Article

Recombinant Expression of a Novel Human Transcriptional Repressor HMBOX1 and Preparation of Anti-HMBOX1 Monoclonal Antibody

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HMBOX1 was a novel transcription factor possibly involving in function of pancreas and cytotoxicity of NK cells. For function determination, recombinant human HMBOX1 protein was obtained and purified, and the monoclonal antibodies against HMBOX1 were prepared. The full-length cDNA fragment encoding HMBOX1 was amplified from NK-92 cells and inserted into prokaryotic expression vector pET22b. The pET22b-HMBOX1-6his vector was then transformed into *E. coli* Rosetta (DE3) and induced by 1 mM IPTG for 4 h at 37°C. The fusion HMBOX1 protein was mainly expressed in inclusion bodies, which was purified and refolded using Ni^{2+}-affinity chromatography. With the purified fusion HMBOX1 protein as antigen, monoclonal antibodies against HMBOX1 were generated, providing a potentially useful tool for further study in HMBOX1 functions. Using these anti-HMBOX1 mAbs, we identified that HMBOX1 is located in both cytoplasm and nucleus and could be detected in 10 human normal tissues, including cerebrum, pancreas, kidney and liver tissues. Moreover, the expression in hepatic carcinoma was significantly lower than that in adjacent tissues. *Cellular & Molecular Immunology*. 2009; 6(4):261-268.

Key Words: HMBOX1, expression, purification, renaturation, monoclonal antibody

Introduction

HMBOX1 (homeobox containing 1), a novel human gene, is composed of 11 exons spanning about 160 kb located at 8p21.2. HMBOX1 has two transcript variants which encode the same protein. The protein contains a homeobox domain in N-terminus and an HNF1-N domain in C-terminus (http://www.ncbi.nlm.nih.gov/Genbank/), and HMBOX1 is highly conserved in human, mouse, rat, chicken and xenopus. Human HMBOX1 is widely expressed in at least 18 tissues, and highly in pancreas, brain, placenta, prostate, thymus and testis. Co-transfection of HEK-293T cells with pM-HMBOX1 plasmid and reporter plasmid $pGAL4_5tkLUC$ indicates that HMBOX1 protein may be one transcription repressor, but how it works is unknown (1).

Homeobox genes are characterized by the possession of the homeobox domain. Most homeodomains have 60 amino acids in length, but HMBOX1 has 78 amino acid (between amino acids 267 and 344) (2, 3). Many homeodomain proteins are transcription factors with important roles in embryonic patterning and cell differentiation. For example, HNF1 (hepatic nuclear factor 1), a member of the homeobox genes, is a major regulator of glucose homeostasis, regulating the expression of albumin, β-fibrinogen and α1-antitrypsin expressed in liver, kidney, and pancreas (4). Recently, we found that HMBOX1 showed negative regulatory effects on NK cell activation (Longyan Wu, et al, unpublished data).

To further study the function of HMBOX1, we successfully cloned and expressed fusion HMBOX1 protein in *E.coli* Rosetta (DE3), and then obtained the purified and renatured proteins by Ni^{2+}-affinity chromatography. Furthemore, we produced and characterized monoclonal antibodies against HMBOX1 that can be applied in immunoblotting and immunohistochemisty assays.

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Abbreviations: HMBOX1, homeobox containing 1; HNF1, hepatic nuclear factor 1; IPTG, Isopropyl-β-D-Thiogalactoside; BSA, bovine serum albumin; PEG, polyethylene glycol; OPD, o-phenylenediamine dihydrochloride; HAT medium, hypoxanthine aminopterin thymidine medium.

Materials and Methods

Bacterial strains and cell lines

Escherichia coli DH5α, *E. coli* Rosetta (DE3), HEK-293T and SP2/0 were stored by our laboratory. *E. coli* Rosetta (DE3) grew in LB medium supplemented with 1 mg/ml ampicillin. HEK-293T cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, GIBCO/BRL, USA), supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. SP2/0 cells were maintained in RPMI 1640 (GIBCO/BRL, USA) supplemented with 15% FBS at 37°C in a 5% CO_2 incubator.

Construction of expression vectors

The cDNA (1336 bp) encoding full-length human HMBOX1 (GenBank accession no. NM_024567.3) was amplified from NK-92 cells by Ex Taq polymerase (Takara Biotechnology, Dalian, China). The PCR began with a step of denaturation (94°C for 60 sec), annealing (57°C for 60 sec) and extension (72°C for 120 sec) by 6 cycles, and followed by 30 cycles of denaturation (94°C for 60 sec), annealing (50.5°C for 60 sec) and extension (72°C for 90 sec). Forward and reverse primers were 5'-GGT ACC GGA TAT TGA TCC GCC TCA TG-3' and 5'-CTC GAG GGT GCT ACG TCT AAA CTA AGT T-3', respectively. Then the PCR amplicon was cloned into pGEM easy T vector (Promega, Beijing, China). With the pGEM-HMBOX1 as template, 30 cycles at 94°C for 60 sec, 58°C for 60 sec and 72°C for 90 sec were performed, using forward primer 5'-CGC GGA TCC TAT GCT TAG TTC CTT TCC AGT GGT T-3' flanked by BamH I restriction site and reverse primer 5'-CCC AAG CTT CCA GTC ATC ATC CAG GGC C-3' linked with a variant stop codon and flanked by Hind III restriction site. This modified HMBOX1 products were also cloned into pGEM easy T vector and confirmed by sequencing. Finally, the target fragment was subcloned into pET22b (Novagen, Merck KGaA, Darmstadt, Germany) by restriction enzymes of BamH I and Hind III (Takara Biotechnology, Dalian, China), recombinant vector pET22b-HMBOX1-6his was constructed.

Expression of fusion HMBOX1 protein

pET22b-HMBOX1-6His was transformed into *E. coli* Rosetta (DE3) using standard procedures. The bacteria were grown in LB medium at 37°C until OD_{600} was about 0.6 and then induced with 1 mM IPTG for 4 h at 37°C. Cells were harvested and disrupted with ultrasonic cell crusher, then supernatant and inclusion bodies were prepared.

Purification and renaturation of HMBOX1-6His by Ni^{2+}-affinity chromatography

Chromatography column was hold with desired amount of Ni^{2+}-charged resin (Novagen, Merck KGaA, Darmstadt, Germany). With regard to protein in inclusion bodies, purification and renaturation of HMBOX1-6His was cooperated by Ni^{2+}-affinity chromatography (5, 6). Firstly, prepared inclusion bodies solubilized in binding buffer containing 6 M urea were loaded in column. Secondly, a gradient of 6 - 0 M urea decreasing every 0.5 M was used to refold the bound protein. Finally, the refolded recombinant protein was eluted with eluting buffer 1 (0.5 M NaCl, 20 mM Tris-HCl, 1 M Imidazol, pH7.9) and the reduced protein eluted with eluting buffer 2 (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazol, 6 M Urea, pH7.9). The samples were then dialysis with N.S and concentrated by ultrafiltration tubes.

Preparation of acid-treated naked Salmonella Minnesota R595 bacteria

Salmonella Minnesota R595 bacteria were cultivated on surface of LB solid medium at 37°C overnight. Cell pellets were collected and incubated with 5% phenol for 5 min to kill bacteria. Then the bacteria were washed by turns with dH_2O twice, acetone twice and diethyl ether, following by vacuum drying. Treated with N.S containing 5% acetic acid and 1% acetic acid in turns, the naked bacteria were washed with N.S and stored after lyophilized.

Production of mouse monoclonal antibodies against HMBOX1

Refolded fusion HMBOX1 proteins (50 μg) were adsorbed to 250 μg of acid-treated naked *Salmonella Minnesota* R595 bacteria (total 200 μl), and the complex was used to immunize 6-8 weeks old BALB/c female mice. The animals were injected intraperitoneally at 2 weeks interval. When the titer of mice serum was higer than $1:10^5$, the mice were given a final booster with antigen complex three days before fusing program.

Splenocytes (1×10^8) were fused with 1×10^7 SP/2 myeloma cells in 50% polyethylene glycol (PEG). Hybridoma cells were selected using HAT medium (20% FCS RPMI1640, 10 mM sodium hypoxanthanine, 40 mM aminopterin, 1.6 mM thymidine). After 10-14 days, when the clones were visible, indirect ELISA was performed to test all wells. The positive wells were then subcloned three times using the limiting dilution method and then the positive clones were frozen and stored.

High concentration anti-HMBOX1 monoclonal antibodies were prepared from ascitic fluid of BALB/c mice. Subsequently, antibodies were purified using hyper- saturated ammonium sulfate suspension and protein A affinity chromatography. Isotypes of the monoclonal antibodies were determined using the Mouse Hybridoma Subisotyping Kit (Calbiochem, Merck KGaA, Darmstadt, Germany) through the ELISA assay.

ELISA

The titers of antibodies were determined using indirect ELISA and the specification of that was performed by competitive ELISA. As previously described (7), fusion HMBOX1 protein (10 μg/ml) was added to microtiter plates, incubated overnight at 4°C. After washed three times, plates were blocked with BSA for 2 h at 37°C and then incubated with various dilutions of mice immunized serum or anti-HMBOX1 monoclonal antibody. After the plates were incubated with conjugated HPR anti-murine antibody (Dako,

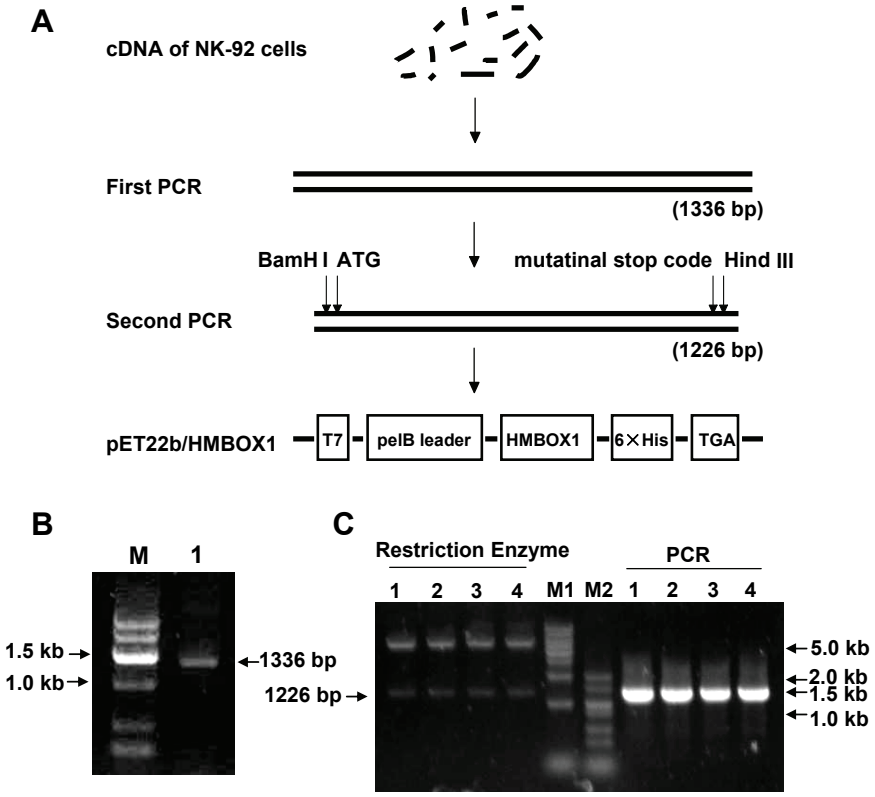


Figure 1. Construction of expression vector pET22b-HMBOX1-6His. (A) Strategy of synthesis of human HMBOX1 coding sequence using two-step PCR assay. The full-length gene of HMBOX1 (1336 bp) was cloned with a first-step PCR from cDNA of NK-92 cells. Using the products of first-step PCR as template, a second-step PCR was performed to amplify target fragment. The gene coding HMBOX1 was then inserted into pET22b vector. (B) Cloning of the full-length HMBOX1 gene from NK-92 cells cDNA. A 1336 bp PCR product was detected on 0.8% agarose gel electrophoresis. Lane M, DNA marker; Lane 1, the PCR product of full-length HMBOX1. (C) Construction of the recombinant vector pET22b-HMBOX1-6His. The recombinant vectors were certificated by PCR and restricted enzyme cutting (BamH I and Hind III). Lanes M1 and M2, DNA marker; Lanes 1-4, four clones of recombinant vector.

Glostrup, Denmark) for 1 h and incubated with OPD (o-phenylenediamine dihydrochloride) as substrate for 15 min, the colorimetric signal was measured at OD_{490nm}.

SDS-PAGE and Western blot

Bacterial extracts containing fusion protein, tissue extracts or purified proteins were separated by SDS-PAGE under reducing or non-reducing conditions on a 12% polyacrylamide gel and then transferred onto PVDF membrane (Millipore, Billerica, Massachusetts, USA) by electroblotting. The membranes were blocked with 5% non-fat milk in TBS/0.1% Tween 20 and then incubated with anti-HMBOX1 mAb as primary antibody for 2 h at room temperature, then washed with TBST 3 times and incubated for 1 h with a secondary anti-murine antibody conjugated HRP (Dako, Glostrup, Denmark). After washing 5 times with TBST, the membranes were detected with enhanced chemiluminescence system (Pierce, Rockford, IL).

Transfection of siRNA or plasmid

Transient transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). According to manufacturer's protocol, HEK-293 cells (1×10^4/well) were seeded in 24-cell plates and incubated for 12 h and treated with Lipofectamine 2000/vehicle (TE), Lipofectamine 2000/siRNA-HMBOX1 (100 nM) and Lipofectamine 2000/pcDNA3.1-HMBOX1 (0.25 mg).

Tissue microarray and immunohistochemistry

Two kinds of tissue microarray from 23 human normal tissues or cancer tissues (OD-CT-HNCom01-001 and OD-CT-Com03-002) were provided by Shanghai Biochip (Shanghai, China). After deparaffinizing and rehydrating tissue sections, heat-induced antigen retrieval was performed. Then the sections were incubated overnight at 4°C with anti-HMBOX1 antibody as primary antibody in a humid chamber. Following the DAB envision system (zsbio, Beijing, China) was applied. Negative controls were treated identically but with the primary antibody omitted.

Statistical analysis

All of values are presented as the mean ± SD for three or more individual experiments. Data were tested with SPSS software (version 10.0, SPSS inc.) for significance. The *p*

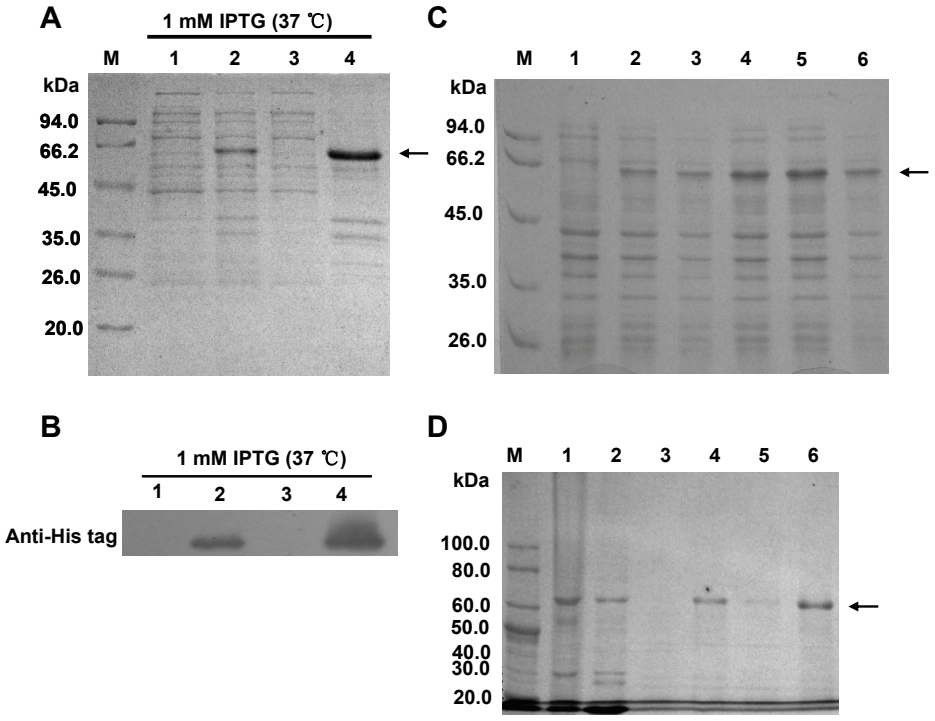


Figure 2. Expression, purification and renaturation of fusion HMBOX1 protein. (A) SDS-PAGE analysis was performed to identify the expression location of fusion HMBOX1 protein. Lane M, protein molecular weight marker; Lane 1, the negative control of cell lysate of Rosetta (DE3)/pET22b; Lanes 2-4, cell lysate, supernatant and inclusion bodies from Rosetta (DE3)/pET22b-HMBOX1 induced with 1 mM IPTG at 37°C for 4 h. (B) Confirmation of the recombinant protein by Western blot assay. A sister SDS-PAGE gel as shown in (A) was transferred onto a membrane and blotted with anti-His mAb. (C) Time gradient of recombinant HMBOX1 expression. Lane M, protein molecular weight marker; Lane 1, the negative control of cell lysate from Rosetta (DE3)/pET22b induced by 1 mM IPTG; Lanes 2-6, cell lysate from Rosetta (DE3)/ pET22b-HMBOX1 induced with 1 mM IPTG at 1 h, 2 h, 3 h, 4 h and 5 h. (D) Purification and renaturation of fusion HMBOX1 by Ni^{+} affinity chromatograph. The samples were analyzed on 12% SDS-PAGE gel. Lane M, protein molecular weight marker; Lane 1, cell lysate of Rosetta (DE3)/pET22b-HMBOX1 induced with 1 mM IPTG at 37°C for 4 h; Lane 2, inclusion bodies; Lane 3, washing buffer; Lanes 4 and 5, eluting buffer 1 containing refolded HMBOX1 protein; Lane 6, eluting buffer 2 containing reduced HMBOX1 protein.

values < 0.05 were considered statistically significant.

Results

Construction of expression vector pET22b-HMBOX1-6His

There are two transcript variants of HMBOX1, differing in the 5'UTR, encode the same protein (420 residues). The relatively high level of human HMBOX1 was shown in NK cells in our study (Longyan Wu, et al, unpublished data). So we cloned the full-length gene containing the coding sequence of HMBOX1 from NK-92 cells by two-step PCR (Figures 1A, 1B) and constructed recombinant expression vector pET22b-HMBOX1-6His. RE and PCR analysis of the clones gave the expected size (Figure 1C). DNA sequencing confirmed that the HMBOX1 gene was successfully cloned into pET22b vector.

Expression, purifications and renaturation of fusion protein

The recombinant plasmid pET22b-HMBOX1-6His was transformed to an expression host *E. coli* Rosetta (DE3) which is a rare codon optimizer strain. As shown in Figure 2A, after induced with 1 mM IPTG at 37°C for 4 h, the fusion protein could be observed in inclusion bodies with an expected distinct band on SDS-PAGE which was not observed in negative control sample. This result could be further confirmed by Western blot assay using anti-His mAb (Figure 2B). Time course study indicated that the expression levels of fusion HMBOX1 protein reached the peak at 4 h (Figure 2C). The molecular weight of fusion HMBOX1 protein is about 60 kDa, which was bigger than estimated size of 4.7 kDa because of containing some amino acids of vector. These results confirmed that the rHMBOX1 protein was successfully expressed in *E. coli* Rosetta (DE3).

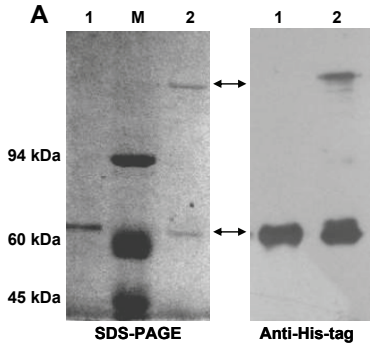
The fusion HMBOX1 protein was presented in an insoluble form as inclusion bodies. To produce the optimum concentration of the fusion protein, the recombinant protein was solubilized and purified with Ni^{2+}-affinity chromatography purification system with routine procedure. At the same time, the reduced protein was refolded directly on column (5, 6). In this course, 81.2% of the fusion protein was refolded (Table 1). The rate of recovery was similar to that by

Table 1. Summary of the purification and refolding of recombinant human HMBOX1 from *E. coli* including bodies

Purification step	Total protein (mg/L)	%Yield
Cell lysate	195.62	
Inclusion bodies	14.60	7.46
Inclusion bodies (after washing)	9.64	4.93
Affinity purified refolded protein	7.37	3.77
Affinity purified unrefolded protein	1.71	0.88

Protein concentration estimated by Bradford assay

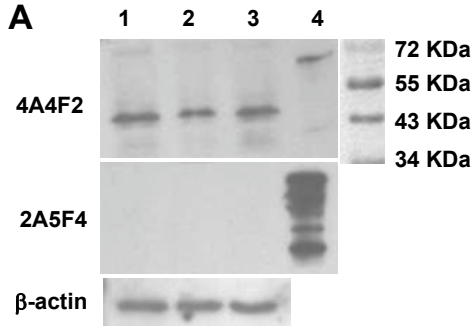
Hancock K. with FPLC system (85%) (5). The final elution products included two types of fusion protein: refolding protein dissolved in elute buffer 1 (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazol, pH7.9) and reduced protein in elute buffer 2 (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazol, 6 M Urea, pH7.9) (Figure 2D). The refolding protein could be demineralized and concentrated by dialysis and ultrafiltration with a final 3.77% yield (Table 1). Although the refolded proteins were concentrated to 1 mg/ml, there is no precipitate appearing, which suggested that the effectiveness of refolding is satisfactory. Additionally, non-reduced SDS-PAGE was performed to identify refolding efficiency of fusion HMBOX1 protein. As shown in Figure 3A, after refolding, the fusion proteins could form dimer pattern. Because there are 3 disulfide bonds in HMBOX1 protein, maybe the dimmer was one functional formation of HMBOX1.



B

1 MLSSFPVVLL ETMSHYTDEP RFTIEQIDLL QRLRRTGMTK HEILHALETL
51 DRLDQEHSDK FGRRSSYGGS SYGNSTNNVP ASSSTATAST QTQHSGMSPS
101 PSNSYDTSPQ PCTTNQNGRE NNERLSTSNG KMSPTRYHAN SMGQRSYSFE
151 ASEEDLDVDD KVEELMRRDS SVIKEEIKAF LANRRISQAV VAQVTGISQS
201 RISHWLLQQG SDLSEQKKRA FYRWYQLEKT NPGATLSMRP APIPIEDPEW
251 RQTPPPVSAT SGTFRLRRGS RFTWRKECLA VMESYFNENQ YPDEAKREEI
301 ANACNAVIQK PGKKLSDLER VTSLKVYNWF ANRRKEIKRR ANIEAAILES
351 HGIDVQSPGG HSNSDDVDGN DYSEQDDSTS HSDHQDPISL AVEMAAVNHT
401 ILALARQGAN EIKTEALDDD

Figure 3. The identification of refolded fusion HMBOX1 protein. (A) Refolded fusion protein identified by 12% SDS-PAGE gel (left), then a sister SDS–PAGE gel was transferred onto a membrane and blotted with anti-His mAb (right). Lane M, protein molecular weight marker; Lane 1, refolded fusion protein identified by reduce SDS-PAGE; Lane 2, refolding fusion protein identified by non-reduce SDS-PAGE. (B) Sequencing of fusion HMBOX1 protein analyzed by LC–MS/MS using a Q-TOF2 mass spectrometer. There were 5 peptides (under lined) used to identify fusion HMBOX1 protein.



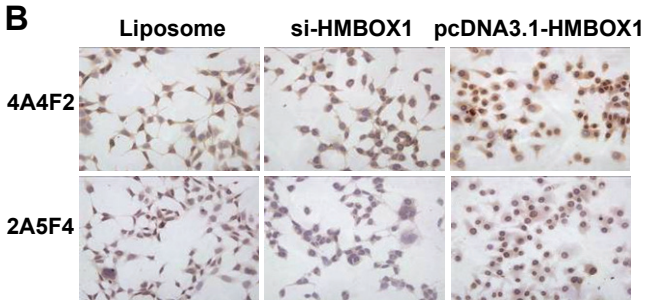


Figure 4. Identification of the specificity of mAbs against HMBOX1. (A) Western blot was performed to analyze cell extracts of HEK-293T cells transfected with liposome (Lane 1), siRNA targeted HMBOX1 gene (Lane 2) or pcDNA3.1-HMBOX1 (Lane 3) with cell extracts of *E. coli* Rosetta (DE3)/pET22b-HMBOX1 as positive control (Lane 4) by using 4A4F2 mAb or 2A5F4 mAb. The β-actin messenger is used as internal control. (B) Immuno-histochemical analyses of HMBOX1 in HEK-293T cells transfected with siRNA targeted HMBOX1 gene or pcDNA3.1-HMBOX1 by using 4A4F2 mAb and 2A5F4 mAb. Original magnification, ×400.

Furthermore, the sequence of fusion HMBOX1 protein was analyzed by LC-MS/MS using a Q-TOF2 mass spectrometer (National Center of Biomedical Analysis, Beijing, China). HMBOX1 was identified with a mowse score of 369 from 5 peptides, covering 14% of the proteins (Figure 3B).

Preparation of murine monoclonal antibodies against human HMBOX1

Through the procedures of immunization, fusion and clone selection, two hybridoma stains, 2A5F4 and 4A4F2, were obtained and both stably produced anti-HMBOX1 mAb. The features of these mAbs were shown in Table 2. The isotype of 2A5F4 mAb is IgG2b/κ and that of 4A4F2 is IgM/κ. The titer and affinity of 2A5F4 from ascitic fluid is 1.024×10^{-5} and $3.67 \times 10^{8} M^{-1}$, respectively. And the titer and affinity of

Table 2. Features of monoclonal antibodies

	2A5F4	4A4F2
Isotype	IgG2b/κ	IgM/κ
Titer	1.024×10^{-5}	5×10^{-5}
Affinity	3.67×10^{8} M^{-1}	3.35×10^{8} M^{-1}
Western blot	No	Yes
IHC	Yes	Yes

4A4F2 from ascitic fluid is 5×10^{-5} and 3.35×10^{8}M^{-1}, respectively.

To verify the specificity of 2A5F4 and 4A4F2 against HMBOX1, HEK-293T cells were transfected with pcDNA3.1-HMBOX1 or siRNA/HMBOX1 which can improve or decrease the expression of HMBOX1. As expected, the expression level of HMBOX1 in HEK-293T cells transfected with pcDNA3.1-HMBOX1 was higher than that in HEK-293T cells, whereas it was lower in HEK-293 cells treated with siRNA/HMBOX1 by Western blot and immunohistochemisty assays using 4A4F2 mAb. But 2A5F4 mAb was only available for immunohistochemisty assay (Figures 4A and 4B). So 4A4F2 mAb can be applied both in Western blot and immunohistochemisty analysis, whereas 2A5F4 mAb was only used in immunohistochemisty experiment (Table 2).

Subcellular location and expression of HMBOX1 in mammalian cells

HMBOX1 is reported as a transcription factor, and mRNA of HMBOX1 could be widely detected in 18 human tissues. In HEK-293T cells transfected with pEGFP-HMBOX1 plasmid, HMBOX1 was mainly accumulated in the cytoplasm, while the fluorescence signal in the nucleus could also be detected (1). In our study, performed by fluorescence method using 4A4F2 mAb, HMBOX1 protein could be detected in both cytoplasm (green) and nucleus (white) of HEK-293T, which meet the characteristic of transcription factor (Figure 5A).

Furthermore, 4A4F2 mAb was used to analyze the expression of HMBOX1 in human tissues by tissue microarray method. As shown in Figure 5B, HMBOX1 expressed widely in human tissues, especially in cerebrum, thyroid gland, stomach, intestine, liver, pancreas, lung, cardiac muscle, testis and prostate. Interestingly, HMBOX1 was prone to express in hepatic cells, islet of Langerhans, muscle tissue and glandular organ (Figure 5B). Moreover, we found that HMBOX1 was mainly observed in the cytoplasm of hepatic cells and the expression levels of HMBOX1 in hepatic carcinoma was much lower than that in adjacent tissues of carcinoma (p = 0.041) (Figures 6A and 6B). It suggested that HMBOX1 may play roles during hepatocarcinoma happening and development.

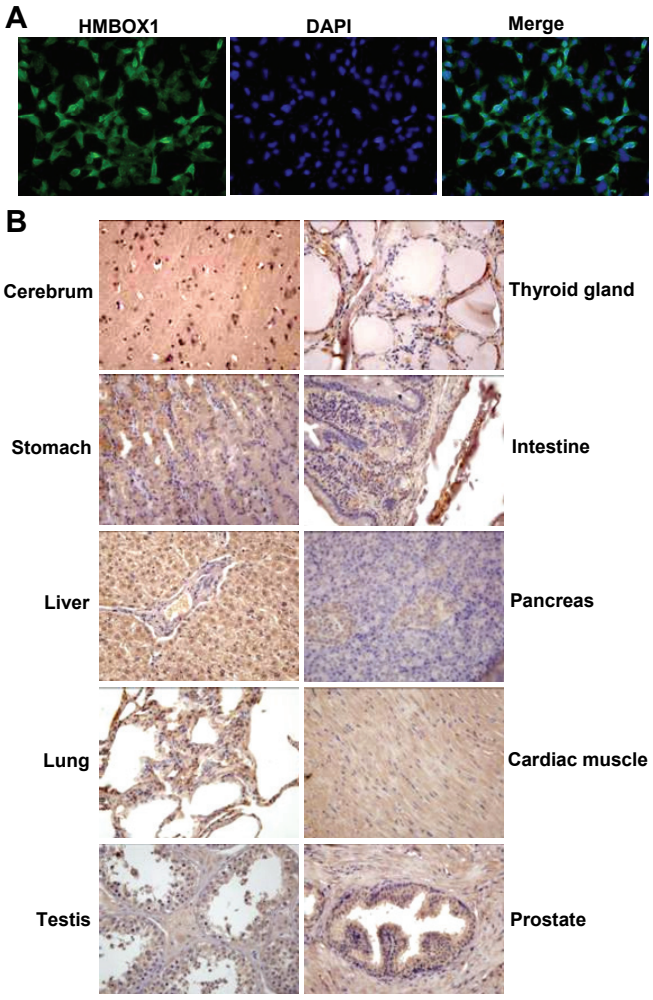


Figure 5. Subcellular location and expression of HMBOX1 in human tissues. (A) Subcellular location of HMBOX1 in HEK-293T cells. HMBOX1 staining (green) with 4A4F2 mAb, nuclear staining with DAPI (blue), overlap between HMBOX1 and nucleus staining (white). Original magnification, ×400. (B) HMBOX1 in human normal tissues was detected by immunohistochemisty. Tissue microarrays containing 23 tissues were taken for staining with 4A4F2 mAb. Original magnification, ×400.

Discussion

HMBOX1 is one novel transcription factor and its structure and function are not well known. In this paper, we obtained fusion HMBOX1 protein by prokaryotic expression system and produced anti-HMBOX1 mAbs which is important for investigating the functional mechanism of HMBOX1.

Over-expression of recombinant protein in *E. coli* may be severely diminished if the codes of the ORF for the protein use "rare" codons that are infrequently used by *E. coli* (8-12). According to the online (http://nihserver.mbi.ucla.edu/RACC/) analysis for coding sequence of human HMBOX1, the result showed that it involves a high percentage of rare codons (about 10%) represented in *E. coli*. For example, there are 26 single rare Arg codons (AGG, AGA and CGA) and 5 tandem rare Arg codon double repeats. At the beginning, we tried to express

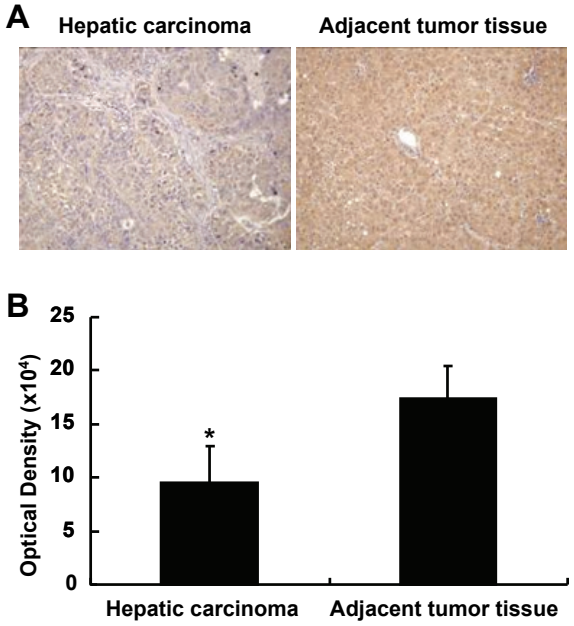


Figure 6. Expression levels of HMBOX1 in hepatic carcinoma and adjacent tissues. (A) Immunohistochemical method was performed to analyze the expression levels of HMBOX1 in hepatic carcinoma and adjacent tissues to carcinoma using 4A4F2 mAb. Tissue microarrays containing hepatic carcinoma and adjacent tissues were taken for staining. Original magnification, ×400. (B) Paired samples *t* test showed a statistical difference between hepatic carcinoma and surrounding tissues tissue. Signals were quantified by NIH image and values indicated as relative density. The data represents means error standard of three independent experiments. *$p < 0.05$.

HMBOX1 in BL21 and M15 stains, but failed. Finally, human HMBOX1 was obtained in Rosetta (DE3), which contains plasmid encoding rare arginine codons AGA, AGG, and CGA, glycine codon GGA, isoleucine codon AUA, leucine codon CUA, and proline codon CCC.

In this study, the fusion HMBOX1 protein was mainly expressed in inclusion bodies. Optimizing many factors that could influence the secretary expression, including growth media, time and temperature, the soluble fusion HMBOX1 protein could be induced with 0.1 mM IPTG at 25°C, but the yield was very low (data not show). So it is necessary to refold the fusion protein and on-column refolding of the bound proteins is performed. Flowed with binding buffer containing 6 M urea to 0 M urea decreased by 0.5 M, the bound protein was refolded. This method not only ensured to facilitate the rapid and efficient refolding of His-tagged recombinant proteins, but also significantly increased the yield of refolded protein (13, 14). Moreover, the application of GSH-GSSG (reduced Glutathione and oxidized Glutathione) also profits the work of protein refolding.

To prepare antibody against HMBOX1, the acid-treated, naked *Salmonella Minnesota* R595 bacteria were used as adjuvant. The antigen, fusion HMBOX1 protein, was adsorbed to naked bacteria formed antigen-bacteria complex which was used to immunize mice. This method can avoid missing of antigen because of emulsification. Moreover, the titer of antibody obtained by this way was comparable to those achieved using Freund's adjuvant, and much less antigen was needed for immunization (15, 16).

It was found that HMBOX1 was widely expressed in human tissues by immunohistochemisty. Like it's homologous factor HNF1 (hepatic nuclear factor 1), a transcription factor with a wide range of functions, which involved in cholesterol, bile acid, and lipoprotein metabolism, HMBOX1 may be one factor involved in fundamental function. Additionally, we found the expression level of HMBOX1 in hepatoma decreased, that was also similar to HNF1. Van wering, et al. reported that hepatoma dedifferentiation and suppression of hepato-specific gene expression is usually accompanied by a decrease in HNF1α expression (17, 18). So HMBOX1 probably correlates with tumorigenesis of hepatic cancer, and it is needed to investigate further.

In conclusion, we developed an efficient protocol for expression, purification and renaturation of human HMBOX1 protein and prepared two monoclonal anti-bodies against HMBOX1 which were available for Western blot and/or immunohistochemisty. These monoclonal antibodies provide a potentially useful tool for further study in HMBOX1 functions.

Acknowledgements

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