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MicroRNA Identity and Abundance in Developing Swine Adipose Tissue as Determined by Solexa Sequencing

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ABSTRACT

MicroRNAs (miRNAs) are small ~22-nt regulatory RNAs that regulate the stability and translation of cognate mRNAs. MiRNAs participate in the regulation of adipogenesis, and identification of the full repertoire of miRNAs expressed in adipose tissue is likely to significantly increase our understanding of adipose tissue growth and development. Here, we adopted a deep sequencing approach to determine the identity and abundance of miRNAs in developing swine adipose tissue. Via this approach, we identified the sequences and relative expression levels of 227 conserved miRNAs (of which 59 were novel) and 66 potential porcine miRNAs. The expression levels displayed a large range, as reflected by the number of sequence reads, which varied from several counts for rare miRNAs to several million reads for the most abundant miRNAs. The abundant miRNAs principally belonged to 32 miRNA gene families, including miR-143, miR-103, let-7, and miR-148. Of the conserved miRNAs, 93 miRNAs were up-regulated and 33 miRNAs were down-regulated in the adult pig adipose tissue. Moreover, we observed sequence variants and seed edits of the miRNAs. KEGG pathway analysis and GO term enrichment suggested that highly expressed miRNAs are involved in adipose tissue development, signal transduction, cell–cell and cell–extracellular matrix communication, neural development and function, and lipid metabolism including carboxylic acid, oxoacid, fatty acid, steroid, glycerolipid, alcohol and phospholipid metabolism. Our results expand the number of known porcine miRNAs and provide a thorough account of the miRNA transcriptome in porcine adipose tissue. J. Cell. Biochem. 112: 1318–1328, 2011.

KEY WORDS: microRNA; ADIPOSE TISSUE; EXPRESSION PROFILES; SOLEXA SEQUENCING; SUS SCROFA

Elucidating the molecular events governing adipogenesis is of utmost importance for both the meat production industry and biomedical research. The development of adipose tissue is dependent on adipocyte hypertrophy (increase in cell size) and adipocyte hyperplasia (increase in cell number) at the cellular level [Hausman et al., 2001]. The accrescence of adipocyte numbers involves both the recruitment and proliferation of adipocyte precursor cells (also known as pre-adipocytes) followed by a complex process involving pre-adipocyte proliferation and differentiation [Hausman et al., 2001]. Both processes are tightly controlled, negatively or positively, by a combination of multiple transcription factors [Gregoire et al., 1998]. Potential regulators of adipogenesis include microRNAs (miRNAs), small (~22 nt) non-protein-coding RNAs which regulate gene expression post-transcriptionally by specific binding to the 3′ untranslated region (3′-UTR) of target mRNAs. Growing evidence has demonstrated that miRNAs play important roles during in vitro as well as in vivo adipogenesis.

The role of miRNAs in lipid metabolism was first reported in *Drosophila*, where deletion of mir-14 increased the accumulation of triacylglycerol and diacylglycerol [Xu et al., 2003]. Thereafter, various studies were completed in human and murine cell models. Esau et al. [2004] first identified a potential role for miR-143 in adipogenesis of human pre-adipocytes, and showed that inhibition of miR-143 decreased adipocyte differentiation. Later, miRNA expression during 3T3-L1 adipocyte differentiation was profiled [Kajimoto et al., 2006]. Other studies showed that miR-103 and the miRNA cluster miR-17–92 enhanced adipogenesis [Wang et al., 2008; Xie et al., 2009], while the let-7 and miR-27 family of genes

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impaired adipogenic differentiation [Karbiener et al., 2009; Sun et al., 2009; Kim et al., 2010]. More recent human studies on the expression of miRNAs in adipose tissue found that the expression of miRNAs was adipose depot-specific [Klöting et al., 2009; Ortega et al., 2010] and that some miRNAs correlated with the morphology of adipose tissue, adipocyte size [Klöting et al., 2009] and metabolic (fasting glucose and/or triglycerides) parameters [Ortega et al., 2010]. In cattle, miRNA transcriptome profiles have been reported for adipose tissue. Gu et al. [2007] identified 154 miRNA sequences from bovine adipose tissue and mammary glands, 54 of which were determined to be adipose tissue-specific. In addition, the expression patterns of 89 miRNAs in back subcutaneous adipose tissues were compared using qRT-PCR analysis, and the expression levels of 18 miRNAs correlated with backfat thickness, with miR-378 showing the strongest correlation [Jin et al., 2010]. These findings suggested that miRNAs play a regulatory role in adipose tissue development, lipid metabolism and adipogenesis in a variety of species. To date, most research evaluating the role of miRNA in adipose tissue has been limited to human and mouse cell lines, while investigations on adipogenic miRNAs in pigs are lagging behind. Thus, studies to identify miRNA expression profiles and gain insight into the potential role of miRNAs during porcine adipose tissue development are required.

The pig (*Sus scrofa*) is among the best animals with respect to adipose accumulation. Investigations into lipid deposition in pigs, especially in the subcutaneous adipose tissues, are important both for the meat production industry and for biomedical research. On one hand, adipose tissue is directly associated with the yield and the quality of meat. On the other hand, the pig is recognized as a potential model system for research into aspects of human health, such as obesity and diabetes, because pigs are closer evolutionarily to humans than mice. Despite the significant role of miRNAs in the regulation of adipic biology, the current release (16.0) of miRBase lists only 211 distinct miRNA sequences in pigs [Griffiths-Jones et al., 2008], significantly fewer than for humans and mice. Although the sequences have not been deposited in the miRBase database, several recent reports have described miRNAs in pigs [Kim et al., 2006; Huang et al., 2008; Sharbati-Tehrani et al., 2008; McDaneld et al., 2009; Li et al., 2010; Xie et al., 2010]. Sixty-four conserved miRNAs have been cloned from porcine adipose tissue [Cho et al., 2010], but the full repertoire of miRNAs expressed in developing adipose tissue remains to be determined. In addition, many studies have demonstrated that miRNAs have a temporal or tissue-specific expression pattern. Thus, in this study, to gain full insight into the potential role of miRNAs during adipose tissue development, the expression profiles of miRNAs were determined by Solexa sequencing in back subcutaneous adipose tissues samples from an obese phenotype of domestic swine at two stages of development.

MATERIALS AND METHODS

ANIMAL COLLECTION AND RNA ISOLATION

The experimental animals used in this study were an obese phenotype, domestic breed of pig known as Rongchang pigs from Sichuan, China. Backfat tissue samples were collected from a 240-day-old pig and a 7-day-old piglet, respectively, and were snap-frozen in liquid nitrogen and stored at -80°C. For Solexa sequencing, samples from three 240-day-old pigs and three 7-day-old piglets were pooled, respectively, and total RNA was isolated from each pooled sample using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA quality and quantity were determined using an Agilent 2100 Bioanalyzer (Agilent, MN).

SMALL RNA LIBRARY PREPARATION AND SOLEXA SEQUENCING

Two small RNA (sRNA) libraries were constructed for the adipose tissue from the 240-day-old pig and the 7-day-old piglet. From each sample, 20 μg of total RNA was used for the library construction following the protocol supplied with the Small RNA Sample Prep Kit (Illumina, protocol version 1004239) with minor modifications. Briefly, the 18–30 base pair fraction of total RNA was excised from 15% criterion Tris–borate–EDTA (TBE)–urea polyacrylamide gels. The sRNA was purified, ligated with 5′-RNA and 3′-RNA adapters and then reverse transcribed and amplified as recommended by the manufacturer. The 70–90 base pair PCR products were separated on 4% low range agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The concentrations of the libraries were determined using a NanoDrop ND-1000 Spectrophotometer and the size and purity were determined using an Agilent 2100 Bioanalyzer in combination with the Agilent DNA 1000 Kit. The purified DNA was used directly for cluster generation and sequence analysis using the Illumina Genome Analyzer (Illumina, San Diego, CA) according to the manufacturer's instructions. The image files generated by the sequencer were then processed to produce digital-quality data. After masking of the adaptor sequences and removal of contaminated reads, clean reads were processed for computational analysis.

SEQUENCE ANALYSIS

Small 35-nt RNA reads were produced using an Illumina 1G Genome Analyzer at BGI-Shenzhen (China). Low quality reads were trimmed using our own perl script. Adaptor sequences were accurately clipped with the aid of a dynamic programming algorithm. After the elimination of redundancy, sequences $\geq$18 nt were used for subsequent analyses.

Sequences, discarding redundancy, were initially searched against miRBase (Release 14.0; http://microrna.sanger.ac.uk/) to identify conserved miRNA homologs in pigs [Griffiths-Jones et al., 2008]. The hits were considered a real match if there was a minimum 16-nucleotide match between the sequence read and the miRNA from the database. Sequences matching a miRNA with the same identifier from different organisms were examined for similarity, and sequences varying only in length and/or a few end nucleotides were grouped under the same miRNA identifier. All sequences that matched a miRNA in miRBase were searched against the porcine genome (http://www.sanger.ac.uk/Projects/S_scrofa/; April 2010). Read sequences that matched genomic sequences (allowing for one or two mismatching end-nucleotides) were selected, and 100 nucleotides of genomic sequence flanking each side of these sequences were extracted. The secondary structures were then predicted using the program mfold [Zuker, 2003] and analyzed by MIREAP (https://sourceforge.net/projects/mireap/)

under default settings. If a hairpin structure with a free energy of hybridization lower than −20 kcal/mol was predicted, the RNA sequence was subjected to MiPred analysis [Jiang et al., 2007], which predicts whether the input RNA sequence is a genuine pre-miRNA-like hairpin sequence.

To identify potential novel miRNAs in pig, all un-annotated sequences were aligned against the porcine genome using the SOAP software [Li et al., 2008a]. Sequences with a perfect match or with one mismatch were retained for further analysis. Potential miRNA hairpin precursors were predicted using the program described above. Stem-loop hairpins were considered typical only when they fulfilled three criteria: mature miRNAs are present in one arm of the hairpin precursors, which lack large internal loops or bulges; the secondary structures of the hairpins are steady, with the free energy of hybridization lower than −20 kcal/mol; and hairpins are located in intergenic regions or introns. Any sequence that fulfilled these criteria was considered a miRNA precursor locus in the porcine genome.

EXPRESSION ANALYSIS USING STEM-LOOP QUANTITATIVE RT-PCR

Total RNA from the back subcutaneous adipose, crural skeletal muscle, heart, liver, lung, pancreas, and spleen of pigs was isolated using Trizol reagent (Invitrogen) following the recommendations of the manufacturer, and real-time quantification of miRNAs was performed by stem-loop RT-PCR [Chen et al., 2005]. Briefly, 1 μg of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Fermentas, Cat. No. K1621) and stem-loop RT primers. The mix was then incubated at 16°C for 30 min, 42°C for 30 min and 85°C for 10 min. Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection system (Applied Biosystems, Foster City, CA) and a standardized protocol. In a 20 μl of reaction mixture, 2 μl of cDNA (1:4 dilution) was used for amplification. The reactions were incubated at 95°C for 3 min, followed by 40 cycles of 94°C for 15 s and 60°C for 40 s. All reactions were performed in triplicate. The threshold cycle (CT) was determined using the default threshold settings. To calculate the expression levels of miRNAs, a series of synthetic miRNA oligonucleotides of known concentration were also reverse transcribed and amplified. The absolute amount of each miRNA was then calculated by referring to the standard curve.

DIFFERENTIAL EXPRESSION ANALYSIS

To compare the miRNA expression between two samples to determine the differentially expressed miRNAs, first, the expression of miRNAs in two samples (piglets adipose tissue and adult pigs adipose tissue) were normalized to obtain the expression of transcripts per million. If the normalized expression of a given miRNA is zero, its expression value will be modified to 0.01. If the normalized expression of a given miRNA is less than 1 in both samples, this miRNA is removed in future differential expression analysis. Then, the fold-change and *P*-value were calculated from the normalized expression using the formula shown below:

P-value formula:

$$p(x|y) = \left(\frac{N_2}{N_1}\right)\frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \qquad C(y \le y_{min}|x) = \sum_{y=0}^{y\le y_{min}} p(y|x)$$

$$D(y \ge y_{max}|x) = \sum_{y\ge y_{max}}^{\infty} p(y|x)$$

The N1 and x represent total count of clean reads and normalized expression level of a given miRNA in sRNA library of piglets adipose tissue sample, respectively. The N2 and y represent total count of clean reads and normalized expression level of a given miRNA in sRNA library of adult pigs adipose tissue sample, respectively.

MiRNA TARGET PREDICTION, KEGG PATHWAY ANALYSIS, AND GO TERM ENRICHMENT

To understand the molecular function of the 15 most abundant differentially expressed miRNAs in adipose tissue, we used the algorithms PicTar [Krek et al., 2005] and TargetScan [Lewis et al., 2003] to predict their target mRNAs. With the use of DAVID bioinformatics resources [Dennis et al., 2003], the genes were classified according to KEGG functional annotations to identify pathways that were actively regulated by miRNA in adipose tissue. In addition, GO term enrichment of the target genes was calculated using the GO::TermFinder Perl module in the Term Enrichment tool [Harris et al., 2004] under the setting conditions (database filter: MGI; *P*-value cutoff: 1E−05; minimum number of gene products: 10).

RESULTS AND DISCUSSION

SOLEXA SEQUENCING OF SMALL RNAS AND THE IDENTIFICATION OF CONSERVED miRNAS

To determine the identity and abundance of miRNA during the development of porcine adipose tissue, two sRNA libraries, one from 7-day-old piglet backfat and the other from 240-day-old adult pig backfat, were sequenced using Solexa technology. After removing the reads of low quality and masking adaptor sequences, a total of 21,632,536 clean reads of 18–30 nucleotides in length were obtained from the two libraries (Table S1-1 in additional file 1). The sequence reads were clustered into unique sequences, of which the vast majority were 22 nt in length (Table S1-2 in additional file 1), consistent with the common size of miRNAs. The total number of clean sequences in the piglet and adult pig adipose tissue sRNA libraries was 149,957 and 270,913 unique reads, respectively. These unique sequences contained 39,522 common sequences between the adult pig and piglet adipose tissue libraries, 110,453 piglet-specific sequences and 231,391 adult pig-specific sequences (Table S1-3 in additional file 1).

To identify the conserved miRNA homologs in pig, the clean sequences were annotated based on their similarities with mature miRNA sequences deposited in miRBase (release 14.0). Combining the data from two libraries identified a total of 227 unique mature

$$\text{Normalized expression(NE)} = \text{Actual miRNA sequencing count/Total count of clean reads} \times 1,000,000$$
$$\text{Fold\_change} = \log_2(\text{adult pigs adipose tissue-NE/piglets adipose tissue-NE})$$

miRNAs, including 201 porcine miRNAs that overlapped between the two libraries, 19 porcine miRNAs that were detected only in the piglet adipose tissue library and seven porcine miRNAs that were detected only in the adult pig library (Table S2-1 and Table S2-2 in additional file 2). At present, the miRBase (release 15.0) contains 175 entries from *S. scrofa* [Griffiths-Jones et al., 2008]. Of the 227 miRNAs identified in this study, 151 of the miRNAs have been deposited in miRBase (release 15.0) (Table S2-3 in additional file 2), 17 have been reported in recent studies [Huang et al., 2008; McDaneld et al., 2009; Reddy et al., 2009; Li et al., 2010; Nielsen et al., 2010; Xie et al., 2010] (Table S2-4 in additional file 2) and 59 were classified as new porcine miRNAs that have not previously been identified in pig (Table S2-5 in additional file 2). Of the 59 new porcine miRNAs, 13 were processed from the hairpin arm opposite to the known mature miRNA deposited in miRBase (Table S2-6 in additional file 2). All conserved porcine miRNAs were classified into known families or currently undefined groups on the basis of sequence similarity. Among the 227 conserved miRNAs, 100 belonged to 32 miRNA gene families, which were detected at high sequence reads in porcine adipose tissue (Table S2-7 and Table S2-8 in additional file 2).

SECONDARY STRUCTURE PREDICTION AND CLUSTERS OF PRECURSOR miRNA SEQUENCES

One important criterion for the discovery of miRNAs is the potential of miRNA precursor sequences to form secondary hairpin structures in which mature miRNA sequences are located on either strand of the stem [Ambros et al., 2003]. Thus, to further validate the miRNA sequences, a BLASTN search was performed against the porcine genome. The genomic sequences with a perfect match to the query sequences and their flanking regions were excised and tested for their folding properties using the program mfold [Zuker, 2003], requiring a free-energy changes $\Delta G \leq -20$ kcal/mol and a read sequence situated on one arm of the hairpin. The stem-loop structure was further interrogated using the program MiPred, which distinguishes between pseudo and real miRNA precursors [Jiang et al., 2007]. The 214 genomic sequences, encompassing the 227 miRNAs identified from the two sRNA libraries above, were predicted to be capable of forming stable hairpin structures characteristic of miRNA precursors. The predicted stem-loop structures of variants of the four most abundant miRNAs are shown in Table I, and the structures of the remaining miRNAs are compiled in Table S3-1 of additional file 3. The data showed that many of the mature miRNAs were derived from opposite sites of the same predicted precursor stem-loop structure. There are more than two predicted hairpin structures for the majority of mature miRNAs (Table S2-3 and Table S2-4 in additional file 2), for example, there are three predicted hairpin structures for ssc-miR-9, and five for ssc-miR-2476. Furthermore, 16 precursors with new chromosomal localizations of the known porcine miRNAs in the miRBase (15.0) were predicted (Table S3-2 in additional file 3).

In animals, genes encoding miRNAs are frequently organized into clusters. The 214 predicted miRNA precursors identified in this study were classified into groups according to their genomic

TABLE I. Predicted Chromosomal Positions and Hairpin Structures of Highly Expressed Porcine miRNAs

miRNA	Chromosomal position	Hairpin secondary structure	ΔG (kcal/mol)	miRNA precurser
ssc-mir-143	Chr2, 136030756-136030831	--- AG G G G U -| AG CCC CC GAG UGCAGUGCU CAUCUC GG UC C GGG GG CUC AUGUCACGA GUAGAG CU AG U AGA AA G G A U G^ GG	-41.10	+
ssc-mir-103-1	Chr16, 52667657-52667739	G C C --| U U C UUGCA CUUA UGCC UC GGCU CU UACAGUGCUGC UUG U GAGU ACGG AG UCGG GA AUGUUACGACG AAC A - C A UA^ - C - UAGGU	-33.60	-
ssc-let-7a-1	Chr3, 38549760-38549851	-- UG U GU UUAGG| ACA C CAC UGGGA GAG AGUAGGUUGUAUAGUU GUC CCCA C GUG AUCCU UUC UCAUCUAACAUAUCAA UAG GGGU A UA CA - UG -----^ A-- C	-39.70	+
ssc-mir-148a	Chr18, 45214541-45214620	- UU - A-| CC - AAU CUU GAGGCAAAGUUCUG AG CACU GACU CUG \ GAG CUCUGUUUCAAGAC UC GUGA CUGA GAU A CA GU A AC^ -- A AGU	-32.50	-

locations on the chromosomes (inter-miRNA distance <10 kb). In total, 82 of the identified miRNAs were found to be in close proximity in 32 different clusters (Table S2-8 in additional file 2), the remaining miRNAs were found to cluster alone. These clusters were distributed across 13 different porcine chromosomes, with an average of 2.5 miRNA genes per cluster. Eight of these clusters comprised of 24 miRNA genes, located on chromosome X. The two largest clusters comprised six miRNA genes, located on chromosome 11 and chromosome X, respectively. Such clustered miRNAs could be transcribed together as polycistronic transcripts with similar expression patterns, and may be functionally related [Baskerville and Bartel, 2005].

SEQUENCE VARIANTS AND EDITING OF BASES IN THE SEED REGION OF THE miRNAS

The Solexa sequencing results revealed that the majority of identified miRNAs showed length and sequence heterogeneity in porcine adipose tissue. The length variations occurred predominantly in the 3′ end of the miRNAs, mainly in the form of missing nucleotides and/or terminal additions of nucleotides. A typical example was miR-186, in which the length varied from 18 to 25 nucleotides (Fig. 1). Additional examples are shown in Additional file 4. Similar observations have previously been reported in pig [Li et al., 2010; Nielsen et al., 2010] and different human and rodent cell types [Landgraf et al., 2007], showing that essentially all miRNAs show length and/or end-sequence variation. These end-sequence variations are intriguing as they may allow miRNA variants to perform distinct roles by influencing miRNA/target mRNA hybrid duplex formation.

The nucleotides at position 2–8 of a mature miRNA is known as the seed region (the so-called "miRNA seed") and this region is highly conserved. The target of a miRNA may alter due to change in the nucleotides in this region [Brodersen and Voinnet, 2009]. Editing of bases in the seed region of the miRNAs has been reported to occur frequently [Kawahara et al., 2007; Liu et al., 2008; Reid et al., 2008]. In our analysis pipeline, miRNAs which might have the editing in the seed region can be detected by aligning un-annotated sRNA tags with porcine mature miRNAs from miRBase (14.0), allowing one mismatch at a certain position. The results showed that the total 17 mature miRNAs in the adult pig and piglet adipose tissue samples displayed single nucleotide substitution in the seed sequence (Table S5-1 and Table S5-3 in additional file 5). The edited sites might be occur at any position in the miRNA seed. A typical example is miR-103 in the adult pig adipose tissue samples, in which the base-edit existed at every position of its seed region (Table S5-1 in additional file 5). The observed occurrence for each possible substitution is summarized in Table S5-2 and Table S5-4 in additional file 5 for the adult pig and piglet samples, respectively. Of the 112 single nucleotide substitutions for the adult pig samples, the most frequent substitutions were C-to-U (16.96%), G-to-U (14.29%), G-to-A (16.96%), A-to-G (13.39%), and T-to-C (9.82%) (Fig. 2), which is similar to a previous report [Ebhardt et al., 2009]. We postulate that the C-to-U and A-to-G-sequencing substitutions in this study may be attributed to (at least in part) C-to-U and A-to-I deaminations.

Fig. 1. Sequence heterogeneity and the predicted hairpin secondary structures of ssc-miR-186. The number of reads for each length and the sequence variants are indicated above the precursor sequence. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

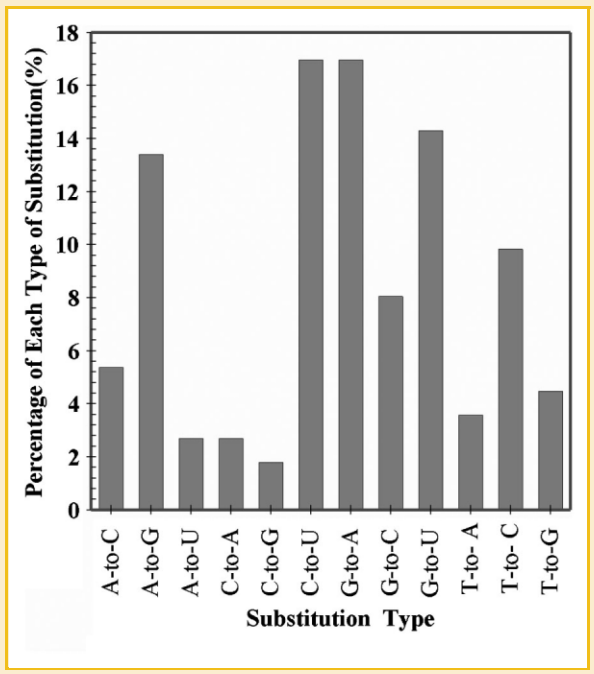

Fig. 2. Histogram displaying the single nucleotide mismatches in the miRNAs seed region sequence when aligning un-annotated sRNAs tags with porcine mature miRNAs from miRBase14.0. The axis represents the percentage comparing of the observed count of each type to the total count of all of substitution type. Substitutions listed on the abscissa are from DNA (genome) to RNA (small RNA sequence). An A-to-G substitution is where a genomically encode adenosine is identified as a guanosine during sequencing. The "uridine" in C-to-U substitution, G-to-U substitution, and A-to-U substitution were observed as a thymine deoxyriboside during sequencing. The data were obtained from the adult pig dataset.

These modifications can be catalyzed by CDARs or ADARs, respectively. Indeed, deamination events of adenosine to inosine (A-to-I) by an ADAR have already been reported in miRNA biogenesis [Luciano et al., 2004; Kawahara et al., 2007; Borchert et al., 2009; Heale et al., 2010]. sRNA sequences not perfectly matching the genome are often detected in high-throughput sequencing of the sRNA, and these mismatched sequences are also often attributed to experimental sequencing errors. However, a recent report demonstrated that "sequencing errors" in sRNA datasets were often the result of post-transcriptional modifications of RNA, not simply technical artifacts [Ebhardt et al., 2009]. Therefore, we presume that the nucleotide substitutions of the other type excepting C-to-U and A-to-G substitutions in this study may be attributed to (at least in part) post-transcriptional modifications of RNA. By these potential RNA editing events observed, it increases the diversity of miRNAs and their targets, and may modulate miRNA function. Remarkable, it seems that the edited probability in seed region of a given miRNA in the adipose tissue invariably correlated with the abundance of miRNA in our data. On the whole, the edited probability of abundance miRNAs in the seed sequence were higher than low abundance miRNAs (data not shown). This indicated that manifold genes are targeted by highly expressed miRNAs in the adipose tissue.

POTENTIAL NOVEL miRNAS

We found many of sequence reads in two sRNA libraries from the adult pig and piglet adipose tissue samples for which there were no matching miRNAs in miRBase (14.0). To find more potential miRNAs, sequences longer than 18 nt were searched against the porcine genome and the flanking regions of the 16,337 sequence reads matching the genome were subjected to secondary structure analysis. According to the criteria for miRNAs mentioned above, 78 short RNA-producing loci corresponding to 66 unique sequence reads were identified that could be folded into step-loop structures (Table S6-1 and Table S6-2 in additional file 6). The short RNA-producing loci were considered as putative new miRNA genes since there is currently insufficient evidence to confirm that they are miRNA genes. Further experiments are in progress to validate these novel miRNAs by Northern blot analysis. Based on these analyses, we identified 66 potential novel miRNAs. Of the 66 identified miRNAs, 5 had been reported in recent studies (Table S6-2 in additional file 6, red lettering) [Li et al., 2010]. With the exception of ssc-miR-new4 and ssc-miR-new16, the absolute sequencing frequencies of these novel miRNAs were much lower in the adult pig and/or piglet adipose tissue samples (Table S6-3 in additional file 6). Of these miRNAs, the 24 novel miRNAs were detected both in the adult pig and piglet adipose tissue samples, 29 miRNAs were identified only in the adult pigs and 14 miRNAs were identified only in the piglets (Table S6-3 in additional file 6). In addition, the length of the novel miRNA sequences varied from 18 to 23 nt, with a distribution peak at 21 nt, and their 5′ ends comprised most frequently uridine (U) (Table S6-4 in additional file 6).

EXPRESSION ANALYSIS OF CONSERVED miRNAS

For the piglet adipose tissue library, more than 9 million sequences corresponding to approximately 75% of all clean reads were assigned to known miRNAs, showing that our sRNA libraries were highly enriched with mature miRNAs. These miRNAs were differentially expressed between the adult pig and piglet adipose tissue libraries. Of the 201 conserved miRNAs, 93 miRNAs were up-regulated, 42 of which were up-regulated more than twofold, and 33 miRNAs were down-regulated in the adult pig adipose tissue (Table S2-1 in additional file 2).

Although these conserved miRNAs were sequenced at varying frequencies, some miRNAs dominated the miRNA library (Table II). In the adult pig adipose tissue library, the most abundant miRNA was miR-143, with almost two million reads corresponding to 20% of all clean reads, and the second most abundant miRNA was let-7a, with 7.7% of all clean reads. The most frequently sequenced miRNA from the piglet adipose tissue library was miR-378, representing 16% of all clean reads. Moreover, some miRNAs such as miR-143, miR-378, miR-103/107, miR-148a, miR-10b, mir-21, miR-30a-5p, mir-101, and miR-199a-3p, were detected with high sequence counts both in the adult pig and piglet adipose tissue libraries, although their abundance differed significantly. In contrast, the sequencing frequency of some miRNAs, including miR-224, miR-122, miR-2411, miR-218a, miR-491, miR-504, miR-324, miR-664-3p, miR-205, and miR-2366, was low in both of the libraries

TABLE II. Abundance and Differential Expression of the Highly Abundant Porcine miRNAs in Developing Adipose Tissue

miRNA name	Sequencing count		Normalized expression		Fold-change [$\log_2$ (No.2-NE/No.1-NE)]	*P*-value	Sig-lable
	No.1	No.2	No.1-NE	No.2-NE			
ssc-miR-143	1015464	1836427	81209.18	201180.93	1.3088	0	**
ssc-miR-378	2019007	530536	161465.02	58120.32	−1.4741	0.014568712	*
ssc-miR-103/107	54192	440597	4333.87	48267.49	3.4773	0	**
ssc-miR-148a	468579	427095	37473.43	46788.34	0.3203	0	
ssc-miR-10b	115300	373685	9220.83	40937.26	2.1504	4.25E−225	**
ssc-miR-21	102197	331977	8172.95	36368.14	2.1537	0	**
ssc-miR-126-3p	90327	212791	7223.68	23311.29	1.6902	4.15E−91	**
ssc-miR-30a-5p	109354	149859	8745.31	16417.08	0.9081	0	
ssc-miR-30d	81664	131245	6530.87	14377.91	1.1385	0	**
ssc-miR-152	65147	109042	5209.97	11945.57	1.1971	0	**
ssc-miR-101	94578	92677	7563.64	10152.78	0.4247	0	
ssc-miR-191	23880	73855	1909.74	8090.83	2.0829	0	**
ssc-miR-10a	38252	63313	3059.11	6935.95	1.181	8.64E−47	**
ssc-miR-199a-3p	66304	57582	5302.5	6308.12	0.2505	0	
ssc-miR-27b	31441	57067	2514.42	6251.7	1.314	0	**
ssc-miR-24	20502	47571	1639.6	5211.41	1.6683	0	**
ssc-miR-199b-3p	49984	43156	3997.34	4727.75	0.2421	0	
ssc-miR-320	16666	38173	1332.82	4181.86	1.6497	0	**
ssc-miR-99b	18279	37334	1461.82	4089.95	1.4843	0	**
ssc-miR-30e-5p	36218	36450	2896.44	3993.1	0.4632	0	
ssc-miR-100	26307	35680	2103.84	3908.75	0.8937	0.270081141	
ssc-miR-423-5p	25356	34727	2027.78	3804.35	0.9077	6.03E−115	
ssc-miR-148b-3p	20718	30085	1656.87	3295.82	0.9922	2.97E−08	
ssc-miR-125b	16518	26321	1320.97	2883.47	1.1262	0	**
ssc-miR-27a-3p	7138	21196	570.84	2322.03	2.0242	3.75E−151	**
ssc-miR-99a	11198	16838	895.53	1844.61	1.0425	0.013042338	*
ssc-miR-181a	15642	16366	1250.93	1792.9	0.5193	6.71E−182	
ssc-miR-16	6730	14987	538.21	1641.83	1.609	0	**
ssc-let-7a	304378	704951	24341.87	77227.52	1.6657	2.96E−06	**
ssc-let-7f	268271	326053	21454.3	35719.17	0.7354	0	
ssc-let-7c	151222	278930	12093.6	30556.83	1.3373	0	**
ssc-let-7g	50178	73011	4012.86	7998.37	0.9951	1.66E−111	
ssc-let-7e	35733	81337	2857.66	8910.48	1.6407	4.08E−05	**
ssc-let-7i	32891	38902	2630.38	4261.72	0.6962	1.02E−282	
ssc-let-7d	13472	19308	1077.39	2115.2	0.9733	0.002789778	

No.1 and No.1-NE represent actual sequencing count and normalized expression level of miRNA in small RNA library of piglets adipose tissue sample, respectively. No.2 and No.2-NE represent actual sequencing count and normalized expression level of miRNA in small RNA library of adult pigs adipose tissue sample, respectively. Fold-change [$\log_2$ (No.2-NE/No.1-NE)]: fold change of miRNAs in pair of samples.
P-value, *P*-value which reflects the significance of miRNA differential between samples. Less *P*-value shows more significance of difference of miRNA between samples; sig-lable, significance label; none, others.
*Fold-change ($\log_2$) > 1 or fold-change ($\log_2$) < -1, and $0.01 \leq P$-value < 0.05.
**Fold-change ($\log_2$) > 1 or fold-change ($\log_2$) < -1, and P-value < 0.01.

(Table S2-1 in additional file 2). It is possible that these miRNAs are expressed at low levels, in certain cell types and/or under certain conditions.

Among the highly abundant miRNAs, some miRNAs such as miR-143, miR-103, let-7, and miR-27, are known to have important regulatory functions in adipose tissue and during adipogenesis. Seven members of the let-7 gene family, including let-7a, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i, were sequenced at high frequency in adipose tissue. The ubiquitously expressed let-7 accounted for 16.9% of all clean reads in adult pig adipose tissue, and 6.9% of all clean reads in piglet adipose tissue (Table S2-7 in additional file 2). These data were in agreement with other studies of miRNAs, showing that the let-7 miRNA is up-regulated during mouse adipocyte differentiation as a regulator of adipogenesis, in part by targeting the transcription factor High Mobility Group A2 (HMGA2) [Sun et al., 2009]. For the most abundant miRNA, ssc-miR-143, that was significantly up-regulated in developing swine adipose tissue (Table II), its mammalian orthologs, which potentially target extracellular signal-regulated protein kinase 5 (ERK5), are also up-regulated during adipogenic differentiation and have been implicated in obesity [Esau et al., 2004; Takanabe et al., 2008; Xie et al., 2009]. Moreover, the abundance of miR-103/107 was more than 3.5-fold up-regulated in adult pig adipose tissue, suggesting its function in adipose biology. Consistently, miR-103 has been shown to accelerate adipogenesis at an early stage, using ectopic expression of miR-103 in pre-adipocytes [Xie et al., 2009]. Regarding the roles of the miR-27 gene family on adipogenesis, several recent observations showed that miR-27 is a negative regulator of adipocyte differentiation that operates via suppression of PPARγ expression during adipogenesis of human multipotent adipose-derived stem (hMADS) cells and 3T3-L1 cells [Karbiener et al., 2009; Kim et al., 2010]. In addition, miR-378, which was the most abundant miRNA in the piglet adipose tissue library, was significantly down-regulated (approximately 1.5-fold) during the adipose tissue development process, which is in agreement with previous observations in bovine adipogenesis [Jin et al., 2010]. This report revealed that miR-378 correlated strongly with bovine backfat thickness and was significantly down-regulated in high backfat thickness fat tissue. These previous observations are therefore in strong agreement with the miRNAs expression

patterns observed in our sequencing analyses, suggesting that these conserved miRNAs may play roles similar to those of their orthologs in regulating the development and function of porcine adipose tissue.

miRNAs that were expressed at relatively low levels in adipose tissue included miR-122, miR-31, miR-125b, and miR-335. It is known that miR-122, a key regulator of cholesterol and fatty-acid metabolism, is specifically expressed in the adult liver [Esau et al., 2006]. The expression of miR-31 and miR-125b has been reported to be down-regulated during the adipogenic differentiation process of adipose-derived stem cells [Tang et al., 2009]. Moreover, a recent report revealed that the expression of miR-335 was up-regulated in the liver and white adipose tissue of obese mice, and was closely correlated with the expression levels of adipocyte differentiation markers in 3T3-L1 adipocytes [Nakanishi et al., 2009]. These findings suggested that these conserved miRNAs may also play a role in regulating the development and function of porcine adipose tissue, even though they were detected at very low sequence counts.

QUANTITATIVE RT-PCR

To validate the sequencing data, we applied stem-loop quantitative RT-PCR to compare the expression levels of the four most abundant miRNAs in adipose tissue and other tissues. The results shown in Figure 3 confirm that these miRNAs are abundant both in piglet and adult pig adipose tissue. The expression levels of miR-143, miR-378, miR-148a, and miR-103/107 in adult pig adipose tissue were approximately 1.2-, −1.7-, −0.9-, and 2-fold higher than the expression levels in piglet adipose tissue, respectively, confirming our sequencing data (Table II). In comparison, miR-143 expression varied substantially among the seven tissues examined (Fig. 3A), being highly expressed in back subcutaneous adipose, spleen, heart, and lung tissue both in piglets and adult pigs and moderately expressed in other tissues. Likewise, the expression of miR-378 was also highly variable among the tissues tested and was found to be abundant in adipose, heart, and lung tissue in piglets (Fig. 3B). Moreover, miR-148a was also abundant in the liver of piglets and adult pigs (Fig. 3C). Remarkably, miR-103/107 showed tissue-specific expression patterns (Fig. 3D). With the exception of adipose

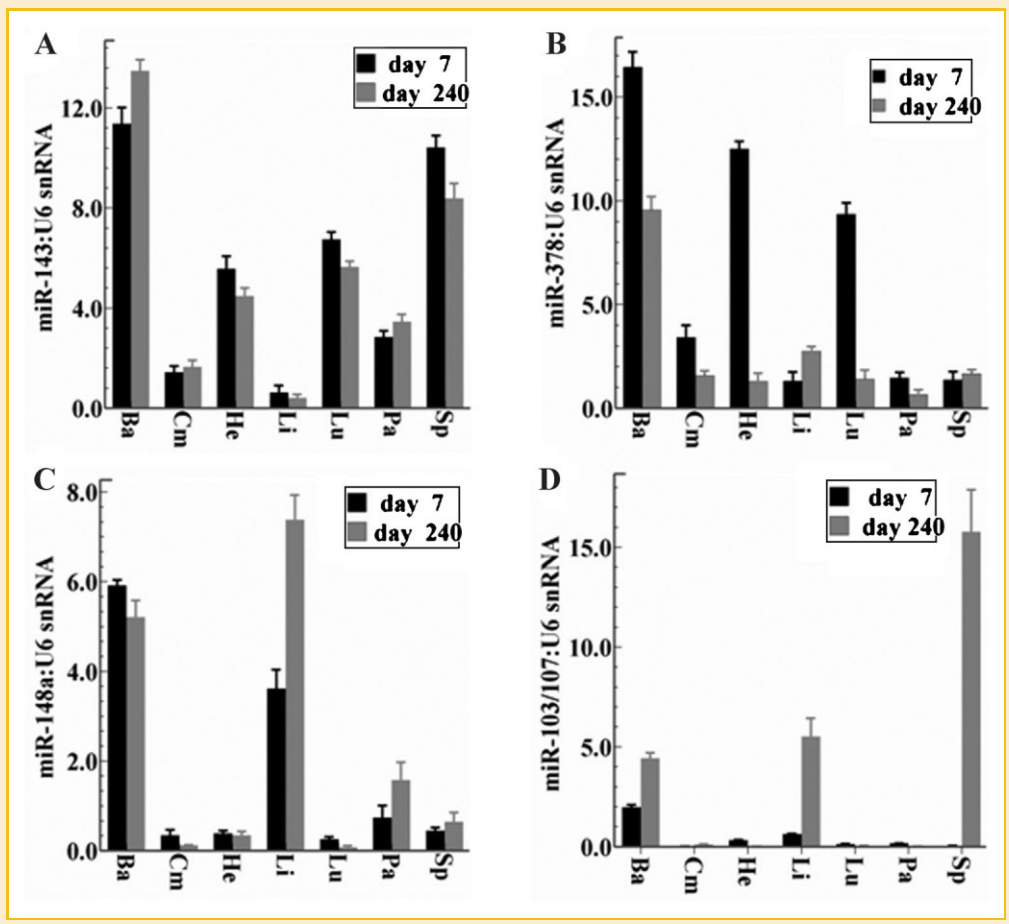

Fig. 3. Tissue expression patterns of the four most abundant porcine miRNAs in developmental adipose tissue. Levels of the miRNAs in seven different porcine tissues were examined using stem-loop RT-qPCR and individually analyzed by the absolute quantification method, as described in the Materials and Methods Section. The data are expressed as the ratio of the level of miRNA to that of U6 small nuclear RNA (snRNA), where U6 snRNA was the internal control. Bars indicate the mean of the values from three replicate experiments. Error bars show the 95.0% confidence interval of the mean. Ba, back subcutaneous adipose; Cm, crura skeletal muscle; Li, liver; Pa, pancreas; Sp, spleen; He, heart; Lu, lung. (A) Expression profiling of miR-143. (B) Expression profiling of miR-378. (C) Expression profiling of miR-148a. (D) Expression profiling o miR-103/107.

tissue, miR-103/107 was only abundant in the liver and spleen of adult pigs and was more weakly expressed in other tissues. These finding suggested that the most abundant miRNAs in developmental adipose tissue may play a regulatory role in the development in other tissues.

MicroRNA TARGET PREDICTIONS, KEGG PATHWAY ANALYSIS AND GO TERM ENRICHMENT

To understand the biological function of the 15 most abundant miRNAs (not including let-7 and miR-378) in adipose tissue, we used the algorithms PicTar [Krek et al., 2005] and TargetScan [Lewis et al., 2003] to predict their target mRNAs. *S. scrofa* genes are not included in the current versions of PicTar and TargetScan and the predictions were therefore based on the human mRNA/miRNA interactions. This approach assumes sequence conservation in the miRNA target sites between orthologues and target sites in the 3′-UTR of genes among closely related animals [Chen and Rajewsky, 2006]. TargetScan predicted 6,917 target genes and PicTar predicted 5,605 target genes. These predictions were then intersected to increase the specificity of the predictions, resulting in 2,149 target genes (Fig. S7-1 in additional file 7). With the use of DAVID bioinformatics resources [Dennis et al., 2003], the predicted target genes were classified according to KEGG functional annotations to identify pathways that were actively regulated by miRNA in adipose tissue (Table III). Intriguingly, the most over-represented miRNA targets belonged to the MAPK signaling pathway, which is known to be closely involved in the inhibition of adipogenesis [Wang et al., 2009]. Another pathway targeted by miRNA in adipose tissue was the Wnt signaling pathway, which is known to be involved in adipocyte lineage commitment [Bowers and Lane, 2008], adipogenesis and metabolism [Prestwich and Macdougald, 2007], and inhibits adipogenesis through beta-catenin-dependent and -independent mechanisms [Kennell and MacDougald, 2005]. In pigs, the Wnt/beta-catenin signaling pathway inhibits adipogenic differentiation potential [Li et al., 2008b] and its related genes appeared to be sequentially expressed during porcine adipose tissue development [Luo et al., 2008]. Likewise, the Hedgehog signaling pathway, also found to be enriched in our results, plays a conserved role from invertebrates to vertebrates, in inhibiting fat formation [Suh et al., 2006]. Another important pathway targeted by the highly expressed miRNAs is the TGF-beta signaling pathway, which is known to be involved in a wide spectrum of cellular functions such as proliferation, apoptosis, differentiation, and migration. It is worth noting that a recent study demonstrated that miR-21, shown to be highly expressed in our study, regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue [Kim et al., 2009]. Furthermore, pathways associated with adheren junctions, regulation of the actin cytoskeleton, focal adhesion, cell cycle, gap junctions, endocytosis and extracellular matrix–receptor interactions, were all significantly enriched, indicating the role of the highly expressed miRNAs in the regulation of cell motility, cell proliferation, the cytoskeleton, cell nutrition, communication between cells and the extracellular matrix. Moreover, enriched pathways for axon-guidance, the neurotrophin signaling pathway and long-term potentiation, suggested that the top 15 miRNAs in our data set participate in nervous system development and function.

TABLE III. KEGG Pathways Enriched for Targets of the 15 Most Abundant miRNAs Expressed in the Developing Adipose Tissue of Swine

Term	Count	*P*-value	Benjamini
Axon guidance	39	1.20E−10	1.90E−08
Neurotrophin signaling pathway	34	3.50E−08	1.80E−06
Wnt signaling pathway	38	5.50E−08	2.10E−06
MAPK signaling pathway	54	1.60E−07	5.00E−06
Melanogenesis	26	4.70E−06	1.20E−04
Adherens junction	22	7.50E−06	1.50E−04
Long-term potentiation	20	1.40E−05	2.20E−04
T cell receptor signaling pathway	24	2.00E−04	2.60E−03
Regulation of actin cytoskeleton	38	3.10E−04	3.70E−03
GnRH signaling pathway	22	3.40E−04	3.80E−03
Focal adhesion	35	7.50E−04	6.90E−03
Ubiquitin mediated proteolysis	24	5.70E−03	4.20E−02
Hedgehog signaling pathway	13	6.20E−03	4.40E−02
ErbB signaling pathway	17	8.30E−03	5.60E−02
Insulin signaling pathway	23	9.70E−03	6.20E−02
Type II diabetes mellitus	11	1.30E−02	7.70E−02
Cell cycle	21	1.60E−02	8.70E−02
Calcium signaling pathway	27	1.80E−02	9.30E−02
TGF-beta signaling pathway	16	1.90E−02	9.30E−02
Gap junction	16	2.20E−02	1.10E−01
Phosphatidylinositol signaling system	14	2.40E−02	1.10E−01
Adipocytokine signaling pathway	13	2.50E−02	1.10E−01
Endocytosis	27	3.00E−02	1.20E−01
ECM–receptor interaction	14	5.90E−02	2.00E−01
mTOR signaling pathway	10	5.90E−02	2.00E−01

It is noteworthy that the insulin signaling pathway and the adipocytokine signaling pathway, that correlate closely with adipose tissue biology, were also found to be enriched. Analysis of the functional annotation chart of target genes in the DAVID bioinformatics resources revealed that some of the biological process involved in the regulation of lipid metabolic, lipid kinase activity, fatty acid beta-oxidation, lipid storage and cholesterol storage, among others, were also enriched (data not shown). These predicted findings were in agreement with our Solexa sequencing results, suggesting that the function of the highly expressed miRNAs may be closely related to the regulation of porcine adipose tissue development. The GO term enrichment of the target genes associated with lipid metabolism was calculated using the Term Enrichment tool [Harris et al., 2004] to gain further insight into the regulation of lipid metabolism by miRNAs (Fig. S7-2 in additional file 7). The GO term of the target genes relevant to carboxylic acid metabolism, oxoacid metabolism, fatty acid metabolism, steroid metabolism, glycerolipid metabolism, alcohol metabolism and phospholipid metabolism, among others, was significantly over-represented, indicating that various aspects of the lipid metabolic process may be regulated by highly expressed miRNAs during adipose tissue development. Collectively, these pathway and biological process analyses illustrate some of the possible roles of the highly expressed miRNAs in adipose tissue biology.

CONCLUSIONS

In summary, we used Solexa sequencing to identify 227 distinct conserved miRNAs, consisting of 168 previously reported miRNAs

and 59 new miRNAs, from porcine adipose tissue at two stages of development. The expression levels of these conserved miRNAs displayed a large range, and those abundantly expressed could be classified into 32 miRNA families. Many of these miRNAs showed differential expression during porcine adipose tissue development. We also analyzed the KEGG pathway and GO term enrichment of the predicted target genes of the 15 most abundant miRNAs using the DAVID bioinformatics resources and the Term Enrichment tool. Identification of the complete set of miRNAs in adipose tissue is necessary to better understand the complexity of gene regulation governing the development and physiology of adipose tissue. Future studies to identify target mRNAs regulated by abundant miRNAs in the developing adipose tissue will also be critical to uncover their biological functions.

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