Clenbuterol, a β2-adrenoceptor agonist, has been proven to be a powerful repartition agent that can decrease fat deposition. Based on results from our previous cDNA microarray experiment of pig clenbuterol administration, a novel up-regulated EST was full-length cloned (4859 bp encoding 1041 amino acids) and found to be the pig homolog of large tumor suppressor 2 (Lats2). We mapped pig Lats2 to chromosome 11p13-14 by using FISH, and western blotting demonstrated that pig Lats2 protein was most abundant in adipose. In Drosophila, Lats2 ortholog was reported as a key component of the Hippo pathway which regulates cell differentiation and growth. Here, we show that pig Lats2 exhibit inverted expression to YAP1, another member of the Hippo pathway which positively regulates cell growth and proliferation, during the differentiation of 3T3-L1 preadipocytes. Our results suggested that Lats2 may involve in Hippo pathway regulating the fat reduction by inhibiting adipocyte differentiation and growth. (BMB reports 2010; 43(2): 97-102)

INTRODUCTION

The capacity of clenbuterol to modulate lipid metabolism in adipose tissue has been demonstrated in many domestic animals (1-3). Despite proven acute toxicity, clenbuterol has been misused to increase lean meat production by commercial animals (4, 5). Being a β2-adrenoceptor agonist, clenbuterol initiates repartition effects mainly by activating the β2-adrenergic receptor/CAMP-dependent pathway (6, 7). However the downstream mechanism by which clenbuterol influences adipose accumulation is still largely unclear.

We attempted to identify novel genes and signaling pathways that are regulated in adipose tissue after clenbuterol administration to pigs (8). Nine differentially expressed ESTs were identified using cDNA microarrays; however, complete sequence information and functional annotation were lacking of these ESTs in databases. In this study, we focused on one novel fragment which we confirmed to be significantly up-regulated in the dorsal subcutaneous adipose tissue of pigs administered clenbuterol by real-time PCR. Using electronic cloning and rapid amplification of cDNA ends (RACE) techniques we obtained the full length sequence of this gene. By examining the homology of this sequence to human and mouse sequences we concluded that this gene was an ortholog of large tumor suppressor 2 (Lats2) in pigs. Lats2 is a mammalian ortholog of Warts (Wts), which was isolated from Drosophila as a tumor suppressor gene. In Drosophila, loss of Wts leads to cell overgrowth in a variety of epithelial structures, including wings, legs and eyes (9, 10). Mammalian Lats2 has been found to control mitosis and cytokinesis, and a deficiency of Lats2 in mouse embryonic fibroblasts leads to centrosome amplification, genomic instability and loss of contact inhibition (11, 12). In Drosophila, it is suggested that Wts and three other tumor suppressor genes, Hippo (Hpo), Salvador (Sav) and Mats, might work together because of the similarities among the phenotypic effects caused by their mutation. A novel kinase cascade has been recently identified (13) in which Sav potentiates Hpo to phosphorylate and activate Wts and then, the partnering of Wats with Mats leads to inactivation of Yorkie (Yki), a transcriptional co-activator that positively regulates cell growth, survival and proliferation (14). Most of the components of the Hippo signaling pathway have been identified in mammalian species, and these components have been found to be highly conserved throughout evolution. The Hpo signaling pathway in mammals is mainly involved in growth control and maintaining tissue homeostasis (15). However, a role for the Lats2 gene in regulating adipose homeostasis has not yet been reported.

Here, we describe the novel pig Lats2 gene, including its cDNA sequence, expression distribution and chromosomal location. Furthermore, we reported a time-shifted relationship between the expression of Lats2 and two adipocyte markers C/EBPα and PPARγ, and an inverted relationship between Lats2 and transcriptional coactivator YAP1 which positively regulates cell growth and proliferation, during the differ-
entiﬁcation process of 3T3-L1 preadipocytes. Our results suggest the potential role of Lats2 involved Hippo pathway in adipocyte differentiation and proliferation, which may lead the observed fat reduction.

RESULTS

Validation of differential expression of unknown EST after clenbuterol administration

Among the differentially expressed expressed sequence tags (ESTs) detected by cDNA microarrays, we found a novel fragment (rpfat_8229) that was signiﬁcantly up-regulated in pig dorsal subcutaneous adipose tissue after clenbuterol administration (8). To validate the ﬁndings obtained from the microarray experiment, the mRNA level of this EST fragment was further measured by real-time RT-PCR. The RT-PCR result agreed with the microarray data, conﬁrming the expression change for rpfat_8229 in the dorsal subcutaneous adipose tissue of both 3- Fig. 1A and 4 (Fig. 1B)-month-old pigs after clenbuterol administration.

Molecular cloning and characterization of pig Lats2

10 EST sequences (BP156775, DB787231, FD640724, FD641802, FD635289, CX056972, FD625790, EW338869, DT333416, and DN110984) from the Sus Scrofa EST database (Version 041709) that overlapped the novel sequence rpfat_8229 with signiﬁcant sequence similarity (Blast threshold set to e-80) were identiﬁed. Using the CAP3 sequence assembly program (16), a 1563-bp contig was obtained based on the 11 EST sequences mentioned above. We BLASTed this contig against the Human/Mouse Lats2 sequences and found that this contig showed signiﬁcant similarity to the 3’-untranslated region of both human and mouse large tumor suppressor 2 (Lats2) genes. Based on the sequence similarity of Lats2 gene in 11 species, exons 4 and 7 of human LATS2 were found to be the most conserved. We thus designed two pairs of primers, one amplifying the sequence from exon 4 to exon 7, and one amplifying the sequence from exon 7 to the end of the assembled contig. The two overlapping fragments (about 2.2 kb and 2.3 kb, respectively) were ampliﬁed by RT-PCR. To identify the remaining upstream and downstream sequences of the full length mRNA, we used 5’ and 3’ RACE. A 213-bp 5’ RACE amplicon and a 970-bp 3’ RACE amplicon were obtained by nested PCR and sequencing. The transcript of pig Lats2 contains 4,839 bp, including a 3,126-bp open reading frame, a 187-bp 5’ UTR and a 1,546-bp 3’ UTR. The polyadenylation signal was identiﬁed as ATTAAA, 14 bp upstream of the poly(A) homopolymer (GenBank accession no. FJ825141) (Supplemental Fig. I). The nucleotide sequence of pig Lats2 shares 79% similarity with that of human Lats2 (NM_014572) and 84% with mouse Lats2 (NM_015771). Human and mouse sequences show 86% identity with each other.

The predicted pig Lats2 protein consists of 1,041 amino acids, and its molecular weight, as predicted by DNAMAN 6.0, is 114 kDa with an isoelectric point of 8.42. Lats2 is a novel mammalian homologue of the Drosophila tumor suppressor gene Warts/Lats2. Warts/Lats2 of Drosophila melanogaster belongs to a new subfamily of protein kinases (9, 10). The members of this subfamily have considerable similarities in function as well as in the structures of their catalytic domains. Multiple alignment of pig Lats2 protein sequence with orthologs from other species (human, chimp, mouse, rat, dog and cow) indicated that a high level of the amino acid identity among functional motifs (Fig. 2A).

Chromosomal location of pig Lats2

To determine the chromosomal location of pig Lats2, we performed FISH. As shown in Fig. 2B, four speciﬁc hybridization signals were observed on two homologous chromosomes (arrows). We preliminarily assigned pig Lats2 to pig chromosome 11p13-14 by comparing the FISH results with G-binding and a standard GTG-banded pig karyotype. This result is supported by evidence that pig chromosome 11 is homologous to human chromosome 13, indicated by previous comparative mapping analyses by the Inland Northwest Research Alliance (INRA), while the human Lats2 is located in the homologous region on chromosome 13q11-12.

Expression analysis of pig Lats2

The distribution of Lats2 mRNA expression among seven tis-
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Fig. 2. Cloning and localization of the pig Lats2 gene. (A) Amino acid sequence alignments among functional motifs of the Lats2 gene products among human, chimp, mouse, rat, dog and cow (GenBank accession nos. NP_055387, XP_509566, NP_056586, XP_224169, XP 534537, and XP 584953, respectively). *, residues or nucleotides identical in all sequences in the alignment; ., conserved substitutions; , semi-conserved substitutions. (B) Chromosomal localization of pig Lats2 by FISH. Photographs of the entire metaphase and G-binding are shown (Left). Arrows indicate specific hybridization signals on the long arm of chromosome 11 compared with a standard GTG-banded pig karyotype (Right).

Fig. 3. Expression analysis of pig Lats2 mRNA and protein. (A) The pig Lats2 mRNA expression level was analyzed by RT-PCR in the indicated tissues. The \( \beta \)-actin gene was used as a loading control. (B) Pig Lats2 protein levels were analyzed by Western blotting in the indicated tissues. Lysates of HeLa cells expressing human Lats2 were used as positive control. \( \beta \)-actin protein was used as an internal control.

Western blotting was used to detect the distribution of Lats2 protein. Because the pig Lats2 protein shares high sequence identity with its human homolog, we used a polyclonal antibody raised against a synthetic peptide corresponding to the N-terminal region between residues 1 and 50 of human Lats2 (NP_055387.1). The levels of pig Lats2 protein were examined among the same seven tissues as mRNA analysis (Fig. 3B). Total protein from HeLa cells was used as a positive control and \( \beta \)-actin protein was used as an internal control. According to the molecular weight of the protein marker and the size of band detected in the positive control, pig Lats2 protein was estimated to be about 120 kDa. We found two major bands at around 120 kDa in most of experimental tissues. We suspected that one of these is the Lats2 protein and that the other is a phosphorylated form of Lats2. Lats2 protein was most abundant in adipose tissue followed by heart and skeletal muscle.

Increased expression of Lats2 during differentiation in 3T3-L1 preadipocytes
Lats2, being a Ser/Thr protein kinase, is an important member of the Hippo pathway which regulates cell differentiation and growth via phosphorylation and inactivation of a transcriptional coactivator. On the other side, it is known that regulating fat lipolysis is one of the main actions of clenbuterol on adipose tissue and, as shown above, we observed that the expression of Lats2 mRNA was significantly increased after clenbuterol treatment. We thus speculated that clenbuterol might function via the mechanism that Lats2 involved Hippo pathway may play a role in the regulation of adipose differentiation and growth. To test this, we utilized 3T3-L1 preadipocytes, a faithful model for studying the process of adipocyte differentiation and metabolism, to trace the changes in the expression of Lats2.

3T3L1 cells were induced to differentiate for 8 days as pre-
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**Fig. 4.** Adipocyte differentiation in 3T3-L1 preadipocytes. 3T3-L1 cells were induced to differentiate from preadipocytes into adipocytes as described. The mRNA expression levels of the adipocyte markers (A) C/EBPα and (B) PPARγ were analyzed at the indicated time points. The mRNA expression levels of the Hippo signaling pathway components (C) Lats2 and (D) YAP1 were analyzed at the indicated time points. Results are from three independent experiments performed in triplicate. Data are expressed as the means±S.E.M.

**DISCUSSION**

Followed by the result of our cDNA microarray experiments, one confirmed up-regulated EST (rpfat_8229), which had not been previously reported, was full-length cloned and characterized. This gene appears to be an ortholog of large tumor suppressor 2 (Lats2) in pig species.

Human Lats2 mRNA was previously reported to be strongly expressed in skeletal muscle and heart, and mouse Lats2 was reported to be moderate in these tissues (12). Here we report the expression of the LATS2 gene in adipose tissue. The western blotting results showed a high level of LATS2 in the pig subcutaneous adipose tissues, which suggests a physiological significance of Lats2 in fat metabolism.

Being a protein kinase, Lats2 is a key component of the Hippo pathway. Orthologs for all Hippo pathway components can be found in most mammalian genomes; however, little is known regarding their physiological functions in this pathway, except in Drosophila. To date, a prevailing hypothesis is that the hippo pathway can be activated to inhibit cell proliferation and promote apoptosis once an organ or tissue approaches its final size, or when cells enter the differentiation phase of development (13). In this way, the Hippo pathway offers a robust mechanism to regulate final tissue or organ size.

Differentiation of 3T3-L1 cells represents a faithful model of preadipocyte differentiation into adipocytes in vivo (18). There are many inducers of adipogenesis (for example, insulin, IGF-I), together with many transcription factors (for example C/EBPα, PPARγ), reinforce the adipogenesis during differentiation. At the same time a variety of inhibitors of adipogenesis (for example, TNF-α, EGF) have been found to suppress preadipocyte differentiation and dedifferentiate mature adipocytes, which can decrease the activity and synthesis of key proteins in carbohydrate and lipid metabolism (19, 20). Adipocyte size can vary according to the amount of lipid stored, however, there is a physical limitation to how large these cells will eventually be. So, inducers and inhibitors should work together during preadipocyte differentiation. Consistently, in the present study we found that Lats2 mRNA levels increased significantly during differentiation in 3T3-L1 preadipocytes, displaying a time-shifted pattern to the expression of C/EBPα and PPARγ. Additionally, we also found that the expression of Lats2 mRNA levels contrasts to the expression of YAP1. Because YAP1 is an important transcriptional coactivator in Hippo pathway (15), the inverted expression pattern between YAP1 and Lats2 in 3T3-L1 adipocytes indicated Lats2 may involve in Hippo pathway which might negatively regulate adipocyte size change. More research is necessary to understand the physiological role of Lats2 in subcutaneous adipose tissue.
cytes can be treated with an adenylyl cyclase stimulator or a β2-adrenergic receptor antagonist can reverse this stimulatory effect of clenbuterol on cAMP and Lats2 expression levels are increased after treatment with clenbuterol, while ICI 118,551, a β2-adrenoceptor antagonist, can reverse this stimulatory effect of clenbuterol on cAMP and Lats2 expression in adipocytes (Supplemental Fig. I and II). This result hints that Lats2 expression in adipocytes is related to a β2-adrenergic receptor-mediated pathway, however further studies are needed to test the causal relationship. For example, adipocytes can be treated with an adenylyl cyclase stimulator or a PKA inhibitor, enabling us to discover the connection between β2-adrenergic receptor/cAMP-dependent pathway and Lats2 expression.

**MATERIALS AND METHODS**

An expanded Materials and Methods section is available in the supplement.

Animal management and tissue collection

Eight Chinese miniature pigs were utilized in this experiment, including four hogs and four sows. Details of animal management and the number of daily doses of clenbuterol were as described previously (8). Dorsal subcutaneous adipose tissue samples taken from each animal were flash frozen in liquid nitrogen and stored at −80°C pending RNA isolation.

Total RNA extraction and cDNA preparation

Total RNA was prepared from pig dorsal subcutaneous adipose tissue or undifferentiated/differentiated 3T3-L1 adipocytes using the QIAZOL Lysis Reagent (RNeasy Lipid Tissue) according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). About 1 μg RNA was reverse transcribed into single-strand cDNA using oligo (dT) 18-mer primers and M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany).

Rapid amplification of cDNA 3’ and 5’ ends

Total RNA from subcutaneous adipose tissue was used for 3’ and 5’ RACE using the 3’/5’-RACE kit (Invitrogen). Details of the amplifying and purification processes were as described previously (21, 22). The more details of our rapid amplification of cDNA 3’ and 5’ ends processes are described in supplemental Materials and Methods.

Fluorescence in situ hybridization (FISH)

For FISH analysis, chromosomes were prepared from a male pig fibroblast using a standard cytogenetic protocol. Chromosomes were subjected to Gband analysis and well-banded metaphases were photographed. A BAC (constructed by our lab using genomic DNA from a male Erhualian pig) clone of the pig Lats2 gene was used as a probe and labeled by nick translation with biotin-14-dATP (Invitrogen, Carlsbad, CA, USA). Details of the hybridization process and image capture procedures were as described previously (23).

Western blotting

Tissues and organs were removed from Chinese miniature pigs, and protein extracts were prepared by complete homogenization of tissues and HeLa cells in immunoprecipitation buffer (Beyotime, CA) according to the manufacturer’s instructions. Equal amounts of protein extracts were mixed with sample buffer and then separated on 7.5% SDS-PAGE gels (40 μg/lane). Details of the western blotting process were as described previously (24). Polyclonal rabbit anti-human Lats2 antibody (Bethyl Laboratories, US), polyclonal rabbit anti-mouse β-actin antibody (Abcam, US) and HRP-conjugated goat anti-rabbit Ig (Amersham) were used.

Cell culture and differentiation

3T3-L1 preadipocytes were purchased from Cell Bank, Chinese Academy of Sciences) maintained in growth media (DMEM containing 10% calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin) at 37°C in 5% CO2. Cells were passaged before reaching confluence. 3T3-L1 preadipocytes were induced to form adipocytes as described (17). On the eighth day of differentiation, 3T3-L1 adipocytes were maintained for 12 h in serum-free medium before various effectors were added. Clenbuterol (Sigma-Aldrich, US), ICI 118,551 (Sigma-Aldrich, US) or both were added to adipocytes; control cells received additions of medium alone. Cells were collected 24 h later.

Assays for intracellular cAMP level

Intracellular cAMP levels were measured using ELISA kits (R&D, US) according to the manufacturer’s instructions. Details of assay were described in supplemental Materials and Methods.

Quantitative real-time RT-PCR

Real-time RT-PCR was performed on a fluorescence thermal cycler (ABI Prism 7900HT Sequence Detection System, Applied Biosystems). A standard two-step procedure was applied. Details of processes are described in supplemental Materials and Methods.

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