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Expression Analysis of miRNAs in Porcine Fetal Skeletal Muscle on Days 65 and 90 of Gestation

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ABSTRACT : MiRNAs (microRNAs) are a class of small non-coding RNA molecules of ~21 nucleotides that down- regulate the expression of target genes at post-transcriptional level. In this study, we first accomplished a preliminary scan of miRNA expression using 65 and 90 day fetal pig skeletal muscle samples by microarray hybridization, and 34 miRNAs showed strong positive signals. Five of these miRNAs were selected for further investigation by real-time RT-PCR. The statistical analyses indicated that three miRNAs exhibited significant differential expression ($p<0.05$) during porcine muscle development from 65 to 90 days of gestation, e.g., *miR-24* and *miR-424* were down-regulated while *miR-133a* was up-regulated. Multi-tissue RT-PCR was performed to detect the expression patterns of the five miRNA precursors. The results showed that most of these precursor miRNAs were ubiquitously expressed in different porcine tissues. (**Key Words :** Pig, MicroRNA, Expression, Skeletal Muscle)

INTRODUCTION

Since the identification of the first miRNA *Lin-4* in *Caenorhabditis elegans*, a great number of miRNAs have been identified in various organisms. MiRNAs are important gene regulators that execute their function via binding target genes and inhibiting translation or directing transcript degradation (Bartel, 2004). Studies revealed that miRNAs are involved in many biological processes including cell proliferation, cell death, stress response, developmental timing, brain morphogenesis, fat metabolism and muscle differentiation, etc. (Lee et al., 1993; Olsen and Ambros, 1999; Ambros, 2003; Xu et al., 2004; Esau et al., 2004; Giraldez et al., 2005; Chen et al., 2006).

Skeletal muscle development is an important physiological process in meat animals, and it directly affects meat production. Muscle mass is mainly determined by muscle fiber number and size in animals. In the pig, muscle fibers are formed in two stages during gestation, including primary and secondary fiber formation, and muscle fiber numbers are fixed before birth (Swatland, 1994). Investigation of genes expressed during skeletal muscle development is elementary in understanding molecular mechanism of muscle growth and can contribute to the discovery of candidate genes associated with meat production and quality traits. There are some reports on gene expression profiles in porcine muscle (Zhao et al., 2003; Zhao et al., 2005; Te Pas et al., 2005; Cagnazzo et al., 2006), however, little is known about the expression of miRNAs related to porcine skeletal muscle development. In a SAGE analysis of gene expression in porcine fetal muscle, we found that there are many genes showed differential expression between 65 and 90 days gestation stages (Tang et al., 2007). In this study, we carried out an initial scan on miRNA expression in porcine fetal muscle using a multi-species miRNA microarray, and further investigated differential expression of five miRNAs by real-time PCR in 65 and 90 days fetal skeletal muscle tissues.

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MATERIALS AND METHODS

miRNA preparation

Fetal skeletal muscle samples were collected from Landrace pig at days 65 and 90 of gestation in Tongcheng

Table 1. Primers used in the real-time PCR experiment

Name	Sequence (5'-3')	Size (bp)	Tm value (°C)
ssc-miR-24	5' -TGGCTCAGTTCAGCAGGAA- 3'	66	60
ssc-miR-30a-5p	5' -TGTAAACATCCTCGACTGGAA- 3'	66	60
ssc-miR-126-3p	5' -TCGTTCCGTGAGTATATAATGC -3'	66	60
ssc-miR-133a	5' -TTGGTCCCCTTCAACCAGCT-3'	66	60
ssc-miR-424	5' -CAGCAGCAATTCATGTTTTGA- 3'	66	60
18s forward primer	5' -TTTCGCTCTGGTCCGTCTTG- 3'	101	60
18s reverse primer	5' -TTCGGAACTGAGGCCATGAT-3'		
Poly (T) adapter	5'-GCGAGCACAGAATTAATACGACTCACTATAGG(T)12VN*-3' V* = A,G,C; N = A,T,G,C		
Reverse primer	5'-GCGAGCACAGAATTAATACGAC -3'		

Table 2. Primers of porcine miRNA precursors and GAPDH

Precursors microRNAs	Primer sequence (5'-3')	Size (bp)	Tm value (°C)
ssc-mir-24	P-PF1: 5' -CCGTGCCTACTGAGCTGAAA -3'	60	50.2
	P-PR1: 5' -GTTCCTGCTGAACTGAGCCA- 3'		
ssc-mir-30a	P-PF2: 5'- CGGCTGTAAACATCCTCGACT- 3'	59	59.8
	P-PR2: 5' -CATCCGACTGAAAGCCCGT -3'		
ssc-mir-126	P-PF3: 5'-GCTGGCGACGGGACATTA-3'	71	50.8
	P-PR3: 5'-CGCATTATATACTCACGGAACG- 3'		
ssc-mir-133a	P-PF4: 5' -TGCTAGAGCTGGTAAAATGGAA -3'	79	50.8
	P-PF4: 5' -AATGCATAGCTACAGCTGGTTG -3'		
ssc-mir-424	P-PF5: 5'-AGGGGATGCAGCAGCAAT-3'	66	62.5
	P-PR5: 5'-ATAGCAGCGCCTCACGTT-3'		
GAPDH	GAPDH-F: 5'-CCTTCATTGACCTCCACTAC-3'	321	60
	GAPDH-R: 5'-GTTGTCATACTTCTCATGGTTC-3'		

pig breeding farm (Hubei, China). First, total RNAs from the 65 and 90 days prenatal longissimus muscle samples were isolated according to the protocol of TRIzol reagent (Invitrogen). Then, the small RNA molecules were isolated and quantified using flashPAGE Reaction Clean-Up Kit (Ambion). After quantifying the small RNAs, the miRNA was Poly (A) tailed directly and a capture sequence was ligated to the Poly (A) tailed miRNAs.

Microarray hybridization and analysis

The Multi-Species microarray, which contains 762 DNA probes targeting the miRNAs of human, mouse and rat deposited in Sanger mirBase database (http://microrna.sanger.ac.uk/sequences/release 7.0) was purchased from Invitrogen Company (USA). Microarry hybridizations were implemented in Shanghai Biochip Company. The tagged miRNAs were purified and hybridized with the NCode™ miRNA microarray following the instructions of the manufacture's instructions. The slide was scanned by Axon scanner. The image and data were analyzed using the methods described in the instruction of the product.

Real-time PCR amplification of miRNAs

Fetal skeletal muscle samples from three 65-day and three 90-day individual piglets were used in the analysis. Real-time PCR amplification procedure was performed using the following method. In brief, 1 μg RNA was polyadenylated with ATP by poly (A) polymerase (Ambion) at 37°C for 1 h in a 20-μl reaction mixture according to the manufacturer's instructions. The polyadenylated RNA was reverse-transcribed with 200 U Superscript III Reverse Transcriptase (Invitrogen) and 0.5 μg poly (T) adapter. Before real-time PCR amplification, each PCR product was sequenced to ensure the correct amplification. For each real-time PCR reaction, 1 μl template cDNA equivalent to ~100 pg total RNA was mixed with 12.5 μl 2×SYBR Green PCR master mix and 5 pmol each of the forward and reverse primer in a final volume of 25 μl. The amplification program was (94°C×30 s, 60°C×30 s, and 72°C×20 s) ×40 cycle. All reactions were performed in triplicates for each sample. Porcine 18S ribosomal RNA (rRNA) (AY265350.1) was used as internal control. Primer sequences, PCR product sizes and anneal temperature (Tm value) were listed in Table 1. T-test was used to determine the expression level differences between the two stages using ΔCt method (Zhao et al., 2006), and the significance level was set at $p<0.05$.

MicroRNA precursor cloning and tissue expression

Tissue samples including heart, liver, spleen, lung, kidney, skeletal muscle and placenta were collected from 90 day porcine fetus. The porcine homolog genomic sequences corresponding to the miRNAs detected by microarray were retrieved using BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). Primer was designed by primer 5.0 based

on the porcine genome sequence (Table 2). To clone the miRNA precursors, PCR reaction was first carried out in 10 μl reaction mixture which containing 5×PCR buffer (Mg^{2+}), 3 pmol each of primer, 75 μM dNTPs, 0.5 U Taq DNA polymerase. The PCR program was as follows, 94°C for 3 min, 4×PCR reaction cycles (94°C for 30 s, 50 to 62.5°C for 30 s, and 72°C for 20 s), 72°C for 5 min. The PCR products were cloned into *pMD-18T* vector (Takara Biotechnology) and sequenced commercially. The semi-quantitative PCR mixture contained 6.7 μl $d2H_2O$, 1 μl of 10×Buffer, 0.2 μl dNTP, 0.6 μl of $MgCl_2$ (25 mM), 0.2 μl of each of the primer, 0.1 μl of Taq DNA polymerase, and 1 μl cDNA. PCR conditions were listed in Table 2. The housekeeping gene, *GAPDH* was used as positive control. Each PCR reaction was repeated for three times. The PCR products were analyzed by electrophoresis on 3% agarose gels.

RESULTS AND DISCUSSION

MicroRNAs expressed in the porcine fetal skeletal muscle tissue

As a preliminary result, 34 porcine miRNAs had high positive signals in 65 and 90 days skeletal muscle tissues using microarray hybridization (Table 3). Among them, sequence and expression patterns of 26 miRNAs have not been reported in porcine tissues yet. While others such as *hsa-miR-143*, *hsa-miR-133b*, *hsa-miR-125b*, *hsa-miR-27a*, *hsa-miR-24*, *hsa-miR-21*, *hsa-miR-19a*, *hsa-miR-18* and *hsa-miR-106a* were concurred with the previous report (Sawera et al., 2005; Wernersson et al., 2005; Kim et al., 2006). The microarry used in the study was designed based on the sequence of miRNAs from human, rat and mouse. Even though there are many probes with strong signals, it is difficult to conclude that there are coordinates exist to these probes because the potential sequence variations may exist in porcine genome. Thus, a BLASTN search was performed to find the genomic sequence coordinates of these miRNAs in the porcine genome. The counterparts of 10 miRNA sequences were found, while the others returned no results due to the current insufficient pig genomic sequences (Table 4). The blast analysis showed that most of the mature miRNA sequences are identical between pig and mouse or human. A few exceptions have length variations at 3' end and it does not affect the efficiency to hybridize with the probes of microarry. *Hsa-miR-424* and *mmu-miR-424* have one base difference within sequence, but only the probe of human miRNA gave signal in the microarray, indicating that the pig *miR-424* sequence is more similar to the human than mouse, and also showing the reliability of the results of the microarray.

In addition, the detected number of miRNA was found to be relative small. There are hithreto 60 porcine miRNAs can be retrieved, including 54 deposited in miRBase and 6 identified by Kim et al. (2006). While only 13.3% (8/60, other 26 miRNAs detected by the microarray have not been reported before) of them have signals in the microarray. Undoubtedly, there should be many miRNAs have been lost during the hybridization, especially for those with low expression. Since the experiment is a preliminary screen only used muscle tissue and we didn't perform any biological duplicated experiments, however, the results still reflected that the expression of miRNAs in fetal muscle.

Real-time PCR revealed differentially expressed miRNAs

After preliminary bioinformatic analysis of miRNAs (e.g. prediction of target genes) which had strong signals, five of the miRNAs detected by the microarray were further investigated by real-time PCR using the method described before (Shi and Chiang, 2005). The PCR products were sequenced to ensure the correct of PCR amplifications (Table 5). The results showed that *miR-24* ($p = 0.0501$) and *miR-424* ($p = 0.0243$) were down-regulated, while *miR-133a* ($p = 0.0496$) was up-regulated between the stage of 65-day to 90-day of gestation. In addition, the expression level of *miR-30a* was higher at 65 day gestation, however the p value of *t-test* did not reach significant ($p = 0.1520$), the *miR-126* showed higher expression at 90 day, the p value was close to significant ($p = 0.1021$). The expression profiles of these miRNAs were shown in Figure 1. Both the results from microarray and the real-time PCR concur with the claims that we have convincingly detected the expression of these miRNAs.

The 65 and 90 gestation days are two important stages during porcine embryo development. The differential expression patterns of specific up or down-expression in different developmental stages reflected the regulation role of these miRNAs. It has been reported that some miRNAs such as *miR-133*, *miR-206* and *miR-1* are related to muscle development, and the miRNAs found in our study could be additional ones that have not been reported. To further investigate the function of these miRNAs, the predicted target genes and their functions were subsequently examined. Interestingly, large number of target genes are likely to relate to cell differentiation, multicellular organism development and growth (*miR-133*, *miR-30a*, *miR-24*, *miR-126*), and assume that miRNAs are involved in porcine skeletal muscle growth and development. However, *miR-133* was up-regulated, *miR-30a* and *miR-24* were down-regulated from 65 to 90 days gestation, these maybe due to the different function of the target genes regulated by these miRNAs. However, there were no reports on *miR-30a*, *miR-24* so far on their function related to growth and development, further study is needed to investigate for these two miRNAs.

Table 3. Putative pig miRNA sequences and their normalized (log10) expression levels in pig fetal skeletal muscles

Porcine miRNAs	Mature Sequences (5′-3′)	Cy5 signal intensity (65 d)	Cy3 signal intensity (90 d)	Probe (accession No. in miRBase)	Already reported?
miR-503	UAGCAGCGGGAACAGUUCUGCAG	2.47	3.00	hsa(MIMAT0002874)	-
miR-424	CAGCAGCAAUUCAUGUUUUGAA	2.89	2.94	hsa(MIMAT0001341)	-
miR-423	AGCUCGGUCUGAGGCCCCUCAG	4.09	3.27	has(MIMAT0001340)	-
miR-377	AUCACACAAAGGCAACUUUUGU	3.91	2.52	hsa(MIMAT0000730)	-
miR-370	GCCUGCUGGGGUGGAACCUGGUU	3.04	3.37	mmu(MIMAT0001095)	-
miR-368	ACAUAGAGGAAAUUCCACGUUU	3.87	4.51	has(MIMAT0000720)	-
miR-341	UCGAUCGGUCGGUCGGUCAGU	3.79	0.00	mmu(MIMAT0000588) rno(MIMAT0000587)	-
miR-320	AAAAGCUGGGUUGAGAGGGCGAA	3.75	3.39	hsa(MIMAT0000510) mmu(MIMAT0000666) rno(MIMAT0000903)	-
miR-299-3p	UAUGUGGGAUGGUAAACCGCUU	3.39	4.10	hsa(MIMAT0000687)	-
miR-206	UGGAAUGUAAGGAAGUGUGUGG	2.60	3.11	hsa(MIMAT0000239) mmu(MIMAT0000462) rno(MIMAT0000879)	-
miR-199a	CCCAGUGUUCAGACUACCUGUUC	2.44	2.94	hsa(MIMAT0000231), mmu(MIMAT0000229) rno(MIMAT0000872)	-
*miR-199a**	UACAGUAGUCUGCACAUUGGUU	2.87	3.42	hsa(MIMAT0000232) mmu(MIMAT0000230)	-
miR-181a	AACAUUCAACGCUGUCGGUGAGU	1.75	2.79	mo(MIMAT0000858)	-
miR-143	UGAGAUGAAGCACUGUAGCUCA	2.46	3.08	hsa(MIMAT0000435) mmu(MIMAT0000247) rno(MIMAT0000849)	-
miR-133a	UUGGUCCCCUUCAACCAGCUGU	2.86	3.64	hsa(MIMAT0000427) mmu(MIMAT0000145)	-
miR-133b	UUGGUCCCCUUCAACCAGCUA	2.91	3.55	hsa(MIMAT0000770) mmu(MIMAT0000769) rno(MIMAT0003126)	(Kim et al., 2006)
miR-127	UCGGAUCCGUCUGAGCUUGGC	2.83	3.61	hsa(MIMAT0000446)	-
miR-127	UCGGAUCCGUCUGAGCUUGGCU	3.73	4.34	mmu(MIMAT0000139)	-
miR-126-3p	UCGUACCGUGAGUAAUAAUGC	2.08	3.01	hsa(MIMAT0000445) mmu(MIMAT0000138) rno(MIMAT0000832)	-
miR-125b	UCCCUGAGACCCUAACUUGUGA	2.05	2.73	hsa(MIMAT0000423) rno(MIMAT0000830)	(Wernersson et al., 2005)
miR-106a	AAAAGUGCUUACAGUGCAGGUAGC	2.73	2.96	hsa(MIMAT0000103)	(Wernersson et al., 2005)
miR-106b	UAAAGUGCUGACAGUGCAGAU	2.53	3.15	hsa(MIMAT0000680) rno(MIMAT0000825)	-
miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	3.26	3.87	hsa(MIMAT0000087) mmu(MIMAT0000128) rno(MIMAT0000808)	-
miR-30d	UGUAAACAUCCCCGACUGGAAG	3.05	3.83	hsa(MIMAT0000245) mmu(MIMAT0000515) rno(MIMAT0000807)	-
miR-27a	UUCACAGUGGCUAAGUUCCGC	2.56	3.23	hsa(MIMAT0000084) mmu(MIMAT0000537) rno(MIMAT0000799)	(Wernersson et al., 2005)
miR-26a	UUCAAGUAAUCCAGGAUAGGC	2.24	2.81	hsa(MIMAT0000082) mmu(MIMAT0000533)	-
miR-24	UGGCUCAGUUCAGCAGGAACAG	3.35	3.71	hsa(MIMAT0000080) mmu(MIMAT0000219) rno(MIMAT0000794)	(Wernersson et al., 2005)
miR-22	AAGCUGCCAGUUGAAGAACUGU	1.85	3.05	hsa(MIMAT0000077) mmu(MIMAT0000531) rno(MIMAT0000791)	-
miR-21	UAGCUUAUCAGACUGAUGUUGA	2.91	2.78	hsa(MIMAT0000076)	(Wernersson et al., 2005)
miR-20	UAAAGUGCUUAUAGUGCAGGUAG	2.29	2.97	mmu(MIMAT0000529)	-
miR-19a	UGUGCAAAUCUAUGCAAAACUGA	3.11	2.91	mmu(MIMAT0000651)	(Sawera et al.,2005 ; Wernersson et al., 2005)
miR-18	UAAGGUGCAUCUAGUGCAGAUA	2.58	3.38	hsa(MIMAT0000072) mmu(MIMAT0000528) rno(MIMAT0000787)	(Sawera et al., 2005 ; Wernersson et al., 2005)
miR-18b	UAAGGUGCAUCUAGUGCAGUUA	2.12	2.88	hsa(MIMAT0001412)	-
miR-1	UGGAAUGUAAAGAAGUAUGUA	3.10	3.30	hsa(MIMAT0000416) mmu(MIMAT0000123)	-
let-7a	UGAGGUAGUAGGUUGUAUAGUU	2.97	2.84	hsa(MIMAT0000062)	-

Table 4. The genomic sequence coordinates of these miRNAs in the porcine genome by BLASTN search

Name	Mature sequences (5′-3′)	Accession (No. In pig genome database)
miR-133b	UUGGUCCCCUUCAACCAGCUA	emb|CT842532.3|
miR-320	AAAAGCUGGGUUGAGAGGGCGAA	emb|CT827951.4|
miR-206	UGGAAUGUAAGGAAGUGUGUGG	emb|CT842532.3|
*miR-199a**	UACAGUAGUCUGCACAUUGGUU	emb|CU234126.1|
miR-133a	UUGGUCCCCUUCAACCAGCUGU	emb|CT842532.3|
miR-106a	AAAAGUGCUUACAGUGCAGGUAGC	emb|CU019588.1|
miR-30d	UGUAAACAUCCCCGACUGGAAG	emb|CT990628.3|
miR-20	UAAAGUGCUUAUAGUGCAGGUAG	emb|CU019588.1|
miR-19a	UGUGCAAAUCUAUGCAAAACUGA	emb|CU019588.1|
miR-18	UAAGGUGCAUCUAGUGCAGAUA	emb|CU019588.1|

Table 5. Sequencing results of real-time PCR products

Name	Sequencing results of real-time PCR products
ssc-mir-24	TGGCTCAGTTCAGCAGGAACAGCAAAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCTG TGCTCGC
ssc-mir-30a	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTGCTTCCAGTCGAGGAT GTTTACA
ssc-mir-126	TCGTTCCGTGAGTATATAATGCAAAAAAAAAA--CCTATAGTGAGTCGTATTAATTCTGT GCTCGC
ssc-mir-133a	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTCCAGCTGGTTGAAGGGGACCAA
ssc-mir-424	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTATTTTTTTTTTTTTTTTTTTCAAAACATGAATTGCTGCTG

Table 6. Sequencing results of miRNA precursors

Name	miRNA precusors sequences
ssc-mir-24	CUCUGCCUCCCGUGCCUACUGAGCUGAAACACAGUUGAUUUGUGCAGACUGGCUCAGUUCAGCAGGAACAGG
ssc-mir-30a	GCGGCUGUAAACAUCCUCGACUGGAAGCUGUGAGGCUGAAGACGGGCUUUCAGUCGGAUGUUUGCAGC
ssc-mir-126	CGCUGGCGACGGGACAUUAUUACUUUUGGUACGCGCUGUGACACUUCAUACUCGUUCCGUGAGUAUAUAAUGCGCUGUC
ssc-mir-133a	CAAUGCUNUGCUAGAGCUGGUAAAAUGGAACCAAAUCGCCUCUUCAAUGGAUUUGGUCCCCUUCAACCAGCUGUAGCUAUGCA
ssc-mir-424	UUGACGAGGGGAUGCAGCAGCAAUUCAUGUUUUGAAGGGCUUUAAAUGGUUCAAAACGUGAGGCGCUGCUAUACCCCCUCG

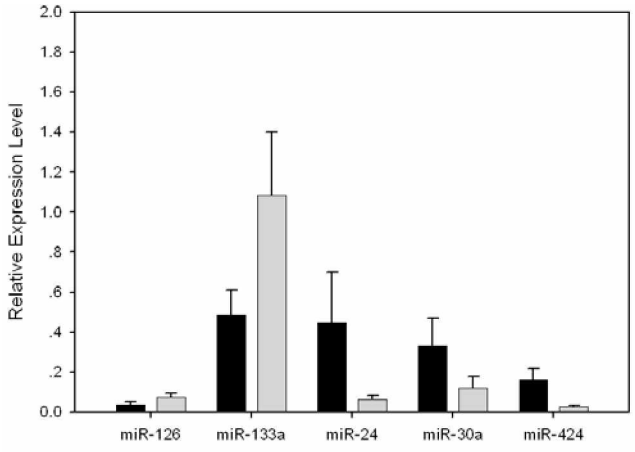

Figure 1. Expression differences of *miR-24*, *miR-30a*, *miR-424*, *miR-126* and *miR-133a* in 65 and 90 day fetal muscle. Dark bars: 65 day fetal muscle samples, gray bars: 90 day fetal muscle samples.

Cloning and expression profiling of miRNA precursors

Six porcine miRNA precursors, *miR-18a*, *miR-24*, *miR-30a-5p*, *miR-126*, *miR-133a* and *miR-424* were successfully cloned and sequenced (Table 6). The results showed that all of the cloned miRNAs were highly conserved in comparison to their homologs from human or mouse. Only few of them have sequence variation in the none-miRNA coding region. To further understand the expression patterns of the miRNAs, the expression levels of the precursors in different tissues (heart, liver, spleen, lung, kidney, skeletal muscle and placenta) were detected by a semi-quantitative RT-PCR assay. The results showed that the precursors of *miR-18*, *miR-24*, *miR-30a* and *miR-126* were ubiquitously expressed in various tissues including heart, liver, spleen, lung, kidney, skeletal muscle and placenta. Precursor of *miR-133a* was specifically expressed in heart and skeletal muscle tissues. Precursor of *miR-424* was moderately expressed in lung, kidney, skeletal muscle, and placenta (Figure 2).

In this study, we investigated the expression of a set of porcine miRNAs, using microarray, real-time PCR and regular RT-PCR technologies, in two stages of porcine fetal skeletal muscle. The differentially expressed miRNAs may be worthy of further investigation on biological roles of miRNAs during muscle development in the pig.

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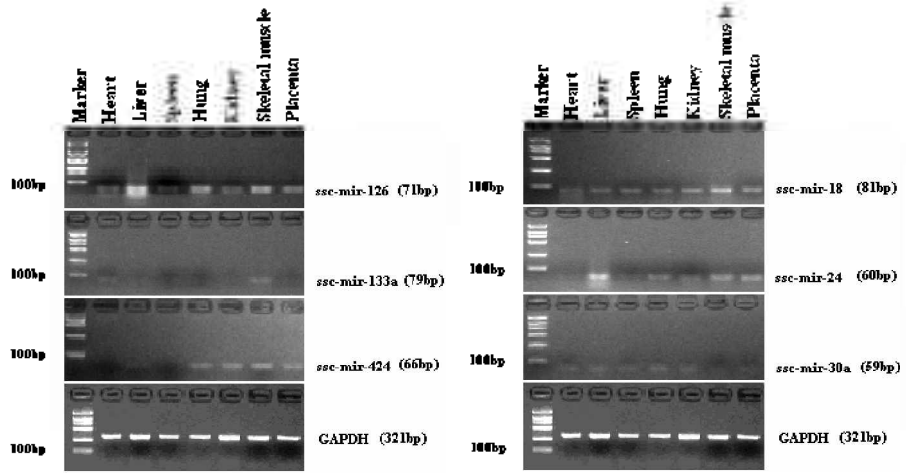

Figure 2. Expression pattern of *miR-126*, *miR-133a*, *miR-424*, *miR-18*, *miR-24* and *miR-30a* precursors in porcine tissues.

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