anti-mouse IgG working dilution for 1 h at 37 °C (Santa Cruz Biotechnology, Santa Cruz, CA). After three more washes, the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) solution was added and incubated at 37 °C for 15 min. The optical density at 450 nm was detected on micro-plate reader (model 680, Bio-Rad, Hercules, CA).

Cell Culture and Transfection

Hela cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Plasmids expressing two isoforms, QKI-5 and QKI-6, and siRNA targeting QKI were transfected in Hela cells using lipofectamine 2000 (Invitrogen). Forty-eight hours later, cells were fixed for immunofluorescence, or its lysates were prepared for Western blot.

Western Blotting

Cell lysates and the purified Ag mixed with loading buffer were boiled for 5 min and applied on SDS-PAGE. The protein in gel was electronically transferred to nitrocellulose membrane. After being blocked by 10% skimmed milk solution for 2 h at room temperature (RT), the membrane was incubated with the prepared first Ab (diluted with Tris-buffered saline) solutions overnight at 4 °C and washed with Tris-buffered saline Tween-20 (TBST) three times. The washed membrane was further incubated with secondary antibody (0.1 μg/ml goat anti-mouse or goat anti-rabbit IgG coupled to infrared (IR) dyes) in TBST for 1 h at RT in the dark. After washing, the bands were visualized by the Odyssey IR imaging system (LI-COR).

Immunofluorescence

Immunofluorescence staining experiments were performed in 12-well plates. Cells were washed in PBS three times and fixed in a 4% formaldehyde/1× PBS solution for 30 min then permeabilized with 0.02% TritonX-100–1× PBS for 20 min and blocked with 1% bovine serum albumin–PBS for 1 h. Incubation was continued overnight at 4 °C with anti-QKI antibody diluted in 0.1% bovine serum albumin at 1:200 followed by washing with PBS and subsequent incubation with Cy3-conjugated anti-mouse immunoglobulin G (IgG;

Sigma) diluted at 1:500 in the dark. One hour later, after the usual washings, cells were nucleus labeled with Hoechst 33258 for 10 min and photographed under a microscope.

Immunohistochemistry

Tissues were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections (4 μm) were prepared for immunohistochemistry (IHC) examination. Briefly, endogenous peroxidases were blocked using 0.75% H_2O_2 in PBS for 30 min, followed by incubation with Serum-Free Protein Block. Incubation with primary antibody anti-QKI (1:300) was performed for 24 h at 4 °C. Immunodetection was performed in a three-step protocol, using streptavidin–horseradish peroxidase complex, with visualization by 3,3-diaminobenzidine.

Results

Expression and Purification of Antigen

Before preparing the polyclonal and monoclonal antibodies, obtaining a highly purified antigen is a prerequisite. In order to achieve a high expression level, a series of IPTG induction times were tested, and 4 h was chosen for cost-effect considerations (Fig. 1a). The expression vector pET28a (+) carrying an upstream 6× histidine tag with OKI-7 was constructed to facilitate protein purification. However, the initial IMAC experiment suggested that single step in affinity-chromatography was not sufficient for optimal purification of this protein (Fig. 1b). Based on the amino acid components of this protein, we performed an Ion-Exchange Chromatography (IEC) combined with reverse phase chromatography, and results were effective as shown (Fig. 1c, d). The purity of final product was up to 95% determined by gel scanning (Fig. 1e). The His tag fused immunogen was further identified by probing with anti-His Ab in Western blot (Fig. 2). Expectedly, the positive band was localized in the 45-kDa region where the purified Ag was present. The intensity of bands was in agreement with their loading amounts.

Preparation of Rabbit Antiserum Following Three-Step Immunization Strategies

By using the above purified immunogen, followed with usual immunization protocol, three groups of rabbit antiserums were produced; only one group from Freund adjuvant vaccination showed positive recognition with the recombinant QKI in Western blot (Fig. 3). As known before, some experiments reported that both polyacrylamide and nitrocellulose were able to act as adjuvants for immunization in Ab production. Considering the cheapness, convenience, and less demanding in purity of immnogen, it is noteworthy to compare the efficiency of the other two alternative types of vaccination with classical Freund one. Unfortunately at our hands, the other two methods, including PAGE gel cuttings and nitrocellulose (NC) membrane mixtures, did not give rise to a good titer antiserum based on the same immunization plan; possibly, we should optimize their immunization strategies by boosting more subsequent immunizations.

Preparation of Mouse Monoclonal Antibody

Following the fusion of immunized spleen cells with Sp20, at least 300 clones were screened and examined by ELISA against the immobilized purified QKI Ag. After three

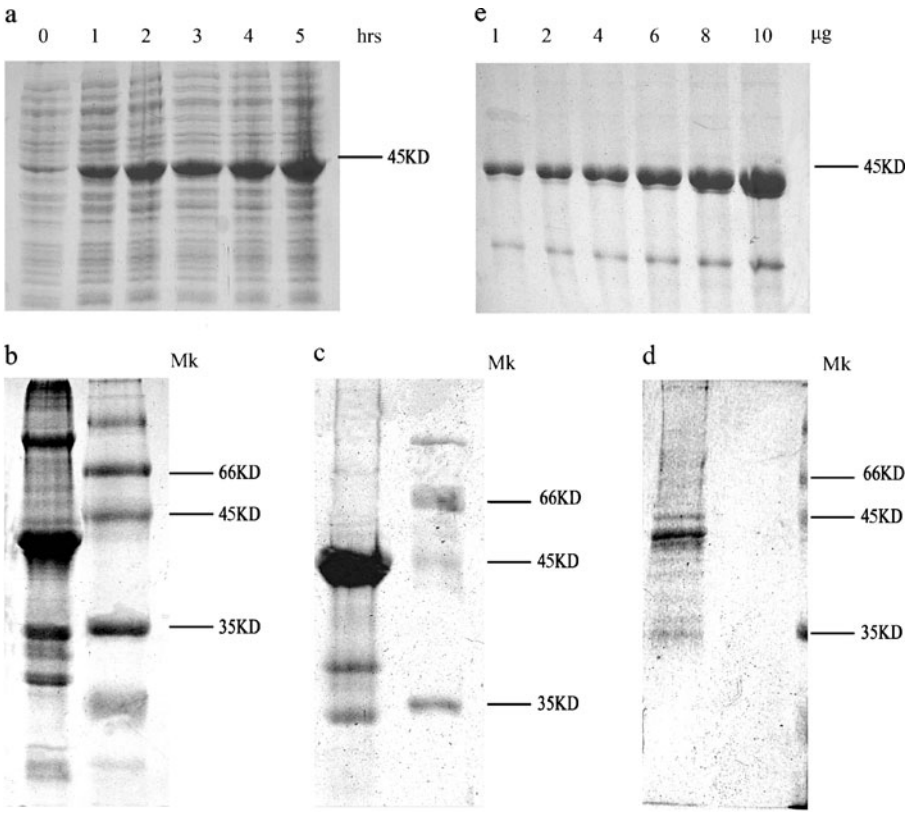

Fig. 1 Expression and purification of 6× His-QKI-7 fusion protein. BL21(DE3) transformed with PET32b (+)-QKI-7 was induced with IPTG at different times (**a**). Products after purification with anti-His affinity chromatography (**b**), hydrophobic chromatography (**c**), and strong ion exchange chromatography (**d**). Purified 6× His-QKI-7 of different amount for SDS-PAGE assay (**e**)

rounds of positive selections with limiting dilution method and one round of negative exclusive screening against His antigen, eight positive McAb clones against QKI were obtained and designated as numbers 1 to 8. Their immunoglobulin isotypes were identified (data not shown). And the clone number 2 antibody was designed QKI-M2, the isotype of which is IgG2a/κ.

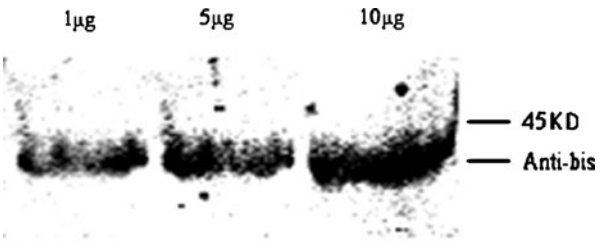

Fig. 2 Determination of recombinant 6× His-QKI7 by anti-His Ab. Purified fusion protein was detected by Ab against histidine. The total amount of protein was denoted

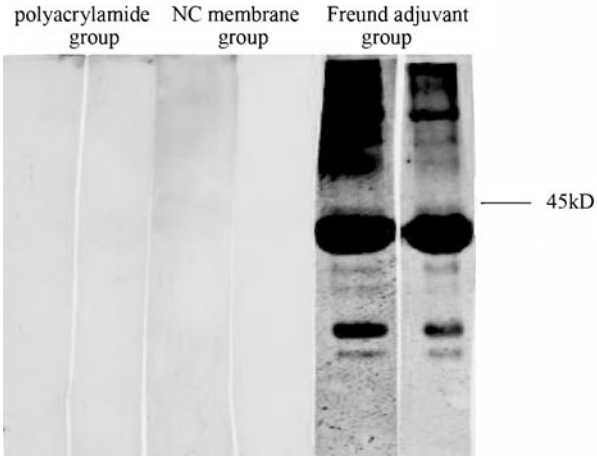

Fig. 3 Determination of Rabbit anti-serum specificity from the three types of immunization plans. Anti-serums immunized with three different types of adjuvants (polyacrylamide, NC membrane, and Freund's adjuvant) were used to detect recombinant 6× His-QKI on Western blot. Only the two `antiserums from Freund's adjuvant group specifically recognized the antigen

Determination of the Specificity of Polyclonal Ab and Monoclonal Ab

After having obtained the Abs above, it is imperative to fully characterize their specificity and applicability. In fact, both polyclonal and monoclonal Abs were able to recognize immunogen by Western blot (Fig. 4); more importantly, they were also able to detect both endogenous and over-expressed isoforms of QKI-5 and -6 in the cell lines by Western blot (Figs. 5a and 6d). In contrast with polyclonal Ab, only monoclonal Ab showed specific immunofluorescence stainings in 293 cell lines after over-expressing QKI-5 or QKI-6, whereas endogeneous QKI signals were predominantly localized in nucleus in Hela cells, consistent with the dominant presence of major nuclear isoform QKI-5. As expected, after over-expressing QKI-5 and QKI-6, respectively, more pronounced positive signals were observed in nucleus or cytoplasm (Fig. 6b, c). After transfecting the short interference RNA targeting QKI, the signals of both Western blot and immunofluorescence assays became much weaker than that in the control groups (Figs. 5b and 6). Collectively, these results validated the specificity of the antibodies and their usefulness.

Immunohistochemistry Examinations by Use of McAb

In order to test the specificity of QKI antibody in in vivo assays, we firstly applied them on normal tissue samples. Previously, QKI mRNA was found to be present in several tissues including lung, kidney, liver, and spleen. Here, we further showed that kidney expressed more mRNA of QKI than other tissues (Fig. 7a). This result is consistent with our IHC data that only the kidney showed the strongest signals, which showed an eccentric distribution,

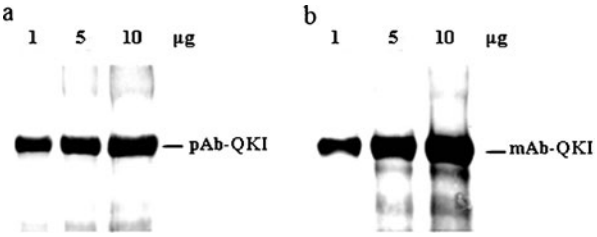

Fig. 4 Both polyclonal (**a**) and monoclonal (**b**) antibody detected the recombinant 6× His-QKI7. The total amount of protein was marked

mainly localizing in the luminal side of urinary tubule structures (Fig. 7b). So far, there is no report demonstrating the presence of QKI in urinary system; our results first disclosed its potential importance in kidney tubules.

Discussion

The selective RNA-binding protein QKI was functionally related with embryonic blood vessel development and postanal myelination in CNS [17]. Structurally, QKI protein contains a highly conserved RNA-binding domain and Src kinase phosphorylated homology region, called SH3 motif, therefore being denoted as a member of STAR family [18]. It functions as a dual relaying factor mediating both upstream kinase signals as well as modulating some specific target mRNA homeostasis [19]. Among the three major isoforms of QKI, nuclear isoform QKI-5 was found to be widely expressed in many cell types during embryonic and postnatal development [20].

Previous reports related to QKI mainly focused on the oligodendrocyte differentiation related with myelination formation in CNS [21, 22]. Recent studies disclosed aberrant QKI expressions in dysmyelinated cortex regions in schizophrenia patients; thus, the biological role of QKI in myelination was directly linked with human disease highlighting the authentic role of QKI in humans [23]. Early developmental phenotype analyses on QKI null and ENU-induced point mutant QKI mice demonstrated that loss of QKI led to defective smooth muscle cell development ending up with vascular remodeling and developmental abnormalities [14, 24]. Nonetheless, lethal phenotype in the above models largely limits the functional determinations of QKI in other adult tissues. Before setting up any tissue- or cell-specific conditional knock-out mice, the expression distribution of QKI probed with Ab in other tissues are prerequisite. Thus, obtaining a good Ab is imperative.

Up to now, QKI-specific Ab is just commercially available but with very limited applications. Therefore, obtaining a good widely applicable Ab is highly demanded. Obtaining a highly purified antigen is the first step for antibody production. Considering convenience of the purification, His-tagged QKI was expressed in *E. coli*. It is important to note that, after one-step purification by use of the Ni^{2+}-chelated IMAC column, the purity and purified mass of target protein is not satisfying. It may be due to structural hindrance of His tag impairing its affinity with Ni+, highlighting the usefulness of including additional

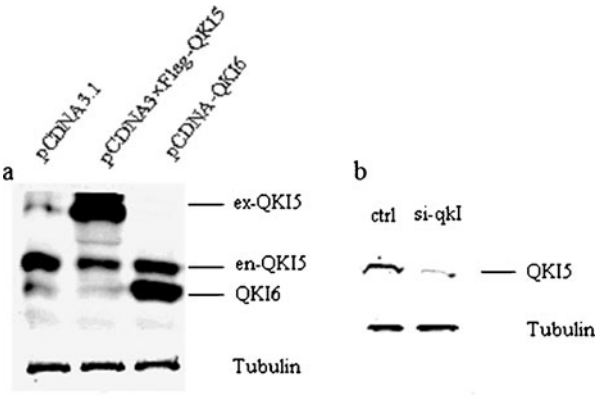

Fig. 5 Specificity determination of polyclonal antibody against QKI. Empty, QKI5, and QKI6 expressing plasmids were transfected into Hela cells (**a**). Scrambled and short interference RNA targeted to QKI were transfected into Hela cells (**b**). Forty-eight hours later, the cell lysates were collected for Western blot probed with rabbit polyclonal antibody

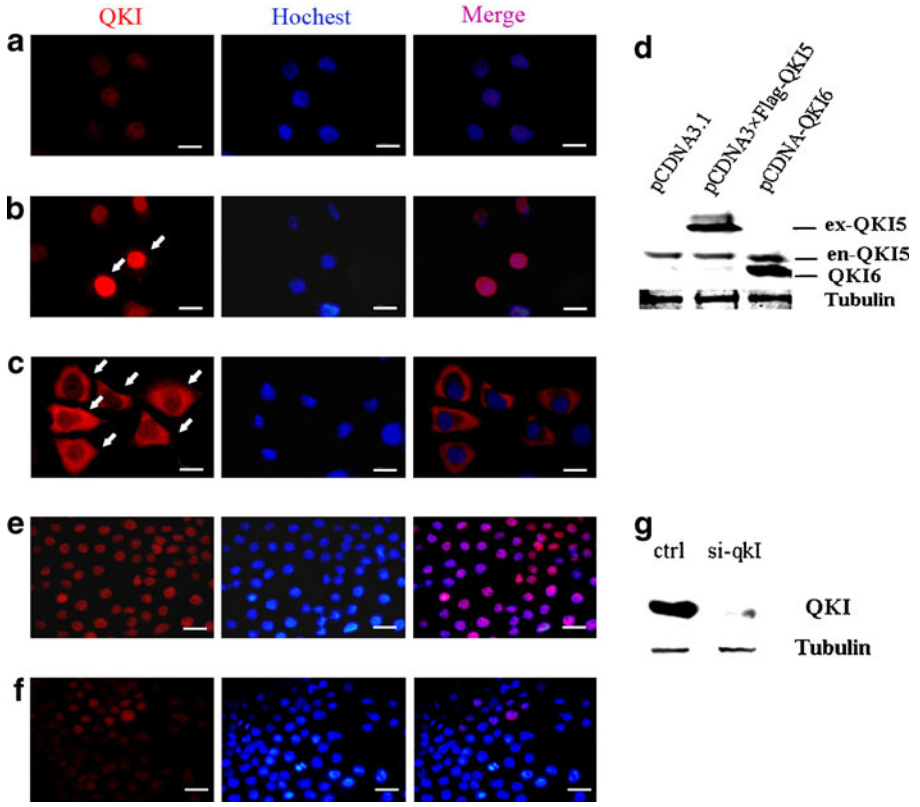

Fig. 6 Specificity determination of monoclonal antibody against QKI. Empty plasmid, plasmids expressing QKI5, and QkI6 were transfected into Hela cells. Forty-eight hours later, the cells were fixed for immunofluorescence (**a**–**c**) or Western blot (**d**) detection by using mouse monoclonal antibody. The *white arrows* indicated the cells in which QKI5 (**b**) and QKI6 (**c**) were overexpresssed, respectively. Scrambled and short interference RNA targeted to QKI were transfected into Hela cells. Forty-eight hours later, the cells were fixed for immunofluorescence (**e**, **f**) or collected for Western blotting (**g**) using mouse monoclonal antibody. *Scale bar* 10 μm in **a**–**c** and 20 μm in **e**, **f**

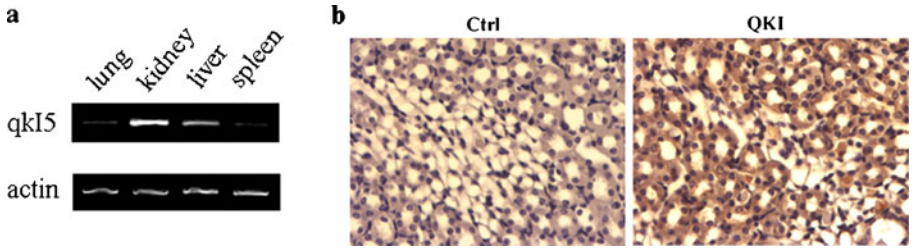

Fig. 7 The expression of QKI in different tissues, including lung, kidney, liver, and spleen. The semiquantitative RT-PCR showed the kidney has higher QKI mRNA expressing level than other tissues tested (**a**). The kidney showed the strong signal by immunohistochemistry (100×); the slides were incubated with mice IgG (*Ctrl*) or QKI monoclonal antibody (*QKI*), respectively (**b**)

His tags at target proteins, which should be helpful in improving efficiency of the one-step affinity purifications [25–27].

For immunization, traditional Freund adjuvant is regarded as the best option, and the mechanisms by which adjuvants promote increased immune response become more fully understood [28–31]. However, in case the purity of antigen is not satisfactory, preparing the specific protein bands mixed with PAGE gel or NC membranes may serve as alternative methods, which are probably responsible for the stronger immunogenicity with fewer side effects [32–34]. Whether these two alternative immunizations are efficient for the Ab production, no conclusive results were reported yet. Under our parallel immunization plan, following the same immunization schedule with similar dosage of Ag except different types of adjuvant applied, only the anti-serum from Freund's group was effective in Western blot detection, whereas two other alternative strategies showed negative results. Our data supported the notion that the type of adjuvant is critical for efficient Ab production. In other words, although the specific protein bands mixed with PAGE gel or NC membranes may serve as immunogen, Freund adjuvant might be still needed for efficient antibody production [35, 36].

In regard to the specificity of Ab, we found that different McAb clones did not show exactly similar staining patterns in immunofluorescence, although all of them displayed enhanced signals in response to the forced expression of exogenous QKI. The differences can be explained by the different clones recognizing different epitopes. It is well known that the epitopes in the Western blot, IHC or immunofluorecence (IF) assays might be different [37], which suggest that we need to screen efficient monoclonal antibodies for different applications. Upon QKI knock down in Hela cells, McAb clone number 2 (QKI-M2) showed specific signal reduction correspondingly. In immunohistochemistry, clone number 2 produced varied staining patterns on several tissues, including liver, lung, kidney, and spleen. Consistent with IHC results, real-time polymerase chain reaction (RT-PCR) results further demonstrated a higher expression level of QKI in kidney. All together, our data provided strong evidence that the QKI-M2 is the good Ab for in vivo immunohistochemistry analysis.

In summary, here we provide a practical workflow for efficient preparation of antibodies against novel protein from efficient purification of the antigen, effective immunization to quickly screen for useful clones of high affinity and specificity. Specially, we here obtained efficient antibodies against QKI, both for Western blot and immunofluorescence assays.

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Conflict of Interest Statement All authors declare that there are no conflicts of interest.

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