

Preparation and Characterization of Polyclonal Antibody Against *Xenopus* PAPC *

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Abstract *Xenopus* Paraxial Protocadherin (PAPC), which was initially identified in a screen for genes present in the Spemann organizer of *Xenopus* embryos, is required for gastrulation, somitogenesis and otic vesicle formation. In order to investigate its function in various developmental events, an antibody was prepared which could specifically recognize *Xenopus* PAPC. Glutathione S transferase (GST) expression system was used to express the fusion protein GST-PAPC. Rabbits were immunized with GST-PAPC fusion protein and the anti-PAPC polyclonal antibody was obtained. In dilution of 1 : 3 000 the antibody recognized a specific band in Western blotting analysis of FL-PAPC transfected HEK 293T cells lysates, which could be specifically blocked by pre-adsorption of prokaryotic expressed GST-PAPC fusion protein. Furthermore, by using immunofluorescence analysis the polyclonal antibody recognized membrane-bound PAPC in FL-PAPC transfected 293T cells and *Xenopus* animal cap cells. By Western blotting analysis, the endogenous 150 ku PAPC protein was detected in *Xenopus* embryos using the anti-PAPC antibody. Take together it could be concluded that a polyclonal antibody specifically against *Xenopus* PAPC was developed.

Key words *Xenopus laevis*, Paraxial Protocadherin (PAPC), prokaryotic expression, polyclonal antibody, Western blotting, immunofluorescent staining

Amphibians, especially the South African clawed frog *Xenopus laevis* and its close relative *Xenopus tropicalis*, are important model organisms in cell and developmental biology. *Xenopus* Paraxial Protocadherin (PAPC) was initially identified in a screen for genes present in the Spemann organizer of *Xenopus* embryos^[1]. The full length of PAPC cDNA is 3 649 base pairs. The complete ORF of PAPC encodes a transmembrane protein containing 979 amino acids with a signal peptide, an extracellular domain, a transmembrane region and an intracellular domain. It is initially expressed in Spemann organizer and then in paraxial mesoderm and otic vesicle in *Xenopus* embryos^[1~3]. PAPC has homophilic adhesion properties and mediates selective cell-cell adhesion and cell sorting, ensuring convergent extension cell movements during gastrulation in *Xenopus* and zebrafish^[1,4]. Surprisingly, it also has signaling functions besides adhesion properties as a protocadherin. The cytoplasmic tails of protocadherins were largely different from each other and from the highly conserved cytoplasmic domain of the classical cadherins. It has been demonstrated that the C-termini

of various protocadherins can interact with components of signaling pathways such as Fyn tyrosine kinase^[5], protein phosphatase 1 (PP1)^[6] and the adapter molecule disabled-1^[7]. *Xenopus* PAPC functionally interacts with the Wnt/PCP pathway that modulates the activity of the small GTPases Rho A and Rac 1 and c-jun N-terminal kinase (JNK). This novel signaling function of PAPC depends on its extracellular as well as intracellular domains, and is essential for the convergent extension cell movements in *Xenopus*^[8,9]. In addition, Kim *et al.*^[2] suggested that *Xenopus* PAPC was partially involved in the establishment of segmental boundaries between posterior and anterior cells in adjacent segments. Furthermore, in our previous study, by careful analysis of the dynamic expression and loss- and gain-of-function of PAPC experiments, we present evidences that PAPC is required for the otic placode

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development in *Xenopus* embryos^[3].

To further investigate the biological functions of PAPC during embryonic development, it is important to study the mutual relationship between PAPC and components of other signaling pathways. Till now, there is neither commercial available anti-PAPC antibody nor reported ones. In the present study, we cloned the coding sequence of C terminal 173 amino acids of PAPC into pGEX4T-3 expression vector, induced the expression of the GST-PAPC fusion protein, and produced a polyclonal antibody against *Xenopus* PAPC by rabbit immunization. The specificity of the anti-PAPC antibody was verified by Western blotting and immunofluorescence analyses. In future this antibody can serve as a useful tool in studies related to PAPC functions.

1 Materials and methods

1.1 Materials

1.1.1 Vectors, cell line and bacteria strain. Expression vector pGEX4T-3 was purchased from Amersham Pharmacia Biotech Company (USA). Host strain *E. coli* DH5 α was from our laboratory; *E. coli* BL21 (DE3) was purchased from Invitrogen Company (USA). HEK 293T cells were purchased from American Type Culture Collection (ATCC). pCS2+FL-PAPC plasmid was a kindly gift from Dr. Robertis, University of California, Los Angeles.

1.1.2 Reagents. Restriction endonucleases were obtained from New England Biolabs (USA). T4 DNA ligase and *Ex Tag* DNA polymerase were purchased from TaKaRa Company limited (Dalian, China). Afi-gel was from Bio-Rad (USA), Lipofectamine 2000 from Invitrogen and protein A-agarose from Santa Cruz (USA). The primers were synthesized by Sangon (Shanghai, China). All other reagents were purchased from Sigma (USA).

1.1.3 Animals. Adult male New Zealand White rabbits (2.5 kg/rabbit) and male and female *Xenopus laevis* were maintained in the experimental animal center, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences.

1.2 Methods

1.2.1 Construction of expression vector pGEX-PAPC. We cloned the coding sequence of C terminal 173 amino acids of PAPC into pGEX4T-3 vector at the *Bam*H I/*Sal* I site and designated it as expression vector pGEX-PAPC. It was generated by Polymerase

ChainReaction (PCR). The pCS2+FL-PAPC plasmid, which contains a complete coding sequence, was used as a template in the PCR assay. Primers, PAPC_24_ *Bam*H I (sense chain): 5' cccggatccCA-AACAGGCATAAAG 3' and PAPC_543_ *Sal* I (antisense chain): 5' gcggtcgcacTCTCTGCGGAGAT-AACG 3' with added restriction sites (underlined) were designed to amplify PAPC antigen region using *Ex Tag* polymerase. The PCR product was cloned into a prokaryotic expression vector pGEX4T-3 at *Bam*H I and *Sal* I sites and transformed into *E. coli* strain BL21 (DE3). The integrity of the recombinant plasmid was verified by DNA sequencing.

1.2.2 Prokaryotic expression and purification of fusion protein GST-PAPC. *E. coli* BL21 (DE3) cells, transformed with the prokaryotic expression plasmid, pGEX-PAPC, were cultured in presence of ampicillin (50 mg/L) in LB medium with vigorous shaking at 37°C until an A_{600} of 0.7~1.0 was attained. The expression of recombinant GST-PAPC protein was induced by addition of 0.1 mmol/L isopropyl-1-thio-D-galactopyranoside (IPTG) and further incubated at 16°C overnight. After centrifugation at 4 000 *g* for 20 min at 4°C, the bacterial pellet was dissolved in lysis buffer (100 mmol/L NaH₂PO₄, 8 mol/L Urea, 10 mmol/L Tris-HCl, pH 8, containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)) and sonicated until clear lysates were obtained. The supernatants and pellets were analyzed by SDS-PAGE and Coomassie brilliant blue staining. The GST-PAPC fusion protein was purified from the bacterial cells lysates by affinity purification with Glutathione-agarose. To determine the amount of the purified GST-PAPC as antigen, absorbance was measured by a spectrophotometer (Eppendorf, Germany).

1.2.3 Production of rabbit polyclonal antibody against PAPC. Two male New Zealand white rabbits were immunized by purified GST-PAPC protein according to Hu *et al.*^[10] 1 ml pre-immune sera were collected from each rabbit as negative sera control. The rabbits were then given hypodermic injections of GST-PAPC (200~300 μ g/rabbit) mixed with complete Freund's adjuvant in a 1:1 ratio. The rabbits were injected subsequently two more times with the protein mixed with incomplete Freund's adjuvant at Day 2 and Day 27. One week after the third injection, rabbit antisera were collected. After passed through GST coupled Afi-gel, the resulting antisera were affinity purified using Afi-gel conjugated with the purified GST-PAPC

protein and stored at -70°C before use.

1.2.4 Cell culture and transfection. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine sera at 37°C , in 5% CO_2 in air. Cells were seeded to a density of about 2×10^6 cells on a 35 mm culture dish and then transiently transfected with 2 μg pCS2+FL-PAPC DNA or pCS2+ vector DNA (as a negative control) using Lipofectamine 2000. Following the transfection period of 3 h, the cells were grown in media containing fresh complete medium and incubated at 37°C for another 48 h. Cellular proteins were extracted for Western blotting analysis.

1.2.5 Embryo manipulation and microinjection. *Xenopus laevis* fertilized eggs were obtained, dejellied and cultured as previous described^[11], staged according to Nieuwkoop and Faber^[12]. Synthetic FL-PAPC mRNA was microinjected into animal pole of *Xenopus* embryos at 4-cell stage. Animal caps were dissected at stage 8, cultured in 1 $\times$ MBS (0.1 mol/L CaCl_2 , 880 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L MgSO_4 , 25 mmol/L NaHCO_3) and harvested at stage 9~10. After MEMFA (0.1 mol/L MOPS, 2 mmol/L EGTA, 1 mmol/L MgSO_4 , 3.7% formaldehyde) fixation the animal caps were stored in methanol/DMSO.

1.2.6 Western blotting analysis. Total proteins were extracted in 500 μl lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40, 50 mmol/L DTT, 1 mmol/L NaF and 2 mmol/L PMSF) from HEK 293T cells or *Xenopus* embryos. 2 μg whole cells extracts and 400 μg

Xenopus embryos lysates were loaded onto an 8% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). Membranes were incubated with the rabbit anti-PAPC polyclonal antibody at 1 : 3 000 dilution for 1 h at room temperature. To test the specificity of the antibody, anti-PAPC antibody was pre-adsorbed with excess of 40 μg purified GST-PAPC protein at 37°C for 1 h, and the pre-adsorbed antibody was used in Western blotting analysis of FL-PAPC transfected HEK 293T cells lysates. The specificity was also assessed with rabbit pre-immune sera at the same dilution of 1 : 3 000. As a loading control, membranes were stripped and then incubated with a mouse anti-GAPDH antibody (Sigma) at 1 : 10 000 dilution.

1.2.7 Immunofluorescence staining. The *Xenopus* animal caps were collected and fixed in MEMFA, embedded in paraffin, sectioned, deparaffinized, and rehydrated by standard procedures. Immunofluorescence staining was performed as described^[13]. The anti-PAPC polyclonal antibody was used in the dilution of 1 : 500. Images were captured digitally and imported into Adobe Photoshop 7.0 for formatting.

2 Results and discussion

2.1 Construction and characterization of expression vector pGEX-PAPC

The sequence of PAPC was compared with the Nucleotide data bases using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>). Analysis of PAPC protein was performed using the SMART

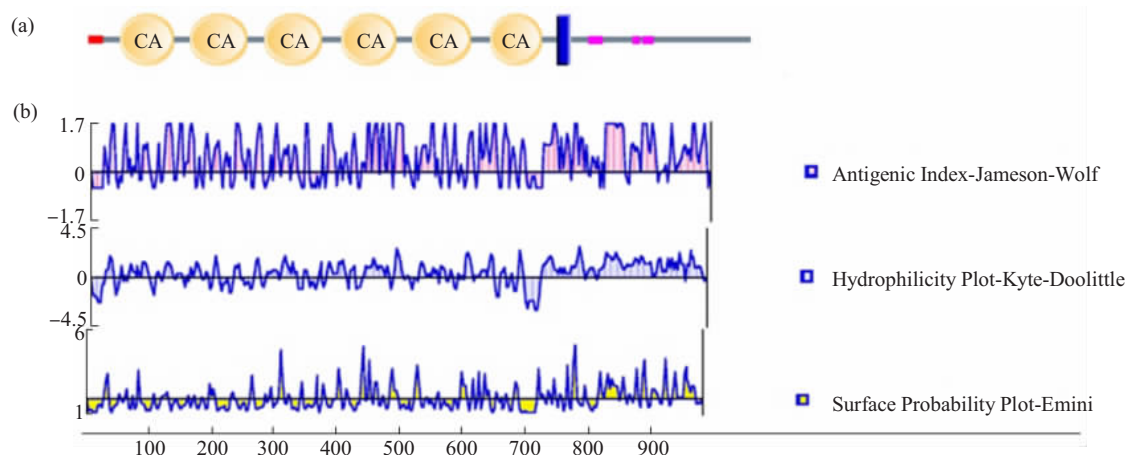


Fig. 1 Predict characters of *Xenopus* PAPC protein

(a) Domain architectures of PAPC protein predicted by SMART tool. Red is signal peptides; cadherin domains occur as six repeats in the extracellular regions; blue is a transmembrane domain; pink is low compositional complexity segments. (b) Antigenic index, surface probability plot and hydrophilicity plot of PAPC protein forecasted by DNASTar Protean software.

package (Simple Modular Architecture Research Tool, <http://smart.embl-heidelberg.de/>)^[14]. The putative PAPC protein has a single transmembrane segment. Following the N-terminal signal peptide, six cadherin domains were predicted in the extracellular regions which were thought to mediate cell-cell contact when bound to calcium (Figure 1a). It seemed that the intracellular domain fit the criterion as an antigen for its uniqueness. Antigenic index, surface probability plot and hydrophilicity plot of PAPC protein predicted by DNASTar Protean software confirmed that the intracellular domain was more adaptive (Figure 1b). So the C terminal 173 amino acids of PAPC were

being chosen as an antigen.

Figure 2a outlines the construction of the expression vector pGEX-PAPC. The C terminal 173 amino acids of PAPC were inserted into the pGEX4T-3 vector in the form of GST tagged at the N-terminus (Figure 2a). PAPC antigen (519 bp) was amplified by PCR and then analyzed by electrophoresis in 1.2% agarose gel (Figure 2b). The constructed pGEX-PAPC expression vector was verified by digestion with restriction endonucleases *Bam*H I and *Sal* I which existed in the cloning site (Figure 2c). DNA sequencing results verified the integrity of the vector again.

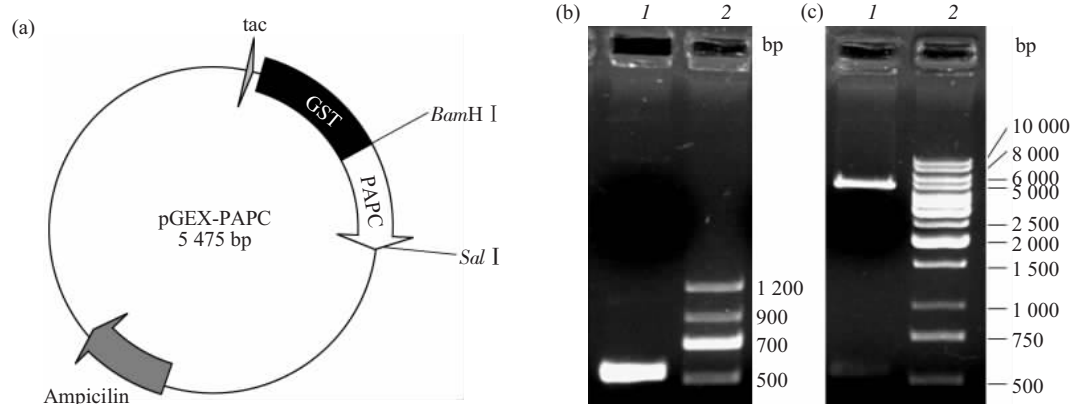


Fig. 2 Construction of expression vector pGEX-PAPC

(a) Construction of expression vector pGEX-PAPC. (b) PAPC antigen fragment amplified from FL-PAPC. 1: PAPC antigen fragment, 2: DNA marker II (Tiangen, China). (c) Verification of expression vector pGEX-PAPC by double enzyme digestion. 1: pGEX-PAPC digested by *Bam*H I and *Sal* I. 2: GeneRuler 1kb DNA ladder (Fermentas, Canada).

2.2 Expression and purification of the fusion protein GST-PAPC

The bacterial strain BL21 (DE3) was transformed with pGEX-PAPC to express the GST-PAPC fusion protein. The expression products were mainly found in the supernatant. As shown in Figure 3a, a band of relative mass 46 ku was detected, which was identical to the theoretical molecular mass (the theoretical molecular of mass PAPC antigen was 20 ku, the theoretical molecular of GST tag was 26 ku). Although there were some degradation bands, the major GST-specific proteins were GST-PAPC (46 ku). Then the GST-PAPC fusion protein was purified from the bacterial cells lysates by affinity purification with Glutathione- agarose. Coomassie brilliant blue staining (Figure 3b) and Western blotting analysis (Figure 3c) showed that the purified protein correspond to the expected molecular weight, 46 ku. From 4 L

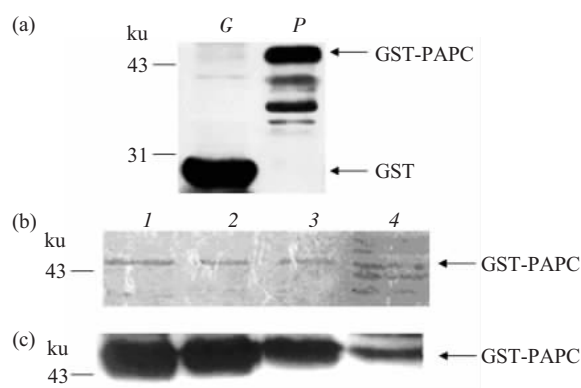


Fig. 3 Prokaryotic expression and purification of the GST-PAPC fusion protein

(a) Western blot analysis of *E. coli* BL21 (DE3) bacterial cells lysates expressing GST or GST-PAPC fusion protein using anti-GST monoclonal antibody. G: pGEX4T-3 transformed bacterial cells induced by IPTG; P: pGEX-PAPC transformed bacterial cells induced by IPTG. (b) Coomassie brilliant blue staining of the purified GST-PAPC protein. (c) Western blotting analysis of the purified GST-PAPC protein. 1: First elution of glutathione-agarose; 2: Second elution; 3: Third elution; 4: Whole bacterial cells lysates. Standard protein molecular weight markers in ku are shown on the left.

IPTG-induced bacterial culture, about 1.8 mg of purified GST-PAPC fusion protein was obtained which was sufficient for all immunization steps.

2.3 Detection of PAPC in transfected culture cells and microinjected *Xenopus* embryos

After immunization of New Zealand White rabbits with GST-PAPC fusion protein, antisera obtained from two rabbits were affinity purified by Afi-gel. The titers of antibody were analyzed by dot blotting (data not shown). As a test of specificity of anti-PAPC antibody, Western blotting analysis was undertaken. The purified antibody displayed very strong immunoreactivities and specificities to lysates of FL-PAPC transfected HEK 293T cells. At 1 : 3000 dilution PAPC protein of 150 ku was detected in Western blotting (Figure 4a, lane 2), and pre-adsorption with 40 μ g purified GST-PAPC protein totally eliminate the detection. (Figure 4a, lane 4). No

immunoreactivity was observed when rabbit pre-immune sera or pCS2 + vector transfected cells lysates were used (Figure 4a, lane 1 and lane 3). Immunofluorescence staining of FL-PAPC transfected HEK 293T cells with anti-PAPC polyclonal antibody also showed membrane bound expression of PAPC, as reported previously (Figure 4b)^[9].

To address the question whether anti-PAPC antibody could detect exogenously expressed PAPC protein in *Xenopus* embryos, synthetic FL-PAPC mRNA was microinjected into animal pole cells of *Xenopus* embryos and then the overexpressed PAPC protein was detected by indirect immunofluorescence staining (Figure 5a). These *Xenopus* animal caps showed a predominant cell membrane localized PAPC with anti-PAPC antibody (Figure 5b).

2.4 Detection of endogenous PAPC in *Xenopus* embryos

RT-PCR and *in situ* hybridization data revealed that PAPC expression is relatively high during somitogenesis^[1]. Hence, using anti-PAPC antibody we also investigated the endogenous expression of PAPC protein in *Xenopus* embryos by Western blotting analysis. Based on previous data, anti-PAPC antibody can specifically recognize FL-PAPC protein in transfected HEK 293T cells lysates (Figure 4a, lane 2). Therefore, we prepared FL-PAPC transfected HEK 293T cells lysates as a positive control for our Western blot analysis (Figure 6, lane 2). In *Xenopus* stage 11 embryos lysates, one major band that corresponded to 150 ku FL-PAPC protein of transfected HEK 293T cells was highlighted (Figure 6, lane 3), which was identical to the molecular mass of endogenous PAPC protein. While this band was absent in *Xenopus* stage 2 embryos extracts when probed with anti-PAPC antibody (Figure 6, lane 1), as well as mRNA level by previous RT-PCR and *in situ* hybridization data^[1]. Since the band profile from *Xenopus* stage 11 embryos extracts compared well with corresponding bands in FL-PAPC transfected HEK 293T positive control, this is assumed to be endogenous PAPC protein. This band was not detectable when pre-immune sera were used (data not shown).

Taken together, we presented here the preliminary data of immunoreactivities and specificities of the anti-PAPC polyclonal antibody. Take advantage of SMART and DNASTar Protean tool, we selected unique C terminal 173 amino acids of PAPC as an antigen. To produce polyclonal antibody,

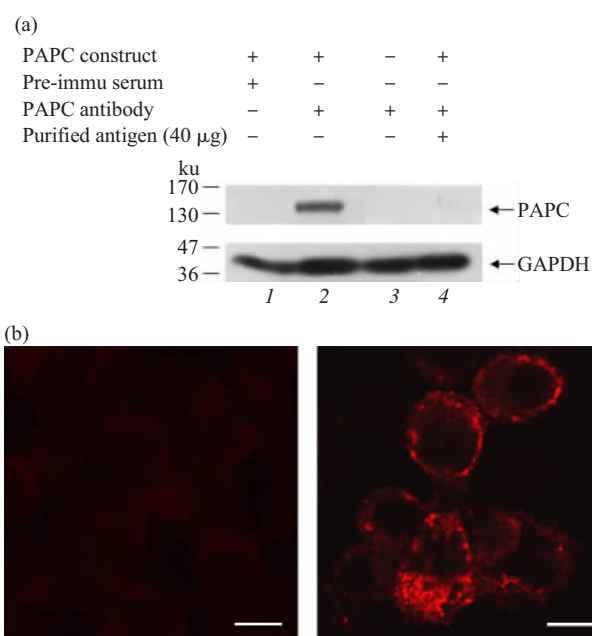


Fig. 4 Characterization of the polyclonal antibody specific to *Xenopus* PAPC in FL-PAPC transfected HEK 293T cells

(a) Western blotting analysis of FL-PAPC transfected HEK 293T cells lysates. Whole cells lysates were separated in 8% SDS-PAGE. 1: Rabbit pre-immune sera (1 : 3000) as a negative control; 2: FL-PAPC transfected cells lysates immunoblotted by anti-PAPC polyclonal antibody (1 : 3000); 3: pCS2+ vector transfected cells lysates; 4: Anti-PAPC antibody was pre-adsorbed with excess of 40 μ g purified GST-PAPC protein and then used in Western blotting analysis. As a loading control, membrane was stripped and then incubated with anti-GAPDH monoclonal antibody (1 : 10000, Sigma). Standard protein molecular mass markers in ku are shown on the left. (b) Cell membrane expression of PAPC shown by immunofluorescence staining of HEK 293T cells transfected with GFP control (left) or FL-PAPC (right) with anti-PAPC polyclonal antibody. Scale bars correspond to 8 μ m in (b).

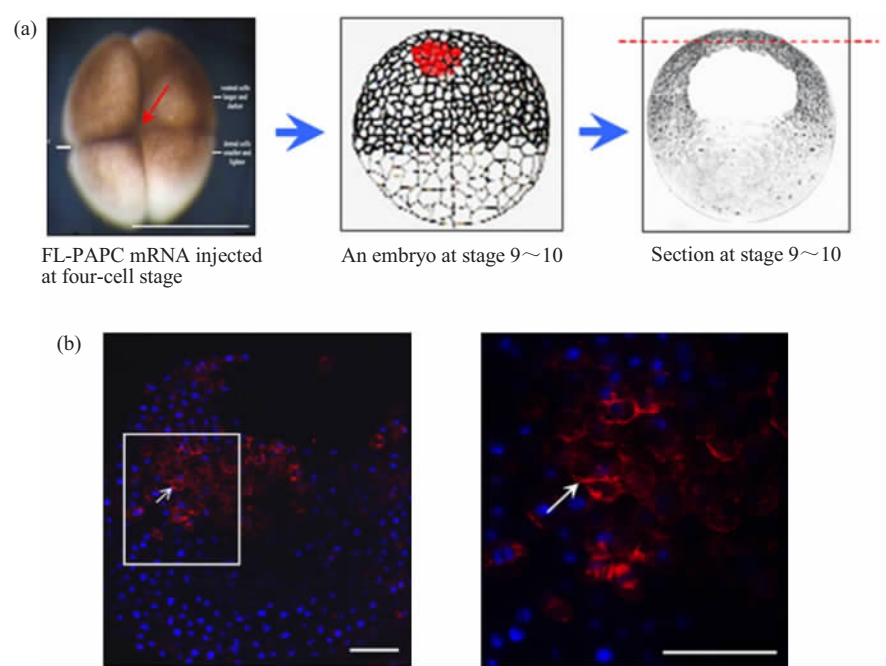


Fig. 5 Characterization of the polyclonal antibody specific to *Xenopus* PAPC in FL-PAPC microinjected *Xenopus* embryos
(a) Flowchart of FL-PAPC injection and immunofluorescence staining. FL-PAPC mRNA was injected into two blastomeres at 4-cell stage. The animal caps were fixed and sectioned when they arrived at stage 9~10. (b) Immunofluorescence of anti-PAPC polyclonal antibody (1 : 500) showed profound membrane bound signals. Picture in the right side is the enlargement of the square in the left one. Nuclei were counterstained with DAPI. Scale bars correspond to 50 μ m in (b). Samples were inspected on the OLYMPUS BX50 microscope.

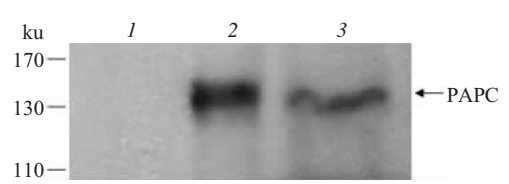


Fig. 6 Characterization of the polyclonal antibody specific to endogenous *Xenopus* PAPC by Western blotting
Western blotting analysis of endogenous PAPC protein in *Xenopus* embryos using anti-PAPC polyclonal antibody. 400 μ g total protein extracts from stage 2 or stage 11 embryos were prepared, electrophoresed through 8% SDS-PAGE, blotted on to PVDF membrane and probed with anti-PAPC antibody (1 : 3000). As in the case of FL-PAPC transfected HEK 293T cells extracts, an endogenous protein of 150 ku was detectable in *Xenopus* stage 11 embryos extracts. 1: *Xenopus* stage 2 embryos lysates as a negative control; 2: FL-PAPC transfected HEK 293T cells lysates as a positive control; 3: *Xenopus* stage 11 embryos extracts. The arrow indicates the 150 ku endogenous PAPC protein. Standard protein molecular mass markers in ku are shown on the left.

GST-tagged PAPC protein was prokaryotic expressed to immunize rabbits. Results from Western blotting and immunofluorescence staining revealed that anti-PAPC polyclonal antibody could specifically recognize untagged PAPC protein in FL-PAPC transfected HEK 293T cells. Immunofluorescence

staining of the *Xenopus* animal cap microinjected with FL-PAPC mRNA showed clear membrane localization of PAPC. By Western blotting analysis, the anti-PAPC antibody detected the endogenous PAPC in *Xenopus* embryos. All these results are strongly supporting that the polyclonal antibody is highly specific to PAPC protein in experiments dealing with immunocytology and Western blotting. Therefore, the information of specific antibodies against PAPC protein will be valuable for the further research.

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非洲爪蟾 PAPC 多克隆抗体的 制备及其特异性鉴定*

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摘要 非洲爪蟾 Paraxial Protocadherin (PAPC) 是一个在爪蟾 Spemann 组织者特异表达的膜蛋白. 它在爪蟾原肠运动阶段的汇聚延伸运动和体节发生阶段的体节边界形成, 以及早期听泡的形态发生和细胞特化过程中都有重要的作用. 为了研究 PAPC 基因在早期胚胎发育过程中的表达及其生物学功能, 需要制备 PAPC 抗体. 应用谷胱甘肽 S- 转移酶 (glutathione S transferase, GST) 表达系统表达 GST-PAPC 融合蛋白, 亲和纯化后用免疫新西兰大白兔, 获得 PAPC 多克隆抗体. 免疫印迹分析发现, 以 1 : 3000 稀释的该多克隆抗体为一抗时, 能够在转染了全长 PAPC 质粒的 HEK 293T 细胞的蛋白质抽提物中, 特异地识别出 150 ku 的印迹条带. 同时, GST-PAPC 融合蛋白可以竞争性抑制该抗体对全长 PAPC 质粒转染细胞的蛋白质抽提物的特异性条带. 用 1 : 500 稀释的该抗体为一抗进行免疫荧光分析时, 发现, PAPC 多克隆抗体能够识别在 HEK 293T 细胞中过表达以及爪蟾动物极细胞中过表达的 PAPC 蛋白, 荧光信号定位在细胞膜上. 免疫印迹分析证明, PAPC 抗体能够识别爪蟾胚胎中内源表达的 PAPC 蛋白.

关键词 非洲爪蟾, Paraxial Protocadherin (PAPC), 原核表达, 多克隆抗体, 免疫印迹, 免疫荧光

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