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Expression Profiling of miRNA-122 and miRNA-221 in Porcine Various Tissues and Developing Testes

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Abstract: MicroRNAs (miRNAs) are endogenous small regulatory RNA molecules that play a crucial role in the regulation of gene expression in eukaryotes. Recent studies indicate that miRNAs are mechanistically involved in mammalian spermatogenesis however, little is known regarding their expression patterns during porcine testis development. Here, researchers characterized the spatio-temporal expression patterns of two miRNAs, miRNA-122 (miR-122) and miRNA-221 (miR-221) in porcine diverse tissues and four stages of testis development by using a Stem-Loop Quantitative RT-PCR Method. The result showed that miR-122 was relative highly expressed in adult porcine testis among all tissues examined except for the liver and had an increasing expression pattern during porcine testis development from birth to adult. Conversely, miR-221 was relative lowly expressed in adult porcine testis among all tissues examined and had a decreasing expression pattern during porcine testis development. The results suggested that miR-122 and miR-221 may play distinct roles during testis development or spermatogenesis. In summary, determination of the spatio-temporal expression patterns of miR-122 and miR-221 in porcine developing testes provides a useful resource for further illustrates their likely roles in pig testis development or spermatogenesis as well as in related mammals.

Key words: miR-122, miR-221, expression pattern, porcine, testis, development

INTRODUCTION

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding regulatory RNAs that modulate gene expression at the post-transcriptional level by degrading targeted mRNAs or repressing mRNA translation (He and Hannon, 2004). Since, the initial discovery that lin-4 acts as a crucial regulatory RNA in *Caenorhabditis elegans* (Lee and Ambros, 1993) increasing evidence has demonstrated that miRNAs play critical roles in a variety of biological processes such as development, proliferation, differentiation, apoptosis and even cancer (Plasterk and Kloosterman, 2006). Most miRNAs are evolutionarily conserved in related species and *miRNA* genes are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) (Lee *et al*., 2004). During two-step processing from the primary transcript, the mature miRNA sequence is loaded into RNA: protein complex called RNA Induced Silencing Complex (RISC) (Khvorova *et al*., 2003). In animals, miRNA-guided RISCs regulate both the translation and stability of mRNAs depending on the complementarity of the miRNA sequence to its target (Chekulaeva and Filipowicz, 2009).

Some miRNAs are expressed in a developmentally regulated or tissue-specific manner. Thus, determination of the spatio-temporal expression patterns of miRNAs in different organisms is a critical step to facilitate the understanding of regulatory mechanisms for many important physiological processes especially during male germ cell development. Spermatogenesis is a complex tune-regulated process, through which diploid germ cells proliferate and differentiate into haploid spermatozoa. Emerging evidence has shown that proper small RNA processing is essential for normal spermatogenesis (Hatfield *et al*., 2005; Maatouk *et al*., 2008). Recently,

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several expression profile studies have identified some miRNAs that are preferentially expressed in human and mouse testis tissues as well as male germ cells (Barad *et al*., 2004; Ro *et al*., 2007; Yan *et al*., 2007; Yu *et al*., 2005). For example, miR-34b expression is much higher in an adult testis compared with a prepuberal testis which indicates that miR-34b may play a role in male germ cell differentiation (Barad *et al*., 2004). In addition, miR-122 is predominately expressed in late-stage male germ cells and it can degrade the Transition Protein 2 (TNP2) transcript which is a post-transcriptionally regulated testis-specific gene involved in chromatin remodeling during mouse spermatogenesis (Yu *et al*., 2005).

The pig (*Sus scrofa*) has considerable agricultural significance and has become an important model system for biomedical research (Rocha and Plastow, 2006). Although, an increasing number of porcine miRNAs has recently been identified in diverse tissues (Huang *et al*., 2008; Mingzhou *et al*., 2010; Nielsen *et al*., 2010; Podolska *et al*., 2011; Sharbati *et al*., 2010; Xie *et al*., 2011), the expression and function of miRNAs in porcine developing testes or male germ cells remains to be elucidated. In the present study, researchers performed a comprehensive expression profiling of both miR-122 and miR-221 in ten porcine tissues and four stages of testis development by using a stem-loop quantitative RT-PCR approach which is much more sensitive than Northern blot. The results show that miR-122 and miR-221 have distinct tissue expression patterns. More importantly, they have a completely converse expression pattern during porcine testis development which suggested that miR-122 and miR-221 may play distinct roles during testis development or spermatogenesis.

MATERIALS AND METHODS

Experiment animal and sample collection: Junmu-1 pig (a white, Chinese indigenous breed) used in the experiments were obtained from the pig farm at Jilin University (Changchun, China). The sample collection protocol was approved by the ethics committee of Jilin University in accordance with the guidelines on animal care and use. The heart, liver, spleen, lung, kidney, stomach, small intestine, adipose, skeletal muscle and testis tissues were obtained from three Junmu-1 boars that were slaughtered at 6 months of age. In addition, testes were obtained from three Junmu-1 boars at postnatal day 1 (birth), 30, 90 and 180, respectively. All samples were frozen in liquid nitrogen immediately and stored at -80°C.

RNA sample preparation: Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's protocol and the RNA concentration and purity were determined photometrically by measuring the absorbance at 260 nm (A260) and the A260/A280 ratio using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Importantly, the RIN (RNA integrity number) of total RNA was >8 (maximum, 10) as determined using the Agilent Technologies 2100 Bioanalyzer.

Table 1: Primer sequences of real-time qPCR assays

miRNA name	Primer	Primer sequence (5'-3')
miR-122	RT	GTCGTATCCAGTGCAGGGTCCGA GGTGCACTGGATACGACACAAACA
	FW	TGCGGTGGAGTGTGACAATGG
	RW	CAGTGCAGGGTCCGAGGT
miR-221	RT	GTCGTATCCAGTGCAGGGTCCGAG GTGCACTGGATACGACAAACCCA
	FW	TGCGGAGCTACATTGTCTGCT
	RW	CAGTGCAGGGTCCGAGGT
U6 snRNA	RT	CGCTTCACGAATTTGCGTGTCAT
	FW	GCTTCGGCAGCACATATACTAAAAT
	RW	CGCTTCACGAATTTGCGTGTCAT

Primer design and synthesis: Primers for miR-122 and miR-221 were designed according to porcine mature miRNA sequences listed in miRBase (http://www.mirbase.org/index.shtml) and using Primer Express Version 2.0 (Applied Biosystems, Foster City, CA) as previous report (Chen *et al*., 2005). For porcine U6 snRNA, primer sequences described previously (Huang *et al*., 2008) were used. All synthetic primers (Table 1) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Stem-loop quantitative RT-PCR: A real-time quantification assay for miRNA was conducted as previously described (Chen *et al*., 2005; Huang *et al*., 2008). Briefly, the assay was performed using stem-loop RT followed by quantitative PCR. Firstly, 1 μg total RNA was reverse-transcribed to cDNA using ReverTra Ace reverse transcriptase (Toyobo Co., Osaka, Japan) and miRNA-specific stem-loop RT primers (Table 1). The mix was incubated at 37°C for 15 min, 85°C for 5 min and then held at 4°C using an Applied Biosystems 9700 Thermocycler. Then, Quantitative PCR was performed on the Agilent technologies Mx3000P/Mx3005P Real-Time PCR Detection System by using a standard SYBR Green Real-time PCR Master Mix (Toyobo: QPK-201). In each reaction, 25 μL reaction mixtures contained 1 μL cDNA (1:10 dilution) were prepared and incubated at 95°C for 5 min followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec in a 96 well optical plate. The melting curve analysis and agarose gel electrophoresis were used to confirm the specific of PCR products. All reactions were run in triplicate and miRNA expression levels were calculated using the $\triangle\triangle$Ct Method (Livak and Schmittgen, 2001) with porcine U6 snRNA as an endogenous reference gene.

RESULTS AND DISCUSSION

MiR-122 relative highly expressed in porcine testis: Previous report has characterized miR-122 as a liver-specific miRNA in mouse (Bennet *et al*., 2006). To analyze the tissue expression patterns of miR-122 in pig, 10 different tissues (testis, heart, spleen, lung, kidney, liver, stomach, small intestine, adipose and skeletal muscle) of adult Junmu-1 pig were examined by using qRT-PCR. The result indicated that miR-122 was significant highly expressed in the liver and relative highly expressed in the testis (Fig. 1). The high expression level of miR-122 in the pig liver is in agreement with previous report (Bennet *et al*., 2006) which suggested its highly conservative expression pattern and function. Notably, researchers also found miR-122 relative highly expressed in the pig testis with barely detected in other tissues examined. This result suggested that miR-122 may also play a role in porcine testis.

MiR-122 increasingly expressed during porcine testis development: To further determine miR-122 expression pattern during porcine testis development, researchers examined the expression of miR-122 at four development stages (postnatal 1 day (birth), postnatal 30 days, postnatal 90 days and adult of Junmu-1 pig testis by using qRT-PCR. The result shown that miR-122 has an increasing expression pattern during porcine testis development (Fig. 2). Moreover, miR-122 was significantly high expressed at adult stage which suggested that it is likely involved in regulating gene expression during porcine testis development or spermatogenesis. Consistently, recent studies have shown that miR-122 is predominately expressed in late-stage male germ cells and it can degrade the Transition Protein 2 (TNP2) transcript which is a post-transcriptionally regulated testis-specific gene involved in chromatin remodeling during mouse spermatogenesis (Yu *et al*., 2005). In addition, this result is also in agreement with findings in the comparative profile of the miRNA transcriptome between sexually immature (30 days) and mature (180 days) pig testes using Solexa deep sequencing which showed that miR-122 was significantly up-regulated in sexually mature (180 days) pig testes.

MiR-221 relative lowly expressed in porcine testis: miR-221 are overexpressed in several types of cancers and has been found to play a critical role in cancer cell proliferation (Pineau *et al*., 2010). In order to characterize the tissue expression patterns of miR-221 in pig, researchers analyzed 10 different tissues by qRT-PCR. The result showed that miR-221 was ubiquitously

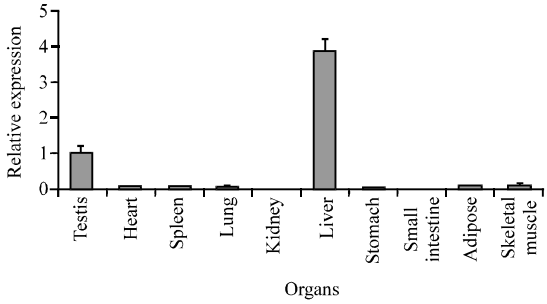

Fig. 1: Tissue expression pattern of miR-122; relative abundance among ten porcine tissues (testis, heart, spleen, lung, kidney, liver, stomach, small intestine, adipose and skeletal muscle) was examined by qRT-PCR for miR-122. Three biological replicates were used. The results are the relative expression levels normalized to U6 snRNA (internal control)

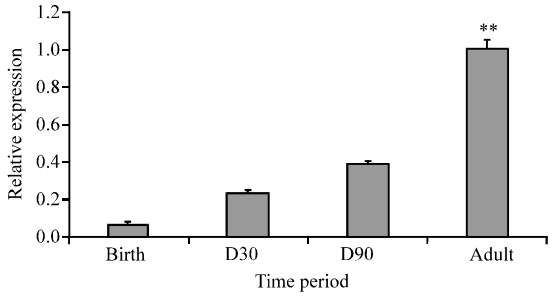

Fig. 2: Expression profiling of miR-122 at four stages of testis development; pig testes at postnatal 1 day (birth), postnatal 30 days (D30), postnatal 90 days (D90) and adult (D180) stages (from left to right) were examined under the internal control of U6 snRNA. Three biological replicates were used

expressed in almost all tissues that researchers analyzed (Fig. 3). It was highly expressed in kidney and moderately in the other tissues but lowly expressed in the testis (Fig. 3).

This data implied that miR-221 may have an important role in kidney. Consistently, miR-221 is overexpressed in the cellular model of aggressive prostate carcinoma and contributing to the oncogenesis and progression of prostate carcinoma through $p27^{Kip1}$ down-regulation (Galardi *et al*., 2007).

MiR-221 decreasingly expressed during porcine testis development: One miRNA is able to regulate the expression of multiple genes because it can bind to its mRNA targets as either an imperfect or perfect complementarity and the biological function of an individual miRNA is cell or tissue specific. Researchers

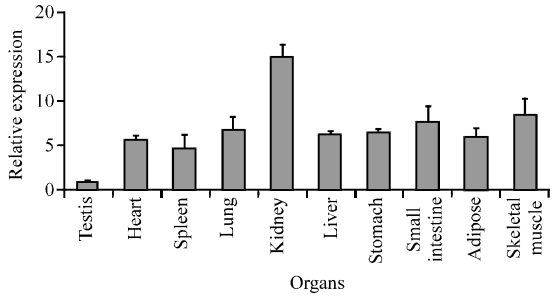

Fig. 3: Tissue expression pattern of miR-221; relative abundance among ten porcine tissues (testis, heart, spleen, lung, kidney, liver, stomach, small intestine, adipose and skeletal muscle) was examined by qRT-PCR for miR-221. Three biological replicates were used. The results are the relative expression levels normalized to U6 snRNA (internal control)

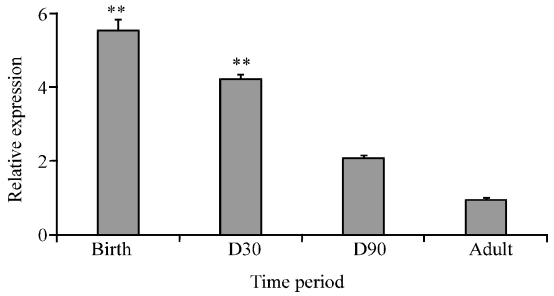

Fig. 4: Expression profiling of miR-221 at four stages of testis development; pig testes at postnatal 1 day (birth), postnatal 30 days (D30), postnatal 90 days (D90) and adult (D180) stages (from left to right) were examined under the internal control of U6 snRNA. Three biological replicates were used

thus further determined miR-221 expression pattern during porcine testis development although, it was relative lowly expressed in porcine testis. Interestingly, miR-221 had a decreasing expression pattern which in contrast to miR-122 during porcine testis development (Fig. 4). Moreover, miR-221 was significantly high expressed at birth stage which suggested that miR-221 may play a role in male germ cell proliferation during spermatogenesis. Similarly, the result is also in agreement with the comparative profile data which showed that miR-221 was significantly down-regulated in sexually mature (180 days) pig testes.

Additionally, this result is consistent with findings in zebrafish showing that most miRNAs are expressed relatively late during embryogenesis (Wienholds *et al*., 2005).

CONCLUSION

In this study, the findings suggest that miR-122 and miR-221 may play distinct roles during testis development or spermatogenesis. Further investigation to identify target mRNAs regulated by them will be critical to uncover their biological functions in pig.

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