·Basic Research·

Expression and regulation of microRNA-29a and microRNA-29c in early diabetic rat cataract formation

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Abstract

• AIM: To determine the role of microRNA (miRNA)-29a and miRNA-29c in the regulation of apoptosis in early rat diabetic cataract formation.

• METHODS: Streptozotocin (STZ) -induced diabetic Sprague-Dawley (SD) rats were used in the study. The expression level of miRNA-29a, miRNA-29c, and BCL2-modifying factor (BMF) in lens epithelial cells (LECs) samples were measured using quantitative real -time polymerase chain redction. Prediction algorithms of miRanda, TargetScan 6.2, and mirRDB to perform a miRNA gene network analysis were used for the potential miRNA-29a and miRNA-29c targets.

• RESULTS: The miRNA-29a and miRNA-29c expression levels were all significantly lower in the control group compared to the 2 and 4wk diabetic samples (*P*<0.01). The network analysis indicated that one miRNA-29a and miRNA -29c targets was BMF. There was significantly higher expression of BMF mRNA compared to the normal controls (*P* <0.01).

• CONCLUSION: Apoptosis occurs in rat LECs following high blood glucose exposure. It is likely that apoptosis during diabetic cataract formation involves the decreased expression of miRNA -29a and miRNA -29c and the increased expression of BMF.

• KEYWORDS: microRNA-29a; microRNA-29c; BCL2-modifying factor; apoptosis; diabetic cataract
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INTRODUCTION

Cataract is a chief reason of blindness worldwide, accounting for nearly half (47.8%) of all blindness cases [1]. The persistence of high blood glucose levels, particularly of galactose, glucose, and xylose, is physiologically toxic, and is most apparent in the eye as the development of cataract [2]. Although the nosogenesis of diabetic cataract is undiscovered, various biochemical pathways, such as oxidative stress, the polyol pathway, and the generation of advanced glycation end products (AGEs) have been implicated[2-3].

Recently, apoptosis has become a hot research topic in the ophthalmological field. Studies indicate that occurrence of lens epithelial cell (LEC) apoptosis is a common cellular basis for the development of non-congenital cataract for both human and animals [4]. Previous evidence also suggests that high glucose (HG) concentrations in culture lead to human LEC apoptosis [5]. Apoptosis and the proliferation of LECs may be induced by factors such as hyperglycemia[5-6].

MicroRNAs are single-stranded RNA molecules of approximately 18-23 nucleotides in length, which play an important part in the regulation of gene expression through base-pairing with the 3'-untranslated region of the target mRNA. miRNAs interact with mRNA targets and change protein expression by triggering the RNA-induced silencing complex[7]. They take part in a complicated of biological and pathological processes [8]. Therefore, their deregulation usually occurs in case of diseases. Studies investigated microRNA expression in lens, retina and other ocular tissues. Many microRNAs showed developmental stage-specific and unique tissue-specific expression patterns, suggesting potential unique functions in ocular tissues [9-11]. Despite growing evidence for the regulatory effects of microRNAs in the diabetic cataract, limited information is available on the consequences of modulating microRNAs expression *in vivo* [2]. Recent findings indicate that miRNA-29 family members (miRNA-29a/b/c) are involved in apoptosis[12-13], and they are expressed in the rat lens [14]. Moreover, miRNA-29a and 29c were evidently down-regulated in cataractous lenses using real-time polymerase chain reaction (RT-PCR) assessment of

Table 1 DNA sequences of primers for RT-PCR

Genes	Genes primers (5'-3')
miRNA-29a-3p	RTprimer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCG
	Forward primer GCGGCGGTAGCACCATCTGAAATC
	Reverse primer ATCCAGTGCAGGGTCCGAGG
miRNA-29c-3p	RT primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCG
	Forward primer GCGGCGGTAGCACCATTTGAAATC
	Reverse primer ATCCAGTGCAGGGTCCGAGG
BMF	Forward primer AGCTTGCTCTCTGCTGACCT
	Reverse primer GCCTTGTCTTCCTGGCTTAC

microRNAs levels, suggesting a possible effect by these microRNAs to cataractogenesis[15].

miRNA-29 target genes that may be involved in pathogenesis of diseases include TCL-1, BCL-2 , MCL-1 and cell cycle and apoptosis related genes [13,15]. We hypothesize that opacification of the eye lens, including apoptotic cell death associated with diabetic cataract formation, may be due to a decrease in the transcription of certain miRNAs and their hybridization with the target mRNAs, inhibiting the translational activities of the latter. In this study, we focused on the interaction between miRNA-29a, miRNA-29c and BCL2-modifying factor (BMF), a pro-apoptotic BCL-2 family member, to examine the role of miRNA-29a and miRNA-29c in the regulation of apoptosis prior to rat diabetic cataract formation.

MATERIALS AND METHODS

Animals Experimental animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No.85-23, Revised 1996). All animal experiments were approved by the Research Ethics Committee of the First Affiliated Hospital, Harbin Medical University. Eight-month-old male Sprague-Dawley (SD) rats (Animal Laboratory Center of the First Affiliated Hospital, Harbin Medical University, China), weighing 220 ±30 g, were fed standard rat chow and had access to water ad libitum. Streptozotocin (STZ)-diabetes was induced with a single intraperitoneal injection of STZ (60 mg/kg) dissolved in citrate buffer (pH 4.5), leading to damage of islet cell and reduced insulin secretion as described [16]. Blood samples for glucose measurements were collected the tail vein at 72h and 2wk after the STZ injection. The control (n=10) rats received a single intraperitoneal injection of 0.1 moL/L citrate buffer solution (pH 4.5).

SD rats with blood glucose ≥16.9 mmoL/L were considered diabetic mellitus (DM) and 100% STZ-diabetes were successfully induced in the study. The experimental groups comprised diabetic rats and control. Lens changes were evaluated by indirect ophthalmoscopy and slit lamp (66 Vision Co., Suzhou, China) weekly. Evaluations were preceded by mydriasis with topical 1% tropicamide hydrochloride. Lens clarity was subjectively classified into the following grades as: 1) clear lenses; 2) equatorial vacuolar; 3) cortical opacities; and 4) total opacity of lens.

The SD rats were quickly euthanized at 2 and 4wk after the STZ-diabetes model was established. Both eyes were immediately enucleated and then dissected to isolate the intact lenses. The content of the isolated lenses was then removed at once and capsules of the lenses were frozen by immersion in liquid nitrogen.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction Total RNA was isolated using the TRIzol reagent (15596-026, Invitrogen, Carlsbad, CA, USA). RNA concentration and quality were confirmed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Quantification of the RNA in the eluate was performed by measuring absorption at 260/280 nm.

The cDNA was synthesized from 1 μg of purified RNA using a transcriptor first strand cDNA synthesis kit (Roche, IN, USA) according to the manufacturer's protocol. The specific primers used for the quantitative polymerase chain reaction (PCR) analysis are listed in Table 1. Primers were designed using the Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). Reactions were performed in 96-well plates with Optical 8-Tube Strips (0.2 mL) (AB4316567, Applied Biosystems). PCR was performed using SYBR Green I (4913914001, Roche, IN, USA) as the reporter dye. The quantitative real-time PCR was performed using an ABI 7500 cycle detection system (Applied Biosystems, Foster City, CA, USA) for 40 cycles for the miRNA and mRNA as follows: 95℃ for 15s and 60℃ for 60s after an initial 15min incubation at 95℃. Each sample was analyzed in triplicate, and the experiment was repeated three times. Data were analyzed using the $2^{-\Delta\Delta CT}$ method[17], and the fold changes of miRNA or mRNA expression were normalized to U6 or glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as endogenous controls.

Determination of miRNA-29a Targeting Sequences by Computational Prediction Computational prediction has already been proven to be an effective and efficient method for predicting miRNA targets. We used the prediction algorithms of miRanda, TargetScan 6.2, and mirRDB to perform a miRNA gene network analysis to predict potential miRNA-29a and miRNA-29c targets. The network analysis indicates that both pro-apoptotic factor genes (BMF and Bak1) and anti-apoptotic factor genes (BCL-w and Mcl1) are putative target genes of miRNA-29a and miRNA-29c. From the target gene list, we selected BMF for further analysis due to its important role in regulating cell proliferation and apoptosis in response to DNA injury[18-19].

Statistical Analysis All data were presented as the mean± standard error (SE). Differences between groups were determined using one-factor analysis of variance (ANOVA). Calculations were performed using SPSS for Windows version 16.0 statistical package (SPSS, Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

RESULTS

Cataract Formation To evaluate the possibility of early cataract formation, each rat was examined weekly for lens opacity. A total of 5% (1/20) of the diabetic rats developed mild cataracts (Grade 2) starting at 2wk. The clarity of the lens then gradually worsened with the duration of hyperglycemia. Of the 20 DM rats, 10% (2/20) of the diabetic rat eyes displayed Grade 2 cataract, 20% (4/20) Grade 3, and 5% (1/20) mature cataracts (Grade 4) at 4wk. At the end of 8w, 2 rats died from DM. Of the 18 DM rats, 27.8% (5/18) were at Grade 2 cataract, 33.3% (6/18) at Grade 3 cataract, and 16.7% (3/18) at Grade 4 cataract. All lenses in the control group appeared to be normal and free of opacities during the 8wk experimental period.

Expression of miRNA-29a During Diabetic Cataract Formation To determine whether miRNA-29a is associated with rat diabetic cataract, we examined miRNA-29a expression in all diabetic rat LEC samples using quantitative real-time PCR. Our data showed that the miRNA-29a expression levels were all significantly lower in the control group compared to the 2 and 4wk diabetic rat samples ($P<0.01$, both) (Figure 1). However, the miRNA-29a expression levels in the LEC samples were different for the 2 and 4wk diabetic rat samples. The miRNA-29a mean of lg ($2^{-\Delta\Delta CT}$) was 0.62 and 0.21 in the 2 and 4wk rat diabetic samples, respectively. The miRNA-29a levels were lower in the samples from the 4wk diabetic rats by 2.95-fold compared to the 2w diabetic rats.

Expression of miRNA-29c During Rat Diabetic Cataract Formation The miRNA-29c expression in diabetic rat LEC samples was also examined using quantitative real-time PCR. The miRNA-29c expression levels were all visibly lower in the control group compared to the 2 and 4wk diabetic rat samples ($P<0.01$). The miRNA-29c levels were decreased by 3.83-fold and 2.76-fold in 2 and 4wk samples from diabetic rats compared to healthy controls ($P<0.01$) (Figure 2).

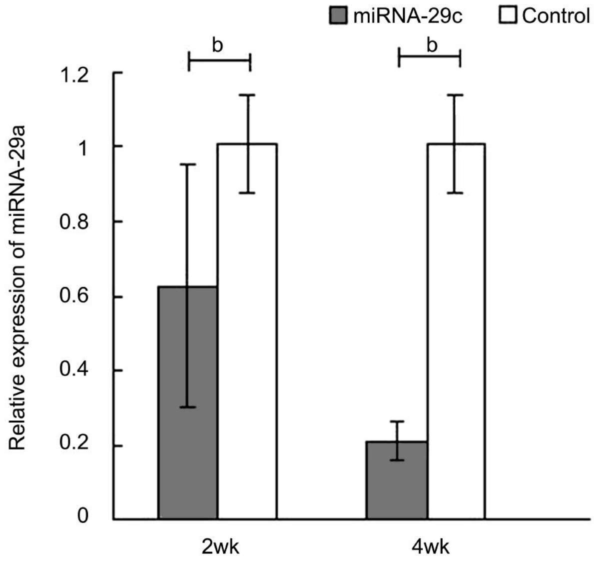

Figure 1 Expression levels of miRNA-29a in LECs samples The miRNA-29a expression in all diabetic rat LECs samples was examined using quantitative real-time PCR. The miRNA-29a mean of lg ($2^{-\Delta\Delta CT}$) was 0.62 and 0.21 in the 2 and 4wk diabetic rat samples, respectively. The miRNA-29a expression levels all decreased significantly in the control group compared to the 2 and 4wk diabetic rat samples ($^bP<0.01$).

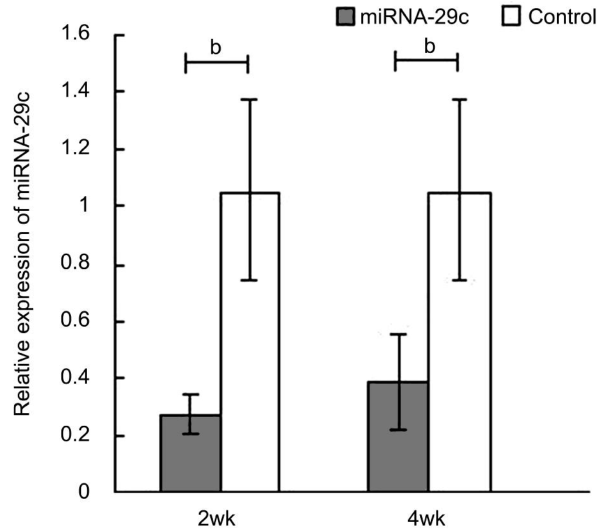

Figure 2 Expression levels of the miRNA-29c in LECs samples The miRNA-29c expression was significantly lower in the samples from diabetic rats compared to healthy controls. The miRNA-29c levels were decreased by 3.83-fold and 2.76-fold in the 2 and 4wk samples from diabetic rats compared to healthy controls ($^bP<0.01$).

Prediction of Potential miRNA-29a and miRNA-29c Targets We used the prediction algorithms of miRanda, mirRDB, and TargetScan 6.2 to fulfill a miRNA gene network analysis to predict the potential miRNA-29a and miRNA-29c targets. The network analysis indicated that one miRNA-29a and miRNA-29c target is BMF. As shown in Figure 3A, there is a sequence region in the 3'-UTR of BMF that is highly conserved among humans, rats and mouse and has identical nucleotides, which is called the "seed"

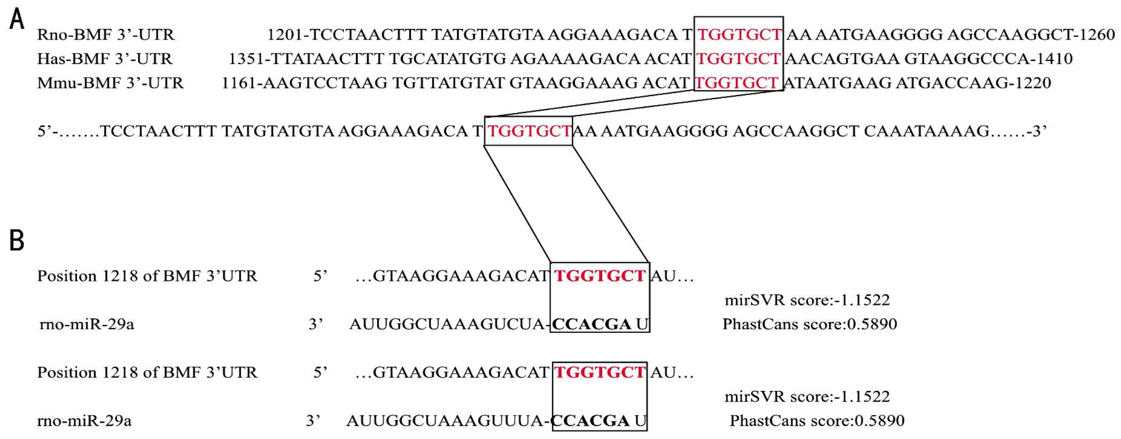

Figure 3 The analysis of potential miRNA-29a and miRNA-29c binding sites within the 3′-UTR of BMF mRNA The bioinformatics algorithms of miRanda, TargetScan 6.2, and mirRDB were used to predict the miRNA-29a and miRNA-29c binding sites in the 3'-untranslated region (3'-UTR) of BMF mRNA. A: A diagram of the 3'-UTR of BMF mRNA was generated to indicate the putative binding sites predicted by the three bioinformatic algorithms. The seed region of this common microRNA binding site is highly conserved among mammals; B: The sketch map shows the target sites for the miRNA-29a and miRNA-29c paralogs in the 3'-UTR of BMF, including the RNA hybrid-free energy calculations and the theoretical miRNA-mRNA duplex pairing.

sequence. The seed sequence is considered the most key sequence for selecting targets of microRNAs. We found that the two paralogs of miRNA-29 (miRNA-29a and miRNA-29c) have a complementary sequence to the seed sequence on BMF with minor divergences (Figure 3B), suggesting that the two paralogs potentially target the BMF mRNA.

BMF mRNA Levels During Rat Diabetic Cataract Formation We explored the BMF mRNA levels in LECs and found that the means of lg ($2^{-\Delta\Delta CT}$) showed significantly higher BMF mRNA levels compared to the healthy controls (P<0.01) (Figure 4). Interestingly, the BMF mRNA level was 3.47-fold higher in 4wk LEC samples from diabetic rats compared to healthy controls.

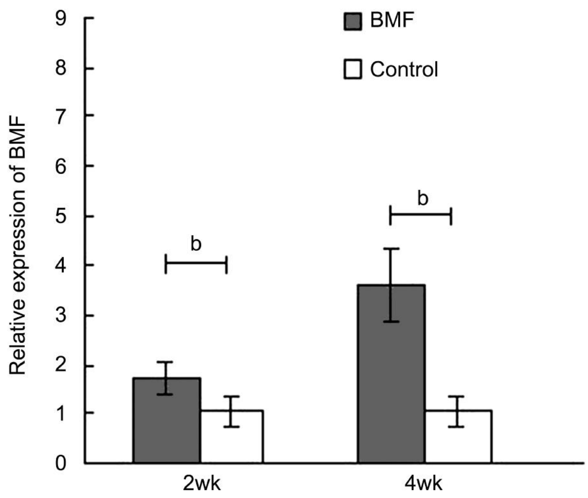

Figure 4 Expression levels of BMF mRNA in LEC samples The means of lg ($2^{-\Delta\Delta CT}$) showed significantly higher BMF mRNA levels compared to the healthy controls and the BMF mRNA level was 3.47-fold higher in the 4wk LEC samples from diabetic rats compared to healthy controls (^bP<0.01).

DISCUSSION

Cataracts are a multifactorial disease associated with several risk factors, among which diabetes is one of the most significant[20]. In addition to the higher incidence, the pace of opacity development as a function of time is also more rapid in diabetic mellitus [21]. Apoptosis is a natural morphogenetic process of lens development. Enhancement or inhibition of apoptosis due to genetic manipulations and mutations, or environmental conditions lead to the formation of abnormal lenses or the absence of the ocular lens [22]. Li *et al* [4] revealed that the cell death of LECs occurred by apoptosis and that the blockade of apoptosis reduced the formation of cataract. Apoptosis is predicted to be a crucial determiner for the normal condition of the lens [6,23]. Apoptotic cells continued to be increased in the STZ-diabetic rat LECs[24-25]. Takamura *et al* [26] validated that when SD rats were fed with a 50% galactose-containing diet, they showed apoptosis in the lens epithelium before the development of cataract. Previous reports using the same animal model indicated that the lens epithelial cells is the most sensitive region of the ocular lens against osmotic stress. LECs showed intracellular vacuole formation 36h after galactose feeding, and resulted in less morphological distortion within 24h[27]. In our study, a total of 5% (1/20) of the diabetic rats developed equatorial vacuoles starting at 2wk. The clarity of the lens then gradually worsened with the duration of hyperglycemia. These results indicate that morphological changes such as equatorial vacuole formation after high blood glucose exposure, occur after 2wk *in vivo*.

MicroRNAs exert their function by regulating the expression of their downstream target genes. The objective was to identify the potential targets of miRNA-29a/c, because several microRNAs regulate an overlapping set of target genes [12-13]. This study found that both pro-apoptotic factor genes (BMF and Bak1) and anti-apoptotic factor genes (BCL-w and Mcl1) are putative direct target genes of miRNA-29 and that mitochondrial pathway is activated in miRNA-29-promoted apoptosis [25,28]. Through interactions with pro- and anti-apoptotic members, BCL-2 family proteins play a key role in controlling cell life and death[29]. In the study by Xia *et al* [28] miRNA-29a recognizes and binds to the 3'-UTR of BCL-w, BMF, and Bak1 and inhibits translation of its target genes. The BMF protein contains the BCL-2 homology region 3 and is a member of the BCL-2 protein family. It is implicated in regulating apoptosis. In present study, the expression of BMF mRNA increased significantly during the development of the diabetic cataract.

miRNAs are a large group of evolutionarily conserved small (18-22 nucleotides) non-coding RNAs, which have emerged as pivotal regulators that alter the transcription of important genes in cell proliferation, differentiation, invasion, migration and apoptosis [30-31]. miRNAs function through the post-transcriptional silencing of the RNA interference pathway. In the present study, the miRNA-29a expression levels were all visibly lower in the control group compared to the 2 and 4wk diabetic rat samples. The study also focused on miRNA-29c expression in the LECs to investigate its role in the formation of diabetic cataracts. Our results show that the miRNA-29c expression levels were also obviously lower in the control group compared to the 2 and 4wk diabetic rat samples and that the level of miRNA-29c expression in the LECs increased with time. However, the increasing expression of miRNA-29c with time in the LECs remains unclear. Interestingly, we observed that the BMF mRNA levels were significantly higher compared to the healthy controls and that the level of BMF mRNA expression increased with time. Although the mechanism by which miRNA-29a/c dynamically modulates BMF remains incompletely understood, our data suggest that decreased miRNA-29a/c expression also promotes cell death, a fundamental feature of cataracts. The decreased expression of miRNA-29a/c with the concomitant enhancement of BMF mRNA expression may partially explain the upregulation of BMF before the formation of the diabetic cataract. These findings suggest that the expression of BMF may be negatively regulated by miRNA-29a/c and may represent a mechanism of pro-apoptosis in LECs.

In conclusion, to understand miRNA-29a/c expression specifically in rat diabetes-induced cataracts, we evaluated miRNA-29a/c expression in LECs prior to diabetic cataract formation. We found that miRNA-29a/c expression is related to rat LECs apoptosis, although our conclusive results are only limited to one pro-apoptotic BCL-2 family member (BMF). Because a previous study [13] showed that not only BCL-2 but also TCL-1 and MCL-1 are activated by miRNA-29 regulation, it would be interesting to investigate the possible interactions between miRNA-29 and other target genes. Based on these results, miRNA-29a and miRNA-29c expressions have a role in rat diabetic cataract formation. Although miRNA-29a and miRNA-29c are powerful mechanisms to inhibit the expression of BMF mRNA, further studies are necessary before this approach can be used for the prevention of human diabetes-induced cataract.

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