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RESEARCH ARTICLE

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An improved yeast two-hybrid approach for detection of interacting proteins

Abstract Yeast two-hybrid approach is popularly used nowadays as an important technical method in the field of studying protein-protein interactions. Although yeast two-hybrid system is obviously advantageous in searching interacting proteins and setting up the network of proteins interaction, not all of proteins can use routine yeast two-hybrid method to search interacting proteins. Many important proteins, such as some nucleoprotein transcriptional factor, carry out the regular method and construct the bait-BD vector to screen the library containing AD vector. However, it usually results in failures because it contains the activate domain and can self-activate the reporter gene. In this study, we changed the research strategy, fused the bait gene (FOXA3) with the AD vector to screen the library containing BD vector, so that we constructed a two-hybrid library containing BD vector and can bypass the interference of self-activation. And we used this two-hybrid library to screen FOXA3, a hepatocyte nuclear factor, and found out an interacting protein: complement component C3.

Keywords yeast two-hybrid system, protein-protein interaction, human liver cDNA library, FOXA3

1 Introduction

HNF is a hepatocyte transcription factor superfamily, which includes at least six gene families, from HNF1 to HNF6(Odom et al., 2004). The HNF3 (FOXA) gene family includes FOXA1, FOXA2, FOXA3, which are expressed in the early mesoderm and have important roles in the regulation of gene expression in the liver and pancreas(Shen et al., 2001). The FOXA1 gene is involved in glucagon secretion and the FOXA2 gene regulates the secretion of insulin in pancreatic β-cells (Katoh and Katoh 2004; Lantz and Kaestner 2005). Shen et al. discovered through studies in knockout mice that *Foxa*3 (hepatocyte nuclear factor 3γ) is required for the regulation of hepatic GLUT2 expression and maintenance of glucose homeostasis during a prolonged fast. Post-implantation expression patterns indicate a role for the mouse *forkhead*/HNF-3 in the determination of the definitive endoderm, chordamesoderm and neuroectoderm (Ang et al., 1993; Monaghan et al., 1993). Many genes are transactivated by FOXA3, including three important enzymes involved in the gluconeogenesis in the liver: phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), fructose-2, 6-bisphosphatase (F2, 6 BPase). At present, it is not known what kind of proteins initiate FOXA3 protein expression in the liver, and what protein interacts with it to transactivate the downstream genes.

In this study, FOXA3 protein was used as bait protein to screen human liver cDNA library through yeast two-hybrid method. The FOXA3-Gal4 DNA-BD (binding domain) fused protein can self-activate and can not be used directly to screen the ready-to-use Gal4 DNA-AD (activating domain) fused cDNA library. On the other hand, the FOXA3-Gal4-AD chimera protein is not able to self-activate. We therefore constructed the Gal4-BD (DNA-BD) fused cDNA library, and screening it with FOXA3-Gal4-AD, found an interacting protein for FOXA3: complement component 3. Preliminary confirmation of the interaction was performed by co-transforming the gene plasmid with the bait plasmid into yeast cells.

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2 Materials and methods

2.1 Plasmids and yeast strains

Information on all plasmids and yeast strains used in the study are provided in Table 1.

Table 1 Strains and plasmids

Strains and plasmids	Relevant characteristics		Source
E. coli DH5α	SupE44 ,lacU169 ,hsdR17 ,recA1 ,endA1 ,gyrA96 ,thi21 ,relA1 ,Nalr		Keep in this lab
E.coli XL10-GOLD	Tet^r△(mcrA)183,endA1,supE44,thi-1,recA1,gyrA96,relA1,lac,the		Stratagene
S.cerevisiae MaV203	*MAT*α, *leu*2-3,112, *trp*1-901, *His33*△200,*ade*2-101,*gal*4△,*gal*80△, *SPAL*10::*URA3*3,*GAL*1::lacZ,*HIS3*$3_{uas\ gal1}$::*HIS3*3@*LYS*2,can1^R ,cyh2^R		Invitrogen
yeast control strain A	MaV203+pPC97+pPC86	no interaction	Invitrogen
yeast control strain B	MaV203+pPC97–RB+pPC86-E2F1	weak interaction	Invitrogen
yeast control strain C	MaV203+pPC97–cyh2^s–dDP+pPC86–dE2F	moderate interaction	Invitrogen
yeast control strain D	MaV203+pPC97–Fos+pPC86–Jun	strong interaction	Invitrogen
yeast control strain E	MaV203+pCL1(full length Gal4) +pPC86	very strong interaction	Invitrogen
pPC86	Gal4 DNA-AD(activating domain), *Trp*1, Amp^r		Invitrogen
pDBLeu86	Gal4 DNA-BD(binding domain), *Leu*2, Km^r		Invitrogen
pPC86-FOXA3	Gal4 DNA-AD fusion of FOXA3, *Trp*1, Amp^r		This study
pDBLeu-FOXA3	Gal4 DNA-BD fusion of FOXA3,*Leu*2,Km^r		This study
pY	Gal4 DNA-BD fusion of a library plasmid, *Leu*2, Km^r		This study

2.2 Reagents and materials

ProQuest^{TM} Gal4 yeast two-hybrid system was purchased from Invitrogen company. Clontech Premium polyA+ RNA (Clontech, USA), and Creator SMART cDNA Library Construction Kit (Clontech, USA) were used to construct the Gal4-BD fused human liver cDNA library. The FOXA3 gene was PCR amplified from the Quick-Clone double-strand cDNA library (BD, USA). The clone reagents included: Taq enzyme and T4 DNA ligation kit (TaKaRa, Dalian, China), DNA restriction enzyme and pGEM-T vector (Promega, USA). Other yeast two-hybrid experiment reagent were SC/-Leu， SC/-Trp, SC/-Ura3-Trp-Leu (SC-UTL), SC/-His3-Trp-Leu (SC-HTL) amino acid dropout mix (Invitrogen, USA) and Yeast Extract, Bacto-Agar, Yeast Nitrogen Base(YNB)W/O amino acids (Difco, USA), Peptone , 3-AT (3-amino-1, 2, 4-triazole)， 212-300 μm beads (Sigma, USA), Tris, PEG3350 (Merck, Germany).

2.3 Construction of the yeast two-hybrid fused plasmids

The FOXA3 gene full-length coding sequence was gotten by using two primers:

P1：5’GAGTCGACGATGCTGGGCTCAGTGAAGA3’(the underlined sequence is *Sal* Ⅰ identification sequence).
P2：5’GCACTAGTCCTGCTAGGATGCATTAAGC3’(the underlined sequence is *Spe* Ⅰ identification sequence) through PCR amplification from the Quick-Clone double-strand cDNA library.

The FOXA3 nucleotide sequence’s accession number was NM_004497. PCR mixture was preheated at 94℃ for 4 min, and then thermal-cycled for 30 cycles (94 ℃ for 30 s, 64 ℃ for 30 s, and 72 ℃ for 2 min), and ended with a final extension at 72 ℃ for 5 min. The purified PCR product was ligated with pGEM-T vector, and then transformed the *E. coli* DH5α, prepared the FOXA3 ORF fragment through *Sal* Ⅰ and *Spe* Ⅰ digestion, and ligated the FOXA3 ORF fragment with pDBLeu and pPC86 yeast two-hybrid vector. Finally, the FOXA3 and BD, AD domains in a same reading frame were confirmed by sequencing.

2.4 Yeast transformation and self-activation test

The yeast strains were transformed as described by Gietz et al. (1992) though with some modifications. A single yeast strain MaV203 colony was inoculated into YPDA media and incubated with shaking at 30℃ overnight. A fraction of the yeast culture was transferred to fresh YPDA medium (initial OD_{600} = 0.01) and incubated at 30℃ for additional hours (usually 4~5 hours) until the OD_{600} = 0.4~0.5. The yeast cells were harvested and suspended with 1x TE/LiAc. The following plasmids were then added: pPC86-FOXA3+pDBLeu and pDBLeu-FOXA3+pPC86, ProQuest^{TM} Gal4 five control plasmids, salmon sperm DNA, and 50% PEG3350. These were incubated at 30℃ for 30 min, 42℃ for 15 min, and 30℃ for 15 min, then plated on the SC-TL culture plates. The plates were incubated for 48~72 hours at 30℃. Replica plates were produced from the SC-Leu-Trp master plate onto SC-UTL, SC-HTL+3AT, and YPDA plates coated with NC membrane. The SC-UTL and SC-HTL+3AT plates were replica cleaned with fresh velvet, and incubated for 24 h at 30℃.

The yeast was incubated at 3AT concentrations of 10, 20, 30, 40, 60, 80 mmol / L. After incubation, the replica clean procedure was repeated and the plates were incubated for 2-3 days (48~72 h) at 30℃.

After incubation for 24 h, yeast on the NC membrane could be assayed for expression of the reporter gene β-galactosidase through X-Gal solution.

2.5 Construction of the Gal4-BD fused human liver cDNA two-hybrid library

BD / Clontech Premium^{TM} adult human liver poly A+ RNA

was reverse transcribed using the PowerScript enzyme of the Creator™ SMART™ cDNA Library Construction Kit. Also included in the reverse transcription system was the SMART primer (5'-AAGCAGTGGTATCAACGCAGA-GTGGCCATTACGGCCGGG-3', the underlined sequence is *Sfi* Ⅰ recognition site). The PowerScript reverse transcriptase has TdT activity and can add several cytosines after finishing the RT reaction. These cytosines base-pair with the oligoguanosines in the SMART primer, so the full-length cDNA was produced containing the *Sfi* Ⅰ digesting site. This was then PCR amplified with Advantage2 Taq polymerase using the CDS primer (5'ATTCTAGAGGCCGAGGCGGCCGACATG-dT_{30}NN3', the underlined sequence is *Sfi* Ⅰ recognition site). The PCR profile was as follows: 95℃ 1 min, 95℃ 15 s, 68℃ 6 min for 20 cycles. The purifed PCR product was digested by *Sfi* Ⅰ (incubate at 50℃ for 2 h), passed through the CHROMA SPIN-400 isolated column, eluted with elution buffer, then ligated with NpDBLeu vector by T4 DNA ligase at 16℃ overnight. The *Sfi* recognition sequence has been added to the multi-cloning site of the NpDBLeu vector and it has been digeseted with the same enzyme. The ligation product was transformed into *E. coli* XL10-GOLD competent cells (Inoue et al., 1990), and produced according to Inoue's method. Resulting colonies were patched from the plate into a glycerol tube and stored at −80℃.

2.6 Screening of yeast two-hybrid

After estimating the titer of human liver cDNA yeast two-hybrid library, appropriate number of culture plates was streaked according to the titer ($3\sim4\times10^4$ colonies per 150mm plate). The plates were incubated at 30℃ for 36 h. The yeast cells were then harvested and the plasmids extracted via alkaline denaturation. The library plasmids were PCR amplified using the sequencing primers of pDBLeu vector to determine the diversity of inserted genes and the efficiency of recombination.

Human liver cDNA-BD two-hybrid library plasmids were transformed into the yeast competent cells containing pPC86-FOXA3 plasmid, and all aliquots of the transformation were plated on 30cm×15cm SC-HTL+3AT plates. At the same time, different titers of transformants were plated onto 3 SC-TL plates (counting plates) to estimate the efficiency of transformation. After incubation at 30℃ for 60 h, all the screening plates were replica cleaned with velvet until the no cell material was visible, then the plates were incubated at 30℃ for 48~60 h. After regrowth, all of the colonies were patched onto the SC-TL plates and incubated for 18 h, then these colonies were replicated onto SC-UTL,SC-UTL+3AT plates, and YPDA plates coated with NC membrane to test the expression of three reporter genes (*Ura*3，*His*3，*LacZ*). The colonies that can grow on the two nutrient deficient screening plates, and can cause blue coloration in the NC membrane in the colony-lift filter assay were considered positive colonies.

2.7 Preparation of positive yeast plasmids and sequence analysis

A single colony was inoculated into SD-Leu liquid media, incubated overnight at 30℃, and then harvested. Preparation of the yeast plasmids was as follows: The lysis buffer STET was added (8% glucose, 5% 1 mol/L pH 8.0 Tris-HCl, 10%0.5 mol/L EDTA, 5%Triton X-100), followed by addition of the same volume saturated phenol/ chloroform and 0.2 g 212~300 μm beaded glass. The solution was then vortexed for 10 min. The plasmids were precipitated using ethanol following two centrifugations. The extracted plasmids were dissolved with TE buffer. The yeast plasmid pY was transformed into *E. coli* DH5α strain, and plated on LB solid media containing kanamycin. The *E. coli* plasmids were extracted and sequenced with the sequencing primer of pDBLeu.

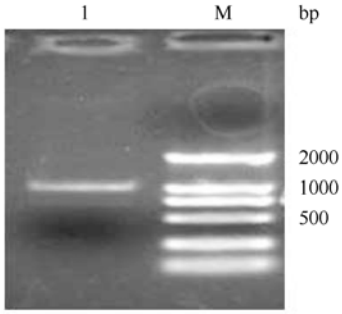
2.8 Co-transformation of the positive yeast plasmids

The plasmid pY, pPC86-FOXA3 and pPC86 were co-transformed into yeast strain MaV203 and plated on the SC-TL plates, followed by incubation for 48 h at 30℃. After colony growth, the colonies were picked and streaked onto fresh SC-TL plates. Five control strains (from very strong positive to negative control) were also streaked. Plates were incubated at 30℃ for 18 h. The expressions of three report genes were analyzed as a measure for self-activation.

3 Results

3.1 Clone of FOXA3 and confirmation

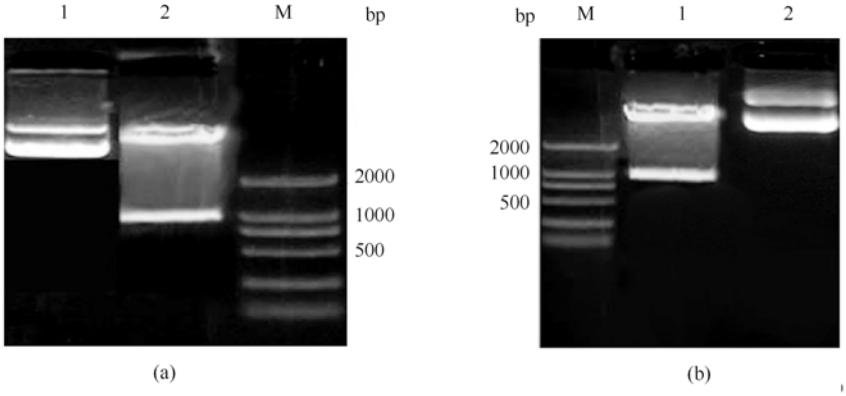
A 1.1 kb DNA band was amplified from the human liver Quick-Clone cDNA library by PCR, as the same length of the ORF fragment of FOXA3 gene (Fig.1). The PCR product was cloned into the pGEM-T vector, and then sub-cloned into the yeast two hybrid pPC86, pDBLeu vectors. The plasmids were



1: *FOXA3* cDNA; M: DNA ladder DL2000

Fig.1 PCR result of *FOXA*3 cDNA

confirmed by digestion with *Sal* Ⅰ and *Spe* Ⅰ (Fig. 2). DNA sequencing of the plasmido confirmed that the inserted gene was FOXA3 (1 053bp, coding 350 amino acids), and it is in the same reading frame with the Gal-AD, Gal-BD of the vectors.



(**a**) 1: pPC86-FOXA3 plasmid, 2: pPC86-FOXA3 digested by *Sa l* Ⅰ and *Spe* Ⅰ, M: DNA marker DL2000
(**b**) 1: pDBLeu-FOXA3 digested by *Sa l* Ⅰ and *Spe* Ⅰ, 2: pDBLeu-FOXA3 plasmid, M: DNA marker DL2000

Fig.2 The result of pPC86-FOXA3 and pDBLeu-FOXA3 digested by *Sa l* Ⅰ and *Spe* Ⅰ

3.2 Self-activation assay and concentration of 3AT test

The resulting colonies from transformation of pDBLeu-FOXA3(BD-FOXA3) and pPC86-FOXA3(AD-FOXA3) into yeast strain MaV203 were of normal size and color, indicating that FOXA3 expression is not harmful to yeast cells. The expression of reporter genes *Ura*3 and *His*3 could be observed by the growth of colonies on SC-UTL, SC-HTL+3AT plates (Table 2). The expression level of the reporter gene *LacZ* was analyzed by using X-Gal solution [Z buffer 2 mL (60 mmol / L, Na_2HPO_4, 40 mmol / L NaH_2PO_4,10mmol / L KCl , 1 mmol / L $MgSO_4$) and β-mercaptoethanol 0.27 mL, X-gal 33.4 μL (100 mg / mL)], which could determine the level of blue coloration in the colonies on the NC membrane.

Table 2 Test of *Ura*3 and *Lac*Z report genes for auto-activation of FOXA3

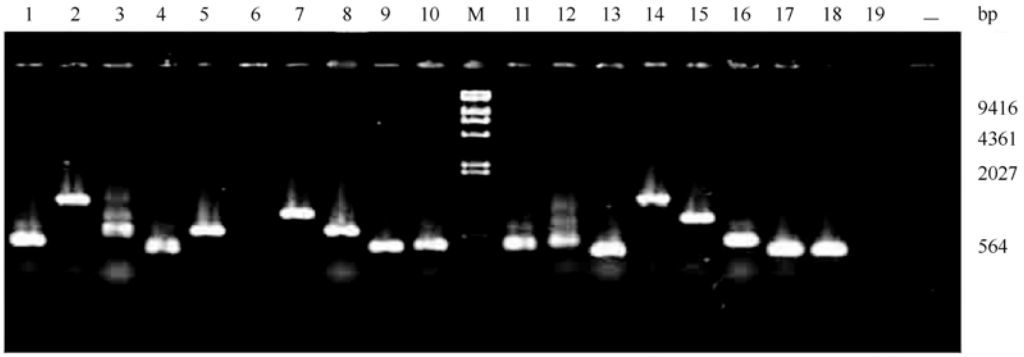
Yeast strain and plasmids	function	*Ura*3 (condition of growth)	*LacZ* (color of colonies)	*His*3 /(mmol·L^{-1}) 3AT					
				10	20	30	40	60	80
MaV203/BD-FOXA3+AD	Self-activatation test	+	blue	+	+	+	+	+	+
MaV203/AD-FOXA3+BD	Self-activatation test	+	white	+	+	±	−	−	−
control strain A	negative control	−	white	+	−	−	−	−	−
control strain B	Weak positive control	−	blue	+	+	+	+	−	−
control strain C	Middle positive. Control	+	blue	+	+	+	+	+	±
control strain D	strong positive control	+	blue	+	+	+	+	+	+
control strain E	Very strong positive control	+	blue	+	+	+	+	+	+

Note: AD meant pPC86, BD pDBLeu, ＋ grow, ± grow weakly, — not grow, blue expression of reporter gene *LacZ*, white no expression

From Table 2, we conclude that fusing the bait gene with Gal4 BD domain following the normal method resulted in self activation of the vector pDBLeu-FOXA3 + pPC86, and expression of the three *Ura3*，*LacZ*，*His*3 reporter genes, even if the concentration of 3AT was 80 mM. At this concentration, the middle strong positive control strain C grew weakly. pDBLeu-FOXA3 + pPC86 could also transactivate the *His*3 gene, allowing the colonies to grow normally. Thus, it is not possible to fuse FOXA3 to Gal4 BD to screen for interacting proteins. We changed the strategy and fused the bait gene to Gal4 AD, and examined if the pPC86-FOXA3 + pDBLeu could also self-activate. Results indicated that the FOXA3-AD plasmid could not transactivate the reporter genes. When the concentration of 3AT was 30 mM, the yeast transformants grew weakly. The growth of colonies was completely inhibited at an AT of 40 mM, and this concentration was used to screen the library.

3.3 Construction of Gal4-BD fused human liver cDNA library

Human liver mRNA was reverse transcribed, PCR amplified, and ligated to the NpDBLeu vector contatining Gal4 BD. Transformation of *E. coli* XL10-GOLD yielded over 3×10^6 transformants, which was sufficient for construction of a yeast two-hybrid library. One μL colonies were re-suspended and patched onto plates. The plasmids were extracted after colony growth and PCR was performed to determine the efficiency of recombination. Figure 3 shows that the inaserted fragments in the library vector were very diverse (Fig. 3). Nineteen plasmids were selected randomly from the libray and PCR assayed. Seventeen lanes contatined the PCR products, indicating that the transformation efficiency was above 90%.

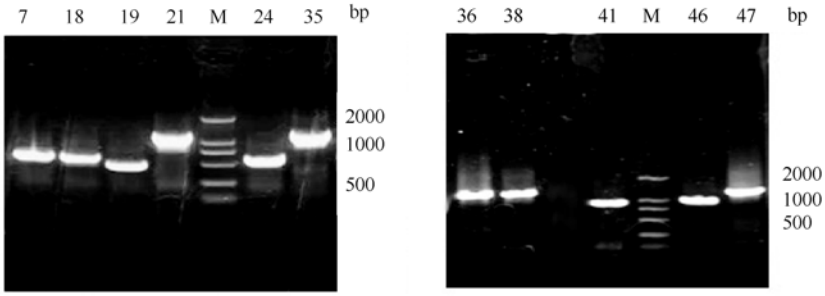


1-19: the plamids of cDNA library chosen by random;—: the negative control, M: λDNA/Hind III marker

Fig. 3 Agarose gel electrophoresis of PCR products from the human liver cDNA-BD yeast two-hybrid library plasmid inserts

3.4 Yeast two-hybrid screening

Following large-scale plasmid preparation using the alkaline denaturation method, we transformed the library plasmids into yeast strain MaV203 containing the pPC86-FOXA3 plasmid. The number of colonies on the counting plates indicated a total of 0.8×10^6 transformants. In total, there were 104 positive candidates following replication of clean plates and incubation for 72 h. The candidates colonies were patched onto SC-TL plates, and then replica plated from the SC-TL master plates onto SC-UTL, SC-HTL+3A plates and YPDA plates coated with NC membrane. Twenty-eight positive colonies were found to activate the three reporter genes (*Ura*3、*LacZ* and *His*3). The 28 positive colonies contained pPC86-FOXA3 and pY (library plasmid, NpDBLeu vector, with kanamycin resistance). These plasmids were extracted, subjected to PCR amplification (Fig. 4) and sequenced. The protein sequences in the three reading frames were obtained from the DNA sequence with the biological computer program Primer Premier 5. The protein sequence in frame with the BD domain of NpDBLeu vector was subjected to BLASTp search (http://www.ncbi.nlm.nih.gov) to find sequence. Excluding false positives, such as collagen, we got a candidate interacting protein: complement component 3 which might interact with FOXA3 protein.

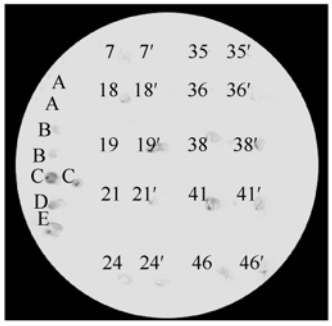


7-47: the candidate positive pre; —: negative control, M: DL2000 DNA marker

Fig. 4 A electrophoresis of PCR products from the pY plasmid inserts

3.5 Co-transformation confirmation assay

The plasmids pY and pPC86-FOXA3, pY and pPC86 plasmids were co-transformed respectively into the yeast strain MaV203. pY+pPC86-FOXA3 was used as confirmation assay for protein interaction, and pY+pPC86 served as a self-activation test for the positive colonies. Colonies were patched onto SC-TL plates following colony growth, then replica was plated and cleaned following the screening procedure mentioned above. As expected, the interacting pair passed the confirmation assay for the reporter genes *Ura3,His3* and *LacZ* (Table 3 and Fig. 5). The 18th colony contained complement component 3 gene (Fig. 5).



7-46:the LacI assay of pasitive preys;
7′-46′:the seH-activcaion assay of positive preys;
A-E:yeast controt strains

Fig.5 Colony-lift filter assay for β-LacZ activity of yeast transformed with two plasmids

4 Discussion

Proteins carry out the function of genes in the cell, and protein-protein interactions are ubiquitous in all kinds of cell activities including the regulation of gene expression, signal transduction, regulation of metabolism, proliferation, differentiation, apoptosis, and so on(Blackstock and Weir 1999). The yeast two-hybrid approach is a popular tool in investigating protein-protein interactions. Proteins obtained through yeast two-hybrid screening often associate closely with the bait protein, and possibly function cooperatively in cell processes. The yeast two-hybrid system can help us understand the functional mechanisms of proteins, especially how these proteins are associated with disease. Although the yeast two-hybrid system has obvious potential in the study of protein interactions and setting up the network of proteins interaction, not all proteins can be screened using this method. Most researchers use the regular method of fusing the bait gene with the BD vector and screening a cDNA library fused with the AD vector. However, this method does not work for some proteins, such as some nucleoprotein transcription factors containing the activating domain because they can self-activate the reporter gene. In this study, the strategy is modified, with the bait gene (FOXA3) being now fused to the AD vector instead of BD to bypass the interference of self-activation. The bait-AD vector is used to screen a library fused with BD. However, such a two-hybrid library is not available on the market. So, our laboratory constructed a human liver cDNA yeast two-hybrid library based on the Invitrogen's GIBCO Gal4 two-hybrid system. This strategy would be advantageous in studying certain kinds of proteins.

In the study, we used FOXA3 as bait to seek its interacting proteins. From 28 positive colonies, we obtained an interacting protein (complement component 3 or C3) by co-transformation confirmation. The C3 gene contains 41 exons and is an important immunological regulatory protein(Fong et al., 1990). Being an immune-related protein, C3 was cloned a long time ago, but recent studies have discovered its novel functions. Ylitalo et al. discovered the role of C3 in fat and sugar metabolism by studying Finland familial combined hyperlipidemia patients(Ylitalo et al., 2002). Faraj et al. (2003)reported that C3 was involed in the regulation of sugar metabolism by insulin from studies with morbidly obese subjects. In 2001, Shen et al. reported that FOXA3 functioned in the maintenance of glucose homeostasis and gluconeogenesis(Shen et al., 2001). In 2004，Cianflone et al. (2004) found out C3 joins the insulin pathway and regulation of blood sugar in the hungry stage. All of these suggest a functional co-relation between FOXA3 and C3. In this study, we discovered via a yeast two-hybrid experiment that FOXA3 and C3 interacted directly, suggesting that FOXA3 can works together with C3 in the regulation of sugar metabolism.

In conclusion, we improved the yeast two-hybrid strategy and constructed a human liver cDNA yeast two-hybrid library containing binding domain. We reported an interacting protein for FOXA3 by screening the two-hybrid library.

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