TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 523-527
© TÜBİTAK
doi:10.3906/biy-1403-38

Expression and purification of porcine *PID1* gene in *Escherichia coli*

Huan WANG, Xiaoling CHEN*, Zhiqing HUANG, Bo ZHOU, Gang JIA, Guangmang LIU, Hua ZHAO
Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, Sichuan, P. R. China

Received: 14.03.2014 • **Accepted:** 17.04.2014 • **Published Online:** 11.06.2014 • **Printed:** 10.07.2014

Abstract: In this study, in order to scale up the production of recombinant porcine phosphotyrosine interaction domain containing 1 (pPID1), a pET-28a (+)-pPID1 expression plasmid was constructed and transformed into *Escherichia coli* Rosetta (DE3). The recombinant pPID1 was then purified and identified by western blotting, and was also analyzed in vitro for its function. The recombinant protein was tagged with only a His6 tag at its C-terminus, which could be conveniently purified by affinity column. The protein could be induced for efficient expression with 0.75 mM IPTG for 8 h at 30 °C, yielding approximately 3 mg/L. In vitro biological activity assay demonstrated that the refolded purified recombinant pPID1 increased 3T3-L1 preadipocyte proliferation. This study provides a reliable technique for the recombinant expression and purification of pPID1 proteins.

Key words: Porcine *PID1*, *Escherichia coli*, expression and purification, identification, 3T3-L1 preadipocytes

1. Introduction

Meat quality in domestic animals is an important economic trait. The quality and the value of the carcass in domestic meat animals are reflected as the ratio between protein and fat content in a carcass. Preadipocytes and adipocytes are important in establishing the overall fatness of a carcass (Hausman et al., 2009). Therefore, it is necessary to identify candidate genes that might contribute to fat deposition, which may help to improve the meat quality.

The phosphotyrosine interaction domain containing 1 (*PID1*, also named *NYGGF4)* was first isolated and characterized from human adipose tissue using suppression subtractive hybridization in 2006 (Wang et al., 2006). There is growing evidence that *PID1* can affect fat cell development in mice and plays a major role in obesity-related insulin resistance (Qiu et al., 2007; Chen et al., 2012; Yu and Guo, 2012). However, very little research has been conducted on the role of porcine *PID1* (*pPID1*). The 3T3-L1 preadipocytes have proven to be a well-characterized in vitro model for the study of adipogenesis (Green and Kehinde, 1974). Qian et al. (2010) reported that *pPID1* mRNA expression level was positively correlated with 3T3-L1 fat content. In our previous study, we reported that *pPID1* modulates 3T3-L1 preadipocytes' proliferation and differentiation (Chen et al., 2013), showing that it may be a functional candidate gene for improving pork quality.

In this study, we report the successful expression of *pPID1* in *E. coli* Rosetta (DE3). The recombinant pPID1 was purified by affinity column and identified by western blotting. Its effect on 3T3-L1 preadipocytes proliferation was also examined. These results will provide a foundation for understanding the function of the porcine *PID1* gene.

2. Materials and methods

2.1. Strains, vectors, and analytical reagents

E. coli DH5α was purchased from TIANGEN Biotech (China) and was used as the host-vector system. *E. coli* Rosetta (DE3) strains were used as the hosts for protein expression. pET-28a(+) plasmid was used to construct the expression vector. Kanamycin sulfate, UNIQ-10 spin column DNA gel extraction kits, and Ni-IDA purification systems were purchased from Sangon Corporation (China). The restriction enzymes and DNA ligation kit were obtained from TaKaRa (China). All DNA markers, 2X Taq PCR master mix, and protein molecular weight marker (low) were purchased from TIANGEN Biotech. The prestained protein molecular weight marker was purchased from Beyotime Company (China). All primers used in this study were synthesized by Sangon Corporation. The monoclonal anti-His (C-term) antibody was from Invitrogen (USA) and the horseradish peroxidase (HRP) conjugated goat antimouse IgG was from Santa Cruz Biotechnology Inc. (USA).

2.2. Plasmid construction for *E. coli* expression

The *pPID1* gene (GenBank accession no. KC524726) was amplified by PCR with the specific primers PIDxR-F (5'-GCTCTAGA*AAAGGAG***ATATA**

* Correspondence: xlchen@sicau.edu.cn

ATGTGGCAGCCGGCC-3') and PIDxh-R (5'-CCGC TCGAGGCCATCATCGGATTC-3') (the restriction enzyme sites *Xba* I and *Xho* I are underlined). The PCR primer sequence was designed to add a ribosome binding site (italicized) and a spacer (bolded) upstream of the *pPID1* gene. The plasmid pcDNA3.1(+)-pPID1 kept in our laboratory, which contained the *pPID1* cDNA, was used as a template. The amplified PCR products were gel-purified, double-digested with *Xba* I and *Xho* I, and ligated into the pET-28a(+) expression vector, resulting in the recombinant plasmid pET-28a(+)-pPID1. Theoretically, the pET-28a(+)-pPID1 vector can express a fusion protein corresponding to the *pPID1* carrying the extra C-terminal sequence His6 tag.

2.3. Expression of recombinant pPID1 protein in *E. coli*

To obtain the recombinant pPID1 protein, the recombinant plasmid pET-28a(+)-pPID1 was transformed into *E. coli* Rosetta (DE3). The empty plasmid pET-28a(+) was used as a control. The strain with the recombinant plasmid was inoculated into 5 mL of LB medium supplemented with 50 μg/mL kanamycin and cultured overnight at 37 °C. The resulting seed culture was then transferred into 50 mL of fresh LB medium containing 50 μg/mL kanamycin in a 250-mL flask. When the optical density OD_{600} reached approximately 0.6, the expression of protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) under certain conditions for any specific purpose. The cells were collected by centrifugation and resuspended in 0.1 M PBS buffer (pH 7.4) containing 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride. The harvested cells were disrupted by sonication. The supernatant and pellet fractions were fractionated by centrifugation at 12,000 × *g* for 30 min at 4 °C and analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Quantitative evaluation of Coomassie-stained protein bands was carried out using the Bio-Rad ChemiDoc XRS imaging system equipped with Quantity One software (Bio-Rad, USA).

2.4. Purification and refolding of recombinant pPID1 protein

Proteins were purified by Ni-IDA affinity chromatography based on the manufacturer's protocol for protein purification under denaturing conditions (QIAGEN, Germany). The purity of the collected samples was verified by 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue R250. The protein concentration was determined using the BCA protein assay kit (Pierce, USA). The purified protein was then step-by-step dialyzed against 0.1 M PBS (pH 7.4) containing 6, 4, 2, and 0 mol/L urea at 4 °C for 24 h.

2.5. Western blot assay

Western blot analysis was performed as described by Huang et al. (2007). Briefly, after 12% SDS-PAGE electrophoresis, the proteins were transferred onto a nitrocellulose membrane using the Semi-Dry Trans-Blot System (Bio-Rad). The membrane was incubated for 1 h in a blocking buffer [3% dried nonfat milk (w/v) in TBST (20 mM Tris-base, 8 g/L NaCl, 0.05% Tween-20, pH 7.6)]. After washing 3 times for 10 min each with TBST buffer, the membrane was then incubated with the monoclonal anti-His (C-term) antibody overnight at 4 °C. The membrane was then incubated for 1 h with HRP-conjugated goat antimouse IgG at room temperature after being thoroughly washed. The specific protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to manufacturer's protocol.

2.6. In vitro assays for biological activity

The 3T3-L1 preadipocytes (ATCC CL-173) were grown in a 5% CO_2 incubator at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, and 100 μg/L streptomycin (Invitrogen). The cultured cells were seeded in a 24-well plate at a density of 1×10^4 cells/well. After 48 h, the medium was removed and replaced with DMEM/0.5% FBS supplemented with different concentrations (0–8 μg/mL) of refolded purified recombinant pPID1. Proliferating 3T3-L1 cells were analyzed after 24 h of recombinant *pPID1* treatment by using the Click-iT EdU (5-ethynyl-2'-deoxyuridine) Alexa Fluor 594 Imaging Kit (Invitrogen) according to the manufacturer's instruction. All results are expressed as mean ± standard error. Statistical analysis of the significance was performed by one-way ANOVA and Tukey's tests using SPSS 11.5. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Cloning of *pPID1* gene and construction of expression vector

Full-length *pPID1* cDNA was obtained by RT-PCR using recombinant plasmid pcDNA3.1(+)-pPID1 as a template and was cloned into expression vector pET28a(+) between the *Xba* I and *Xho* I restriction sites. The recombinant plasmid pET28a(+)-pPID1 was confirmed by colony PCR and sequencing. The construct of the expression vector is shown in Figure 1. The recombinant pET28a(+)-pPID1 plasmid encoding pPID1 with a His6 tag in the C-terminal was used for heterologous expression of the protein in the *E. coli* Rosetta (DE3) strain.

3.2. Optimization of recombinant pPID1 expression and solubility in *E. coli*

To optimize the expression of recombinant pPID1, the growth temperature, the IPTG concentration, and the time after induction by IPTG were varied. The cell lysates obtained under different conditions were separated into soluble and insoluble fractions by centrifugation

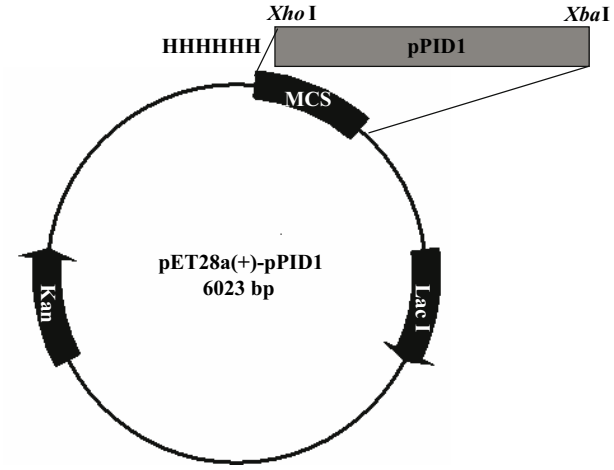

Figure 1. Map of the pET28a(+) expression vector and the recombinant pPID1 construct [pET28a(+)-pPID1]. This vector allows C-terminal fusion of recombinant protein with a His6 tag. The *Xba* I restriction site is located at the 5'-end of the DNA fragment and a ribosome binding site is behind the *Xba* I restriction site. The *pPID1* gene was cloned between the *Xba* I and *Xho* I restriction sites, in frame with the His6 tag. The gene is under the control of a T7 promoter, and the plasmid contains a kanamycin-resistant gene for selection and maintenance of the recombinant construct.

and were analyzed by SDS-PAGE. As shown in Figure 2, insoluble recombinant protein with a molecular mass of approximately 26 kDa, consistent with the size of the predicted recombinant pPID1 protein, was effectively expressed in the *E. coli* Rosetta (DE3) when the culture was induced with 0.75 mM IPTG for 8 h at 30 °C.

3.3. Purification and identification of recombinant pPID1

The expression of recombinant pPID1 was performed at 30 °C for 8 h of induction with 0.75 mM IPTG. The protein was loaded into a Ni-IDA affinity column to bind the polyhistidine-tagged recombinant pPID1, washed with 20 mM imidazole, and eluted with 250 mM imidazole. The purity of recombinant pPID1 was greater than 90% (Figure 3A) and approximately 3 mg/L of pure recombinant pPID1 was obtained. The protein was further confirmed by western blotting analysis using the monoclonal anti-His (C-term) antibody (Figure 3B).

3.4. In vitro activity of the recombinant pPID1 protein

The purified recombinant pPID1 protein was renaturated in suspension as described (Chen et al., 2008). In order to determine whether recombinant pPID1 protein was functionally active, we used the in vitro 3T3-L1 cellular assay. As shown in Figure 4, compared with the control group, the recombinant pPID1 protein accelerated proliferation of 3T3-L1 preadipocytes.

4. Discussion

E. coli has been used extensively as the host for foreign protein expression due to its rapid growth rate, capacity for continuous fermentation, and relatively low cost. The pET28a(+) vector is used for T7 promoter-driven expression of recombinant proteins and the expression system is by far the most commonly used system. However, it contains the addition of a 19 amino acid N-terminal fusion tag with a His6 tag, followed by a thrombin protease cleavage site and 2 stop codons in the vector at the C-terminal cloning site. In the present study, we constructed the recombinant plasmid containing the *pPID1* gene with only a His6 tag fusion partner in the C-terminal while still conserving the His tag, which would be useful for purification purposes. The recombinant pPID1 was successfully expressed in *E. coli*. The recombinant pPID1 protein was identified by western blotting analysis, indicating that the expressed protein was the target protein.

There is considerable evidence suggesting that N-terminal fusion His tags cause a drop in peptides' activities (Huang et al., 2008; Zhao et al., 2011). By contrast, other studies suggest that the N-terminal is not crucial for the activities of fusion proteins (Franklin and Clarke, 2001; Wu et al., 2011). In this study, the recombinant pPID1 only has a His6 tag at its C-terminus. We analyzed its function in an in vitro cellular assay using 3T3-L1 preadipocytes. The result showed that recombinant pPID1 could significantly promote proliferation of the 3T3-L1 preadipocytes, which was similar to the findings of Chen et al. (2013). Our data suggested that the refolded purified recombinant pPID1 protein was active.

In summary, the *E. coli* expression system has been developed to produce recombinant pPID1 with only a His6 tag fusion partner at its C-terminal. The refolded purified recombinant pPID1 could promote the proliferation of 3T3-L1 preadipocytes. This study provides a basis for further functional analysis of the *PID1* in vivo and the underlying mechanism of the effects of *PID1* on the intramuscular fat deposits.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31201811) and the Specific Research Supporting Program for Discipline Construction in Sichuan Agricultural University.

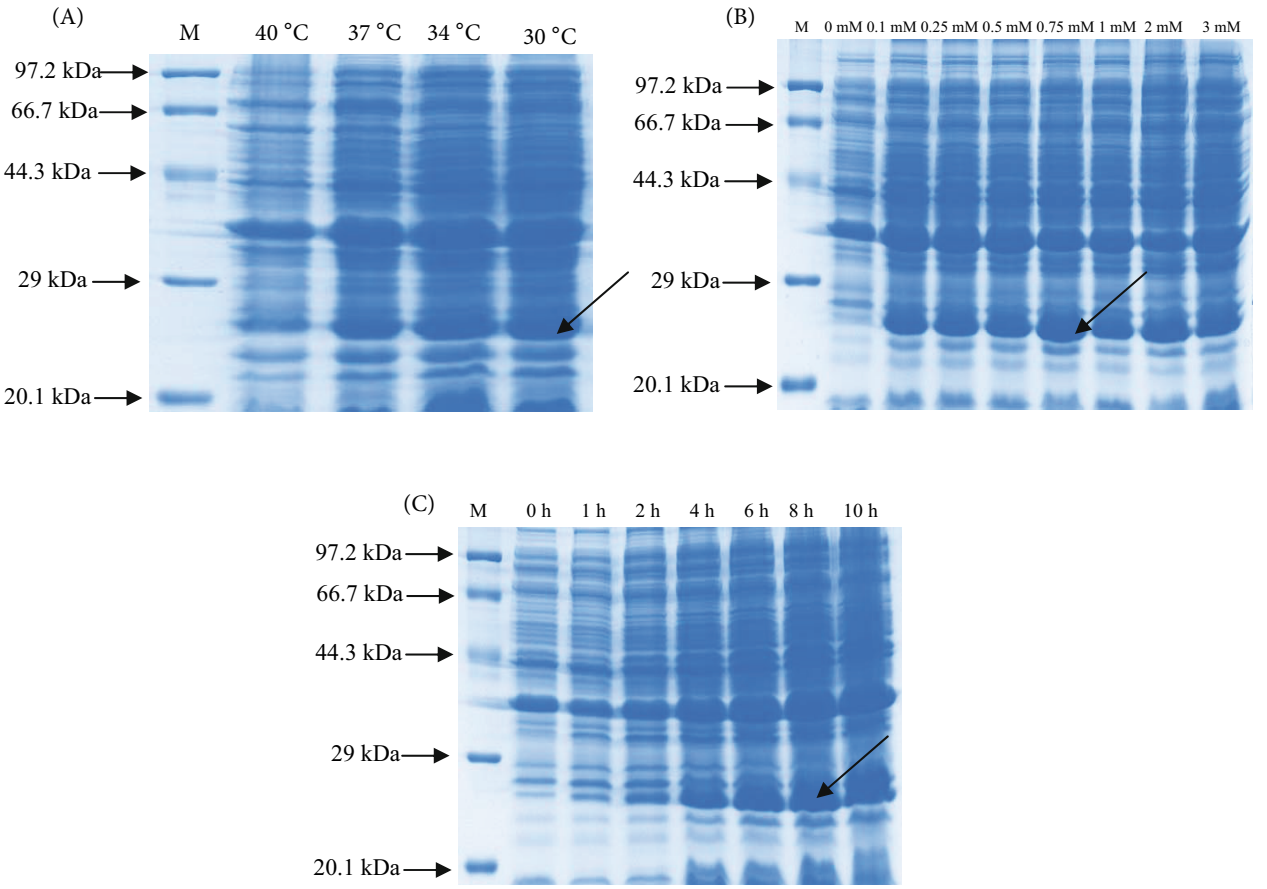

Figure 2. Optimization of the induction conditions for the expression of recombinant pPID1 in *E. coli*. A) Optimization of induction temperature. The *E. coli* Rosetta (DE3)/pPID1 expression system was induced for 4 h at 30 °C, 34 °C, 37 °C, and 40 °C, respectively. B) Optimization of IPTG concentrations. Final IPTG concentrations of 0, 0.1, 0.25, 0.5, 0.75, 1, 2, and 3 mM were added to each tube and inducted 4 h at 30 °C. C) Optimization of induction time. The bacterial culture harboring the pET28a(+)-pPID1 expression plasmid was induced at 0.75 mM IPTG and incubated for 0, 1, 2, 4, 6, 8, and 10 h at 30 °C, respectively. M: Protein size markers. The arrow indicates the target protein.

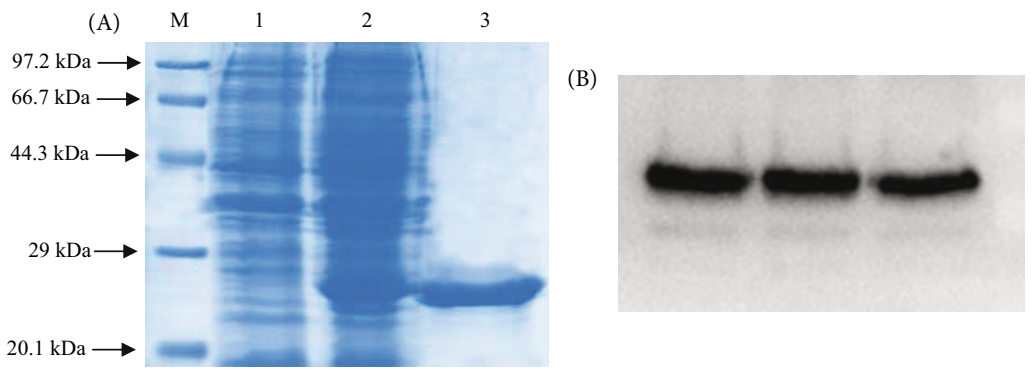

Figure 3. Purification and identification of the recombinant pPID1 protein. A) pPID1 was purified by Ni^{+}-IDA column and purified pPID1 was separated on SDS-PAGE and stained with Coomassie Blue R250. Lane M: Protein size markers; Lane 1: control (–) pET-28a (+); Lane 2: recombinant protein induced with IPTG; Lane 3: purified pPID1 protein. B) The purified pPID1 protein was detected by western blotting analysis using monoclonal anti-his (C-term) antibody.

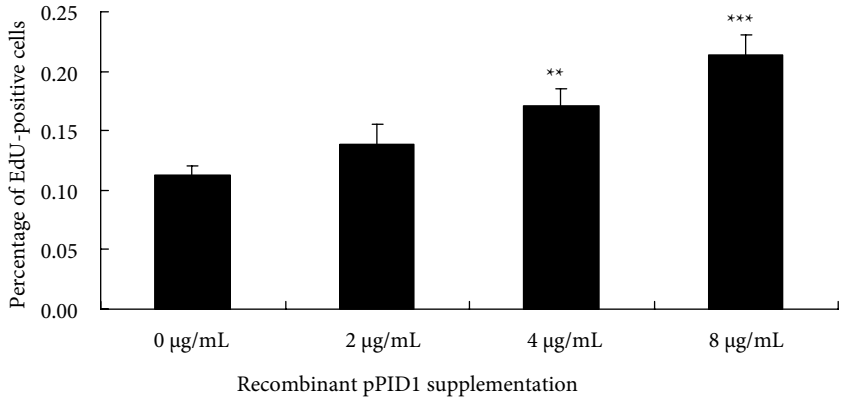

Figure 4. Effect of recombinant pPID1 on 3T3-L1 preadipocytes' proliferation. 3T3-L1 preadipocytes were cultured in DMEM/10% FBS medium for 48 h and subjected to treatment in DMEM/0.5% FBS media by adding 0 μg/mL (control), 2 μg/mL, 4 μg/mL, or 8 μg/mL of recombinant pPID1. Cell proliferation was evaluated by EdU proliferation assay after 24 h of recombinant pPID1 treatment. The percentage of EdU-positive 3T3-L1 preadipocytes was quantified. Results are presented as mean ± standard error (n = 6). **: $P < 0.01$, ***: $P < 0.01$, as compared with the control.

References

Chen X, Huang Z, Chen D, Jia G, Mao X, Wu X (2012). Role of NYGGF4 in insulin resistance. Mol Biol Rep 39: 5367–5371.

Chen X, Huang Z, Wang H, Jia G, Liu G, Zhao H, Nie H (2013). Porcine phosphotyrosine interaction domain containing 1 modulates 3T3-L1 preadipocyte proliferation and differentiation. Biologia 68: 1010–1014.

Chen X, Lu W, Cao Y, Li D (2008). Prokaryotic expression, purification and characterization of *Aspergillus sulphureus* β-mannanase and site-directed mutagenesis of the catalytic residues. Appl Biochem Biotech 149: 139–144.

Franklin K, Clarke AJ (2001). Overexpression and characterization of the chromosomal aminoglycoside 2'-N-acetyltransferase of *Providencia stuartii*. Antimicrob Agents Ch 45: 2238–2244.

Green H, Kehinde O. Sublines of mouse 3T3 cells that accumulate lipid. Cell 1974; 1: 113–116.

Hausman GJ, Dodson MV, Ajuwon K, Azain M, Barnes KM, Guan LL, Jiang Z, Poulos SP, Sainz RD, Smith S et al. (2009). Board-invited review: the biology and regulation of preadipocytes and adipocytes in meat animals. J Anim Sci 87: 1218–1246.

Huang L, Ching CB, Jiang R, Leong SS (2008). Production of bioactive human defensin 5 and 6 in *Escherichia coli* by soluble fusion expression. Protein Expres Purif 61: 168–174.

Huang ZQ, Chen DW, Zhang KY, Yu B, Chen XL, Meng JH (2007). Regulation of myostatin signaling by c-Jun N-terminal kinase in C2C12 cells. Cell Signal 19: 2286–2295.

Qian Y, Zeng YQ, Du JF, Cui JX, Li H, Chen QM, Song YP, Chen W (2010). CDS cloning and relationship between intramuscular fat content and mRNA expression of *PID1* gene in pig. Hereditas 32: 1153–1158.

Qiu J, Ni Y, Gong H, Fei L, Pan X, Guo M, Chen R, Guo X (2007). Identification of differentially expressed genes in omental adipose tissues of obese patients by suppression subtractive hybridization. Biochem Bioph Res Co 352: 469–478.

Wang B, Zhang M, Ni YH, Liu F, Fan HQ, Fei L, Pan XQ, Guo M, Chen RH, Guo XR (2006). Identification and characterization of *NYGGF4*, a novel gene containing a phosphotyrosine-binding (PTB) domain that stimulates 3T3-L1 preadipocyte proliferation. Gene 379: 132–140.

Wu J, Wang C, He H, Hu G, Yang H, Gao Y, Zhong J (2011). Molecular analysis and recombinant expression of bovine neutrophil b-defensin 12 and its antimicrobial activity. Mol Biol Rep 38: 429–436.

Yu Z, Guo X (2012). NYGGF4 as a new therapeutic target for obesity-associated insulin resistance. Med Hypotheses 78: 432–434.

Zhao T, Lv J, Zhao J, Huang X, Xiao H (2011). Expression of human globular adiponectin-glucagon-like peptide-1 analog fusion protein and its assay of glucose-lowering effect in vivo. Int J Med Sci 8: 203–209.