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A MicroRNA Catalog of Swine Umbilical Vein Endothelial Cells Identified by Deep Sequencing

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Abstract

MicroRNAs (miRNAs) are endogenous ~22 nt RNAs that play important regulatory roles in targeting mRNAs for cleavage or translational repression. Despite the discovery of increasing numbers of human and mouse miRNAs, little is known about miRNAs from pig. In this study, we sought to extend the repertoire of porcine small regulatory RNAs using Solexa sequencing. We sequenced a library of small RNAs prepared from immortalized swine umbilical vein endothelial cells (SUVECs). We produced over 13.6 million short sequence reads, of which 8 547 658 perfectly mapped to the pig genome. A bioinformatics pipeline was used to identify authentic mature miRNA sequences. We identified 154 porcine miRNA genes, among which 146 were conserved across species, and 8 were pig-specific miRNA genes. The 146 miRNA genes encoded 116 conserved mature miRNAs and 66 miRNA*. The 8 pig-specific miRNA genes encoded 4 mature miRNAs. Four potential novel miRNAs were identified. The secondary structures of the 154 miRNA genes were predicted; 13 miRNAs have 2 structures, and miR-9 and miR-199 have 4 and 3 structures, respectively. 36 miRNAs were organized into 19 compact clusters. miR-206, miR-21 and miR-378 were the relatively highly expressed miRNAs. In conclusion, Solexa sequencing allowed the successful discovery of known and novel porcine miRNAs with high accuracy and efficiency. Furthermore, our results supply new data to the somewhat insufficient pig miRBase, and are useful for investigating features of the blood-brain barrier, vascular diseases and inflammation.

Key words: microRNA, sequencing, Solexa, pig, umbilical vein endothelial cells

INTRODUCTION

MicroRNAs (miRNAs) are an abundant class of ~22 nt noncoding RNAs that play important regulatory roles in animals and plants (Bartel 2004). In animals, they regulate gene expression by targeting 3′ untranslated regions of messenger RNAs with imperfect complementary pairing. Recently, the number of newly discovered miRNAs has increased rapidly, and many of them are highly conserved across species (Griffiths-Jones *et al*. 2006). Although the role of most miRNAs remains unknown, recent studies indicated that miRNAs have important roles in the regulation of gene expression, protein synthesis suppression, and diverse cellular processes (Kloosterman and Plasterk 2006; Grimson *et al*. 2007; Niwa and Slack 2007).

Traditional sequencing of small RNA cDNA libraries is expensive, time consuming and labor intensive. Moreover, it can only identify highly expressed miRNAs; those at low-levels can not be detected. Recent developments in the next generation of sequencing technology have allowed the identification of millions of small RNAs in samples from various organ-

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isms (Burnside *et al*. 2008; Moxon *et al*. 2008; Rathjen *et al*. 2009). With the advent of deep sequencing, we have an opportunity to identify low abundance miRNAs, which might not be detected by hybridization methods. The Solexa (Illumina Inc., USA) system provides approximately two orders of magnitude greater depth than current competing technologies (Berezikov *et al*. 2006b), yielding several million sequences from a single flow cell lane.

As an important model organism in biomedical research, it is critical to identify a comprehensive set of miRNAs in pig. The latest miRNA database (miRBase 15.0) contains 175 porcine miRNA genes, which encode 202 distinct mature miRNAs and no miRNA*, although Cho *et al*. (2010) has cloned two miRNA*s (Griffiths-Jones *et al*. 2006). 54 of these miRNAs were computationally predicted on the basis of sequence homology to known miRNAs from other species (Wernersson *et al*. 2005; Griffiths-Jones *et al*. 2008). Kim *et al*. (2006, 2008) predicted 58 porcine miRNAs by a homology search of known human and mouse miRNAs against the pig genome, and directly cloned 25 miRNA,14 of which were previously unreported. In recent studies, research groups (McDaneld *et al*. 2009; Nielsen *et al*. 2010) have identified 12, 57 and 212 miRNAs from porcine skeletal muscle. Reddy *et al*. (2009) identified 120 conserved miRNA homologs in pig heart, liver and thymus. From porcine skeletal muscle and adipose tissue, Cho *et al*. (2010) obtained 89 distinct miRNAs using small RNA cloning, which consisted of 74 previously reported miRNAs and 15 new conserved miRNAs. 332 intestinal miRNAs were discovered in porcine intestine, of which 201 represented assumed novel porcine miRNAs (Sharbati *et al*. 2010). Furthermore, the total number of swine miRNAs is currently lower than that identified in human (1 240), mouse (799) or even zebrafish (661) (Griffiths-Jones *et al*. 2008a). This suggests that there may be large numbers of undiscovered miRNAs in pig.

Immortalized swine umbilical vein endothelial cells (SUVECs) are commonly used as an *in vitro* model for studying features of the blood-brain barrier, vascular diseases and inflammation (Hong *et al*. 2007). In this study, we aimed to investigate the repertoire of small regulatory RNAs expressed in pig by Solexa sequencing and to construct a library of small RNAs isolated from the SUVEC cell line. The data generated will provide indications of the biological functions of miRNAs expressed by endothelial cells.

MATERIALS AND METHODS

Cell culture and RNA isolation

Immortalized swine umbilical vein endothelial cells (SUVECs) (Hong *et al*. 2007) were cultured in M199 (Gibco, USA) medium containing 20% fetal calf serum (Hyclone) at 37°C and 5% CO_2. For Solexa sequencing, total RNA was extracted using Trizol (Invitrogen Inc., USA), according to the manufacturer's instructions. The RNA concentration and purity were determined by absorbance at 260 nm and the OD_{260}/OD_{280} ratio using a NanoDrop ND1000 spectrophotometer, USA. RNA samples were stored at -80°C.

Solexa sequencing

This work was carried out by the BGI-Sequencing Corporation, China. Briefly, RNA was subjected to polyacrylamide gel electrophoresis (PAGE), and small RNA molecules less than 30 nt were isolated and ligated with adapters at the 5′ and 3′ ends. The samples were used as templates for cDNA synthesis, which was then amplified by PCR. Fragments of about 90 base pairs were isolated from agarose gels, sequenced by the Solexa (Illumina Inc.) technique and used for sequencing analysis and cluster generation. The image files generated by the sequencer were processed to produce digital-quality data. After masking of adaptor sequences and removal of contaminated reads, clean reads were processed for computational analysis.

Analysis of sequencing data

The 3′ adaptor sequences were removed from the raw sequence reads and the clean reads were mapped onto the *Sus scrofa* genome using SOAP (Li *et al*. 2008). We then matched our reads to the miRBase 15.0 to identify identical, or highly similar, mature miRNAs from other species. To identify the actual miRNA genes, we used MiRAlign and MiPred (Jiang *et al*. 2007) online

softwares (to distinguish between pseudo and real miRNA precursors) to test the results. We then used MIREAP software to identify both known and novel miRNAs from small RNA libraries that were deep sequenced by Solexa technology. MIREAP fully considers miRNA biogenesis, sequencing depth, and structural features to improve the sensitivity and specificity of miRNA identification (http://sourceforge.net/projects/mireap/). To analyze RNA secondary structures, all precursor sequences of identified miRNA sequences were predicted by mfold (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi) (Zuker 2003). Stem-loop hairpins were considered typical only when they fulfilled three criteria: mature miRNAs are in one arm of the hairpin precursors, which lack large internal loops or bulges; the secondary structures of the hairpins are steady, with a free energy of hybridization lower than -20 kcal mol^{-1}; and hairpins are located in intergenic regions or introns. Finally, based on the genomic locations of the precursors, gene clusters were analyzed.

RESULTS

Deep sequencing of small RNAs from SUVECs

Immortalized swine umbilical vein endothelial cells (SUVECs) were cultured and the extracted RNA was pooled. A library of small RNAs was constructed after trimming the reads of low quality sequences, adaptor sequences and contaminants (Hong *et al*. 2007). We obtained 13 612 934 total clean reads of 18-30 nt in length, which represented 863 648 unique sequences. The most abundant size class was 22 nt (20.93%), followed by 23 nt (14.59%), which was consistent with the accepted size of miRNAs (Fig. 1). 8 547 658 (62.97%) reads perfectly mapped to the pig genome (*S. scrofa* 9) using SOAP (Li *et al*. 2008). The small RNAs were then classified into different categories and annotated (Appendix A). 4 411 560, 705 891, 89 629, and 23 246 were degradation products of rRNA, tRNA, snRNA, and snoRNA, respectively, and these groups of RNAs were not characterized or further analyzed (Appendix A).

Identification and selection of miRNA precursors

After annotation, there were 5 932 143 reads assigned

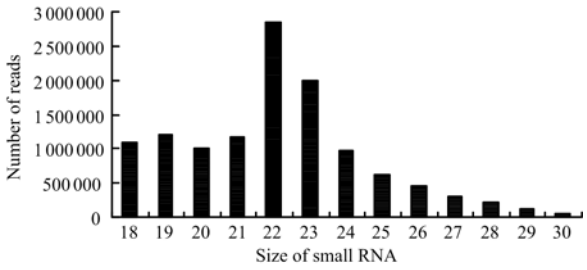

Fig. 1 Size distribution of sequenced small RNAs.

to the miRNA group. Sequence similarity searches against the miRBase 15.0 revealed that 75 738 sequences representing 949 unique mature miRNAs, which were identical or highly similar to known miRNAs from other species, including human, mouse, zebrafish, and other model animals (Appendix B). However, an important feature of miRNAs is their pre-miRNA sequence stem-loop hairpin structure (Berezikov *et al.* 2006a). To find genuine miRNAs, we scanned the *S. scrofa* genome for hairpin structures using the MIREAP software. In total, 190 loci were identified that generated typical stem-loop hairpin structures (Appendix C). However, there are also many random inverted repeats in eukaryotic genomes that can also fold into dysfunctional hairpins, which are termed "pseudos" (Jiang *et al.* 2007). To overcome this problem, we used MiPred online software to distinguish pre-miRNAs from other similar segments, resulting in the removal of 5 loci with free energy lower than -20 kcal mol^{-1} and 31 pseudos. Subsequently, we searched for paralogs or orthologs using MiRAlign (requiring MFE=-20 kcal mol^{-1}, delta length=15, minimal sequence similarity score is 70). Ultimately, 154 loci remained as candidate miRNA genes (Appendix D), comprising 146 conserved miRNA genes and 8 pig-specific miRNA genes (named ssc-mir-s1, s2, s3, and so on) (Appendixes D and E), which encoded 116 and 4 mature miRNAs, respectively. Among the genes encoding the 120 mature miRNAs, 17 exist as 2 copies, 4 are present as 3 copies, and 1 is present in 4 copies. The duplicate genes are distributed on the same or separate chromosomes and they produced identical mature miRNAs (Appendix E).

The dsRNA-specific endonuclease known as DICER, excises a 21-23 nt small RNA duplex from a hairpin-like miRNA precursor, producing an equal ratio of miRNA/miRNA*. Subsequently, the strand with lower

thermodynamic stability in its 5′ end is preferentially incorporated into the RISC complex and, unlike the other strand, is protected from rapid degradation (He and Hannon 2004). In many cases, miRNA* can not be detected by conventional methods due to their rapid turnover; however, the deep sequencing procedure allows many of them to be identified. In this study, 66 miRNA* were also detected (Appendix E).

Potential novel miRNAs

To identify novel miRNAs in the sequencing data, we used the following criteria: (1) no mismatched with porcine genome; (2) not identified in other species; and (3) embedded within a canonical stem-loop hairpin precursor. According to these criteria we found 4 potentially novel mature miRNAs and their genes, using MIREAP (Appendix F). These miRNAs lacked annotations in miRBase but had perfect matches to the pig genome. Further experiments were performed to confirm the novel miRNAs candidates.

Secondary structure prediction

One of the important criteria for distinguishing miRNAs from other endogenous small RNAs is the ability of the flanking sequences to adopt a hairpin precursor structure. We used mfold online software (requiring that the folding temperature is fixed at 37°C and the read sequence situated on one arm of the hairpin) to predict the secondary structures of the 154 miRNAs (Appendix G, where red represents miRNA sequence and green represents miRNA* sequence). These were the 146 conserved miRNAs and the 8 pig-specific genes encoding the 4 predicted novel miRNAs. In general, all of the precursor sequences identified were in the typical size range (70-80 nt) for animal miRNA precursors (Bartel 2004). Most of the mature miRNAs were derived from either 5′ strand, 3′ strand, or both strands of a stem region in the hairpin structure of an miRNA precursor. Among all the hairpin structures, there are 13, including miR-101a (Nielsen *et al.* 2010), with predicted hairpin structures as a result of the fact that they match 2 different genomic sites that fold into precursor stem-loop structures. miR-9 and miR-199 have 4 and 3 structures, respectively (Appendix G).

miRNA gene clusters in the pig genome

Animal miRNA genes are frequently clustered on the genome, and many of them are from the same polycistronic primary transcript, resulting in similar expression patterns and functions (Altuvia *et al.* 2005; Bartel 2004). We identified 36 miRNA genes organized into 19 compact clusters, including 16 pairs, 2 triplets, and 1 group of 5 (Fig. 2). Two identical copies of cluster miR-15a-miR-16 were located on 2 different chromosomes (11 and 13). Identical clusters of miR-181a-miR-181b were also located on 2 chromosomes (1 and 10). The corresponding porcine miRNAs are separated by 1 787 and 92 nt, respectively. Interestingly, 5 miRNAs (ssc-miR-24, ssc-miR-27a, ssc-miR-27b, ssc-miR-23, and ssc-miR-23b) were located on chromosome 2 and chromosome 10. In the clusters miR-24-miR-27a-miR-23 and miR-24-miR-27b-miR-23b, the corresponding miRNAs are separated by 77 and 109 nt, and 468 and 109 nt, respectively. The 1 cluster containing 5 miRNAs (miR-17-miR-18a-miR-19a-miR-19b-miR-92a) was located on chromosome 11, except for the missing ssc-miR-91 which was involved in the *S. scrofa* miR17-92 miRNA cluster (GenBank: AY87597.1) and identified by Sawera *et al.* (2005).

Expression analysis of miRNA in SUVECs

The sequencing frequency of the miRNAs was used to establish the miRNA expression profile (Appendix E). We found that, although the count number of miRNAs and miRNA* were variable, most miRNA* showed weak expression and their expression levels were much lower than their corresponding miRNAs, which is consistent with the idea that miRNA* strands are short-lived. Among the relatively highly expressed miRNAs in SUVECs, we noticed that miR-21 represented almost 15.3% of all reads, miR-378 represented 11.6% and miR-206 represented 8%, which is consistent with their expression in human endothelial cells and porcine skeletal muscle (Kuehbacher *et al.* 2007; Nielsen *et al.* 2010).

DISCUSSION

Endothelial cell function is necessary for the vascular

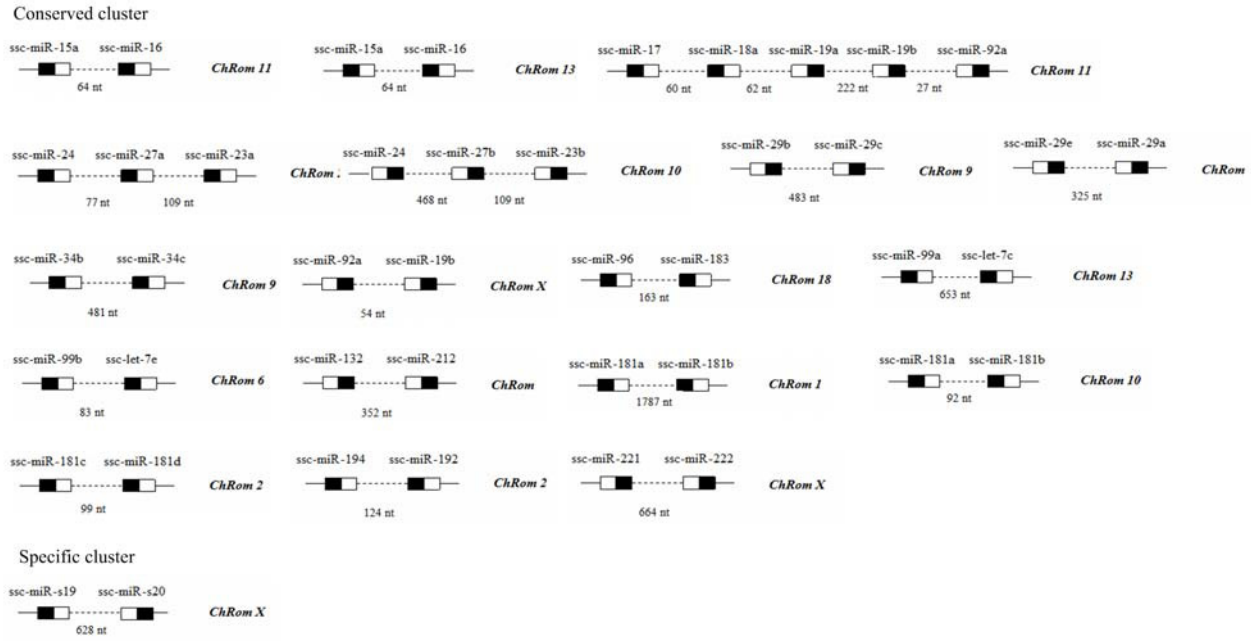

Fig. 2 miRNA gene cluster. The precursor structure is indicated as a box, and the location of the miRNA within the precursor is shown in black.

system. Endothelial cells show important endocrine and exocrine functions and regulate the coagulation through release of anticoagulant and pro-coagulant substances. Cytokines released by immune and inflammatory cells stimulate endothelial cells to participate in mechanisms of pathogenesis. We chose the Solexa method to identify new miRNAs in SUVECs to provide information on the biological function of miRNAs associated with endothelial cells.

Previously, miRNAs were defined as non-coding RNAs that fulfill a combination of expression and biogenesis criteria. Firstly, a mature miRNA should be expressed as a distinct transcript of approximately 22 nt that is detectable by Northern blot analysis or other experimental means, such as cloning from size-fractionated small RNA libraries. Secondly, a mature miRNA should originate from a precursor with a characteristic secondary structure, such as a hairpin or fold-back that does not contain large internal loops or bulges.

We obtained 13 612 934 reads from SUVECs using Solexa, and 62.79% of them were matched with the porcine genome. We identified 146 conserved miRNA genes in SUVECs that encode 116 mature miRNAs. All of these conserved miRNAs meet the expression and structure criteria required for miRNA annotation, and many have additional supporting evidence, such as genomic clustering and cloning of their miRNA* sequences. Unfortunately, the species-specific expression patterns of non-conserved pre-miRNAs are not very clear in eukaryotes (Bentwich *et al*. 2005). To overcome this problem, we used MiPred, which identified 27 pseudo-pre-miRNAs and 8 real pre-miRNAs from among 35 pre-miRNA-like hairpins. Thus, 8 miRNA genes constitute the final collection of non-conserved miRNA genes and these encode 4 mature miRNAs. All of these miRNAs meet the expression and structural criteria, including genomic clustering and cloning of miRNA* sequences.

miR-206, miR-378, miR-21, miR-24, and miR-34c were all highly expressed in the small RNA library (Table 1). Indeed, previous research has indicated the functions of several of these miRNAs elevated miR-206 levels in MCF-7 cells ultimately resulted in reduced cell proliferation and enhanced apoptosis (Adams *et al*. 2009) and were upregulated in myocardial infarction model (Shan *et al*. 2009). miR-378 enhances cell survival, reduces caspase-3 activity, and promotes cell survival, tumor growth and angiogenesis by targeting suppressor of fused (SuFu) and Fus-1 (tumor suppressor candidate 2, TUSC2) expressiones *in vitro* and *in vivo* (Lee

et al. 2007). miR-21 is over expressed in human umbilical vein endothelial cells (HUVECs) that had decreased apoptosis, and increased eNOS phosphorylation and nitric oxide (NO) production, implying that miR-21 influences endothelial biology by decreasing apoptosis and activating the NO pathway (Weber *et al*. 2010). In addition to miR-21 plays an important role in vascular cell proliferation and apoptosis, several experiments have proved that the target genes of miR-21 are involved in the pathogenesis of cardiovascular diseases (Table 2) (Cheng and Zhang 2010). miR-34c restored cell cycle progression and expression of cyclin D1 and Cdk4, as well as having a significant role downstream of p63 in controlling epidermal cell proliferation (Antonini *et al*. 2010). More related validated target genes were listed in Table 2. These miRNAs seems to be indicating that they might play a fundamental role in biological function and pathological mechanism of SUVECs.

Table 1 High expression mature miRNA (cloning counts>50 000)

ID	Mature miRNA sequence	Cloning counts
ssc-miR-21	TAGCTTATCAGACTGATGTTGAC	736 578
ssc-miR-378	ACTGGACTTGGAGTCAGAAGGC	443 263
ssc-miR-34c	AGGCAGTGTAGTTAGCTGATTGC	173 049
ssc-miR-183	TATGGCACTGGTAGAATTCACT	148 190
ssc-miR-24	TGGCTCAGTTCAGCAGGAACAG	77 631
ssc-miR-99b	CACCCGTAGAACCGACCTTGC	72 760
ssc-miR-206	TGGAATGTAAGGAAGTGTGTG	57 175

Table 2 High expression miRNAs and their target genes

ID	Target genes
miR-21	PDCD4, phosphatase, PTEN, SPRY1, SPRY2
miR-378	SuFu, Fus-1
miR-34c	c-Met, p53
miR-183	Integrin l, kinesin
miR-24	DND1, FAF1, MYC
miR-99b	-
miR-206	SRC-1, SRC-3, GATA-3

-, no target gene.

The research on miRNA has been highly active in disease rapidly developed in human, but is lagged behind in pigs. One of the reasons was that porcine miRNAs were released much less than those announced in humans. When the study was under way, a few of miRNA was registered in miRBase. Compared with miRBase 15.0, 21 new conserved miRNAs were identified in out study, and 86 coincided with muscles and adipose tissue. These 21 miRNAs have not been conformed to specially express in umbilical vein endothelial cells, since miRNAs of other porcine tissues have not been conformed. But some of the miRNAs identified in the present study might aid further research into the regulation of virus infection in endothelial cells, not only porcine diseases but also humans'. This is because vascular endothelial cells have been demonstrated to be involved in several viral diseases, such as classical swine fever virus (CSFV), Ebola virus (EBOV) and dengue virus (DENV). Especially classical swine fever is a highly contagious disease of pigs characterized by fatal hemorrhagic fever. It rapidly spreads across national borders, causing serious losses in the pig industry would wide. Recently, it was reported that cellular microRNAs not only inhibit viral protein expression, but others might also promote viral proliferation and replication (Browne *et al*. 2005; Cullen 2006). For example, HCV was upregulated by miR-122 (Jopling *et al*. 2005) and miR-24 was identified to be involved in HCV entry, replication, and propagation (Liu *et al*. 2010). Nevertheless, so far no report is on porcine contagious disease which bring about acute economic losses in agriculture.

CONCLUSION

Our results have identified 116 conserved miRNAs and 4 novel pig-specific miRNAs. The miRNAs identified by the direct sequencing method used in our study might be candidates for investigating the biological functions of miRNAs associated with the blood-brain barrier, vascular diseases, and inflammation.

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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