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Molecular Cloning and Expression of Forkhead Transcription Factor O1 Gene from Pig *Sus scrofa*

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ABSTRACT : Foxo1 plays an important role in the integration of hormone-activated signaling pathways with the complex transcriptional cascade that promotes preadipocyte differentiation of clonal cell lines from rodents. We isolated the full-length cDNA of porcine FoxO1 gene using RACE, confirmed by visual Northern blotting. The deduced amino acids indicated 94% and 90% identities with the corresponding human and mice aa. Analysis of the aa sequence, showed that it included a Forkhead domain (aa 167-247), a transmembrane structure domain (aa 90-113), a LXXLL motif (aa 469-473), and 51 Ser, 8 Thr, and 4 Tyr phosphorylation sites, indicating a potential important role for FoxO1 transcriptional activity *in vivo*. Using the IMpRH panel, we mapped FoxO1 gene to chromosome 11p13. Our data provide basic molecular information useful for the further investigation on the function of FoxO1 gene. Time-course analysis of FoxO1 expressions indicated that levels of mRNA and protein gradually increased from day 0 to 3, and it reached almost maximal level at day 3, then decreased from day 5 to 7 in porcine primary preadipocyte differentiation. After induction by IGF-1, GPDH activity and accumulation of lipid increased, however, expressions of FoxO1 mRNA and protein were inhibited in a dose dependent manner. These results suggest that FoxO1 takes part in porcine preadipocyte differentiation and expressions of FoxO1 were regulated by IGF-1. (**Key Words :** Pig, Forkhead Transcription Factor O1, Gene Cloning, Expression, Preadipocyte, IGF-1)

INTRODUCTION

Foxo1 is a member of the evolutionarily conserved Foxo subfamily of forkhead transcription factors (Kaestner et al., 2000; Birkenkamp et al., 2003), which are thought to be important in mediating effects of insulin and growth factors on glucose homeostasis and processes such as cell differentiation (Nakae et al., 2003), glucose metabolism (Nakae et al., 2002; Daitoku et al., 2003), cell-cycle regulation (Stahl et al., 2002), apoptosis (Dijkers et al., 2002), and oxidative stress resistance (Kops et al., 2002). Foxo1 is expressed in tissues involved in energy metabolism such as liver, muscle, and adipose tissue, where its function is inhibited by insulin and insulin-like growth factor 1 (Farmer et al., 2003). However, the sequence and characterization of porcine FoxO1 gene have been remained poorly understood.

Members of the forkhead box O (Foxo) family of transcription factors, including Foxo1 (previously described as FKHR), Foxo3a (FKHRL1), Foxo4 (AFX), and Foxo6, are phosphorylated by insulin/IGF-1 through a PI3K/Akt-dependent pathway and excluded from nucleus to inhibit their transcriptional activity (Accili et al., 2004). Foxo1 cause lipid metabolism through induction of G6Pase, apoC-III, and Igfbp-1 expression (Nakae et al., 2001; Nakae et al., 2002; Altomonte et al., 2004). Phosphorylation of three highly conserved protein kinase B sites, corresponding to Thr_{24}, Ser_{256}, and Ser_{319} in human FOXO1, suppresses transactivation and promotes nuclear exclusion of FOXO proteins by multiple mechanisms (MacDougald et al., 1995).

The process of adipocyte differentiation has been extensively characterized in cultures of clonal cell lines of preadipocytes, such as mouse 3T3-L1 and 3T3-F442A (Gregoire et al., 1998; Rosen et al., 2000). *In vitro* adipogenesis requires a sequence of events, including growth arrest of proliferating preadipocytes, coordinated reentry into the cell cycle with limited clonal expansion, and growth arrest associated with terminal differentiation. These processes are accompanied by characteristic changes in gene expression. Foxo1 plays an important role in coupling insulin signaling to preadipocyte differentiation (Nakae et al., 2003).

Although sequences and functions of FOXO1 and Foxo1 in humans and rodents have been revealed, porcine FoxO1 has not been reported hitherto. In the present

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Table 1. The major relevant primers used in these experiments

Name	Sequences	Length (nt)
5'-CDS1	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'	30
5'-CDS2	5'-$(T)_{25}$N-1N-3'	27
3'-CDS	5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN-3'	57
UPMS	5'-CTAATACGACTCACTATAGGGC-3'	22
UPML	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'	45
FP1	5'-GCTCAGTCCACGTCTGCCGTCAATG- 3'	25
FP2	5'-GTCATTATGGGGAGGAGAGTCGGAAGT-3'	27
LPF	5'-CAGACCGTCCCTTCGCACC- 3'	19
LPR	5'-GCAGCGTCGGGGTTGGCG- 3'	18
OligodT*Sal*IA	5'-CTGCGCCAGAATTGGCAGGTCGAC$(T)_{25}$V-3'	50
OligodT*Sal*IB	5'-CTGCGCCAGAATTGGCAGGTCGAC-3'	24
CapFinderB1	5'-GAGAGAACGCGTGACGAGAGACTGACAGGGGGGGGH-3'	36
CapFinderB2	5'-GAGAGAACGCGTGACGAGAGACTGACAG-3'	28
FP3F	5'-GGCAGCCCTGATTCTTCTAC-3'	20
FP3R	5'-GCCCAGATGTTGCGTAAGTC-3'	20
FP4F	5'-GCAAATCGAGTTACGGAGGC-3'	20
FP4R	5'-AATGTCATTATGGGGAGGAGAGT-3'	23
APF	5'-GCGGCATCCACGAAACTAC-3'	19
APR	5'-TGATCTCCTTCTGCATCCTGTC-3'	22

H = A, C, or T; V = A, G or C, and N = A, C, G, or T. UPM = UPML (0.4 μM)+UPMS (2 μM).

experiment, we got the full-length cDNA for porcine FoxO1 gene, analyzing the putative FoxO1 aa sequence, and mapping FoxO1 gene to porcine chromosome set. We also investigated time-course expression of FoxO1 in procine preadpocytes induced by IGF-1. The successful cloning and time-course expression of FoxO1 will enhance the understanding of the involvement of the gene in regulating differentiation of preadipocytes in pigs.

MATERIALS AND METHODS

Animals and tissue collection

Male crossbred pigs (Duroc×Yorkshire×Landrace) were from the experimental farm of Northwest A&F University. Dorsal subcutaneous adipose tissue was collected from 180-day-old healthy animals. All the samples were immediately homogenized in liquid nitrogen and total RNA was isolated according to the manufacturer's instruction of TRIZOL® reagent (Invitrogen). The total RNA was treated with DNase I. Animals were cared for according to the recommended code of practice and killed using an acceptable method approved by the local Animal Care Committee following the guideline.

Cloning the full-length cDNA of porcine FoxO1

A cDNA library was constructed from the adipose tissues of piglet at day 180, using the SMART™ RACE cDNA Amplification Kit (CLONTECH). 5'-CDS1 and 5'-CDS2 were used for getting 5'-RACE-Ready cDNA, 3'-CDS for 3'-RACE-Ready cDNA (Table 1).

A special PCR primer FP1 was designed based on the fragment from pig (designed according to accession no. DQ673620). The PCR reaction to get 3'-end was performed by using FP1 and universal primer UPM with the 3'-RACE-Ready cDNA library mixture as template. The PCR program was 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s and the final extension step at 72°C for 10 minutes. The generated PCR products were gel-purified and cloned into the pMD-18T vector (TaKaRa, Japan). After transformed into the competent cells of *Escherichia coli* JM-109, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates. There positive clones were sequenced in both directions.

To get the 5'-end of the FoxO1 cDNA, a specific primer FP2 was designed according to the sequence (accession no. DQ673620). PCR was performed using 5'-RACE-Ready cDNA library mixture as the template with the primer set of FP2 and universal primer UPM. The PCR program was 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s and the final extension step at 72°C for 10 minutes. All the resulted sequences were verified and subjected to cluster analysis.

Virtual northern blot analysis

The transcript of FoxO1 was detected by virtual Northern blots. Total RNAs were extracted from adipose in 180-day-old pig, and reversely transcribed into cDNA by using an anchored oligodT primer (OligodT*Sal*IA) and CF primer (CapFinderB1) (Table 1) at the elevated temperatures (48°C) for 1 h. The PCR reaction was performed in a PTC-100 Programmable Thermal Controller Cycler (Biorad USA), using OligodT*Sal*IB and CapFinderB2 (Table 1) as primers in a 25-μl reaction

volume containing 1 U TaKaRa Ex Taq, 200 μM dNTPs, 2.5 mM $MgCl_2$ and 20 pmol of each primer and 1 μl of reverse transcribed cDNA products. The LA-PCR program was 95°C, 1 min; 60°C, 1 min; 68°C, 12 min; 7 cycles of 95°C, 30 s; 60°C, 30 s; 68°C, 12 min; 7 additional cycles of 95°C, 30 s; 60°C, 30 s; 68°C, 14 min; and 7 additional cycles of 95°C, 30 s; 60°C, 30 s; 68°C, 16 min. The PCR product (2-5 μg of amplified cDNA) were separated on a 0.6% agarose gel, denatured and subsequently blotted onto a nylon membrane in a conventional Southern transfer (downward with 10×standard SSC for 1-2 h) and UV-crosslinked. The probe was labeled with Digoxin by PCR amplification with primers FP3 and FP4 (Table 1). Prehybridization, hybridization, washing and detection were performed according to standard protocols (Franz et al., 1999; Adomas et al., 2006).

Sequence analysis

The sequence was searched for similarity using BLAST program at web servers of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The FoxO1 deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignment of porcine FoxO1 with human FOXO1 and mouse Foxo1 was performed with the ClustalW Multiple Alignment Program (http://www.ebi.ac.uk/clustalw) and Multiple Alignment show (http://www.bio-soft.net/sms/index.html). Phylogenetic tree was constructed by a CLUSTALW alignment and MEGA2 Neighbor-Joining.

Chromosomal localization

LP1 and LP2 primers used for locating FoxO1 gene in porcine chromosome set were designed according to the sequence of accession no. EF453379. LP1 primer is located in 5' untranslated region and LP2 primer in CDS region. Chromosomal localization of porcine FoxO1 gene was performed using IMpRH7000-rad as the method described by Wang et al. (2005) and Yu et al. (2007).

Cell culture

Dorsal subcutaneous adipose tissue was aseptically removed from the 1-day-old crossbreed piglet, and the preadipocytes were isolated according to the method used by Li et al. (2006). Briefly, adipose tissue was removed under sterile conditions from the dorsal subcutaneous depot in the neck and rinsed with KRB (Krebs Ringer Bicarbonate, containing 3% BSA, 10 mM glucose, 50 U penicillin/ml and streptomycin) buffer. The tissue was cut with scissors into approximately 1 mm^3 sections and then incubated in a digestion buffer comprised of Dulbecco's modified Eagle's medium/F12 (DMEM/F12, a 50:50 mixture of DMEM/F12, Gibco), 100 mM Hepes, 20 g/L bovine serum albumin (BSA, Sigma), pH 7.4, containing 1 g/L collagenase (Type I, Gibco). A fivefold excess of digestion buffer (room temperature, excluding collagenase) was added to the digestion flask after incubating for 60 min at 37°C in a shaking water bath. To remove undigested tissue and large cell aggregates, flask contents were mixed and filtered through 200 μm nylon mesh filters. The filtered cells were centrifuged at 800 g for 5 min to separate the floating adipocytes from the pellet of stromal-vascular cells. The stromal-vascular cells were then incubated with erythrocyte lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) at room temperature for 10 min, followed by centrifugation. The stromal-vascular cell pellet was washed twice with DMEM/F12 medium supplemented with 15 mM $NaHCO_3$, 50 U penicillin/ml and streptomycin. After washing, the cells were resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS vol/vol, Sigma). Finally, aliquots were seeded in culture plates at a density of 5.0×10^4 cells/cm^2 and maintained at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every other day. Recombinant human IGF-1 (CytoLab Ltd.) was dissolved in phosphate-buffered saline (PBS) with 0.1% BSA and added to the medium in the concentrations as shown in each figure throughout the differentiation period (from day 0 to day 7). Cells were harvested at day 0, 1, 3, 5, and 7.

Oil Red O staining

Cultures were washed twice with PBS on day 5, fixed with 10% formalin in PBS, stained with 0.5% Oil Red O and photographed.

GPDH activity analysis

Control cells and cells treated with 50 ng/ml IGF-1 and 100 ng/ml IGF-1 were carefully washed twice with ice-cold PBS on day 1, 3, 5, 7, 9 and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 by sonication for measurement of GPDH-specific activity. GPDH activity was determined according to the procedure of Wise and Green. Protein concentration was measured with the method of Lowry et al. and 1 U of GPDH activity was defined as the amount of protein required for consuming 1 nmol NADH/min/mg protein.

Time-course expression of FoxO1 mRNA

Total RNA was extracted from harvested porcine preadipocyte cultures at different time points using TRIZOL® reagent (Invitrogen, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using oligodT primers (Invitrogen, USA). Reverse transcription was performed by the M-MLV reverse transcriptase (Promega, USA) with DNase I (Promega, USA) treated total RNA as template according to the manufacturer's instructions.

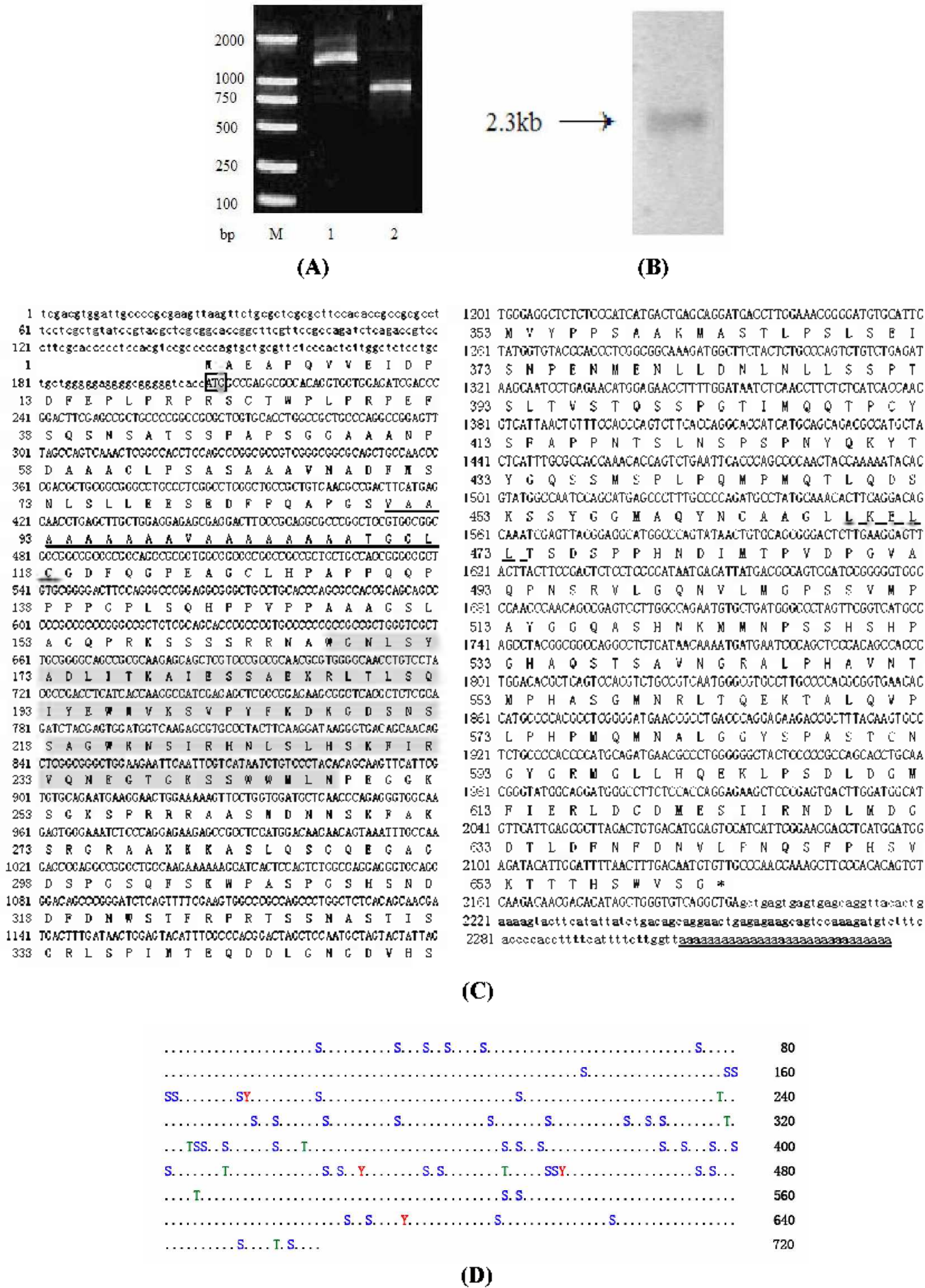


Figure 1. Cloning and sequencing of porcine FoxO1. (A) The 5`and 3`RACE fragment gel of porcine FoxO1. 1 lane and 2 lane indicated 5`and 3`RACE fragment, respectively. (B) Detection of FoxO1 mRNA by Virtual Northern Blots. Lane contains 1 μg of total RNA from adipose tissue of pig at day 180. The Marker indicates a 2.3 kb transcript in the adipose. (C) Alignment of the predicted aa sequences of pig FoxO1. The start codon (ATG) is boxed and the stop codon (TGA) is marked with an asterisk. Transmembrane structure domain is double bold line (aa 90-113). Forkhead DNA domain is indicated as shaded residues (aa 167-247). An LXXLL motif (aa 469-473) in porcine FoxO1 is under dash line. The polyA signal sequence is under bold line. (D) Predicted phosphorylation site in FoxO1 aa sequence. S = serine; Y = tyrosine, T = threonine.

The expressions of FoxO1 mRNA in different time points were investigated by real time qRT-PCR with an ABI Prism 7700 Sequence Detection System (Applied Biosystems). β-Actin was used as an internal control. The qRT-PCR primers were shown in Table 1, FP4F, FP4R for FoxO1 and APF, APR for β-actin. The PCR reactions were performed in a 20 μl total volume, which contained 1 μl of cDNA, 10 μl SYBR Green PCR Master Mix (TOYOBO), 0.5 μl each primer set (10 μM), and 8 μl H_2O. The PCR profiles consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 25 s, 58°C for 30 s, extension at 72°C for 1 min. The qRT-PCR was performed in triplicate. The threshold cycle (CT) value for each sample was determined using the automatic setting on the ABI Sequence Detection System. The CT values were exported into a Microsoft Excel Sheet for subsequent data analyses. The differences in the CT values of target gene with the corresponding internal control β-actin gene, ΔCT (CT_{gene}-CT_{actin}), were calculated. The relative expression level of target gene to β-actin was described using the equation $2^{-\Delta CT}$.

Time-course expression of FoxO1 protein

Western blotting analysis of porcine FoxO1 was performed with the procedure (Nakae et al., 2002). Briefly, harvested cells were homogenized in a lysis buffer (10 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 0.5 mM DTT, 10 mM KCl, 10 mM NaF, 1 mM Na_3VO_4, and 0.5 mM PMSF). After 15 min at 4°C, we added NP-40 to a final concentration of 2%, following centrifugation (12,000 rpm for 1 min at 4°C). Protein was quantified in the supernatant using Bradford assay. For Western blot analysis of FoxO1, A 50 μg aliquot was separated through 8% SDS polyacrylamide gel and electrotransferred to PVDF membranes, unbound sites were blocked 2 h at room temperature with 5% (W/V) nonfat milk in Tris-buffered saline containing 20 mmol/L Tris-HCl (pH 7.6), 140 mmol/L NaCl, and 0.1% (W/V) Tween-20. The membranes were incubated with the specified primary antibody goat anti-human FKHR(N-13) (Santa Cruz Biotechnology) dilution at 1:500 in TBS buffer containing 5% nonfat dry milk overnight at 4°C. After 4 washes, the blots were incubated with secondary antibodies linked to horseradish-peroxidase labeled donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed in a chemiluminescence system and then visualized by exposure to Kodak X-ray film. The accuracy of protein loading on the gel was verified by re-probing with polyclonal antibody to β-actin (Imgenex Corporation). Densitometry was analyzed using the AlphaImager 2200 (Alpha Innotech).

Statistical analyses

Data were expressed as means±SE calculated from 4 replicates, and were statistically analyzed using SPSS 11.5 statistical software package (SPSS Inc.). Statistical significance was set at $p<0.05$.

RESULTS

Cloning and sequencing of porcine Foxo1

The 3'-end and the 5'-end PCR products of the FoxO1 cDNA were detected on a 1% agarose gel (Figure 1A). In the 3'-end PCR product of 830 bp, including stop codon TAA and a poly (A) tail were identified in this fragment. A fragment of 1,680 bp was amplified from the 5'-end of FoxO1 cDNA by using the primers of FP2 and UPM. A 2,335 bp nucleotide sequence representing the complete cDNA sequence of FoxO1 gene was obtained by cluster analysis of the above two fragments. Sequence analysis showed that it contained our previous published partial sequence of porcine FoxO1 (GeneBank accession no. DQ673620). Virtual Northern blot was used to confirm the length of this gene, and a single transcript of the approximate 2.3 kb was detected (Figure 1B).

The nucleotide sequence of FoxO1 was shown in Figure 1C. The full length cDNA sequence was of 2,335 bp, contained a 5'-untranslated region (UTR) of 205 bp, followed by an open reading frame (ORF) of 1,989 bp, a 3'-UTR of 141 bp including a poly (A) tail. Searching for sequence homology of the FoxO1 with other known sequences by BLASTn revealed that it was closely matched with human *Homo sapiens* FOXO1 (GeneBank accession no. AF032885, identity = 90%). The sequence of the FoxO1 gene was deposited in GenBank under GeneBank accession no. EF453379.

Analysis of the FoxO1 amino acid sequence

The ORF of FoxO1 was of 1,989 bp encoding a polypeptide of 662 aa with an estimated molecular mass (MM) of 69.93 kDa and a predicted isoelectric point (PI) of 6.295. There were a Forkhead domain (aa 167-247), and a trarsmembrane structural domain (aa 90-113). Searching for sequence similarities of the FoxO1 with known proteins by BLASTp revealed that it was closely matched with human *Homo sapiens* FOXO1 (accession no. AAC39591, identity = 94%).

Phosphorylation sites predicted that there were 51 Ser, 8 Thr, and 4 Tyr sites in the FoxO1 aa sequence. We identified an LXXLL (where L is a leucine and X any amino acid) motif at aa 469-473 inporcine FoxO1, which is highly conserved among FoxO family members in human *Homo sapiens*, murine *Mus musculus*, and *Xenopus laevis*. This finding suggests that the LXXLL motif may have an

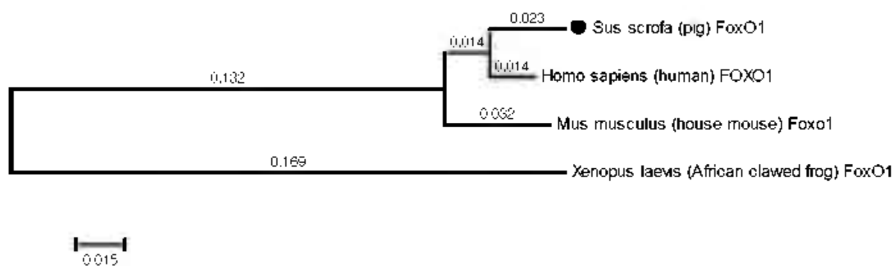


Figure 2. Phylogenetic tree analysis of pig FoxO1, *Homo sapiens* FOXO1, *Mus musculus* Foxo1, and *Xenopus laevis* FoxO1. Phylogenetic tree was obtained from a CLUSTALW alignment and MEGA2 Neighbor-Joining of 4 sequences. The bar indicated the distance.

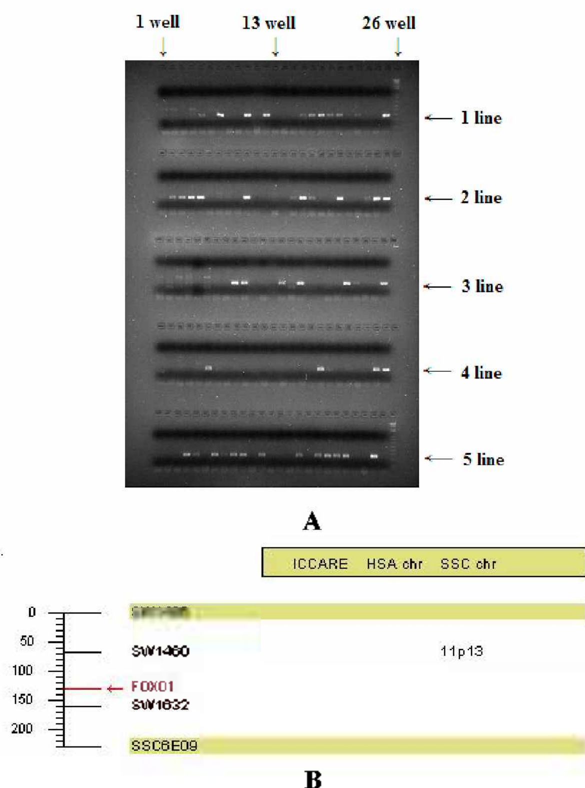


Figure 3. Mapping of porcine FoxO1 gene. (A) The pig×hamster radiation hybrid (IMpRH) panel of porcine FoxO1. There are 5 lines on the gel, 26 wells on each line (comb), 1-24 are hybrid DNAs, 25 is pig positive control, 26 is the 1kb+ ladder (if there is a ladder, otherwise it is empty). For the last line, 1-22 are the hybrid 97-118 DNAs, 23 is the Hamster DNA control, 24 is pig DNA control, and 25 is a water (negative) control. 26 is the ladder. (B) Figure of mapping of porcine FoxO1 gene. Porcine FoxO1 gene was closely linked the SW1632 and mapped to SSC 11p13. (More details reference to http://imprh.toulouse.inra.fr/4DACTION/LanceMap).

important role in the function of FoxO proteins. Based on overall aa sequences of *Homo sapiens*, *Mus musculus*, and *Xenopus laevis*, a phylogenetic tree was constructed (Figure 2). The results showed that FoxO1 was most similar to human *Homo sapiens* FOXO1.

Chromosomal localization

Analyses of INRA-Minnesota porcine radiation hybrid (IMpRH) panel (Figure 3), according to the results of PCR typing and reference to stuff in INRA website (http://imprh.toulouse.inra.fr/), revealed that retention fraction rate was 27% and porcine FoxO1 gene was mapped to SSC 11p13 and closely linked the SW1632 (0.32 cR, LOD = 12.17).

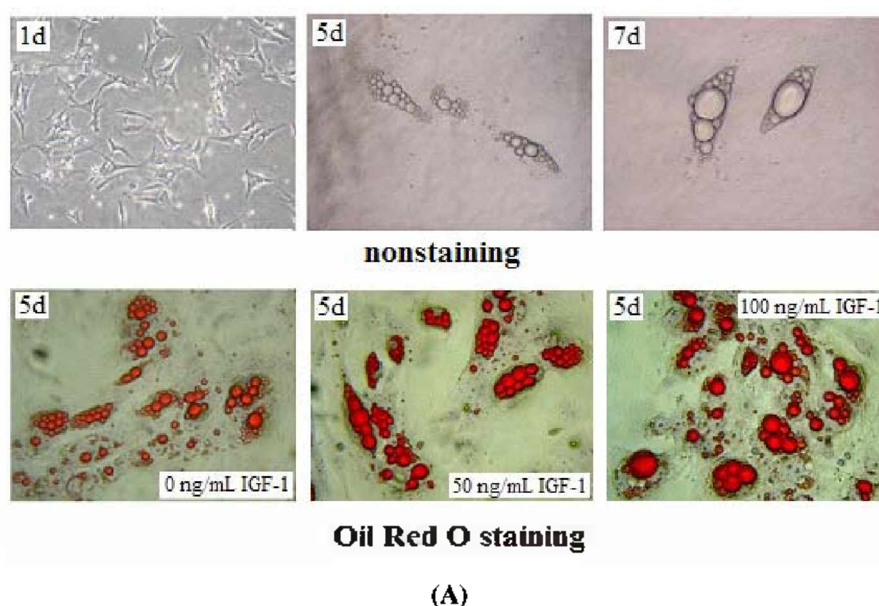
Porcine preadipocyte morphological changes and GPDH activity analysis

Primary porcine preadipocytes were at day 1, 5, and 7 (Figure 4A). At day 1, there were not lipid droplets in cells. At day 5, there were many small lipid droplets in cells. From day 5 to 7, the number of lipid droplets gradually decreased and the size of lipid droplets gradually enlarged in cells. Using Oil Red O staining, we found that in 100 ng/ml IGF-1 treatment group the number of lipid droplets notably decreased and the size of lipid droplets significantly enlarged in cells at day 5. The IGF-I by time- and dose-depended manner promoted GPDH activity throughout the differentiation period (Figure 4B), indicating significant differences at day 3, 5, and 7.

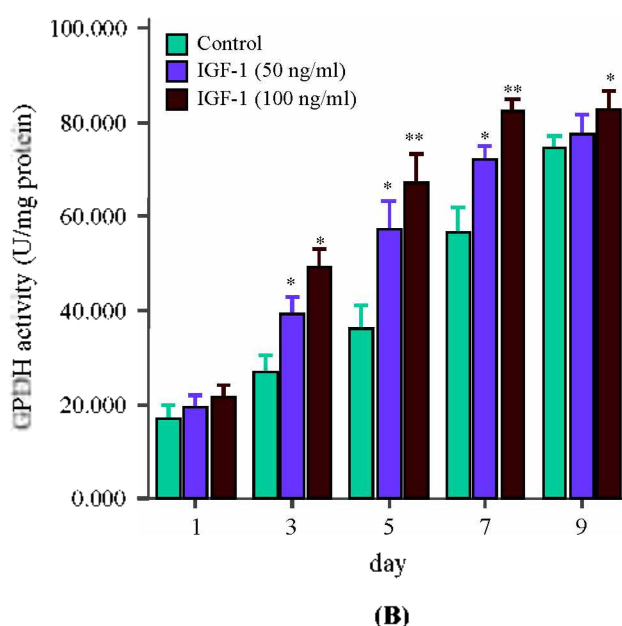
Foxo1 time-course expression in porcine preadipocytes differentiation

Expressions of FoxO1 mRNAs and protein quickly increased from day 0 to 3, and reached almost maximal levels at day 3, then gradually decreased from day 5 to 7. Compared with the relative expression level of FoxO1 mRNA at day 0, those at day 1, 3, 5, and 7 were 1.87-fold, 5.42-fold, 4.29-fold, and 2.23-fold, respectively (Figure 5A). Compared with the relative expression levels of FoxO1 mRNA in 0 ng/ml IGF-1 group at day 0, 1, 3, 5, and 7, those in 50 ng/ml IGF-1 group were 1.00-fold, 0.78-fold, 0.66-fold, 0.63-fold, and 0.70-fold, in 100 ng/ml IGF-1 group 1.00-fold, 0.65-fold, 0.48-fold, 0.54-fold, and 0.64-fold, respectively, and significant difference in preadipocyte differentiation at day 3 and 5 ($p<0.05$).

No protein expression was at day 0, then faintness at



(A)



(B)

Figure 4. Morphological changes and GPDH activity of porcine preadipocyte. (A) Adipocytes at day 1, 5 and 7. At day 5, cells were stained using Oil Red O. The magnification was 200×. (B) GPDH activity analysis of porcine preadipocyte at day 1, 3, 5, 7 to 9. Values are means±SE for 4 replicated cultures. * $p<0.05$ vs. control, ** <0.01 vs. control.

day 1, and peaked at day 3, then gradually decreased from day 5 to 7 (Figure 5B). Compared with the relative expression levels of FoxO1 protein in 0 ng/ml IGF-1 group at day 3, 5, and 7, those in 50 ng/ml IGF-1 group were 0.30-fold, 0.38-fold, and 0.43-fold, in 100 ng/ml IGF-1 group 0.12-fold, 0.16-fold, and 0.19-fold, respectively, and significant difference in preadipocyte differentiation at day 3, 5 and 7 ($p<0.05$). Therefore, after induced by IGF-1, porcine primary preadipocyte differentiation was promoted, however, expressions of FoxO1 mRNA and protein were inhibited in a dose dependent manner.

DISCUSSION

In the present study, the full-length cDNA of FoxO1 was cloned from pig and verified by virtual Northern blots. The full-length cDNA of FoxO1 was of 2335 bp, contained an ORF (nucleotides 206-2194) encoding a protein of 662 aa with an estimated MM of 69.93 kDa and a predicted PI of 6.295 (acidic). There was a putative 80 aa Forkhead domain and a putative 24 aa transmembrane domain in the deduced amino acid sequence of FoxO1, but no predicted signal peptide was found in the aa sequence, indicating that FoxO1 should belong to one of forkhead transcription

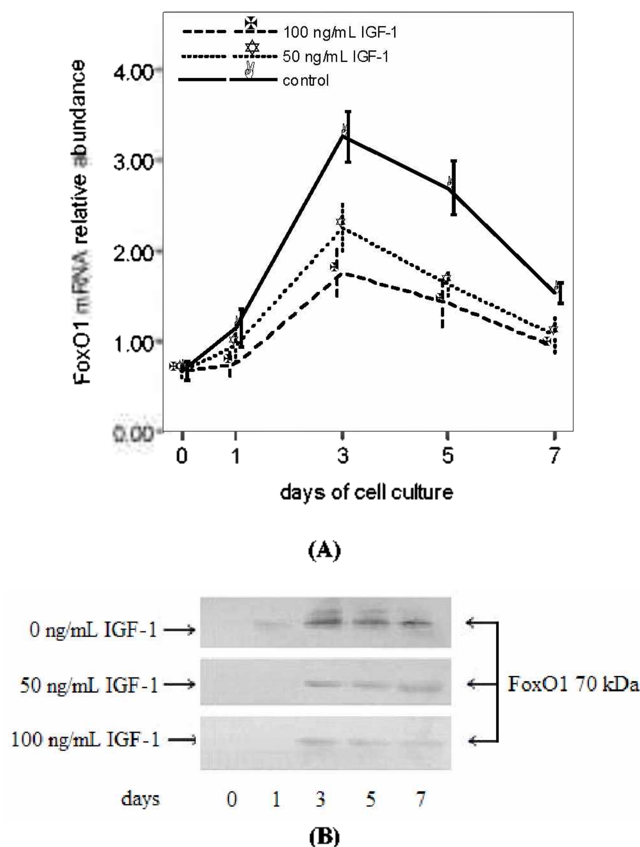


Figure 5. Time-course expressions of porcine FoxO1 gene in preadipocytes. (A) Time-course analysis of FoxO1 mRNA in porcine preadipocytes. Expression of each mRNA was quantified by scanning densitometry of autoradiogram and normalized with β-actin. Data were presented as FoxO1 mRNA relative abundance. Columns and error bars indicate mean (SD) (n = 4). (B) Time-course expressions of FoxO1 protein in porcine preadipocyte. Extracts were prepared at different time points, and equal amounts of protein extract applied to each lane.

factor O subfamily. Meanwhile, the full-length cDNAs of human FOXO1 (GeneBank accession No AF032885) and mouse Foxo1 were of 5,723 bp and 3,185 bp, respectively, which were longer than that of porcine FoxO1. The ORF of FOXO1 was 1,965 bp in length encoding a protein of 654 aa residues with MM of 70 kDa and a PI of 6.314 (acidic). The ORF of Foxo1 was 1,956 bp in length encoding a protein of 651 aa residues with MM of 69.58 kDa and a PI of 6.314 (acidic). Comparison of porcine FoxO1 with another mammal including human FOXO1 and mouse Foxo1, indicated that they are all forkhead transcription factor O 1, and they high share about 90% identity at the aa sequences. In the phylogenetic tree, porcine FoxO1 was clustered with human FOXO1 firstly and then grouped with mouse Foxo1. The results demonstrated that porcine FoxO1 had a nearer relation with human FOXO1 than mouse Foxo1.

Foxo subfamily (Kaestner et al., 2000) is phosphorylated in an insulin-responsive manner by Akt/PKB and Sgk (Brunet et al., 2001). Phosphorylation leads to Foxo inhibition through nuclear exclusion. Our predicted results of phosphorylation sites indicated that there were 51 ser sites, 8 thr sites and 4 tyr sites in porcine FoxO1 aa sequence. Interestingly, the putative Thr_{319} of porcine FoxO1 phosphorylation sites were different with the Ser_{319} of human FOXO1 and mouse Foxo1 aa sequence, indicating Thr_{319} were new special important phosphorylation sites which were possibly phosphorylated by Akt/PKB. Of course, the phosphorylated site of porcine FoxO1 should be studied further, because protein phosphorylation is central to FoxO1 transcriptional regulation signal pathways, and this special difference may have functional relevance to porcine special FoxO1 signal pathway. Analysis using SMS soft revealed that porcine Foxo1 protein had a transmembrane helix from aa 90 to 113, indicating the transmembrane helix possibly related to FoxO1 shuttled between nuclear and cytoplasm when it was phosphorylated by PI3K/Akt. However, more work will be required to address these presumptions.

In present experiment, we found a LKELL motif (aa 469-473) of porcine FoxO1 aa sequence. The LXXLL (aa 459-463) motif of Foxo1 have an important role for its transcriptional activity *in vivo* (Nakae et al., 2006). Foxo family members have a transactivation domain in their C terminal region. Control of gene expression is a complex process that involves assembly of multiple transcription factors at the distal enhancer region and basal transcriptional machinery at the core promoter region of target genes (Hampsey et al., 1999). Gene expression involves the sequential assembly of an array of coregulatory proteins that include coactivators. Coactivator complexes are recruited via specific protein-protein interactions, often mediated by specific interaction motifs in the transactivation domains of transcription factors (Chen et al., 2001). The LXXLL motif was originally identified in a variety of coactivators, such as p300/CREB-binding protein (p300/Cbp) and RIP-140. It is described as a signature motif that mediates the recruitment of coactivators by the nuclear hormone receptors (Heery et al., 1997; McInerney et al., 1998). Specific LXXLL motifs of nuclear receptor coactivators (NcoAs) mediate ligand-dependent interaction with nuclear receptors as well as p300/Cbp (McInerney et al., 1998). Thus, LXXLL motifs have evolved to serve overlapping roles that are likely to permit both receptor-specific and ligand-specific assembly of a coactivator complex, and these recognition motifs underlie the recruitment of coactivator complexes required for nuclear receptor function. Therefore, we can infer that the LKELL motif of porcine FoxO1 have the same role for its transcriptional activity as that of mouse Foxo1.

The present study shows that porcine FoxO1 gene is

closely linked to maker SW1632 by method of the IMpRH panel. Therefore, FoxO1 gene is mapped to 11p13, while human FOXO1 is mapped to 13q13-q14.1 (Anderson et al., 1998) and porcine FoxO1 is mapped to 11p11-15 (Yu et al., 2007). Nucleinic acid sequence of FoxO1 gene includes sequences of 3' and 5' untranslated regions. Variations in untranslated region of genes are widely different. In our experiment, one of the primers is designed in 5' untranslated region and the other in CDS region for locating FoxO1 gene in porcine chromosome set. The results indicate that these primers are contributed to gene location. Our data provide basic molecular information useful for the further investigation on the function of FoxO1 gene.

Real-time RT-PCR analysis indicates that Foxo1 is the most abundant in murine white and brown adipose tissue (Nakae et al., 2002). Foxo1 mRNA is abundant in mice embryonic stage, and in adult mice, Foxo1 mRNA is mainly distributed in adipose (Furuyama et al., 2000). Above dada implies that Foxo1 plays an important role in development of porcine adipose.

In vitro differentiation of committed preadipocytes follows a well-characterized sequence: upon reaching confluence on day 0, cells undergo limited clonal expansion on days 1-2, postmitotic growth arrest on days 3-4, and terminal differentiation on days 4-10 (Nakae et al., 2003). The present experiment showed the morphologic change of porcine preadipocytes on days 0-7. However, compared with the clonal cell lines, *in vitro* differentiation of porcine primary preadipocytes was delayed, upon reaching confluence on day 1-2, cells undergo limited clonal expansion on days 3-4, postmitotic growth arrest on days 5-7, and terminal differentiation on days 7-12.

IGF-I is a single-chain peptide consisting of 70 amino acids coded for by a gene on the long arm of chromosome 12. Circulating IGF-I is synthesized primarly in the liver but also by adipocytes, suggesting autocrine/paracrine effects (Peter et al., 1993). The main biological effect of IGF-I is to stimulate growth and differentiation at the cellular level, but it also exerts a variety of insulin-like effects in different types of derived cells *in vitro* (Sara et al., 1990; Frosch et al., 1985; Wabitsch et al., 2000).

Most peptide growth factors stimulate myoblast proliferation and prevent differentiation (Coolican et al., 1997). Our data showed that IGF-1 treatment throughout the differentiation period promoted GPDH activity and accumulation of lipid by time- and dose-depended manner. However, its treatment in the early phase of differentiation only modestly increased these differentiation markers. These results suggested that IGF-1 continuously promoted porcine preadipocyte differentiation throughout the differentiation period. In 3T3-F442A preadipocytes, mRNA levels of the Foxo1 are comparably low (Nakae et al., 2003). In our present experiment, during differentiation, FoxO1 relative mRNA abundance increases up to 5.42-fold over basal levels, peaking at day 3, to then partially decline. Although protein levels of FoxO1 are comparably low, quickly increased from day 0 to 3, and reached almost maximal levels at day 3, then gradually decreased from day 5 to 7. IGF-1 treatment of insulinoma cells protected against free fatty-induced apoptosis and was associated with increased phosphorylation of both Akt and Foxo1 (Wrede et al., 2002). IGF-1 stimulated the protein metabolism of myotubes during human myotube hypertrophy by inducing a downregulation of Foxo1-atrogin-1 protein degradation pathway (Virginie et al., 2007). Interestingly, IGF-1 inhibited FoxO1 mRNA and protein expressions in a dose-dependent manner during porcine preadipocyte differentiation. Capability of porcine deposit fat is superlative among domestic animals. The different species possibly result in important difference of the gene expression. These data indicate that FoxO1 has a unique expression pattern in differentiation of porcine primary preadipocyte using IGF-1 treatment.

In conclusion, we got porcine FoxO1 gene full-length cDNA, and FoxO1 gene was mapped to 11p13. From analysis of porcine FoxO1 aa sequence, we presumed physiologic function of porcine FoxO1 was similar to that of human FOXO1 and rat Foxo1. In different time points of porcine preadipocyte culture, FoxO1 has a unique expression pattern, so we can infer that FoxO1 takes part in porcine preadipocytes differentiation. The successful cloning and time-course expression of FoxO1 will enhance the understanding of the involvement of the gene in regulating differentiation of preadipocytes in pigs.

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