Distinctive Genes Determine Different Intramuscular Fat and Muscle Fiber Ratios of the *longissimus dorsi* Muscles in Jinhua and Landrace Pigs

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Abstract

Meat quality is determined by properties such as carcass color, tenderness and drip loss. These properties are closely associated with meat composition, which includes the types of muscle fiber and content of intramuscular fat (IMF). Muscle fibers are the main contributors to meat mass, while IMF not only contributes to the sensory properties but also to the plethora of physical, chemical and technological properties of meat. However, little is known about the molecular mechanisms that determine meat composition in different pig breeds. In this report we show that Jinhua pigs, a Chinese breed, contains much higher levels of IMF than do Landrace pigs, a Danish breed. We analyzed global gene expression profiles in the *longissimus dorsi* muscles in Jinhua and Landrace breeds at the ages of 30, 90 and 150 days. Cross-comparison analysis revealed that genes that regulate fatty acid biosynthesis (e.g., fatty acid synthase and stearoyl-CoA desaturase) are expressed at higher levels in Jinhua pigs whereas those that regulate myogenesis (e.g., myogenic factor 6 and forkhead box O1) are expressed at higher levels in Landrace pigs. Among those genes which are highly expressed in Jinhua pigs at 90 days (d90), we identified a novel gene porcine FLJ36031 (*pFLJ*), which functions as a positive regulator of fat deposition in cultured intramuscular adipocytes. In summary, our data showed that the up-regulation of fatty acid biosynthesis regulatory genes such as *pFLJ* and myogenesis inhibitory genes such as *myostatin* in the *longissimus dorsi* muscles of Jinhua pigs could explain why this local breed produces meat with high levels of IMF.

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**Introduction**

The Jinhua pig, named after Jinhua City in Zhejiang Province of eastern China, is a traditional, slow-growing breed with a high IMF content and is popular for its superior quality pork. Jinhua ham, a type of dry-cured ham produced from the meat of Jinhua pigs, is the most famous brand name's in China and Jinhua ham was awarded first prize in the 1915 Panama International Merchandise Exhibition. Jinhua pigs show strong competency of oxidative metabolism and adipogenesis, which are believed to induce more satisfactory features in muscles, such as favorable meat color, marbling and flavor [1,2]. In contrast, Landrace pigs, a commercial breed of Danish origin selected over many generations for rapid growth and enhanced carcass yield, show low activities of oxidative metabolism and adipogenesis which lead to trace amounts of fat depot. As a consequence, Landrace pigs produce comparatively less flavorful pork [3–5]. Thus, these two pig breeds serve as ideal models to study porcine growth performance and meat quality.

Skeletal muscle is the primary abundant porcine tissue that comprises 20% to 50% of total body mass among different pig breeds, and is the main tissue responsible for meat production in pigs. It is also the major metabolic tissue and contributes up to 40% of the resting metabolic rate in adult pigs [6]. Skeletal muscle is a heterogeneous tissue that is composed of four muscle fiber types including oxidative (type I and IIa) and glycolic (type IIb) fibers [7]. Muscle with a higher content of oxidative fibers contains a higher percentage of lipids, capillaries, myoglobin and mitochondria [8]. Favorable meat traits such as color, flavor and tenderness have been found to be closely associated with a higher content of oxidative fibers in muscles [9,10]. In addition, individuals with muscles that are abundant in oxidative fibers are less likely to produce pale, soft, exudative (PSE) meat. Therefore, understanding the molecular processes that govern the development and phenotypic characteristics of skeletal muscle is instrumental in the breeding of pigs with high meat quality.

Microarray technology can simultaneously examine the differential expression of a large number of genes in a given tissue [7,11] and has been widely used to compare gene expression profiles for the identification of candidate genes responsible for relevant phenotypes [12–14]. For example, microarray analysis showed that sexual dimorphism of adipose tissue is determined by differentially regulated sex-specific genes regardless of diet [15]. In contrast,
comparison of global gene expression profiles using Affymetrix Mu11K SubB containing 6516 probe sets revealed only 49 differentially expressed genes in the *quad* (white muscle) and the *soleus* (red muscle) [16]. Based on a home-made porcine cDNA microarray carrying 5,500 cDNA clones, Bai et al. identified 115 differentially expressed genes between the *psoas* (red muscle) and the *longissimus dorsi* (white muscle) of a 22-week-old Berkshire pig [17]. Over the past decade, a tremendous amount of porcine transcriptomics data has been obtained using the pig cDNA microarray [18–20], while the Affymetrix porcine genome array showed particularly superior performance for swine transcriptomics [21,22]. However, reports on the comparison of global gene expression patterns in the skeletal muscles of different pig breeds at different developmental stages are lacking. In this study, a global gene expression profiling investigation was conducted to identify differentially expressed genes in *longissimus dorsi* muscles of Jinhua and Landrace pigs at three developmental stages using the Affymetrix GeneChip® Porcine Genome Array containing oligonucleotides representing approximately 23937 transcripts from 20201 porcine genes. We found that genes involved in adipogenesis and myogenesis were differentially expressed in Jinhua and Landrace pigs. To validate the potential utility of our microarray data, we characterized the expression and function of a novel gene, *pFLJ*, that is one of the genes up-regulated in Jinhua pigs at the age of d90 using both drug and gene-specific small interfering RNA (siRNA) treatment approaches in cultured intramuscular adipocyte precursor cells. Our results showed that knockdown of *pFLJ* expression down-regulated the genes involved in fat biosynthesis and reduced fat deposition, suggesting that *pFLJ* is a novel regulator of adipogenesis in the muscle.

**Results and Discussion**

**Comparison of Carcass Traits and Meat Quality Features between Jinhua and Landrace Pig Breeds**

The overall appearance of a typical adult Jinhua pig is very different from that of a Landrace pig (Figure 1A). Growth

![Jinhua pig (d150) and Landrace pig (d150)](image)

**Figure 1. The Landrace breed grows faster than does the Jinhua breed.** (A) Photographs showing three Jinhua pigs and one Landrace pig at d150. (B) Comparison of the body weight of Jinhua and Landrace pigs at the age of d30, d60, d90, d120 and d150, respectively. Landrace pigs gained weight much faster than Jinhua pigs. Pigs were slaughtered at around the age of d30, d90 and d150 (nine individuals per stage) and d60 and d120 (three individuals per stage) for each breed. Data are presented as means ± standard error. *P*<0.05, **P**<0.01. doi:10.1371/journal.pone.0053181.g001
performance, meat quality and carcass traits in Jinhua and Landrace pigs at the same age (d30, d60, d90, d120, d150, days of age) were compared. Our results showed that from the age of d30 to d150, on average, Jinhua pigs gained approximately 40 kg in weight, while Landrace pigs gained about 70 kg (Figure 1B), demonstrating that the Jinhua were apparently growing more slowly than the Landrace. Analysis of the lean meat ratio (LMR) and loin meat area (LMA) showed that both were significantly lower in Jinhua pigs aged from d30 to d150 (Table 1). In contrast, Jinhua pigs exhibited significantly greater back fat thicknesses (BFT) and fat meat ratios (FMR) (Table 1, P < 0.01). For example at d150, BFT and FMR in Jinhua pigs were about 2- and 2.4-fold higher, respectively (BFT: 23.7 mm in Jinhua versus 12.0 mm in Landrace; FMR: 32.4% in Jinhua versus 13.3% in Landrace) (Table 1).

It was previously reported that the Chinese Dahe pig breed exhibited a lower incidence of PSE meat [24]. We determined the pH45 value at 45 min post mortem [23]. A high pH displayed higher pH values (6.08) than the western crossbred Jinhua Pigs have a High Content of IMF

Analysis of the color parameters showed that there was a significant tendency for the a* value in muscle redness; b*, yellowness) are used as an index of meat quality. Table 1. Determination of carcass traits and meat quality in Jinhua and Landrace pigs at the age stage of 30, 60, 90, 120 and 150 days age.1

| Items                | 30                  | 60                  | 90                  | 120                 | 150                 |
|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                      | Jinhua (n = 9)      | Landrace (n = 9)    | Jinhua (n = 9)      | Landrace (n = 9)    | Jinhua (n = 9)      |
| BFT (mm)             | 9.33 ± 1.23a        | 2.15 ± 0.45a        | 11.00 ± 1.07a       | 6.24 ± 0.63a        | 20.03 ± 0.91         |
|                      | 6.00 ± 0.58b        | 21.90 ± 0.76        | 10.33 ± 0.88        | 23.70 ± 0.92        | 12.00 ± 1.00a       |
| FMR (%)              | 14.86 ± 1.03a       | 6.70 ± 0.94a        | 15.60 ± 2.37a       | 7.01 ± 0.24a        | 26.21 ± 1.13         |
|                      | 7.67 ± 0.54a        | 29.58 ± 1.30        | 8.36 ± 0.29a        | 32.40 ± 1.75         | 13.26 ± 1.26b       |
| LMR (%)              | 44.27 ± 0.52a       | 51.97 ± 2.45a       | 47.19 ± 1.28        | 62.01 ± 1.23a       | 42.73 ± 1.13a        |
|                      | 70.77 ± 1.81a       | 40.78 ± 0.64        | 69.59 ± 1.48        | 41.01 ± 1.48        | 46.84 ± 2.08b        |
| LMA (cm²)            | 0.48 ± 0.01         | 0.87 ± 0.08         | 1.06 ± 0.10         | 1.02 ± 0.21         | 1.47 ± 0.01c         |
|                      | 3.19 ± 0.05         | 1.72 ± 0.05         | 4.04 ± 0.06         | 2.49 ± 0.05         | 5.40 ± 0.21b         |
| PH45                 | 5.59 ± 0.19         | 6.12 ± 0.12         | 6.26 ± 0.25         | 6.50 ± 0.11         | 6.31 ± 0.06          |
|                      | 6.52 ± 0.09         | 6.15 ± 0.02         | 6.46 ± 0.13         | 6.39 ± 0.04         | 6.32 ± 0.36          |
| Color                | 44.46 ± 0.09a       | 41.11 ± 0.63a       | 44.07 ± 0.24        | 44.54 ± 0.47        | 44.12 ± 0.93         |
|                      | 43.12 ± 0.28        | 42.91 ± 1.08        | 40.77 ± 0.85        | 44.25 ± 0.96         | 39.77 ± 0.26c        |
| a*                   | 12.27 ± 0.92        | 15.17 ± 0.68        | 9.71 ± 0.31         | 11.52 ± 0.37        | 9.96 ± 0.68          |
|                      | 10.02 ± 0.39        | 10.50 ± 0.39        | 10.22 ± 0.09        | 8.52 ± 0.66a        | 10.33 ± 0.39         |
| b*                   | 11.66 ± 0.71        | 11.54 ± 0.53        | 10.28 ± 0.31        | 10.74 ± 0.12        | 11.02 ± 0.36         |
|                      | 11.31 ± 0.032       | 10.86 ± 0.33        | 8.74 ± 0.14         | 9.92 ± 0.35         | 9.45 ± 0.34          |

1. Results are presented as means ± standard error.
2. BFT = back fat thickness.
3. FMR = fat meat ratio.
4. LMR = lean meat ratio.
5. LMA = longissimus muscle area.
6. PH45 = pH value at 45 min postmortem.
7. Color = meat color. l*, a*, b* represent as lightness, redness and yellowness, respectively.
8. a and AbMeans with different superscripts of capital or lowercase letter at the same row of the same age are significantly different (P < 0.05 or P < 0.01).
Figure 2. The Jinhua breed has a higher IMF content than the Landrace breed. (A) Oil Red O staining of longissimus dorsi muscles in Jinhua and Landrace pigs, respectively. Oil Red O stained IMF displayed a red color. (B) Comparison of IMF contents in longissimus dorsi muscles in Jinhua and Landrace pigs at the age of d30, d60, d90, d120 and d150, respectively. Pigs were slaughtered at around the age of d30, d90 and d150 (nine individuals per stage) and d60 and d120 (three individuals per stage) for each breed. Data are presented as means ± standard error. **P<0.01. Scale bars, 100 µm.
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Table 2. Summary of the number of genes up- or down-regulated in longissimus dorsi muscles in Jinhua or Landrace pigs at age of d90 and d150.1

|               | Jinhua pigs |               | Landrace pigs |               |
|---------------|-------------|---------------|---------------|---------------|
| d90-up        | 177         | d90-down      | 242           |
| d150-up       | 101         | d150-down     | 389           |
| d90- & d150-up| 37          | d90- & d150-down| 109         |
| d90-up        | 106         | d90-down      | 231           |
| d150-up       | 93          | d150-down     | 387           |
| d90- & d150-up| 11          | d90- & d150-down| 64         |
| d90-up, Jinhua vs Landrace | 2          | d90-down, Jinhua vs Landrace | 8 |
| d150-up, Jinhua vs Landrace | 6          | d150-down, Jinhua vs Landrace | 57 |

1Number of genes was obtained by comparing the expression profiles between d30 and d90 or d30 and d150 in each breed. Details are listed in Tables S2 (d90-up in Jinhua pigs), S3 (d90-down in Jinhua pigs), S4 (d150-up in Jinhua pigs), S5 (d150-down in Jinhua pigs), S6 (d90-up in Landrace pigs), S6 (d90-down in Landrace pigs), S7 (d150-up in Landrace pigs), and S8 (d150-down in Landrace pigs).
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at d30, 37 genes were both d90-up and d150-up, 109 genes were d90-down and d150-down, two genes were d90-up but d150-down, and six genes were d30-down but d150-up (Table 2).

In contrast, in *longissimus dorsi* muscles of Landrace pigs, 106 d90-up, 231 d90-down, 93 d150-up, 383 d150-down genes were identified, respectively, when compared with expression at d30 (Table 2; Table S5, S6, S7, S8). Clustering analysis of microarray data showed that, in comparison to expression at d30, 31 genes were both d90-up and d150-up, and 64 genes were d90-down and d150-down. Interestingly, no gene was found to be d90-up but d150-down or d90-down but d150-up (Table 2).

The fact that no or only a limited number of genes belonged to the d90-up/d150-down or d90-down/d150-up categories in both breeds suggests that the transcriptome operates sequentially to support the development of *longissimus dorsi* muscle during the d30 to d150 period. This provides a possible explanation for the continuous gain in muscle mass during this developmental window.

We also compared the d90-up and d90-down genes in Jinhua pigs with those of Landrace pigs. The results showed that only 0.7% of d90-up and 1.7% of d90-down genes were shared in these two breeds (Table 2). For d150-up and d150-down genes, only 3.2% of d150-up and 7.9% of d150-down genes were common to the two breeds (Table 2). These data clearly indicates that different genes are mobilized in these two breeds to govern the development of their respective *longissimus dorsi* muscles.

### Table 3. Summary of the number of genes differentially expressed in *longissimus dorsi* muscles in Jinhua and Landrace pigs at age of d30, d90 and d150.1

| Age (d) | Jinhua-up | Jinhua-down |
|---------|-----------|-------------|
| 30d     | 176       | 199         |
| 90d     | 276       | 155         |
| 150d    | 525       | 670         |

1 Number of genes was obtained by comparing the expression profiles between Jinhua and Landrace pigs of the same age. Details are listed in Tables S10 (d30 Jinhua-up), S11 (d90 Jinhua-up), S12 (d150 Jinhua-up), S13 (d30 Jinhua-down), S14 (d90 Jinhua-down), S15 (d150, Jinhua-down).

Identification of Genes Differentially Expressed in Jinhua and Landrace Pigs during Muscle Development

The global expression profiles in *longissimus dorsi* muscles at d30, d90 and d150 in Jinhua pigs were compared with those in Landrace pigs at corresponding stages. A total of 375, 431 and 1195 genes were identified at d30, d90 and d150 age of stage, respectively, with at least 2.0-fold difference (*P* value, 0.05) between two breeds (Table 3). Among these, 176, 276 and 525 genes corresponding to the stages of d30, d90 and d150 were up-regulated in Jinhua pigs (Jinhua-up genes) (Table 3; Table S9, S10, S11), and 199, 155 and 670 genes corresponding to the stages of d30, d90 and d150 were down-regulated (Jinhua-down genes) (Table 3; Table S12, S13, S14).

Among the differentially expressed genes identified by microarray in *longissimus dorsi* muscles of Jinhua and Landrace pigs at d90, 16 Jinhua-up genes (AY589691.1, CO993113, BF712908, CN153105, BF078710, BX924812, CF365450, NM_213785, NM_213938.1, NM_214392, BQ600160, BI399912, U83916.1, CF176622, NM_214294.1, NM_214236.1) were selected for validation by quantitative polymerase chain reaction (qPCR). Our results showed that with the exception of NM_214392 all of the selected genes were confirmed to be Jinhua-up genes (Figure 3).
However, we noticed that, although the patterns of differential expression of the examined genes were qualitatively similar between microarray and qPCR analysis (which shows the reliability of our microarray analysis), the fold changes obtained by the two approaches differed. We reasoned that this may be due to the greater accuracy of quantitation provided by qPCR compared with microarrays or to differences in the scope of magnitude of measurement of the two techniques [32].

### Table 4. List of representative genes for adipogenesis and myogenesis differentially expressed in *longissimus dorsi* muscle in Jinhua and Landrace (L) pigs at d30.

| Probe ID       | Gene ID     | Gene name                        | Gene Symbol | J/L Z score |
|----------------|-------------|----------------------------------|-------------|-------------|
| **Adipose metabolism related genes (Jinhua-up)** |             |                                  |             |             |
| Ssc.16159.1.S1_at | NM_213781.1 | stearoyl-CoA desaturase           | SCD         | 5.97        |
| Ssc.5538.1.S1_at | CN153105    | similar to Carbonic anhydrase 2   | LOC100154873| 6.60        |
| Ssc.1225.1.S1_at | CK455955    | Similar to acetyl-Coenzyme A acyltransferase 1 | LOC100152367| 3.15        |
| Ssc.17347.1.S1_at | NM_214349.1 | pyruvate carboxylase             | PC          | 2.78        |
| Ssc.22959.1.S1_at | BX676168    | phosphoenolpyruvate carboxykinase 1 | CH242-37G9.2| 6.63        |
| Ssc.1147.2.S1_at | BF712908    | Lipoprotein lipase               | LPL         | 2.79        |
| Ssc.6784.1.S1_at | AY686758.1  | lipase, hormone-sensitive        | LIPE        | 3.08        |
| Ssc.18175.1.A1_at | CN166778    | fatty acid synthase              | FASN        | 6.13        |
| Ssc.4360.1.A1_at | CB471223    | fatty acid binding protein 3     | FABP3       | 2.61        |
| Ssc.18549.1.S1_at | AYS89691.1  | C1Q and collagen domain containing adiponectin | ADIPOQ     | 3.18        |
| Ssc.11096.1.S1_at | NM_213938.1 | 3-oxoadic Co transferase 1       | OXCT1       | 2.59        |
| Ssc.4021.1.S1_at | BG608754    | 1-acylglycerol-3-phosphate O-acyltransferase 1 | SBAB-649D6.6| 2.24        |
| **Adipose metabolism related genes (Jinhua-down)** |             |                                  |             |             |
| Ssc.4292.1.S1_at | BF193243    | similar to Peroxisomal biogenesis factor 19 | LOC100154884| −2.94       |
| Ssc.8799.1.A1_at | AJ658284    | Similar to Apolipoprotein O-like  | LOC100153260| −3.11       |
| Ssc.1013.1.A1_at | BIII99912   | pyruvate dehydrogenase kinase, isozyme 4 | PDK4        | −3.65       |
| Ssc.8139.1.S1_at | CB475937    | Phytanoyl-CoA 2-hydroxylase      | PHYH        | −2.12       |
| Ssc.2143.1.A1_s_at | CF789622     | phosphoglycerate dehydrogenase   | CH242-38B5.2| −2.95       |
| Ssc.1942.1.S1_at | CN166665    | lipin 1                          | LPIN1       | −3.12       |
| Ssc.9365.1.S1_at | NM_213883.1 | insulin-like growth factor 2     | IGF2        | −2.65       |
| Ssc.1858.1.S1_at | CN155220    | beta glucuronidase               | GUSB        | −2.42       |
| Ssc.1326.1.S1_at | BX924410    | eukaryotic translation initiation factor 4E binding protein 1 | EIF4EBP1 | −2.90       |
| Ssc.217.1.S1_at | NM_214060.1 | esterase D                       | ESD         | −2.39       |
| **Muscle development related genes (Jinhua-up)** |             |                                  |             |             |
| Ssc.1025.1.A1_at | BI400362    | phosphodiesterase 4B, CAMP-specific | PDE4B        | 3.72        |
| Ssc.0906.1.S1_at | BF075680    | Mdfic family inhibitor domain containing | MDFIC       | 2.13        |
| Ssc.9984.1.A1_at | BIII99508   | Kruppel-like factor 4            | KLF4        | 2.09        |
| Ssc.657.1.A1_at | NM_214214.1 | chemokine (C-C motif) ligand 2   | CCL2        | 3.78        |
| Ssc.1901.1.A1_at | CO939491    | cardiac muscle alpha actin 1     | ACTC1       | 2.52        |
| Ssc.9013.1.S1_at | NM_213878.1 | calponin 1, basic, smooth muscle | CNN1        | 2.82        |
| **Muscle development related genes (Jinhua-down)** |             |                                  |             |             |
| Ssc.12900.1.A1_at | BI404128    | similar to Peripheral plasma membrane protein CASK | LOC100153146| −2.32       |
| Ssc.1032.1.A1_at | BI400288    | similar to myosin regulatory light chain interacting protein | LOC100155795| −2.12       |
| Ssc.21763.1.A1_at | CK456888    | gamma Sarcoglycan                | SGC          | −4.34       |
| Ssc.715.1.S1_at | NM_214236.1 | myoglobin                        | MB          | −2.78       |
| Ssc.16626.1.S1_at | AY188502.1  | myogenic factor 6                | MEF6        | −2.03       |
| Ssc.73.1.S1_at | NM_214014.1 | forkhead box O1                  | FOXO1       | −2.06       |
| Ssc.7146.1.A1_at | BII82779    | ATP-binding cassette, sub-family A, member 1 | ABCA1 | −3.57       |
| Ssc.3715.3.A1_at | CA778869    | Solute carrier family 7          | SLC7A7      | −2.19       |

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Adipose Deposition Related Genes are Differentially Activated in Jinhua and Landrace Pigs

A high IMF ratio is considered to be the major factor that contributes to the flavor of Jinhua meat. We noted that the IMF ratio in Jinhua pigs (2.25) was ~76% higher than that in Landrace pigs (1.28%) at d90 (Figure 2B), suggesting that, in addition to muscle development, IMF development program in Jinhua pigs must be activated at this time-point. We analyzed the differentially expressed genes in the two breeds at d30, d90 and d150 to elucidate the relationship between differential gene expression patterns and phenotypic differences in their longissimus dorsi muscles. Table 4, Table 5, and Table 6 (for pigs at d30, d90 and d150, respectively) listed the representative differentially expressed genes known to be related to adipose deposition and muscle development based on the OMIM database of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/omim/) and relevant publications that described their biological function.

We first examined the genes related to adipose deposition. At d30, genes related to adipose deposition were clearly more active in Jinhua than in Landrace pigs (Jinhua-up genes) (Table 4). These include stearoyl-CoA desaturase (NM_213781.1), acetyl-Coenzyme A acyltransferase 1 (CK453595), lipoprotein lipase (BF712908) [33–35], hormone-sensitive lipase (AY686758.1) [36–38], fatty acid synthase (CN166778) [39–41], fatty acid binding protein 3 (CB471223) [42–44], C1Q and collagen domain containing adiponectin (AY589691.1) [45] and 1-acylglycerol-3-phosphate O-acyltransferase 1 (BG668754) etc. At d90 and d150, more adipose deposition-related genes were classified as Jinhua-up genes, including cavelolin 2 (BF191227) [46–49], C-4 to C-12 straight chain acyl-Coenzyme A dehydrogenase (NM_214039.1) [49,50], lipoprotein lipase (AY666760.1) and 3-oxoacid CoA transferase 1 (NM_213938.1) etc at d90 (Table 5), and solute carrier family 27 member 4 (fatty acid transporter) (CN156506), nitrilase 1 (BX672917) [52], ribosomal protein L32 (NM_001001636.1), ribosomal protein L23 (AP296004) [53], ribosomal protein L12 (BP172489), Claudin 7 (CK450245) and carboxylesterase (NM_214246.1) [54–56] etc at d150 (Table 6). These expression signatures correlate well with the fact that Jinhua pigs have a high IMF content.

In contrast, the longissimus dorsi muscles of Landrace pigs were found to express genes (Jinhua-down) such as insulin-like growth factor 2 (NM_213803.1) [57,58], insulin-like growth factor binding protein 3

| Table 5. List of representative genes for adipogenesis and myogenesis differentially expressed in longissimus dorsi muscle in Jinhua and Landrace (L) pigs at d90. |
|---|
| **Probe ID** | **Gene ID** | **Gene name** | **Gene Symbol** | **J/L** | **Z score** |
| Adipose metabolism related genes (Jinhua-up) | | |
| Ssc.1680.1S1_at | CK451176 | similar to WW domain containing E3 ubiquitin protein ligase 1 | LOC100157283 | 2.04 |
| Ssc.6238.2S1_at | BI400300 | similar to adenylosuccinate synthetase 1 | LOC100155691 | 2.39 |
| Ssc.1013.1A1_at | BI399912 | pyruvate dehydrogenase kinase, isozyme 4 | PDK4 | 2.16 |
| Ssc.16335.1S1_at | AY686760.1 | lipoprotein lipase | LPL | 3.05 |
| Ssc.9637.1S1_at | NM_213909.1 | glutamate-ammonia ligase (glutamine synthetase) | GLUL | 4.25 |
| Ssc.31165.1S1_at | BF191227 | cavelolin 2 | CAV2 | 2.81 |
| Ssc.1203.1S1_at | AU055626 | C1Q and collagen domain containing adiponectin | ADIPOQ | 2.42 |
| Ssc.142.1S1_at | NM_214039.1 | acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain | OXCT1 | 2.31 |
| Ssc.777.1S1_at | AF414124.1 | 11-beta hydroxysteroid dehydrogenase isofrom 1 | HSD11B1 | 2.43 |
| Adipose metabolism related genes (Jinhua-down) | | |
| Ssc.4292.1S1A1 at | BF193243 | similar to Peroxisomal biogenesis factor 19 | LOC100154884 | 2.22 |
| Ssc.6779.1A1_at | AJ658284 | Similar to Apolipoprotein O-like | LOC100153260 | 2.16 |
| Ssc.6498.1A1_at | BI360380 | similar to Peroxisomal biogenesis factor 19 | LOC100154884 | 2.22 |
| Ssc.15800.1S1_at | NM_214099.1 | insulin-like growth factor binding protein 5 | IGFBP5 | 2.09 |
| Ssc.9365.2S1_at | CK463136 | insulin-like growth factor binding protein 5 | IGF2 | 2.42 |
| Muscle development related genes (Jinhua-up) | | |
| Ssc.1664.1A1_at | BG382637 | Kruppel-like factor 4 | KLF4 | 2.06 |
| Ssc.9984.1A1_at | BI399508 | similar to Peroxisomal biogenesis factor 19 | KLF4 | 2.96 |
| Ssc.235.2S1_at | M20160.1 | calpastatin | CAST | 2.22 |
| Ssc.335.1S2_at | AF188635.1 | myostatin | MSTN | 2.25 |
| Muscle development related genes (Jinhua-down) | | |
| Ssc.715.1S1_at | NM_214236.1 | Myoglobin | MB | −2.56 |
| Ssc.11858.1S1_at | CN163410 | fibromodulin | FMD | −2.41 |
| Ssc.1901.1A1_at | CO939491 | cardiac muscle alpha actin 1 | ACT1 | −4.48 |
| Ssc.1029.1S1_at | BX666372 | capping protein (actin filament) muscle Z-line, beta | CAPZB | −2.23 |
| Ssc.7538.1S1_at | BQ604786 | cadherin 1, type 1, E-cadherin (epithelial) | CDH1 | −2.97 |

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Table 6. List of representative genes for adipogenesis and myogenesis differentially expressed in *longissimus dorsi* muscle in Jinhua and Landrace (L) pigs at d150.

| Probe ID | Gene ID | Gene Name | Gene Symbol | J/L Z score |
|----------|---------|-----------|-------------|-------------|
| **Adipose metabolism related (Jinhua-up)** | | | | |
| Scs.2430.1.51_at | CN156586 | similar to solute carrier family 27 | LOC100155567 | 2.18 |
| Scs.1294.3.51_at | BX672817 | similar to nitrilase 1 | LOC100155270 | 2.07 |
| Scs.11142.9.51_at | AW359358 | Similar to carbonic anhydrase IX | LOC100152792 | 2.19 |
| Scs.3284.1.51_at | NM_001001636.1 | ribosomal protein L32 | RPL32 | 2.65 |
| Scs.805.1.51_at | AJ296004 | ribosomal protein L23 | RPL23 | 2.57 |
| Scs.939.1.51_at | BP172489 | ribosomal protein L12 | RPL12 | 2.24 |
| Scs.13910.1.51_at | BX667169 | phenylethanolamine N-methyltransferase | PNMT | 2.20 |
| Scs.37.1.51_at | NM_214000.1 | haptoglobin | HP | 3.51 |
| Scs.18918.1.51_at | CF365816 | glutathione peroxidase 2 | GPX2 | 4.59 |
| Scs.204.1.51_at | NM_21423.1 | cytochrome P450 3A29 | CYP3A29 | 7.95 |
| Scs.825.1.51_at | CK450245 | claudin 7 | CLDN7 | 3.10 |
| Scs.19471.1.51_at | CF365558 | Carboxylesterase 1 (monocyte/macrophage serine esterase 1) | CES1 | 2.25 |
| Scs.760.1.51_at | NM_214246.1 | carboxylesterase | CES3 | 2.92 |
| Scs.16162.1.51_at | NM_214224.1 | 4-hydroxyphenylpyruvate dioxygenase | HPD | 2.12 |
| **Adipose metabolism related (Jinhua-down)** | | | | |
| Scs.1008.1.51_at | BF703815 | wingless-type MMTV integration site family, member 10B | WNT10B | −2.11 |
| Scs.1049.1.51_at | NM_213781.1 | stearoyl-CoA desaturase | SCD | −7.06 |
| Scs.11488.1.51_at | BF919324 | similar to Peroxisomal biogenesis factor 19 | LOCl00154884 | −2.43 |
| Scs.15928.1.51_at | CF753539 | insulin-like growth factor binding protein 7 | IGFBP7 | −2.36 |
| Scs.15950.1.51_at | CN163405 | insulin-like growth factor binding protein 6 | IGFBP6 | −3.32 |
| Scs.16169.1.51_x_at | BP152514 | insulin-like growth factor 2 | IGF2 | −3.74 |
| Scs.16473.1.51_at | NM_214281.1 | fumarate hydratase | FH | −2.40 |
| Scs.16671.1.51_at | CB285696 | fatty acid binding protein 2, intestinal | FABP2 | −2.02 |
| Scs.17914.1.51_at | CK461797 | Cellular retinoic acid binding protein 1 | LOCl00169745 | −3.29 |
| Scs.17991.1.51_at | NM_214438.1 | caveolin 1 | CAV1 | −2.00 |
| Scs.18061.1.51_at | CF178743 | calstabin 1 | LOC733663 | −2.14 |
| Scs.18223.1.51_at | BQ599486 | C1q and tumor necrosis factor related protein C1QTNF3 | 3 | −3.46 |
| Scs.18206.2.51_a_at | BF080387 | ATP citrate lyase | ACL | −2.34 |
| Scs.18318.1.51_at | BI401144 | arachidonate 5-lipoxygenase-activating protein | ALOX5AP | −3.42 |
| **Muscle development related (Jinhua-up)** | | | | |
| Scs.13859.1.51_at | CN069994 | Unc-45 homolog B | UNC45B | 2.47 |
| Scs.2464.1.51_at | BI400766 | Stanniocalcin 1 | STC1 | 2.53 |
| Scs.18944.2.51_at | CF180682 | similar to ankyrin repeat domain 2 (stretch responsive muscle) | LOCl00155185 | 3.74 |
| Scs.20874.3.51_at | BP165311 | similar to Alpha-centractin (Centrosome-associated actin homolog) (ARP1) | LOCl00156619 | 2.23 |
| Scs.9781.1.51_at | NM_213910.1 | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | SERPINE1 | 2.19 |
| Scs.16060.1.51_at | AF128841.1 | sarcolumenin precursor | CBPG | 3.24 |
| Scs.21716.1.51_at | BG834768 | protein phosphatase 1 catalytic subunit alpha LOC733611 isoform | | 2.29 |
| Scs.23978.1.51_at | BF80704 | phosphatase and actin regulator 3 | CH242-60A21.1 | 2.50 |
| Scs.27601.1.51_at | AY579430.1 | paired box 3 | PAX3 | 2.62 |
| Scs.10199.3.51_at | CF364321 | dystrobrevin binding protein 1 | DTNBP1 | 2.23 |
Insulin-like growth factor binding protein 6 (NM_214099.1) [59,60], insulin-like growth factor binding protein 7 (CN163405) [59], lipin 1 (CF175359) [61], and peroxisomal biogenesis factor 19 (BF193243) from d30 to d150 (Table 4, Table 5, Table 6). These genes are known to be involved in regulating fatty acid oxidation [64–66], suggesting that the longissimus dorsi muscles of Landrace pigs have stronger active in fatty acid oxidation than deposition.

Muscle Development Related Genes are Differentially Expressed in Jinhua and Landrace Pigs

In contrast to the strong expression of genes related to adipose deposition, some key genes related to muscle development, including myogenic factor 6 (AY188502.1), forkhead box O1 (NM_214014.1) [67,68], -sarcoglycan (CK456888) [69,70], myosin regulatory light chain interacting protein (BI400288) and peripheral plasma membrane protein CASK (BI404128) [71,72] were expressed at a lower level in Jinhua (Jinhua-down) than in Landrace pigs at d30 (Table 4). In addition, myogenic differentiation 1 (NM_001002824.1) [73,74] was also expressed at a lower level in Jinhua than in Landrace pigs at d150. In fact, Jinhua pigs appeared to express genes that slow down muscle development at d30 and d90. For example, MyoD family inhibitor domain containing factor (BF075680) [75] and myostatin (AF188635.1) [76,77] were expressed at a higher level in Jinhua than in Landrace pigs at d30 and d90, respectively (Table 4 and Table 5). Consequently, many genes encoding muscle components were expressed at a lower level in Jinhua pigs (Jinhua-down) throughout the developmental stages of d30-d150, including myoglobin (NM_214236.1) [78,79], fibromodulin (CN163410) [80], -capping protein (actin filament) muscle Z-line (BX666372) [81,82], cardiac muscle alpha actin 1 (CO939491), and fibrinogen-like 2 (BI402879) (Table 4, Table 5, Table 6). This observation provides an explanation for the slow growth rate of Jinhua pigs.

Interestingly, some other factors which might be related to adipose deposition or muscle development were also found to be Jinhua-up, such as Kruppel-like factor 4 (BI399508) [83,84], smooth muscle calponin 1 (NM_001002824.1) [85,86], and myogenic differentiation 1 (NM_001002824.1) [87,88].

### Table 6. Cont.

| Probe ID | Gene ID | Gene Name | Gene Symbol | J/L Z score |
|----------|---------|-----------|-------------|-------------|
| Ssc.12333.1.A1_at | CF366197 | similar to fibronectin type III domain containing 1 | LOCL00154276 | -3.22 |
| Ssc.15316.1.S1_at | NM_001002824.1 | myogenic differentiation 1 | MYOD1 | -2.41 |
| Ssc.16494.1.A1_at | CB468993 | fibronectin | FN1 | -4.14 |
| Ssc.16525.1.S1_at | CN163410 | fibromodulin | FMD | -5.33 |
| Ssc.16854.1.A1_at | BI402879 | fibrinogen-like 2 | FGL2 | -2.40 |
| Ssc.1664.2.S1_at | NM_001001771.1 | fibrillin 1 | FBN1 | -3.59 |

### Table 7.

| Probe ID | Gene ID | Gene Name | Gene Symbol | J/L Z score |
|----------|---------|-----------|-------------|-------------|
| Ssc.16679.1.S1_at | BF079341 | similar to Bone morphogenetic protein 1 (BMP-1) | LOC100156461 | 2.44 |
| Ssc.3139.1.A1_at | CK456262 | Regulator of G-protein signaling 2, 24kDa | RGS2 | 2.63 |
| Ssc.11281.1.A1_at | BI181438 | proenkephalin | PENK | 2.19 |
| Ssc.396.1.S1_a_at | NM_214119.1 | diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein) | DBI | 2.59 |
| Ssc.9707.1.A1_at | BX666261 | BTG family, member 2 | BTG2 | 2.32 |
| Ssc.2798.2.S1_at | BX916748 | Zinc finger, AN1-type domain 5 | ZFAND5 | -2.06 |
| Ssc.29341.1.A1_at | BQ399924 | similar to Zinc finger protein 22 | LOC100156567 | -2.10 |
| Ssc.29341.1.A1_at | C0954104 | similar to F-box and leucine-rich repeat protein 4 | LOC100156082 | -2.65 |
| Ssc.23226.1.S1_at | CK452343 | similar to E2F-associated phosphoprotein | LOC100153549 | -2.14 |
| Ssc.10025.3.S1_at | BI181438 | similar to CCAAT/enhancer-binding delta protein | LOC100153946 | -2.38 |
| Ssc.22985.1.S1_a_at | CK457158 | similar to BTB (POZ) domain containing 1 | LOC100154013 | -2.55 |
| Ssc.3931.1.S1_at | NM_213946.1 | four and a half LIM domains 3 | FH3 | -2.18 |
| Ssc.4368.3.S1_at | BP463181 | F-box protein 32 | FBXO32 | -4.87 |

This provides an explanation for the slow growth rate of Jinhua pigs.
(NM_213878.1) and chemokine (C-C motif) ligand 2 (NM_214214.1) at d30 (Table 4), Kruppel-like factor 4 (BI339050), Kruppel-like factor 9 (BG3802637) [85] and calpain 1 (M20160.1) [37, 36] at d90 (Table 5), and ankyrin repeat domain 2 (stretch responsive muscle) (CFI79329) [87], stamatin-like 1 (BP141278) [88, 89] and Unc-45 homolog B (CN069994) [90] at d150 (Table 6). It would be of great interest in future studies to determine how these factors contribute to the differences between Jinhua and Landrace pigs in growth rate and meat composition of the longissimus dorsi muscles.

Transcription Factors and Signaling Molecules are Differentially Expressed in the longissimus dorsi Muscles in Jinhua and Landrace Pigs

Further analysis of the differentially expressed genes led us to identify a number of known transcription factors and signaling molecules that have not previously been reported to function in the development of longissimus dorsi muscles. Among these, we found that (bone morphogenetic protein 1 (BMP-1), regulator of G-protein signaling (RGS2) and proenkephalin (PENK) were up-regulated whereas four and a half LIM domains 3 (FHL3), F-box protein 32 (FBXO32) and a gene similar to CCLAT/enhancer-binding delta protein (LOC100153896) were down-regulated in Jinhua pigs at 30d (Table 7). Transcription regulators SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (SMARCA5), a gene similar to T-box 3 protein (LOC1001532741) and growth arrest and DNA-damage-inducible alpha (GADD45A) were up-regulated while selenoprotein X 1 (SEPX1), homoeobox protein A10 (HOXD10/4) and DNA-cytosine-5-methyltransferase 3 alpha (DNMT3A) were down-regulated in Jinhua pigs at d90 (Table 8). Interestingly, we noted that BMP2 and BMP receptor type 1B (BMPR1B) which mediate BMP signaling were up-regulated while secreted frizzled-related protein 4 (SFRP4) and dickkopf homolog 3 (DKK3) which mediate Wnt signaling were down-regulated at d150 (Table 9), suggesting that key developmental signaling pathways are differentially mobilized in Jinhua and Landrace pigs. It will be of our great interest in the future to study how these transcription factors and signaling molecules control/regulate the distinct developmental events in Jinhua and Landrace pigs.

pFLJ Encodes a Novel Protein and is Highly Expressed in the longissimus dorsi Muscle of Jinhua Pigs at d90

The microarray data allowed us to search for novel genes involved in the adipogenesis process in muscles. We noted that one unknown gene corresponding to an expressed sequence tag (EST) with accession number BI184304 was expressed at a much higher level in Jinhua than in Landrace at d90. We cloned the full length cDNA corresponding to BI184304 through 5’- and 3’-rapid amplification of cDNA ends (RACE; data not shown) and found that this gene encodes a previously uncharacterized protein named FLJ in humans [91]. A database search revealed that FLJ is highly conserved among different species and pig FLJ (pFLJ) shares 93%, 83%, 92% and 92% homology with human, mouse, chimpanzee and rhesus monkey FLJ, respectively (Figure 4A).

qPCR was performed to examine the expression of pFLJ in different organs/tissues in Jinhua pigs. Our results showed that pFLJ is expressed at high levels in the brain, kidney, longissimus dorsi muscle and subcutaneous fatty tissue (SF) but at a much lower level in the heart, liver, spleen and lung, demonstrating that pFLJ is differentially expressed in pigs (Figure 4B). We then examined the expression of pFLJ in the longissimus dorsi muscles in Jinhua pigs at d30, d60, d90 and d120. Our results showed that the transcript levels of pFLJ sharply increased from d30 to d90, peaked at d90 and then decreased to a lower level at d120 (Figure 4C), thus pFLJ exhibits a dynamic expression pattern during skeletal muscle development.

### Table 8. List of genes encoding transcription factors and signaling molecules differentially expressed in longissimus dorsi muscle in Jinhua and Landrace pigs at d90.

| Probe ID | Gene ID | Gene Name | J/L Z score |
|----------|---------|-----------|-------------|
| Regulatory factors (Jinhua- up) |
| Ssc.1091.1.A1_at | CK464481 | TPS3KR binding protein | 2.02 |
| Ssc.1287.1.S1_at | CB287966 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 | 2.68 |
| Ssc.1913.1.A1_at | CN163609 | slowmo homolog | 2.40 |
| Ssc.6578.1.S1_at | BI467852 | similar to T-box 3 protein | 2.11 |
| Ssc.26039.1.S1_at | BX926726 | similar to RAR-related orphan receptor A | 2.00 |
| Ssc.1303.1.S1_at | CK463456 | membrane-associated ring finger (C3HC4) 6 | 2.45 |
| Ssc.20913.1.S1_at | CN161066 | growth arrest and DNA-damage-inducible, alpha | 2.37 |
| Ssc.4368.3.S1_at | BP463181 | F-box protein 32 | 2.34 |
| Regulatory factors (Jinhua- down) |
| Ssc.15733.1.S1_at | CF176266 | similar to Transmembrane emp24 domain-containing protein 3 precursor (Membrane protein p24B) | 0.20 |
| Ssc.1300.2.S1_at | CK455870 | similar to Leukocyte elastase inhibitor (LEI) (Serpin B1) (LNPI) | 0.22 |
| Ssc.5520.1.S1_at | BJ182015 | similar to Chromosome 9 open reading frame 16 | 0.22 |
| Ssc.101.1.A1_at | NM_214023.1 | secreted phosphoprotein 1 | 0.26 |
| Ssc.26254.1.S1_at | BX926970 | Homeobox protein A10 | 0.27 |
| Ssc.1704.1.S1_at | BX915676 | alpha DNA (cytosine-5-) -methyltransferase 3 | 0.05 |

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pFLJ is a Positive Regulator of Fat Deposition in Intramuscular Adipocytes

Because its expression levels and its dynamic expression pattern in the longissimus dorsi muscle differ between Jinhua and Landrace pigs, we wondered whether pFLJ might be involved in the process of adipogenesis. To address this question, we first established a protocol to culture intramuscular adipocyte precursor cells in vitro. These cells could be successfully induced to differentiate into adipocytes at 4 days, as judged easily by Oil Red staining (data not shown). qPCR revealed that pFLJ was expressed at a higher level in the differentiated adipocytes (data not shown). SR141716 (rimonabant, an antagonist of cannabinoid receptor 1 of mammals and commonly used as an inhibitor for fat deposition) was added in the differentiated adipocytes (data not shown). SR141716 significantly down-regulated the transcript levels of pFLJ (Figure 5A) and fat contents were determined at 24- and 48-hour after treatment, respectively. Our data showed that SR141716 significantly down-regulated the transcript levels of pFLJ (Figure 5A) and fat deposition (Figure 5B) 48 hours after treatment.

The above data suggest a probable role of pFLJ in fat deposition. To test this supposition, three siRNAs (fs1, fs2, fs3) were designed to targets the pFLJ transcript specifically. qPCR showed that these three siRNAs efficiently knocked down the transcript levels of pFLJ in cultured intramuscular adipocytes (Figure 6A), with fs1 showing the strongest effect at 36 hours after treatment (Figure 6B). These cultured cells were treated with pFLJ siRNA fs1 and control siRNA NS and the contents of total triglyceride (fat) in the treated cells and free glycerol in the culture medium 36 hours after treatment were measured. We found that the total triglyceride level was significantly down-regulated (Figure 6D), which in turn resulted in an elevation in free glycerol levels in the medium (Figure 6E). We then examined the transcript levels of fatty acid synthesis (FAS), acetyl-CoA carboxylase (ACC), adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in the siRNA treated cells. FAS and ACC encode two key enzymes for the synthesis of fat while ATGL and HSL gene products are responsible for the hydrolysis of fat. We found that transcript levels of all four genes were significantly down-regulated (Figure 6C). We therefore concluded that pFLJ is a positive regulator of fat deposition in cultured intramuscular adipocytes, probably by regulating the expression of genes that are essential for fat biosynthesis.

Conclusion

In summary, our results revealed that genes that regulate adipogenesis and myogenesis are differentially expressed in Jinhua and Landrace pigs, with Jinhua pigs expressing higher levels of adipogenesis and myogenesis genes. More importantly, from the microarray data, a novel gene, pFLJ, was identified as a positive factor in the regulation of fat deposition in intramuscular adipocytes. pFLJ exhibited dynamic spatial and temporal expression patterns in Jinhua pigs, with high expression in the muscle at d90. Down-regulation of pFLJ by either drug treatment or siRNA-mediated gene knockdown reduced fat deposition concomitantly with the down-regulation of genes responsible for fat biosynthesis. This

Table 9. List of genes encoding transcription factors and signaling molecules differentially expressed in longissimus dorsi muscle in Jinhua and Landrace pigs at d90.

| Probe ID      | Gene ID       | Gene Name                  | Gene Symbol   | J/L Z score |
|---------------|---------------|----------------------------|---------------|-------------|
| **Transcriptors (Jinhua - up)** |               |                            |               |             |
| Ssc.810.1.S1_at | AY550058.1    | scavenger receptor class B member 2 | Scarb2        | 2.52        |
| Ssc.11352.1.A1_at | BI185713     | karyopherin alpha 7 (importin alpha 8) | KPNAt7       | 2.35        |
| Ssc.15865.1.A1_at | AY101066.2    | karyopherin alpha 3 (importin alpha 4) | KPNAt3       | 2.49        |
| Ssc.20913.1.S1_at | CN161066     | growth arrest and DNA-damage-inducible, alpha | GADD45A      | 3.07        |
| Ssc.66.1.S3_at | CO950299     | bone morphogenetic protein receptor, type IB | BMP1 RB       | 2.10        |
| Ssc.4190.1.S1_at | CA779219      | bone morphogenetic protein 2 | BMP2         | 2.19        |
| **Transcriptors (Jinhua - down)** |               |                            |               |             |
| Ssc.10160.1.A1_at | BG609515      | transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma) | CH242-255C19.1 | -2.44       |
| Ssc.1020.1.S1_at | AJ583828.1    | toll-like receptor 1 | TLR1         | -2.04       |
| Ssc.10245.2.A1_a_at | CN163698     | tissue factor pathway inhibitor | LOC100155066 | -2.57       |
| Ssc.10822.1.S1_at | CK455045      | similar to tumor suppressor candidate 3 | LOC100156093 | -2.68       |
| Ssc.11131.1.S1_at | C7F95993      | similar to Sushi repeat-containing protein SRPX | LOC100156108 | -3.67       |
| Ssc.11310.2.A1_at | BG600663      | similar to pleckstrin 2 | LOC100154251 | -2.19       |
| Ssc.11559.2.A1_at | CN032097      | similar to PDZ and LIM domain 2 | LOC100152859 | -2.14       |
| Ssc.11618.2.S1_at | BE235724      | similar to neunin | LOC100154738 | -6.44       |
| Ssc.11862.1.A1_at | BP109598      | similar to KLC4 protein | LOC100157157 | -2.37       |
| Ssc.12963.1.S2_at | CK457158      | similar to BTB (POZ) domain containing 1 | LOC100154013 | -2.50       |
| Ssc.13079.2.S1_at | CB286263      | similar to Baiap2l2 protein | LOC100154063 | -2.15       |
| Ssc.140.1.S1_at | BG382598      | secreted frizzled-related protein 4 | SFRP4         | -3.55       |
| Ssc.1714.1.S1_at | CD949346      | dickkopf homolog 3 | DKK3          | -2.15       |
| Ssc.18231.2.S1_at | BI399410      | AXL receptor tyrosine kinase | AXL           | -2.78       |
| Ssc.1850.1.A1_at | CO938780      | angiopoietin-like 2 | ANGPTL2       | -3.22       |

Table: List of genes encoding transcription factors and signaling molecules differentially expressed in longissimus dorsi muscle in Jinhua and Landrace pigs at d90.

The above data suggest a probable role of pFLJ in fat deposition. To test this supposition, three siRNAs (fs1, fs2, fs3) were designed to targets the pFLJ transcript specifically. qPCR showed that these three siRNAs efficiently knocked down the transcript levels of pFLJ in cultured intramuscular adipocytes (Figure 6A), with fs1 showing the strongest effect at 36 hours after treatment (Figure 6B). These cultured cells were treated with pFLJ siRNA fs1 and control siRNA NS and the contents of total triglyceride (fat) in the treated cells and free glycerol in the culture medium 36 hours after treatment were measured. We found that the total triglyceride level was significantly down-regulated (Figure 6D), which in turn resulted in an elevation in free glycerol levels in the medium (Figure 6E). We then examined the transcript levels of fatty acid synthesis (FAS), acetyl-CoA carboxylase (ACC), adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in the siRNA treated cells. FAS and ACC encode two key enzymes for the synthesis of fat while ATGL and HSL gene products are responsible for the hydrolysis of fat. We found that transcript levels of all four genes were significantly down-regulated (Figure 6C). We therefore concluded that pFLJ is a positive regulator of fat deposition in cultured intramuscular adipocytes, probably by regulating the expression of genes that are essential for fat biosynthesis.

Conclusion

In summary, our results revealed that genes that regulate adipogenesis and myogenesis are differentially expressed in Jinhua and Landrace pigs, with Jinhua pigs expressing higher levels of adipogenesis genes and Landrace expressing higher levels of myogenesis genes. More importantly, from the microarray data, a novel gene, pFLJ, was identified as a positive factor in the regulation of fat deposition in intramuscular adipocytes. pFLJ exhibited dynamic spatial and temporal expression patterns in Jinhua pigs, with high expression in the muscle at d90. Down-regulation of pFLJ by either drug treatment or siRNA-mediated gene knockdown reduced fat deposition concomitantly with the down-regulation of genes responsible for fat biosynthesis. This
observation strongly suggests that up-regulation of pFLJ together with other factors (e.g. myostatin, a myogenesis inhibitory gene) in the longissimus dorsi muscles of Jinhua pigs might play a key role in determining their high rate of IMF. Future efforts will be needed to determine the functional mechanism of pFLJ in this process. Therefore, transcriptomes for adipogenesis and myogenesis in the longissimus dorsi muscles are mobilized differentially in Jinhua and Landrace pig to produce meats with different ratios of muscle fiber to intracellular fat.

Materials and Methods

Ethics Statement

This study did not involve non-human primates. All experiments described in the study were performed in full accordance with the guidelines for animal experiments released by the National Institute of Animal Health with a permit (License No: GB/T 14925-94).

Animals

Sixty six castrated Jinhua (Jinhua II breed) and Landrace (Danish breed) pigs were raised and had ad libitum access to commercial diets (nutrients levels according to the NRC) under similar conditions during the whole experimental period. Nine individual pigs from each breed at each stages (d30, d90 and d150) and three individuals per breed at each stages (d60 and d120) were slaughtered. The longissimus dorsi muscles at the last rib were collected after exsanguinations and were subsequently divided into four portions for use in the measurement of intramuscular fat, determination of meat color, determination of pH values, and isolation of total RNA. For RNA extraction, the excised samples were directly frozen in liquid nitrogen and stored at −80°C until use.

Determination of Meat Quality

At each stage (d30, d60, d90, d120 and d150), experimental pigs were individually weighed and average bodyweights of all pigs of each breed at each stage were obtained. The BFT value was averaged from the fat thickness values measured on the first rib, last rib and the last lumbar vertebrae for each individual pig using a sliding caliper (Messschieber 0–150 mm mit Momentfeststellung Nonius 1/20 mm, Wollschlaeger). The FMR or LMR were calculated as the ratio of weight of fat meat or lean meat to the
Figure 5. SR141716 down-regulates pFJ expression and inhibits fat deposition in cultured intramuscular adipocytes. (A and B) qPCR analysis of pFJ expression (A) and measurement of total triglyceride (B) in cultured adipocytes 24 and 48 hours after SR141716 treatment. The qPCR values are shown as expression fold changes after normalization against the control 18s rRNA. Data are presented as means ± standard error. Gene ID was as shown. Cells were stained with Oil-Red O to determine lipid accumulation (total triglyceride), *: P < 0.05, **: P < 0.01. doi:10.1371/journal.pone.0053181.g005

Microarray Hybridization
Total RNA from a total of 34 pigs at d30, d90 and d150 of age stage (nine pigs for each breed at each stage) was extracted. RNA samples from three pigs of the same breed at the same age stage were pooled as one sample for one gene-chip hybridization. Microarray data from three samples for each breed at each stage were obtained for data analysis. A total of 18 microarrays were used in the experiment, corresponding to the 18 pooled RNA samples from longissimus dorsi muscles. The GeneChip Porcine Genome Array (Affymetrix, Santa Clara, CA) contains 23937 probes sets interrogating 23256 transcripts, representing 20201 genes. RNA labeling and Affymetrix Gene Chip microarray hybridization were conducted according to the Affymetrix Expression Analysis Technical Manual. Array scanning and data extraction were carried out following procedures recommended by Affymetrix.

Microarray Data Analysis
To quantify the intensities from the same probe sets on different arrays, these were scaled so that the median intensities for all arrays were the same. We then calculated the average intensity for each probe in all replicate arrays and this mean intensity was used for downstream analysis. When comparing gene expression between different breeds at the same time-point and in the same tissue, Lowess intensity dependent normalization was performed for each array pair. Z-scores were then calculated as described previously [93] and Z-scores ≥2 or ≤-2 was used as the cut-off value for selection of up- or down-regulated genes. Hierarchical and K-means clustering of differentially expressed genes was done using Cluster 2.10 and viewed in TreeView 1.50 from Eisen Lab (http://rana.lbl.gov/EisenSoftware.htm).

qPCR
Primer sequences, melting temperatures and expected product sizes for the genes analyzed are shown in Additional file 1 (Table S1). The sizes of the PCR products were confirmed using agarose gel electrophoresis (1.8%). The specificity of the PCR products was judged based on a single peak observed in dissociation/melting curves. All RNA samples prepared for gene-chip hybridization were also used in qPCR. qPCR was performed using SYBR green I nucleic acid dye on an BIO-RAD CFX96 Real-Time PCR System (BIO-RAD, Foster City, CA, USA) to quantify the target genes expression levels. Data are expressed as the ratio between expression of the target gene and that of the housekeeping gene 18s rRNA. All qPCR reactions followed this thermal profile: after an initial denaturation at 94°C for 2 minutes, amplification was performed with 40 cycles of 94°C for 30s and annealing for 40 s at temperatures specific for each target genes. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. At the end of the PCR run, melting curves were generated and analyzed to confirm non-specific amplification, and the mean value of each triplicate was used for further calculations. To calculate the mRNA expression of selective genes, the ΔΔCt values was used for detection of their mRNA.
related to internal control 18s rRNA expression using the
2^−ΔΔCt method [94].

Cloning of the pFLJ Gene

To obtain the full-length cDNA sequence of pFLJ, RACE
technology was carried out to clone the 5′-ends of pFLJ by
using the SMARTTM RACE cDNA Amplification Kit and
GeneRacer Kit (Invitrogen Biotechnology Co. Ltd., Shanghai,
China). Briefly, for 5′-RACE, 5′ phosphates and the 5′ cap
structure were removed from the total RNA from porcine
tissues, the GeneRacer RNA Oligo sequence (5′-CGACUG-
GAGCAGGGAGCAUGACAGUAAAGAGAUAGA-
AA-3′) to the 5′ end of the prepared mRNA was ligated and
the 5′ RACE cDNA template was then obtained by reverse-
transcribing the ligated mRNA according to the manufacturer's
instructions. Four steps were required to obtain the full length
of pFLJ36031 cDNA. The first reaction of PCR was performed
using a combination of sm-FLG-R1 (5′-GCCACCAATGAC-
CAAAAGCATTGATGAA-3′) and 10*UPM using the 5′
RACE cDNA template. The PCR condition was as follows:
94°C for 2 min, 5 cycles of 94°C for 30 s and 72°C for 1.5 min, 5
cycles of 94°C for 30 s and 72°C for 1.5 min, 25

Figure 6. pFLJ functions as a positive regulator of fat deposition in intramuscular adipocytes. (A) Cell images to verify transfection
efficiency. Cells were transfected with pSilencer TM 4.1-CMV neo plasmids carrying the sequences fs1, fs2 and fs3. Transfection efficiency was
assessed by expression of the reporter gene EGFP (green color) harbored by the plasmid. (B) qPCR analysis of pFLJ expression in cultured adipocytes
24 hours after siRNA treatment. fs1, fs2 and fs3: pFLJ specific siRNAs; ns: negative control siRNA. (C) qPCR analysis of FAS, ACC, ATGL and HSL in
cultured adipocytes treated with fs1 siRNA. The qPCR values are shown as expression fold changes after normalization against the control 18s rRNA.
Data are presented as means ± standard error. *: P<0.05, **: P<0.01 (C and D) Measurement of total triglyceride (as before) in the cultured
adipocytes or free glycerol (the free glycerol release was normalized to total cellular protein and expressed relative to the control group) in the
culture medium 36 hours after treatment with fs1 siRNA. ab means every two columns with different letters are significantly different (P<0.05).
doi:10.1371/journal.pone.0053181.g006
cycles of 94°C for 30 s, 65°C for 30 s and 68°C 3.0 min. Then the product was further identified using another primer (sm-
FLG-R2:5’-GCCCTGATCAACGATTTCCTGTTGCTCTCA-3’) that is located on the downstream of sm-FLG-R1. The PCR condition used was: 94°C 2 min, 30 cycles: 94°C 30 s 66°C 30 s and 68°C 1.5 min. The gene-specific primer sm-FLG-R1 was designed based on the FLJ EST available in GenBank. The resulting PCR product obtained from this step was isolated, cloned, and sequenced. The three subsequent 5’-RACE products were gel-purified, cloned, and sequenced. By ligation of the four overlapping cDNA fragments, full-length pFLJ cDNA was obtained. Primer pairs used for qPCR were: sense: 5’-aactgtcgctggccgacagca-3’; antisense: 5’-age ctc acc aac ggt tcc ag-3’.

siRNAs Targeting pFLJ
Three potential siRNA target sites in pFLJ (FS1:5’-aactgtcgctggccgacagca-3’; FS2:5’-aactgtcgctggccgacagca-3’. FS3:5’-aactgtcgctggccgacagca-3’) were determined using the Qiagen siRNA design programme, and the sequence was BLAST-confirmed for specificity. Oligonucleotides to produce plasmid-based siRNA were cloned into pSilencerTM 4.1-CMV neo plasmid (Ambion) and all constructs were confirmed by sequencing. For RNA interference experiments, porcine intramuscular adipocytes were transfected with empty plasmid (wt), negative control siRNA (ns), or pFLJ-siRNA (fs1, fs2 and fs3). Transfections were performed using LipofectamineTM 2000 (Invitrogen Life Technologies) according to the manufacturer’s protocol. A final concentration of 2000 ng/ml siRNA was used to treat the cultured intramuscular adipocytes. Negative control siRNA (Neg-siRNA, ns, 5’-aactgtcgctggccgacagca-3’) was supplied by Ambion.

In vitro Culture of Intramuscular Adipocyte Precursor Cells and Induction of Adipocytes
For in vitro culture of intramuscular adipocyte precursor cells, D (Duroc) ×L (Landrace) ×Y (Yorkshire) pigs from d5 to d7 of age were overdosed with sodium thiopental and exsanguinated. The longissimus dorsi muscle was removed and porcine pre-adipocytes were prepared by previously published methods [95,96]. Briefly, longissimus dorsi muscle tissue was cut with scissors into approximately 1 mm sections under sterile condition and digested with collagenase type II for 45 hours, at 37°C in a 120rpm shaking water bath. The digested material collected was first centrifuged at100 g for1 min, and the resulting floating adipocytes were collected in Dulbecco’s Modified Eagle Medium (DMEM) at 37°C. The number of intramuscular pre-adipocytes isolated in suspension was determined as described previously. The pre-adipocytes were seeded on six-well (35-mm) tissue culture plates in complete media (DMEM/F12+10% fetal bovine serum [FBS]+100 Upenicillin+100 Ustreptomycin) and cultured at 37°C under a humidified atmosphere of 95% air and 5% carbon dioxide according to previous study [42]. Intramuscular preadipocytes were induced to differentiate into intramuscular adipocytes when the cells were completely fused and were then treated with a final concentration of 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 1μmol/L dexamethasone (DEX) and 1.7μmol/L insulin of complete medium. The culture medium was changed to complete medium containing a final concentration of 10 mg/L insulin after 48 hours.

Statistical Analysis
All experimental data of comparisons between two pig breeds were analyzed using one-way analysis of variance (ANOVA, Statistical Product and Service Solutions (SPSS) 16.0). Data are represented as means±standard error; *P<0.05 and **P<0.01 displayed here indicate statistically significant difference.

Supporting Information
Table S1 177 genes upregulated in longissium dorsi muscles of jinhua pig at d90 compared with that at d30 age stage (Jinhua-d90-LD-up vs d30).

Table S2 242 genes downregulated in longissium dorsi muscles of jinhua pig at d90 compared with that at d30 age stage (Jinhua-d90-LD-down vs d30).

Table S3 101 genes upregulated in longissium dorsi muscles of jinhua pig at d150 compared with that at d30 age stage (Jinhua-d150-LD-up vs d30).

Table S4 389 genes downregulated in longissium dorsi muscles of jinhua pig at d150 compared with that at d30 age stage (Jinhua-d150-LD-down vs d30).

Table S5 106 genes upregulated in longissium dorsi muscles of Landrace at d90 compared with that at d30 age stage (Landrace-d90-LD-up vs d30).

Table S6 231 genes downregulated in longissium dorsi muscles of Landrace at d90 compared with that at d30 age stage (Landrace-d90-LD-down vs d30).

Table S7 93 genes upregulated in longissium dorsi muscles of Landrace at d150 compared with that at d30 age stage (Landrace-d150-LD-up vs d30).

Table S8 383 genes downregulated in longissium dorsi muscles of Landrace at d150 compared with that at d30 age stage (Landrace-d150-LD-down vs d30).

Table S9 176 genes upregulated in longissium dorsi muscles of Jinhua pig versus Landrace at d30 of age stage (Jinhua-d30-LD-up).

Table S10 276 genes upregulated in longissium dorsi muscles of Jinhua pig versus Landrace at d90 of age stage (Jinhua-d90-LD-up).

Table S11 525 genes upregulated in longissium dorsi muscles of Jinhua pig versus Landrace at d150 of age stage (Jinhua-d150-LD-up).

Table S12 199 genes downregulated in longissium dorsi muscles of Jinhua pig versus Landrace at d30 of age stage (Jinhua-d30-LD-down).

Table S13 242 genes downregulated in longissium dorsi muscles of Jinhua pig versus Landrace at d150 of age stage (Jinhua-d150-LD-down).
Table S13  155 genes downregulated in longissimus dorsi muscles of Jinhua pig versus Landrace at d90 of age stage (Jinhua-d90-LD-down). (XLS)

Table S14  670 genes downregulated in longissimus dorsi muscles of Jinhua pig versus Landrace at d150 of age stage (Jinhua-d150-LD-down). (XLS)

Table S15  Primer sequences. (XLS)

Author Contributions

Conceived and designed the experiments: JRP ZYW. Performed the experiments: TW ZQY IJL. Analyzed the data: ZHZ JRP ZYW JC. Contributed reagents/materials/analysis tools: ZHZ. Wrote the paper: JRP TW ZHZ YZW.

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