



Differential Expression of miR-34c and Its Predicted Target Genes in Testicular Tissue at Different Development Stages of Swine

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ABSTRACT: To verified the target genes of miR-34c, bioinformatics software was used to predict the targets of miR-34c. Three possible target genes of miR-34c related to spermatogenesis and male reproductive development: zinc finger protein 148 (*ZNF148*), kruppel-like factor 4 (*KLF4*), and platelet-derived growth factor receptor alpha (*PDGFRA*) were predicted. Then, the expression of miR-34c and its target genes were detected in swine testicular tissue at different developmental stages by quantitative polymerase chain reaction. The results suggested that the expression of *PDGFRA* has the highest negative correlation with miR-34c. Then immunohistochemical staining was done to observe the morphology of swine testicular tissue at 2-days and 3, 4, 5-months of age, which indicated that *PDGFRA* was mainly expressed in the support cells near the basement membrane during the early development stages of testicular tissue, but that the expression of *PDGFRA* was gradually reduced in later stages. Therefore, western blot analyzed that the highest expression of *PDGFRA* was generated in 2-days old testicular tissues and the expression levels reduced at 3 and 4-months old, which correlated with the results of immunohistochemical staining. In conclusion, *PDGFRA* is a target gene of miR-34c. (**Key Words:** miR-34c, *PDGFRA*, Testicular Tissue, Differential Expression)

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, non-coding RNAs of about 20 to 25 nucleotides long that have a regulatory function in eukaryotes (Lee et al., 2004). Mature miRNAs assemble into RNA-induced silencing complexes, which target mRNA through complementary base pairing to regulate target genes by target mRNA degradation or repression of the target mRNA translation (Reinhart et al., 2000). And miRNAs play regulatory roles in many physiological processes, such as development, virus defense, hematopoiesis, organogenesis, cell proliferation and apoptosis, fat metabolism, viral replication and tumor formation (Chang et al., 2014; Mello et al., 2014; Ning et al., 2014). Numerous studies have shown that miRNAs are

essential for normal spermatogenesis and male fertility.

MiR-34c, is an important miRNAs as it regulates sperm production and male fertility (Geremia et al., 1977). In mice, the expression of miR-34c is significantly different in mature and immature testicular tissues, which suggest that miR-34c could play an important regulatory role in the developmental process of male germ cells. High-throughput sequencing suggests that the expression of miR-34c also is significantly different between mature and immature swine testis (Lian et al., 2012). But few reports have investigated the target genes of miR-34c. Therefore, in the present study, the expression of miR-34c and three of its predicted target genes in Junmu No.1 swine testicular tissues at different developmental stages were investigated using quantitative polymerase chain reaction (PCR), western blot and immunohistochemical methods.

MATERIALS AND METHODS

Samples

A total of 24 samples, which included 2-days, 3-month

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to 7-month, 9-month, and 12-month old swine testicular tissue were collected from the Jilin University swine farm. Animal experiments were done under the guidance of Jilin University Animal Care and Use Committee.

Bioinformatics prediction

Three target genes of miR-34c (zinc finger protein 148: *ZNF148*, kruppel-like factor 4: *KLF4*, and platelet-derived growth factor receptor alpha: *PDGFRA*) were predicted by four kinds of bioinformatics software (microRNA.org, miRDB, miRGen, TargetScan). Amplification of miR-34c and target genes was done as described in the manual (Takara, Dalian, China). The cDNA sequences were retrieved from the database (<http://www.Ensemble.org>) and (<http://www.ncbi.nlm.nih.gov/>).

RNA isolation, cDNA synthesis, and real-time PCR

Swine-specific primers were designed according to mature miR-34c sequence. Primers of miR-34c and its target genes were designed for quantitative reverse-transcription polymerase chain reaction according to the sequence of NCBI and miRBase14.0. U6 was used as the control (Ren et al., 2009). Primers of *ZNF148* gene and reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed as previously reported (Zhou et al., 2012). The primers for *KLF4* were designed using Primer 5.0 software. All primer sequences are listed in Table 1 and were synthesized (Sangon, Shanghai, China).

Testis tissue samples from healthy swine specimens were frozen in liquid nitrogen. Trizol reagent was used to extract total RNA. The cDNA was synthesized by reverse transcription with an RT-PCR Kit (Takara) according to the instruction. The qPCR reaction mixture included cDNA (2 μ L), PCR-Master Mix (10 μ L), PCR-F-Primer (0.5 μ L), PCR-R-Primer (0.5 μ L), and RNase-free H₂O (7 μ L) in a

total volume of 20 μ L. The qPCR was performed in a reaction under the following procedure: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. The expression of miR-34c and genes were detected using the SYBR Green I (Takara) and were analyzed by Eppendorf AG-5341 fluorescence quantitative instrument. The data were analyzed using the SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA) and $2^{-\Delta\Delta Ct}$ method was used according to the following formula:

$$\Delta\Delta Ct = [Ct(\text{positive}) - Ct(\text{reference})] - [Ct(\text{control}) - Ct(\text{reference})]$$

Here, $2^{-\Delta\Delta Ct}$ refers to the relative expression ratio.

Validation of immunohistochemical staining

To further investigate the expression of *PDGFRA* at different developmental stages of swine testis, immunohistochemical staining was done in testicular tissues from 2-day, 3-month, 4-month and 5-month old animals. For dewaxing and hydration, tissue sections were sequentially placed in xylene (10 minutes \times 2), 100% ethanol (5 minutes \times 2), 90% ethanol, 80% ethanol, 70% and 50% ethanol (5 minutes at each concentration), distilled water (5 minutes), and 0.01 phosphate buffered saline (PBS, PH 7.4; 5 minutes \times 3). After high-pressure antigen retrieval in a pressure cooker, the tissue sections were stained with a chromogenic DAB based *PDGFRA* IHC kit according to manufacturer's protocol (Beijing Biosynthesis Co., Beijing, China).

Western blot detection

Total protein was extracted from testicular tissue at different developmental stages using RIPA buffer (Boster, Wuhan, China) following the manufacturer's instructions. Protein concentration was determined using the BCA

Table 1. Sequences of primers for real-time PCR

Gene	Primer	Primer sequence (5'-3')
ssc-miR-34c-5p	RT-Primer	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGCAATCAG
	F-Primer	CCCGCCAGGCAGTGTAGTTA
	R-Primer	CTCAACTGGTGTCTGGAGTC
U6	F-Primer	CTCGCTTCGGCAGCAC
	R/RT-Primer	AACGCTTCACGAATTTGCGT
<i>ZNF148</i>	F-Primer	GCCTTTAGAACGAACACTATCAC
	R-Primer	AGGTACTTCTGTATGAAACGC
<i>KLF4</i>	F-Primer	TCGGACCACCTGCCTTACA
	R-Primer	TTGGGAACCTGACCATGATTGTAG
<i>PDGFRA</i>	F-Primer	TCCAGCAGTTCTACCTTCATCA
	R-Primer	AGGAGTCTATGCCAATGTCGTC
<i>GAPDH</i>	F-Primer	GTTTGTGATGGGCGTGAAC
	R-Primer	ATGGACCTGGGTTCATGAGT

PCR, polymerase chain reaction; zinc finger protein 148(*ZNF148*), kruppel-like factor 4(*KLF4*), platelet-derived growth factor receptor alpha (*PDGFRA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Protein Assay Kit (Boster). Total protein (35 µg per sample) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Bio-Rad Laboratories Inc, Hercules, CA, USA). Immunoblotting was conducted using the following primary antibodies with the suggested dilutions from the manufacturer: anti-PDGFR α (Abcam, London, UK); anti- β -actin (Abcam). The antibodies were diluted with 5% BSA (Albumin from bovine serum) and the suggested dilutions were 1:200 and 1:1,000. The immunoblots were developed using an ECL Advanced Western Blotting Detection Kit (Invitrogen, Grand Island, NY, USA).

RESULTS

The differential expression of miR-34c and its target genes in swine testes at different developmental stages

Expression of miR-34c at different developmental stages in the swine testicular tissue is shown in Figure 1. The expression of miR-34c was very low at 2-days and 3-months of age, but the expression level increased significantly at 4-months and reached a plateau between 5 to 7-months of age. The expression level dropped at 9-months and then came back at 12-months. The relative expression levels were significantly different between 2-days and 3-months and the levels of 4, 5, 6, 7, 9, 12-months of age ($p < 0.01$). However, there were no significant differences among the expression levels at 5, 6, 7, 9, 12-months of age ($p > 0.05$).

The relative expression level of *ZNF148* was gradually upregulated from 2-days to 6-months old. The expressions were down regulated from 7-months to 9-months, but upregulated again at 12-months. The relative expression levels before 5-months were significantly different from the expression levels of 6, 7, and 12-months ($p < 0.05$). No

significant differences existed among the expression levels at 4, 5, 6, 7, 9, and 12-months ($p > 0.05$).

For *KLF4*, the expressions were relatively low and stable from 2-days to 4-months, but significantly increased to the peak value at 5-months. The expression was down regulated from 6-months to 9-months and upregulated again at 12-months. The results showed that the relative expression levels at 2-days, 3-months, and 4-months were significantly different from the level at 5-months ($p < 0.05$). No significant differences exist among the expression levels at 5, 6, 7, 9 and 12 months of age ($p > 0.05$).

For *PDGFR α* , the expression reached the highest level at 2-days and then down regulated to the lowest level at 4 months. The expression levels maintained relatively lower from 5-months to 12-months.

Location expression of *PDGFR α* gene in testis

As shown in Figure 2A, *PDGFR α* is highly expressed in the surrounding cells of the spermatid basement membrane (including Sertoli cells and spermatogonial stem cells) in 2-days testicular tissues. A small amount of *PDGFR α* was also expressed in Leydig cells and mesenchymal cells.

Also, Figure 2B to 2D show the expression of *PDGFR α* in 3-months to 5-months swine testicular tissues. The locations of expressions were similar to 2-days testicular tissue, but the staining signals were weaker.

The protein of *PDGFR α* gene differentially expresses in swine testes at different developmental stages

As shown in Figure 3, the protein of *PDGFR α* gene was differentially produced at different developmental stages of testis, which was detected by western blot. The highest expression level was at swine testicular tissue of 2-days old, and gradually decreased from 3-months to 4-months.

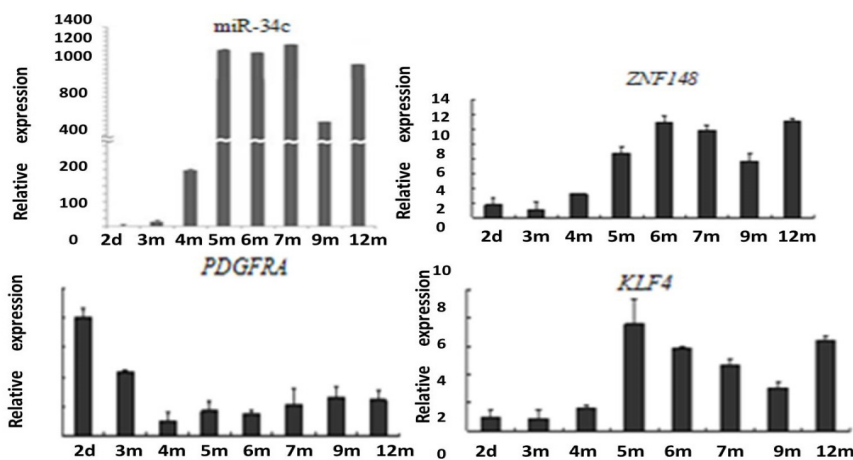


Figure 1. mRNA expressions of miR-34c and target genes In this Figure, we detected the expressions of miR-34c and target genes zinc finger protein 148 (*ZNF148*), kruppel-like factor 4 (*KLF4*), and platelet-derived growth factor receptor alpha (*PDGFR α*) in swine testis at 2-days, 3- to 7-month-old, 9-month-old, and 12-month-old by qPCR.

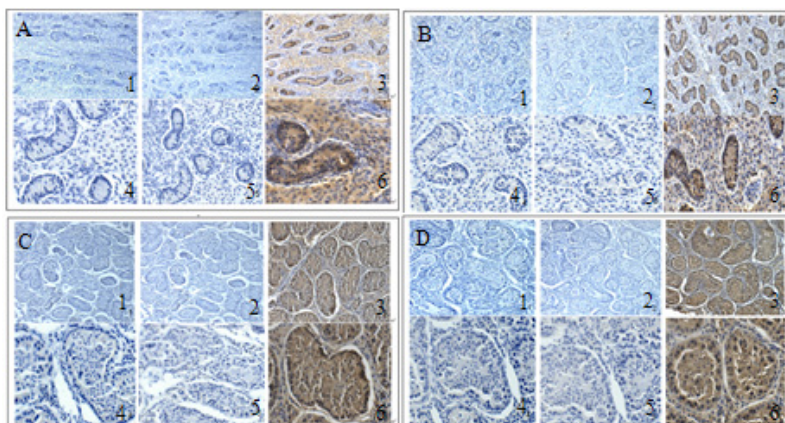


Figure 2. Tissue sections of immunohistochemical staining. In this Figure, immunohistochemical staining was done about *PDGFRA* gene in testicular tissues at 2-days, 3-month-old, 4-month-old, and 5-month-old. (A) Two days swine testis. (B) Three months swine testis. (C) Four months swine testis. (D) Five months swine testis. And (1 to 3) respectively represent testis observations ($\times 100$), also (4 to 6) respectively represents testis observations under ($\times 400$). (1, 4) were treated using PBS, (2, 5) were treated using immune serum as negative control, (3, 6) used *PDGFRA*-specific antibody. *PDGFRA*, platelet-derived growth factor receptor alpha.

However, the expression level upregulated again at 5-months of age.

DISCUSSION

miR-34c is involved in many important biological processes, such as the early development of somatic cell nuclear transfer bovine embryos, as a putative tumor suppressor in high-grade serous ovarian cancer, growth and invasion of colorectal cancer cells and the permeability of blood-tumor barrier. It is also required for spermatogenesis, but not for the first cleavage division in mice. Studies have shown that miR-34c has a significantly higher expression in mature mouse testis than in immature testis (Yan et al., 2007; Yoshida et al., 2007), suggesting that miR-34c plays an important role in germ cell development (Sette et al., 2000; Bouhallier et al., 2010). At the same time, studies have shown that miR-34c plays important role in these processes by regulating the target genes. So it is extremely valuable to predict and verify the targets of miR-34c.

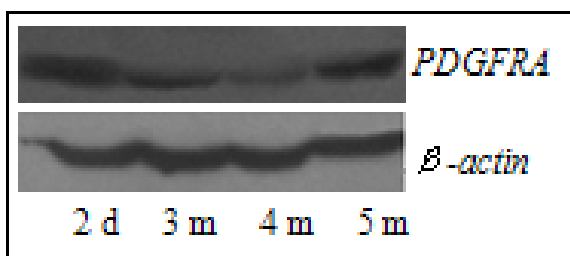


Figure 3. Protein expression of *PDGFRA* gene. In this Figure, the protein expression of *PDGFRA* gene was detected by western blot in testicular tissues at 2-days, 3-month-old, 4-month-old and 5-month-old. *PDGFRA*, platelet-derived growth factor receptor alpha.

The bioinformatics database, is an important tool in predicting target genes, which can be assessed through matching the degree of conformation between the sequence of miRNAs and *UTR* sequence of genes. Based on this principle, we predicted three target genes (*ZNF148*, *KLF4*, and *PDGFRA*) of miR-34c regarding spermatogenesis and male fertility.

Then, we detected the expression of miR-34c and targets at different development stages of testicular tissue. Our results show that miR-34c was more highly expressed in swine testicular tissues after 4-months of age. While the predicted target gene of miR-34c, *PDGFRA*, was highly expressed in 2-days old testicular tissues. The expressions of *KLF4* were relatively low and stable from 2-days to 4-months, but significantly increased to the peak value at 5-months. The relative expression level of *ZNF148* was gradually upregulated from 2-days to 6-month old. SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA) analyzed that the expression of *ZNF148* and *KLF4* were positively correlated with the expression of miR-34c. However, miR-34c had a significant negative correlation with *PDGFRA* ($R = -0.6$, $p < 0.001$), suggesting that *PDGFRA* gene may be a true target gene of miR-34c. Furthermore, the detected protein expression of *PDGFRA* show that it is differentially expressed at different developmental stages of testis, which is consistent with the results detected by fluorescence quantitative PCR. The following results of immunohistochemistry sections showed that the expression levels of *PDGFRA* in 2-days and 3-month were significantly higher than in 4-months and 5-months, which is consistent with the earlier results and provided further evidence that *PDGFRA* was a target gene of miR-34c. In addition, the expression of miR-34c was upregulated rapidly indicating that mi-34c was important for the growth

of seminiferous tubules. While, the expression of *PDGFRA* was mainly in cells surrounding the basement membrane, mainly in Sertoli cells, indicating that *PDGFRA*, as a target gene of mi-34c, may play an important role in the development of Sertoli cells in testis during early development stages.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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