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A Novel Porcine Gene-*erlin2*, Differentially Expressed in the Liver Tissues from Meishan and Large White Pigs

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Abstract The messenger RNA differential display technique was performed to investigate the differences of gene expression in the liver tissues from Meishan and Large White pigs. A fragment of one differentially expressed gene was isolated and sequenced. A complete complementary DNA (cDNA) sequence was obtained using the rapid amplification of cDNA end method. Nucleotide sequence of the gene is not homologous to any of the known porcine genes. The sequence prediction analysis revealed that the open reading frame of this gene encodes a protein of 339 amino acids which have high homology with those of the ER lipid-raft-associated 2 isoform 2 (ERLIN2) of eight species—human (97%), rhesus monkey (97%), rat (96%), horse (97%), cattle (97%), mouse (97%), dog (95%), and red jungle fowl (90%)—so that it can be defined as the swine *erlin2* gene. The phylogenetic tree analysis revealed that the swine *erlin2* gene has a closer genetic relationship with the *erlin2* genes of human and rhesus monkey. The tissue expression profile analysis indicated that the swine *erlin2* gene is differentially expressed in detected tissues from Meishan and Large White pigs. Our experiment suggested that the swine *erlin2* gene might play an important role in the superabundant fat deposition of Chinese pigs.

Keywords Pig · *erlin2* gene · mRNA differential display · RACE

Introduction

Messenger RNA (mRNA) differential display first described by Liang and Pardee [1] is a fast and efficient method for isolating and characterizing altered gene expression in

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different cell types. It was statistically shown that 80–120 primer combinations would be sufficient to cover all the transcript populations in the cell [2]. This technique possesses the following advantages over other similar techniques: it is based on simple and established methods; more than two samples can be compared simultaneously and only a small amount of starting material is needed [3].

Chinese indigenous pig breeds such as Meishan, Erhualian, and Tongcheng often have some conspicuous flaws such as superabundant fat and too low grow rate while exotic pig breeds such as Large White, Landrace, and Duroc always have lower fat rate, higher lean meat rate, and higher grow rate. Therefore, Chinese indigenous pigs are always named fat-type pigs while exotic pigs are always named lean-type pigs [4]. Phenotypic variances are mainly determined by the genetic differences. So that detecting the genetic differences between Chinese indigenous pig breeds and exotic pig breeds or finding out the differentially expressed genes between Chinese indigenous pig breeds and exotic pig breeds which determine these phenotypic variances is necessary for pig breeders.

It is well known that liver is an important lipid metabolism tissue and a lot of its gene expressions are associated with the fat deposition. Hennessy et al. [5] indicated that a diet high in saturated fat and cholesterol may increase the accumulation of triglyceride and cholesterol in the liver, each resulting in the suppression of hepatic low-density lipoprotein receptor mRNA levels. Ye et al. [6] revealed that the mRNA expressions of apoA I, apoA IV, apoA V, apoB100, and Angptl 3 in the liver of apoE(−/−) mice change significantly, and these genes are relevant to the complicated lipid metabolism network and involved in the early stage of atherogenesis. Zou et al. [7] also found that the changes of PPARalpha, LXRalpha, and their target genes aggravated lipid metabolic disorder in the liver and further accelerated the development of atherosclerosis of apolipoprotein E and low-density lipoprotein receptor double-deficient mice on a stress of high-fat and cholesterol diet.

Our present study was carried out with the mRNA differential display technique to isolate the differentially expressed genes in the liver tissues from one fat-type pig breed, Meishan, and one lean-type pig breed, Large White. This would be helpful to understand the molecular mechanism why Chinese indigenous pigs have too much fat deposition while exotic pigs have not.

Materials and Methods

Sample Collection, RNA Isolation, and First-Strand cDNA Synthesis

The liver samples were collected from 120-day-old Large White (five males and five females) and Meishan (five males and five females) pigs for mRNA differential display and semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) identification. The tissues including back fat, liver, small intestine, muscle, spleen, lung, and kidney were collected from one 200-day-old Large White pig and one 200-day-old Meishan pig for the later tissue expression profile analysis. These tissues were immediately frozen in liquid nitrogen and stored at −80 °C. The total RNA was extracted from tissues mentioned above using the total RNA extraction kit (Gibco, Grand Island, NY, USA). Before the first-strand complementary DNA (cDNA) synthesis, DNase I treatment of the total RNA was

performed. First-strand cDNA synthesis was conducted by RNA reverse transcription as previously described [7].

Differential Display

The differential display PCR amplification of each reverse-transcription product was carried out with ten arbitrary primers and nine oligo(dT) primers as previously described [7, 8]. The PCR products were then separated on the 8% nondenaturing polyacrylamide gel and stained by silver stain described previously [8, 9].

Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed for porcine *erlin2* gene identification and expression profile analysis as described earlier [9–12]. To eliminate the effect of cDNA concentration, we repeated the RT-PCR four times using 100-, 200-, 300-, 400-, and 500-ng cDNA as templates, respectively. We selected the housekeeping gene *beta-actin* (DQ845171) as the internal control. The control primers used were: 5′-TGC TGTCCCTGTACGCCTCTG-3′ (forward primer 1) and 5′-ATGTCCCGCACGA TCTCCC-3′ (reverse primer 1). The PCR product is 220 bp in length. The following expressed sequence tag (EST) or gene-13-specific primers were used to perform the RT-PCR for identification and tissue expression profile analysis: 5′-GGTGG TGAACTTCCTGGTC-3′ (forward primer 2) and 5′-TGAAATCTTCTTCTCCGTCT-3′ (reverse primer 2). The PCR product is 460 bp in length. The 25-μl reaction system was: 2-μl cDNA (100–500 ng), 5 pmol each oligonucleotide primer (forward primers 1 and 2, reverse primers 1 and 2), 2.5 μl 2 mmol/l mixed dNTPs, 2.5 μl 10× Taq DNA polymerase buffer, 2.5 μl 25 mmol/l $MgCl_2$, 3.0 units of Taq DNA polymerase, and finally added with sterile water to a volume of 25 μl. The PCR program initially started with a 94 °C denaturation for 4 min, followed by 30 cycles of 94 °C/50 s, 52 °C/50 s, and 72 °C/50 s, then 72 °C extension for 10 min, and finally 4 °C to terminate the reaction.

The quantification of the PCR products was carried out with the use of Glyco BandScan software (PROZYME®, San Leandro, CA, USA) and the ratio of erlin2 to beta-actin was calculated using the common EXCEL program. Difference significance of ratios of erlin2 to beta-actin was analyzed with the least square method (GLM procedure, SAS version 8.0).

5'- and 3'-RACEs

5'- and 3'-rapid amplifications of cDNA ends (RACEs) were performed as the instructions of BD SMART™ RACE cDNA Amplification Kit (BD Sciences, San Jose, CA, USA). The gene-specific primers (GSPs) were: 3'-RACE GSP: 5'-AAACTGGCTTTG CAGCAGGATCTGA-3', 5'-RACE GSP: 5'- TTCCATCAGCTCATAGTTCCTGCGG-3'.

RACE touchdown PCRs were carried out with five cycles of 94 °C 30 s and 72 °C 3 min, followed by five cycles of 94 °C 30 s, 70 °C 30 s, and 72 °C 3 min, and finally with 30 cycles of 94 °C 30 s, 68 °C 30 s, and 72 °C 3 min to terminate the reaction. The RACE PCR products were then cloned into PMD18 T-vector (TaKaRa, Dalian, China) and sequenced bidirectionally with the commercial fluorometric method. At least five independent clones were sequenced for each PCR product.

Sequence Analysis

The cDNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and analysis were performed using the

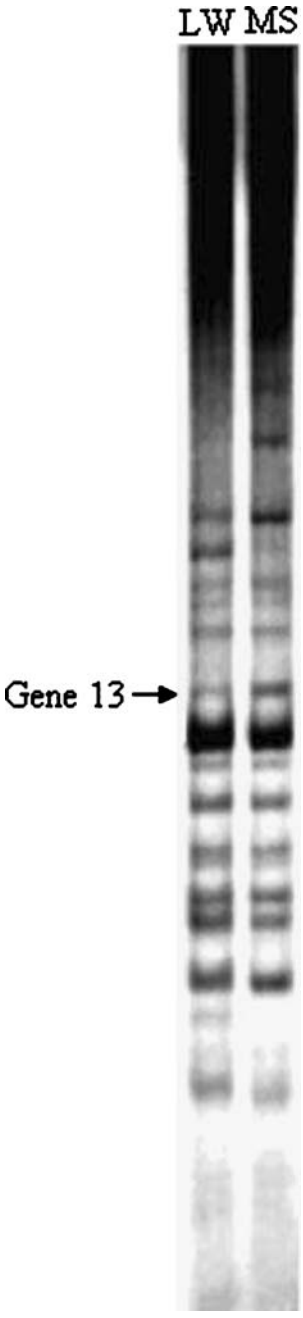

Fig. 1 The differential expression analysis of gene 13. The *arrow* indicates the cDNA profile for the gene13 on a polyacrylamide gel of 8%, stained with silver nitrate. *LW*—Large White; *MS*—Meishan

Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://www.ebi.ac.uk/clustalw).

Results and Discussion

mRNA Differential Display

From the mRNA differential display, one band, nominated as gene 13, later identified as the *erlin2* gene, was found to be moderately expressed in the liver of Meishan pigs while was weakly expressed in the liver of Large White pigs as shown in Fig. 1.

Semiquantitative RT-PCR

The differentially expressed gene band was recovered from gel and used as the template for the reamplification, which was performed with the corresponding oligo(dT) primer and the arbitrary primers used in the mRNA differential display. The resulting PCR product was 498 bp. This was in agreement with the result of the mRNA differential display. The purified PCR product was then cloned into the T-vector and the recombinant plasmid was sequenced. Semiquantitative RT-PCR was then conducted using the EST-specific primers and the results are presented in Fig. 2.

Semiquantitative RT-PCR results indicated that gene 13 was weakly expressed in the liver of Large White pigs and moderately expressed in the liver of Meishan pigs. This also coincided with the result of mRNA differential display.

5'- and 3'-RACEs

Through 5'-RACE, one PCR product of ~900 bp was obtained. The 3'-RACE product was ~1,000 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1,729-bp cDNA complete sequence was finally obtained.

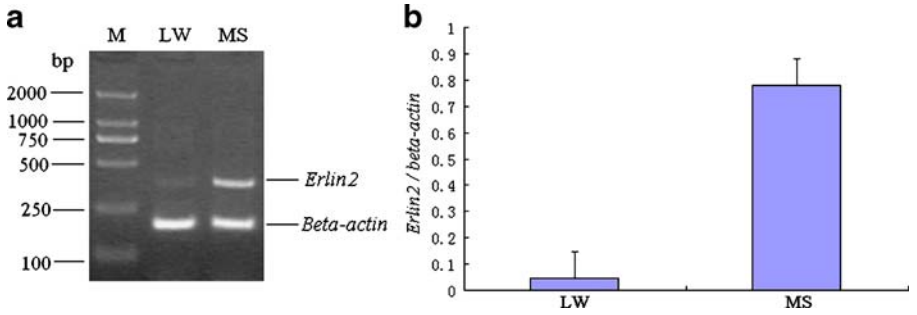

Fig. 2 Semiquantitative RT-PCR identification of gene 13 (*erlin2*). **a** The semiquantitative RT-PCR analysis of gene 13 (*erlin2*) on the agarose gel of 1% stained with ethidium bromide. **b** *Error bars* indicate standard deviations ($n=5$) of relative *erlin2* mRNA expression levels to beta-actin. *LW*—*Large White*; *MS*—Meishan. The *erlin2* to beta-actin ratios are the averages of five semiquantitative RT-PCRs using 100-, 200-, 300-, 400-, and 500-ng cDNA as templates. The signals of the PCR product were measured by BandScan software version 4.50

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Pig               MAQLGAVVAVAASFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Human             MAQLGAVVAVASSFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Rhesus monkey     MAQLGAVVAVASSFLCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Rat               MAQLGAVVAVASSFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Horse             MAQLGAIVAVATSFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Cattle            MAQLGAVVAVAASFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Mouse             MAQLGAVVAVASSFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Dog               MAQLGAVVAVATSFFCASLFSAVHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
Red jungle fowl   MAQLGAIAALVLSFLAAAFLSAIHKIEEGHIGVYYRGGALLTSTSGPGFHLMLPFITSYK
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Pig               SVQTTLQTDEVKNVPCGTSGGVMIYFDRVEVVNFLVPHAVYDIVKNYTADYDKALIFNKI
Human             SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPNAVYDIVKNYTADYDKALIFNKI
Rhesus monkey     SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPNAVYDIVKNYTADYDKALIFNKI
Rat               SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPHAVYDIVKNYTADYDKALIFNKI
Horse             SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPNAVYDIVKNYTADYDKALIFNKI
Cattle            SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPHAVYDIVKNYTADYDKALIFNKI
Mouse             SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPNAVYDIVKNYTADYDKALIFNKI
Dog               SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLVPNAVYDIVKNYTADYDKALIFNKI
Red jungle fowl   SVQTTLQTDEVKNVPCGTSGGVMIYFDRIEVVNFLIQSAVYDIVKNYTADYDKALIFNKI
                  ****************************:******:  **********************

Pig               HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Human             HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Rhesus monkey     HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Rat               HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Horse             HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Cattle            HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Mouse             HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Dog               HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTSMAPGLVIQAVRVTKPNIPEAIR
Red jungle fowl   HHELNQFCSVHTLQEVYIELFDQIDENLKLALQQDLTTMAPGLIIQAVRVTKPNIPETIR
                  ************************************:*****:************:**

Pig               RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Human             RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Rhesus monkey     RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Rat               RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Horse             RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Cattle            RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITFGQKVMEKETEK
Mouse             RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Dog               RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKVAQVAEITYGQKVMEKETEK
Red jungle fowl   RNYELMESEKTKLLIAAQKQKVVEKEAETERKKALIEAEKIAQVAEITYGQKVMEKETEK
                  ****************************************:*******:***********

Pig               KISEIEDAAFLAREKAKADAECYTAMKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Human             KISEIEDAAFLAREKAKADAECYTAMKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Rhesus monkey     KISEIEDAAFLAREKAKADAECYTAMKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Rat               KISEIEDAAFLAREKAKADAECYTALKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Horse             KISEIEDAAFLAREKAKADAECYTAMKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Cattle            RISEIEDAAFLAREKAKADAECYTAMKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Mouse             KISEIEDAAFLAREKAKADAECYTALKIAEANKLKLTPEYLQLMKYKAIASNSKIYFGKD
Dog               KISEIEDAAFLAREKAKADAECYTAMKLAEANKLKLTPEYLQLMKYRAIASNSKIYFGKD
Red jungle fowl   RISEIEDAAFLAREKARADAECYTAMKVAEANKLKLTPEYLQLMKYKAIAANSKIYFGKD
                  :***************:********:*:******************:***:*********

Pig               IPNMFMDSAGSLGKQFEGLT-DKLSFGLEDEPLEAGTEEN--
Human             IPNMFMDSAGSVSKQFEGLA-DKLSFGLEDEPLETATKEN--
Rhesus monkey     IPNMFMDSAGSVSKQFEGLA-DKLSFGLEDEPLETATKEN--
Rat               IPNMFMDSAGGLGKQSEGLS-DKLGFGLEDEPLETATKDN--
Horse             IPNMFVDSAGGLGKQFEGLA-DKLGFGLEDEPLEADPEEN--
Cattle            IPNMFMDSAGGVGKQFEGLA-DKLSFVLEDEPMEADSEN---
Mouse             IPNMFMDSAGGLGKQFEGLSDDKLGFGLEDEPLEAPTKEN--
Dog               IPNMFVDSAGSLGKQFEGLA-DKL--ILDDESLDADPEEN--
Red jungle fowl   IPNMFMDYAGSQSKFAEGLAEGIHEEDGAGPSEDTKLLHNTN
                  *****:* **. .*  ***: .       . . ::   .
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Fig. 3 The alignment of the proteins encoded by gene 13(*erlin2*) from pig and eight other kinds of ERLIN2 proteins from human, rhesus monkey, rat, horse, cattle, mouse, dog, and red jungle fowl

Sequence Analysis

The nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) revealed that this gene was not homologous to any of the known porcine genes and it was then deposited into the GenBank database (accession number FJ436386). The sequence prediction was carried out using the GenScan software. An open reading frame encoding 339 amino acids was found in this 1,729-bp cDNA sequence. Poly-A signal was from 1,489 to 1,494 bp (consensus: AATAAA). The theoretical isoelectric point (pI) and molecular weight (Mw) of this deduced protein of this swine gene were also computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). The pI of swine ERLIN2 protein was 5.36. The molecular weight of this putative protein was 37,803.47.

These putative proteins were also blasted using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and its conserved domains were identified as Band_7_3 domain.

Further, BLAST analysis of these proteins revealed that swine ERLIN2 protein has high homology with the ERLIN2 of eight species—human (NP_009106, 97%), rhesus monkey (XP_001088868, 97%), rat (NP_001099558, 96%), horse (XP_001493841, 97%), cattle (NP_001040041, 97%), mouse (NP_705820, 97%), dog (XP_848949, 95%), and red jungle fowl (XP_424380, 90%; Fig. 3).

From the sequencing and structural results described, this gene can be defined as the swine *erlin2* gene. Based on the results of the alignment of seven different species of ERLIN2, a phylogenetic tree was constructed using the ClustalW software (http://www.ebi.ac.uk/clustalw), as shown in Fig. 4 The phylogenetic tree analysis revealed that the swine *erlin2* gene has a closer genetic relationship with the *erlin2* genes of human and rhesus monkey than those of rat, horse, cattle, mouse, dog, and red jungle fowl.

Tissue Expression Profile

The tissue expression profile analysis indicated that the swine *erlin2* gene is moderately expressed in liver, spleen, lung, and kidney, weakly expressed in small intestine and muscle and hardly expressed in back fat of a 200-day-old Large White pig. The tissue expression

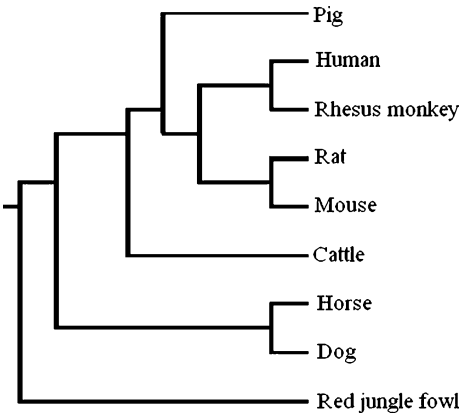

Fig. 4 The phylogenetic tree for nine kinds of *erlin2* genes from pig, human, rhesus monkey, rat, horse, cattle, mouse, dog, and red jungle fowl

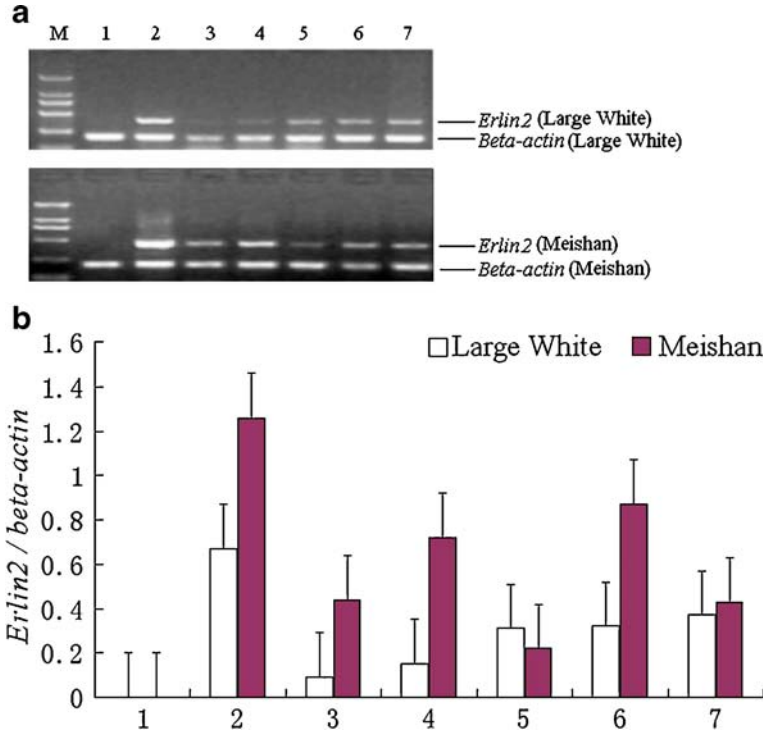

Fig. 5 Tissue expression profile of the swine *erlin2* gene. **a** The tissue expression profile analysis of the swine *erlin2* gene on the agarose gel of 1% stained with ethidium bromide. **b** *Error bars* indicate standard deviations (n=5) of relative *erlin2* mRNA expression levels to *beta-actin*. *M*, DL2000 marker, the marker molecular weight was same as in Fig. 2; *1*, back fat; *2*, liver; *3*, small intestine; *4*, muscle; *5*, spleen; *6*, lung; *7*, kidney. The *erlin2* to *beta-actin* ratios are the averages of five semiquantitative RT-PCRs using 100-, 200-, 300-, 400-, and 500-ng cDNA as templates. The signals of the PCR product were measured by BandScan software version 4.50

profile analysis also revealed that the swine *erlin2* gene is highly expressed in liver, moderately expressed in spleen, lung, kidney, small intestine, and muscle and hardly expressed in back fat of a 200-day-old Meishan pig. The swine *erlin2* gene expression of Meishan pig is higher than that of Large White pig in the liver, lung, kidney, small intestine, and muscle tissues except for spleen (Fig. 5).

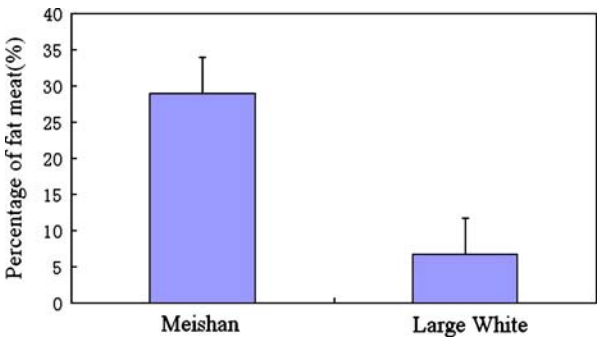

Fig. 6 Comparison of the percentage of fat meat (Meishan *vs* Large White)

ERLIN2 is a member of the band 7 domain of flotillin (reggie) like proteins. Many of these band-7-domain-containing proteins are lipid-raft-associated. Individual proteins of this band 7 domain family may cluster to form membrane microdomains which may in turn recruit multiprotein complexes. Microdomains formed from flotillin proteins may in addition be dynamic units with their own regulatory functions [13, 14]. Up until today, the swine ERLIN2 has not been reported.

From the results obtained above, we found that the *erlin2* gene was differentially expressed in the liver from Meishan and Large White pigs. Meishan is a fat-type pig breed, comprising much more body fat than lean meat or muscle. On the other hand, Large White is a typical lean-type pig breed, presenting the opposite phenotype than that described for the Meishan breed (Fig. 6).

To the percentage of fat meat, the two divergent pig breeds show the trend of Large White-low and Meishan-high. It is very interesting that the expression of the swine *erlin2* gene in the liver shows the same trend of Large White-low and Meishan-high. As we know, ERLIN2 is a kind of protein associated with the lipid raft. All these evidences above suggested that Meishan pigs had much more ERLIN2 protein expressed in the liver tissues than Large White pigs and Meishan pigs had much more ERLIN2-associated lipid raft capacity in the liver tissues than Large White pigs. So that Large White pigs have lower percentage of fat meat than Meishan pigs.

We also found that the swine *erlin2* gene also differentially expressed in other tissues through tissue expression profile analysis; especially, the swine *erlin2* gene expression of Meishan pig is higher than that of Large White pig in most tissues except for spleen; might these differential expressions also be associated with the fat metabolic process of these tissues? This also deserves to be studied.

In this experiment, we obtained the complete cDNA sequence of the swine *erlin2* gene and found that this gene is differentially expressed in the liver tissues from Meishan and Large White pigs. Our results suggested that the swine *erlin2* gene might play an important role in the superabundant fat deposition of Chinese pigs.

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