Expression and cellular localization of microRNA-29b and RAX, an activator of the RNA-dependent protein kinase (PKR), in the retina of streptozotocin-induced diabetic rats

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Purpose: The apoptosis of retinal neurons plays a critical role in the pathogenesis of diabetic retinopathy (DR), but the molecular mechanisms underlying this phenomenon remain unclear. The purpose of this study was to investigate the cellular localization and the expression of microRNA-29b (miR-29b) and its potential target PKR associated protein X (RAX), an activator of the pro-apoptotic RNA-dependent protein kinase (PKR) signaling pathway, in the retina of normal and diabetic rats.

Methods: Retinas were obtained from normal and diabetic rats within 35 days after streptozotocin (STZ) injection. In silico analysis indicated that RAX is a potential target of miR-29b. The cellular localization of miR-29b and RAX was assessed by in situ hybridization and immunofluorescence, respectively. The expression levels of miR-29b and RAX mRNA were evaluated by quantitative reverse transcription PCR (qRT–PCR), and the expression of RAX protein was evaluated by western blot. A luciferase reporter assay and inhibition of endogenous RAX were performed to confirm whether RAX is a direct target of miR-29b as predicted by the in silico analysis.

Results: We found that miR-29b and RAX are localized in the retinal ganglion cells (RGCs) and the cells of the inner nuclear layer (INL) of the retinas from normal and diabetic rats. Thus, the expression of miR-29b and RAX, as assessed in the retina by quantitative RT–PCR, reflects their expression in the RGCs and the cells of the INL. We also revealed that RAX protein is upregulated (more than twofold) at 3, 6, 16, and 22 days and downregulated (70%) at 35 days, whereas miR-29b is upregulated (more than threefold) at 28 and 35 days after STZ injection. We did not confirm the computational prediction that RAX is a direct target of miR-29b.

Conclusions: Our results suggest that RAX expression may be indirectly regulated by miR-29b, and the upregulation of this miRNA at the early stage of STZ-induced diabetes may have a protective effect against the apoptosis of RGCs and cells of the INL by the pro-apoptotic RNA-dependent protein kinase (PKR) signaling pathway.

The discovery of microRNA (miRNA) added a new layer of complexity to the regulation of gene expression [1]. MiRNAs are endogenous, noncoding RNAs (19–25 nucleotides in length) that regulate gene expression via binding to specific sites at the 3′-untranslated region (3′-UTR) of the target mRNAs, thereby causing translational repression or mRNA degradation. Recent computational predictions indicate that miRNAs may regulate approximately 30% of human protein-coding genes [2]. In mammals, the functions of specific miRNAs have been described in important cellular processes, such as cell-cycle regulation [3] and hematopoietic [4] and adipocyte [5] differentiation. The abnormal expression of miRNAs has recently been associated with several diseases. However, little is known about the role played by miRNAs in ocular diseases [6,7].

Diabetic retinopathy (DR) is one of the most common complications of diabetes, and nearly all people with type 1 and more than half with type 2 diabetes develop retinopathy [8]. Accumulating evidence indicates that apoptosis of the retinal neurons precedes the microvascular alterations in the retina of diabetic patients and streptozotocin (STZ)-induced diabetic rats [9,10]. It has also been demonstrated that apoptosis of the retinal neurons plays a critical role in the pathogenesis of DR [11], but the molecular mechanisms underlying this phenomenon are currently unclear.

Recent findings indicated that microRNA-29b (miR-29b) is involved in apoptosis [12,13]. Based on the finding that the miR-29 family is expressed in the rat retina [14] and that one miRNA has several targets, we hypothesize that miR-29b could regulate the genes in the pro-apoptotic pathways that are involved in the apoptosis of the retinal neurons of STZ-induced diabetic rats. The activation of the RNA-dependent protein kinase (PKR) is reported to be involved in the cell death of the retinal ganglion cells treated with tunicamycin [15]. These authors showed that the
activation of PKR is due to the endoplasmic reticulum (ER) stress induced by tunicamycin. Moreover, it was demonstrated that ER stress has a role in the early stage of diabetic retinopathy [16]. Interestingly, the in silico analysis revealed that one of the potential targets of miR-29b is RAX (PKR-associated protein X), the only known physiologic activator of PKR. It has been suggested that RAX may be a direct stress-sensitive activator of PKR and could induce apoptosis by activating the PKR-signaling pathway under stress conditions [17]. In this context, it is possible that the sustained hyperglycemic state and consequent ER stress observed in diabetes could induce the expression of RAX in the retinal neurons of diabetic animals.

In the present study, we used STZ-induced diabetic rats because this experimental model of diabetes displays many of the morphological and functional changes in the retinal neurons that are evident in human DR [18]. We investigated the cellular localization and expression of miR-29b and RAX in the retina of normal and diabetic rats to elucidate their possible involvement in the apoptosis of retinal neurons.

**METHODS**

**Animals and treatment with streptozotocin:** Male Wistar rats weighing 130 to 150 g were housed in suspended wire-bottom cages in a room kept at 25±2 °C with a 12:12 h light–dark cycle and were provided with food and water ad libitum. The animals were randomly divided into groups of eight. For the induction diabetes, STZ (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.01 M citrate buffer, pH 4.5, and was injected within 5 min of its preparation. The rats were fasted overnight, anesthetized with isoﬂurane, and injected with STZ in the jugular vein at a dose of 45 mg/kg bodyweight. The control rats were injected with the citrate buffer [19]. Blood glucose levels were measured by the colorimetric oxidase glucose method (Labtest, Lagoa Santa, Brazil) 24 h after the injection of STZ or citrate buffer. Only animals with blood glucose values ≥400 mg/dl were used. All experiments were performed between 8:00 AM and 10:00 AM. The STZ-injected and control rats were sacrificed by rapid cervical dislocation at the indicated time points after treatment. The retinas were dissected and used either for the analysis of RAX mRNA and miR-29 expression and protein analysis or processed for in situ hybridization and immunofluorescence. The care and treatment of the animals received prior institutional approval (protocol 012/2008) from the Ethical Commission of Ethics in Animal Research of the School of Medicine at the University of São Paulo, Ribeirão Preto, SP, Brazil.

**Tissue fixation:** For in situ hybridization and immunofluorescence, enucleated eyes were first immersion fixed in 4% buffered paraformaldehyde prepared in 0.2 M phosphate buffer (pH 7.3) at 4 °C for 24 h, transferred to cassettes, and subsequently treated with 70% ethanol for 2 h, 99% ethanol for 1 h, absolute ethanol for 30 min, xylene at room temperature for 1 h, xylene at 37 °C for 10 min, then in a paraffin bath at 56 °C for 1 h. The fixed samples were subjected to vacuum pressure for 30 min and then embedded in paraffin. The paraffin blocks were cut into 5-μm serial sections that were placed on Superfrost™ (Cole-Parmer, Vernon Hills, IL) slides. Cellular integrity was assessed by histological staining using Harris hematoxylin and eosin–phloxine. For this, the sections were adhered to the slides in an oven at 60 °C, deparaffinized in xylene, and rehydrated through graded ethanol washes (100%, 95%, 80%, and 70%) for 10 min each followed by a wash in distilled water for 5 min. Subsequently, the sections were stained with hematoxylin for 1 min, washed in distilled water, and stained with eosin for 2 min. The slides were dehydrated and mounted in Permount (Fisher Scientific, Pittsburgh, PA). Other sections of each sample were processed for immunofluorescence and in situ hybridization.

**Immunofluorescence:** For immunostaining, the slides were incubated in 0.1 M glycine (dissolved in PBS) 0.01 M phosphate-buffered saline, pH 7.4) for 30 min and blocked for 1 h in 1% goat serum, 2% BSA (BSA), and 0.05% Triton X-100 in PBS at room temperature in a humidity chamber. The slides were incubated in the primary anti-PACT (PKR activator) goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:50 in blocking buffer under the same conditions for 3 h. After three 5-min washes in PBS, slides were incubated in a 1:2,000 Alexa Fluor 594 rabbit antigen fluorescent secondary antibody solution (Molecular Probes, Invitrogen) in a humidified chamber for 1 h at room temperature in the dark. The slides were washed ten times in PBS, mounted with Vectashield mounting medium with with the 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and coverslips were applied. The slides were viewed with a Leica DM5500B fluorescent microscope (Leica Microsystems, Bannockburn, IL). The images were captured with the Leica DFC 340 FX software (Leica Microsystems) and processed using using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA). The specificity of antibody staining was confirmed by incubating the adjacent sections in the absence of the primary antibody.

**In situ hybridization:** To perform in situ hybridization of miR-29b, a locked nucleic acid (LNA)-modified, digoxigenin (DIG)-labeled probe was generated by Exiqon (Vedbaek, Denmark). Such LNA probes have previously been shown to provide exceptional miRNA hybridization specificity that is sensitive to 1 or 2 nucleotide mismatches [20]. We optimized the miRNA in situ hybridization protocol described by Ryan et al. [21]. In this protocol, deparaffinized sections were treated for 15 min with 0.1% H2O2 to block endogenous peroxidase activity and treated for 5 min with proteinase K (10 μg/ml) followed by 30 s in 0.2% glycine. The sections were refixed in 4% paraformaldehyde for 10 min, washed twice in PBS, and prehybridized for 2 h in the hybridization buffer (50% formamide, 5×300 mM NaCl, 30 mM sodium citrate, pH 7.0, 0.4% Tween, 9.2 mM citric acid [pH 6],...
50 μg/ml heparin, 500 μg/ml yeast RNA). The tissues were hybridized overnight at room temperature in the presence of 20 nM of the DIG-labeled probe. The slides were washed twice in 2× SSC for 5 min, followed by three 30-min washes in 50% formamide plus 2× SSC at 37 °C. After five washes in 0.1% Triton X-100 in PBS, the slides were incubated in blocking solution (2% sheep serum, 2 mg/ml BSA in PBST: 0.1M PBS, 0.5% Tween-20, pH 7.4) followed by an overnight incubation at 4 °C in a 1:1,000 of anti-digoxigenin monoclonal antibody conjugated with biotin (Abcam, Cambridge, UK). The slides were washed seven times for 5 min in PBS.

Immunological detection was performed with the TSA (Tyramide Signal Amplification) Biotin System (Perkin Elmer, Boston, MA) for 10 min at room temperature. The specificity of the antibody staining was confirmed by incubating adjacent sections in the absence of the miR-29b antibody. As a positive control, a U6 probe generated by Exiqon was substituted for the miR-29b probe.

RNA isolation: Total RNA was extracted from the retinas of normal and diabetic rats using Trizol-LS Reagent (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation. RNA concentration was assessed using a biophotometer (Eppendorf, Hamburg, Germany), and a ratio of 2.0 for sample absorbance at 260/280 nm was considered acceptable.

Analysis of microRNA-29b expression by quantitative reverse transcription PCR: The expression analysis of mature miR-29b was performed with quantitative reverse transcription PCR (qRT–PCR) for the retina samples at various intervals after STZ treatment and for the control retinas. Briefly, 2.5 μl of total RNA (10 ng) was supplemented with the RT primer mix with the RT primer (TaqMan MicroRNAs Reverse Transcription Kit, Applied Biosystems, Foster City, CA). For β-actin assays, RT reactions were performed using 500 ng of RNA and random hexamers (Applied Biosystems). All reactions were incubated in the Thermo Hybird PCR Express (Middlesex, UK) at 42 °C for 1 h. After the RT reaction, the cDNA products were diluted to 1:4. In a 10-μl PCR reaction, 4.0 μl of the diluted cDNA was added to 5.0 μl of 2× PCR master mix (TaqMan Universal PCR master mix; Applied Biosystems) and 1.0 μl of the miR-29b primers and TaqMan probe mix; Applied Biosystems). The same procedure was used for the β-actin assays except for the use of 4.5 μl of the diluted cDNA and 1.5 μl of the human β-actin primers and probe mix (predeveloped TaqMan assay; Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 10 min, using an Applied Biosystems 7500 Sequence Detection system. The cycle threshold (ΔCt) values were calculated, and the data were normalized to the housekeeping gene β-actin (ACTB) [22]. Real-time PCR was performed in triplicate in three independent experiments, and the results were expressed as means±standard error of the mean (SEM).

Prediction of microRNA-29b targets: We used three algorithms to computationally predict potential targets of miR-29b. The algorithms included miRanda, TargetScan 5.0, and FindTar. The computationally predicted miRNA–target pairs were downloaded to a local database. We selected the best predicted targets of miR-29b by comparing the results from the three target prediction databases [23].

Analysis of the PKR activator RAX mRNA expression by quantitative reverse transcription PCR: Standard SYBR Green qRT–PCR was performed to detect the RAX transcript levels in the total cellular RNA from the retinas of the hyperglycemic and the normoglycemic rats. After RNA isolation, 1.2 μg of RNA from the individual samples was reverse transcribed using the Superscript III Reverse Transcriptase Kit (Invitrogen). The quantitative PCR (qPCR) assays were performed using an Applied Biosystems 7500 Sequence Detection system. The thermal cycling conditions for PCR were as follows: 95 °C for 3 min and 40 cycles of amplification comprising 95 °C for 12 s, 60 °C for 30 s, and 72 °C for 30 s. The reaction mixture consisted of 2 μl cDNA, SYBR Green PCR Master Mix (Applied Biosystems), and primers to RAX (sense 5′-AGC GGG ACC TTC AGT TTG G-3′ and antisense 5′-CTT GGT CTG CGT GCC GTA CTC-3′). These primers were specific for the published RAX cDNA sequence (GenBank NM_001024780.1), designed using Primer Express version 2 software (Applied Biosystems), and optimized for use at a final concentration of 2.5 μM. ACTB mRNA was analyzed in the same run using specific primers (sense 5′-TGG AAT CCT GTG GCA TCT ATG AAA C-3′ and antisense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′) as recommended by the manufacturer (Applied Biosystems). The Ct values were calculated, and the data were normalized to the housekeeping gene mRNA levels (ACTB). qPCR was performed in triplicate in three independent experiments with ±SEM reported. A linear standard curve analysis for each primer pair at a maximum of a 10+ dilution of the control-pooled cDNA and a primer amplification efficiency (E) greater than 0.96 was obtained for all the PCR experiments. The heat dissociation of the amplified DNA detected a single peak in all cases, indicating that a single specific PCR product had been synthesized. This was confirmed by electrophoresis of the PCR products in which a single band of the expected molecular weight was observed.

Western blot analysis: The retinas were lysed directly on ice in 200 μl of lysis buffer consisting of 20 mM Tris-HCl (pH 7.6), 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM dithiothreitol, 1% Triton X-100, and 20% glycerol. The reagents used to prepare lysis buffer were purchased from Sigma Chemical Co., St. Louis, MO. The lysates were centrifuged at 10,000 × g for 20 min, and the supernatant was stored at −70 °C. The soluble proteins were quantitated following the Lowry method [24]. Total cellular
proteins (30 μg) were separated by electrophoresis through a 10% sodium dodecyl sulfate PAGE (SDS–PAGE)-resolving gel with an SDS–PAGE stacking gel. After electrophoresis, the proteins were transferred onto a Hybond-C-supported nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) by semidry electroblotting. The membranes were blocked with 3% BSA in 100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20 (TBST) for 1 h and incubated in a 1:300 dilution anti-PACT goat polyclonal antibody (Santa Cruz Biotechnology) in blocking buffer for 1 h. After washing the membranes in TBST for 20 min, 1:2,000 dilution rabbit antigoat immunoglobulin secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) in TBST was added, and the membranes were incubated at room temperature for 60 min. After washing the membranes for 1 h in diluted in TBST, Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham Biosciences, Little Chalfont, UK) were added to the membranes for 2 min, and the membranes were developed on Hyperfilm (GE Healthcare, UK). The western blots were repeated three times; qualitatively similar results were obtained each time. Equal loading and transfer were ensured by reprobing the membranes for β-actin. The films were documented and the loading and transfer were ensured by reprobing the membranes for β-actin. The films were documented and the densities were assessed with the paired Student t test, and p<0.05 was considered statistically significant. Analysis of the data was performed with GraphPad Prism 4.02 software (GraphPad, San Diego, CA).

Luciferase reporter assay: The reporter plasmid containing the miR-29b complementary sequence was constructed with a fragment of the 3′ UTR of RAX mRNA containing the predicted miR-29b binding site. This fragment was amplified by RT–PCR using primers (sense 5′-GCT GAG TGT GGC ATC CAT TT-3′ and antisense 5′-CCA CTT CAC AAA GCT TG TG CAT-3′) that produced a 141-bp amplicon spanning nucleotides 189–330 and containing one potential target site for miR-29b. The amplicon was cloned in the pCR® 2.1 TOPO vector (Invitrogen) to incorporate the SacI–XbaI restriction enzyme sites. After the appropriate digestion and purification (QIAquick Gel Extraction and PCR Purification Kit; Qiagen), the product was subcloned into the XbaI site of site of pISO [25,26], downstream of the firefly luciferase-coding sequence, to generate the pISORAX plasmid. The plasmid was purified (Plasmid Purification Mini/Midi Kits; Qiagen), and successful cloning was confirmed by DNA sequencing. For transfection, human embryonic kidney cells (HEK293) were seeded in complete Dulbecco’s minimal essential medium (DMEM; Gibco-Invitrogen, Paisley, UK) supplemented with 15% fetal bovine serum. After 24 h, the cells were transfected with Lipofectamine 2000 reagent (Invitrogen), 100 ng of the pISO reporter construct (pISO-REPORT-3′-UTR/RAX or pISO-REPORT empty), 20 ng of the Renilla luciferase control vector, pRL-TK (Promega, Lyon, France); and either a synthetic miR-29b mimic (Dharmacon, Denmark) or a short double-stranded RNA (dsRNA; Dharmacon), as a negative control at two doses (50 nM or 75 nM). The sequence (5′-GAT AGC AAT GAC GAA TGC GTA-3′) of the dsRNA control is not complementary to the 3′-UTR of RAX mRNA. The efficiency of transfection was monitored by reporter activity, which was measured using the dual luciferase assay kit (Promega) and a luminometer (Lecteur Microplaque-Mithras LB940). We measured firefly luciferase activity 24 and 48 h after transfection and normalized the results to the Renilla luciferase activity. We performed at least three independent experiments for each assay, and the results were expressed as the mean±SEM.

Analysis of protein and mRNA expression levels of RAX, an activator of PKR, in the presence of a microRNA-29b mimic: To confirm the results of the luciferase reporter assay, CJ4 rat fibrosarcoma cells were seeded in six-well plates at a density of 1×10^4 cells/well in DMEM supplemented with 15% fetal bovine serum. After 24 h, when they reached a confluence of 80%–85%, the cells were transfected at two doses (50 nM and 75 nM) with either the synthetic miR-29b mimic or the dsRNA negative control (described above) and 30 μl of Lipofectamine (Invitrogen, Carlsbad, CA) 2000. The cells were lysed at 24 and 48 h after transfection. Protein expression levels and RAX mRNA expression levels were assessed by western blot analysis and qRT–PCR, using the standard procedures already mentioned. We performed at least three independent experiments for each assay, and the results were expressed as the mean±SEM.

Statistical analysis: Data are presented as mean±SEM. Differences between the control and the treated retinas were assessed with the paired Student t test, and p<0.05 was considered statistically significant. Analysis of the data was performed with GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel.

RESULTS

Cellular localization of microRNA-29b in the retina: We used in situ hybridization to determine the cellular localization of miR-29b in the retina of diabetic rats. The cellular localization of miR-29b is shown in the control rat retinas (Figure 1A) and the diabetic rat retinas on days 6 (Figure 1B), 28 (Figure 1C), and 35 (Figure 1D) post-STZ injection. The miR-29b signal was weak at 6 days but strong at 28 and 35 days. The miR-29b staining was observed in the cytoplasm, and there was no change in the cellular localization of miR-29b in the retina of normal and diabetic rats.

Expression of microRNA-29b in retina: The distribution of miR-29b was strongest and primarily seen in the ganglion cell layer (GCL). Very little signal was detected in the inner nuclear layer (INL), and little if any expression was detected in the outer nuclear layer (Figure 1C,D). The analysis of the expression of miR-29b in the retina by qRT–PCR reflects the expression seen in the RGCs and cells of the INL, as indicated.
in Figure 1C. We observed that miR-29b is upregulated (>3 fold) at 28 (p=0.01) and 35 (p=0.05) days after injection of STZ (Figure 2). The expression profiles of miR-29a and miR-29c were similar to that found for miR-29b (data not shown).

**Computational prediction of potential microR-29b targets:**

The results of the computational analysis indicated that one of the miR-29 targets is RAX mRNA. As shown in Figure 3A, there is a region of sequence in the 3'-UTR of RAX that is highly conserved across all mammals and has identical nucleotides from the second to the seventh base; this is known as the “seed” sequence. The seed sequence is considered to be the most critical sequence for selecting targets of miRNAs. We found that the three paralogs of miR-29 (miR-29a, miR-29b, and miR-29c) have a complementary sequence to the seed sequence on RAX with minor divergences (Figure 3B), suggesting that the three paralogs potentially target RAX mRNA. The localization of the binding sites of these paralogs of miR-29 to on the RAX 3'-UTR overlap, and there is only a single target site on RAX for each miRNA studied.

**Cellular localization of PKR associated protein X (RAX) protein in the retina:**

The analysis by immunofluorescence demonstrated that RAX protein is expressed in the same specific retinal cell types as that observed for miR-29b.
expression (Figure 1B and Figure 4B). The RAX-positive cells are the same in the retinas of normal and diabetic rats (Figure 4A,B); however, there is strong cytoplasmic expression of RAX at 6 days after STZ injection when compared to normal rats. In the retinas of diabetic animals at 28 and 35 days after STZ injection, RAX expression was strongly reduced to the level of normal rats (Figure 4A,C,D).

Expression of PKR associated protein X (RAX) mRNA and protein in the retina: PKR associated protein X (RAX) mRNA expression in the retinal cells was significantly higher (>3.5-fold) than that of the control group at 3 (p=0.04), 6 (p=0.004), and 22 (p=0.001) days after injection of STZ, and the expression levels returned to that of the control by 28 days (Figure 5A). The expression of RAX protein was increased (>1.5 fold) at 3, 6, 15, and 22 days (p=0.001, p=0.01, p=0.002,
p<0.001, respectively) and decreased to a level below that of the control group at 35 days after STZ injection (p<0.001; Figure 5B).

Analysis of the microR-29b target by luciferase reporter assay: To investigate whether miR-29b directly regulates RAX expression, we used a luciferase reporter assay. HEK293 cells were co-transfected with the plasmids (pISO-RAX or pISO empty) and a synthetic mimic of miR-29b or dsRNA (negative control). The levels of luciferase activity were measured at 24 and 48 h after transfection to determine the repressive effects of this miRNA. The dual-luciferase activity assay revealed that there was no difference in luciferase activity between the cells transfected with the negative control and the miR-29b oligonucleotides (Figure 6B,C).

The effects of overexpression of microR-29b on PKR associated protein X (RAX) mRNA and protein levels: When a specific mRNA is a target of an miRNA, a change in the concentration of the miRNA in the cell causes a change in the amount of the mRNA and/or protein of the target in the cell. CJ4 cells were transfected with a synthetic mimic of miR-29b or dsRNA (negative control) in concentrations of 0, 50, and 75 nM, and the expression of RAX protein was evaluated at 24 and 48 h after transfection. The results revealed no significant changes in the protein expression of...
RAX (Figure 7A,B). Although there is little information [27] in the literature showing the regulation of gene expression by miRNAs in mammalian cells through the degradation of the mRNA target, we decided to investigate this possibility in our system. With this objective, CJ4 cells were transfected in the same conditions above and the total RNA was extracted from the transfected cells at 24 and 48 h. The analysis by qRT–PCR revealed that there was no significant change in the expression level of RAX mRNA (Figure 8).

DISCUSSION

It was previously demonstrated that RGCs undergo apoptosis in STZ-induced diabetic rats [9,10]. Recent studies have indicated that miR-29b is involved in apoptosis [12,13]; therefore, the first step was to investigate the cellular localization of miR-29b in the retinas of normal and diabetic animals. We observed, by in situ hybridization, that miR-29b is highly expressed in the neurons of the RGC of diabetic rats. The analysis of the expression of miR-29b in the retina by qRT–PCR showed that miR-29b is upregulated at 28
and 35 days after STZ injection. Considering the localization results from in situ hybridization, it is reasonable to assume that the miR-29b expression detected by qRT–PCR reflects its expression in RGCs and the cells of the INL of the rat retina.

The next step was to examine the potential targets of miR-29b, as it is known that several miRNAs regulate an overlapping set of target genes [12,13,28]. The computational prediction programs suggested that RAX is a target of the miR-29 family (miR-29a, miR-29b, and miR-29c), and miR-29b showed the highest score of prediction. It was reported that RAX, an activator of PKR, and its human ortholog PACT are 98% identical and ubiquitously expressed in mammalian cells [17,29]. Recently, several studies have shown that RAX is upregulated during cellular stress [17,30-32]. Moreover, it was found that PKR plays a role in the regulation of important cellular processes, including the apoptosis of RGCs and neurons in patients with Parkinson, Alzheimer, and Huntington’s diseases [33-37]. Interestingly, PKR is a stress-responsive kinase, and it has been described that apoptosis in mammalian cells occurs in response to stress [15]. Thus, it was found that PKR has a significant role in ER stress-dependent apoptosis through the eIF-2α/ATF4/CHOP signaling pathway [38]. These authors also observed that ER stress does not induce PKR expression but rather activates the pre-existing PKR via the induction of the PKR-activating protein PACT. More recently, it was suggested that hyperglycemia induces ER stress in the tubular cells of the kidney in patients with established diabetic nephropathy [39]. In addition, the intracellular accumulation of glucosamine observed in the cells of diabetic animals may promote the misfolding of proteins in the ER lumen and consequently ER stress [40]. On the basis of these observations, we speculated that RAX is upregulated in the retinal neurons of STZ-induced diabetic rats. To test this hypothesis, we investigated the cellular localization and the expression of RAX protein in the retina of STZ-induced diabetic rats.

Clearly, the miRNA and its mRNA target must be expressed in the same cells, and they are expected to have an inverse correlation of expression. The analysis of the cellular localization of RAX by immunofluorescence indicated that RAX is also strongly expressed in the neurons of the ganglion cell layer of the retinas of diabetic animals. These findings suggest that, if the miR-29b target site in the 3′-UTR of RAX mRNA is accessible in vivo, the expression of RAX may be negatively regulated by miR-29b. Interestingly, the
upregulation of miR-29b at 28 and 35 days after the injection of STZ is accompanied by the downregulation of RAX protein. This suggests that RAX expression is negatively regulated by miR-29b and may represent a mechanism of protection of retinal neurons against apoptosis, which occurs around 35 days after STZ-induced diabetes [41]. Recently it was found that miR-29b has a protective effect against the deposition of extracellular matrix in the trabecular meshwork cells when induced by chronic oxidative stress, as observed in glaucoma [15].

To gain more insight into the role played by miR-29b in the retina during the early stage of STZ-induced diabetes, we investigated whether RAX is a direct target of miR-29b. The luciferase assay and the overexpression of miR-29b did not validate RAX as a direct target of miR-29b. Thus, it is possible that in the retina of diabetic rats miR-29b is acting on another target rather than RAX or this PKR activator is an indirect target of miR-29b. Not all the targets predicted by computational programs are experimentally validated [42]. In addition, an inverse correlation of expression of an miRNA and its putative target may suggest that miRNAs function indirectly. Moreover, it has been observed that miRNAs act as indirect regulators of gene expression through the regulation of transcription factors [43]. It is estimated that approximately a quarter of the putative targets of miRNAs are transcription factors, suggesting that changes in the
expression of an miRNA may modulate the activity of specific transcription factors and alter gene regulatory circuits in physiologic and pathological conditions [44].

Recently, it was reported that miR-29b indirectly regulates DNA methyltransferase 1 (DNMT1) by targeting the transcription factor Sp1 which binds to the GC boxes in the promoter of the DNMT1 gene in mice and humans [45-47]. Interestingly, the promoter of PACT and RAX contains GC boxes, indicating that the expression of these PKR activators is regulated by Sp1 [48]. These findings prompted us to perform in silico analysis to search binding sites for miR-29b in the 3′-UTR of rat Sp1 mRNA. Our results revealed that the binding sites for miR-29b are conserved in human and rat, suggesting that miR-29b may also regulate Sp1 expression in rat (data not shown). It should be emphasized that Sp1 is involved in the transcription of several genes regulated by miR-29b [28].

Based on the results of this work and that from the literature, it is reasonable to speculate that RAX expression is indirectly regulated by miR-29b in the retinal neurons of the rat (Figure 9). Thus, the downregulation of RAX protein that was observed in the rat retina at 28 and 35 days after injection of STZ could be explained by the inhibition of Sp1 expression due to the upregulation of miR-29b. Moreover, the downregulation of RAX could result in a decrease of the activated PKR level with subsequent reduction of the activity of the pro-apoptotic PKR signaling pathway. It is important to note that the apoptosis of the retinal neurons in our experimental model of diabetes occurs around 35 days after STZ injection [10]. Therefore, it is possible that the upregulation of miR-29b at 28 and 35 days may represent part of an adaptive response to protect the retinal neurons against apoptosis by the PKR signaling pathway in diabetic rats. However, additional experiments are required to confirm our working hypothesis, such as the validation of rat Sp1 as a direct target of miR-29b and the evaluation of the expression of Sp1 and phosphorylated (activated) Sp1 in the retinas of normal and diabetic rats. The levels of activated Sp1 should be examined to determine if the phosphorylation of pre-existing Sp1 can explain the apparently miR-29b-independent induction of RAX observed at 6 days after injection of STZ.
To our knowledge, this is the first evidence for miRNA involvement in the apoptosis of retinal neurons. It should be emphasized that the apoptosis of retinal neurons plays a crucial role in the pathogenesis of DR [9,10]. Moreover, our results provide a new focus for future studies and may contribute to the development of new strategies for the treatment of DR, such as the intravitreal injection of miR-29b. Recent studies suggest a potentially therapeutic use of miRNAs and small interfering RNAs for human diseases. This represents a promising and exciting area of biomedical research.

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REFERENCES

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116:281-97. [PMID: 14744438]
2. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20. [PMID: 15652477]
3. Wang Y, Baskerville S, Shenoy A, Babiarz JE, Baehner L, Chen CZ. Ling Li, Harvey FL, David PB. MicroRNAs regulate the G1-S transition and promote rapid proliferation. Cell 2005; 120:15-20. [PMID: 15652477]
4. Esau C, Kang X, Peralta E, Hanson E, Marcusson E, Blelloch R. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. Nat Genet 2005; 37:266-71. [PMID: 15917565]
5. Li B, Wang HS, Li GG. Zhao M H. The role of endoplasmic reticulum stress in the early stage of diabetic retinopathy. Acta Diabetol 2010; (Nov):19. [PMID: 19367364]
6. Chen CZ. Ling Li, Harvey FL, David PB. MicroRNAs Modulate Hematopoietic Lineage Differentiation. Science 2004; 303:83-6. [PMID: 14657504]
7. Wei M, Leslie O, Maree TS, Fraser BR, Katrina S, Andrew JH, Darryl B, Lindsay B. The Streptozotocin-Diabetic Rat as a Model of the Chronic Complications of Human Diabetes. Heart Lung Circ 2003; 12:44-50. [PMID: 16352106]
8. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate function. Cell 2004; 116:281-97. [PMID: 14744438]
9. Groisman R, Souidi M, Cuvellier S, Harel-Bellan A. The role of the apoptosis of retinal neurons plays a crucial role in the pathogenesis of DR [9,10]. Moreover, our results provide a new focus for future studies and may contribute to the development of new strategies for the treatment of DR, such as the intravitreal injection of miR-29b. Recent studies suggest a potentially therapeutic use of miRNAs and small interfering RNAs for human diseases. This represents a promising and exciting area of biomedical research.

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REFERENCES

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116:281-97. [PMID: 14744438]
2. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20. [PMID: 15652477]
3. Wang Y, Baskerville S, Shenoy A, Babiarz JE, Baehner L, Blelloch R. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. Nat Genet 2005; 37:266-71. [PMID: 15917565]
4. Li B, Wang HS, Li GG. Zhao M H. The role of endoplasmic reticulum stress in the early stage of diabetic retinopathy. Acta Diabetol 2010; (Nov):19. [PMID: 19367364]
5. Chen CZ. Ling Li, Harvey FL, David PB. MicroRNAs Modulate Hematopoietic Lineage Differentiation. Science 2004; 303:83-6. [PMID: 14657504]
6. Wei M, Leslie O, Maree TS, Fraser BR, Katrina S, Andrew JH, Darryl B, Lindsay B. The Streptozotocin-Diabetic Rat as a Model of the Chronic Complications of Human Diabetes. Heart Lung Circ 2003; 12:44-50. [PMID: 16352106]
7. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate function. Cell 2004; 116:281-97. [PMID: 14744438]
8. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate function. Cell 2004; 116:281-97. [PMID: 14744438]
9. Groisman R, Souidi M, Cuvellier S, Harel-Bellan A. The role of
during mammalian myoblast differentiation. Nat Cell Biol 2006; 8:278-84. [PMID: 16489342]

27. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 2006; 20:515-24. [PMID: 16510870]

28. Luna C, Li G, Qiu J, Epstein DL, Gonzalez P. Role of mir-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. Mol Vis 2009; 15:2488-97. [PMID: 19956414]

29. Patel RC, Sen GC. PACT, a protein activator of the interferon-induced protein kinase, PKR. EMBO J 1998; 17:4379-90. [PMID: 9687506]

30. Bennett RL, Blalock WL, Abtahi DM, Pan Y, Moyer SA, May WS. RAX, the PKR activator, sensitizes cells to inflammatory cytokines, serum withdrawal, chemotherapy, and viral infection. Blood 2006; 108:821-9. [PMID: 16861340]

31. Bennett RL, Blalock WL, May WS. Serine 18 Phosphorylation of RAX, the PKR activator, is required for PKR activation and consequent translation inhibition. J Biol Chem 2004; 279:42687-93. [PMID: 15299031]

32. Patel CV, Handy I, Goldsmith T, Patel RC. PACT, a stress-modulated cellular activator of interferon-induced double-stranded RNA-activated protein kinase, PKR. J Biol Chem 2000; 275:37993-8. [PMID: 10988289]

33. Bando Y, Onuki R, Katayama T, Manabe T, Kudo T, Taira K, Toyohara M. Double-strand RNA dependent protein kinase (PKR) is involved in the extrastriatal degeneration in Parkinson's disease and Huntington's disease. Neurochem Int 2005; 46:11-8. [PMID: 15567511]

34. Page G, Rioux Bilan A, Ingrand S, Lafay-Chebassier C, Pain S, Rajewsky N, Bender TP, Rajewsky K. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 2007; 131:146-59. [PMID: 17923094]

35. Watanabe MAE, Souza LR, Murad JM, De Lucca FL. Activation of the RNA-dependent protein kinase of lymphocytes by regulatory RNAs: implications for immunomodulation in HIV infection. Curr HIV Res 2005; 3:329-37. [PMID: 16250880]

36. Delgado André N, De Lucca FL. Non-coding transcript in T cells (NTT): antisense transcript activates PKR and NF-kappaB in human lymphocytes. Blood Cells Mol Dis 2008; 40:227-32. [PMID: 17928244]

37. Murad JM, de Souza LR, De Lucca FL. PKR activation by a non-coding RNA expressed in lymphocytes of mice bearing B16 melanoma. Blood Cells Mol Dis 2006; 37:128-33. [PMID: 16857398]

38. Lee ES, Yoon CH, Kim YS, Bae YS. The double-strand RNA-dependent protein kinase PKR plays a significant role in a sustained ER stress-induced apoptosis. FEBS Lett 2007; 581:4325-32. [PMID: 17716668]

39. Lindenmeyer MT, Rastaldi MP, Ikehata M, Neusser MA, Kretzler M, Cohen CD, Schlöndorff D. Proteinuria and hyperglycemia induce endoplasmic reticulum stress. J Am Soc Nephrol 2008; 19:2225-36. [PMID: 18776125]

40. Werstuck GH, Khan MI, Femia G, Kim AJ, Tedesco V, Trigatti B, Shi Y. Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated atherosclerosis in a hyperglycemic mouse model. Diabetes 2006; 55:93-101. [PMID: 16380481]

41. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin. J Clin Invest 1998; 102:783-91. [PMID: 9710447]

42. Sethupathy P, Megraw M, Hatzigeorgiou AG. A guide through present computational approaches for the identification of mammalian microRNA targets. Nat Methods 2006; 3:881-6. [PMID: 17060911]

43. Xiao C, Calado DP, Galler G, Thai TH, Patterson IC, Wang J, Rajewsky N, Bender TP, Rajewsky K. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 2007; 131:146-59. [PMID: 17923094]

44. Sabia R, Goodman CD, Huzarewich RL, Robertson C, Booth SA. A miRNA signature of prion induced neurodegeneration. PLoS ONE 2008; 3:e3652. [PMID: 18987751]

45. Kishikawa S, Murata T, Kimura H, Shiota K, Yokoyama KY. Regulation of transcription of the Dnmt1 gene by Sp1 and Sp3 zinc finger proteins. Eur J Biochem 2002; 296:2961-70. [PMID: 12071960]

46. Liu S, Liu Z, Xie Z, Pang J, Yu J, Lehmann E, Huynh L, Vukosavljevic T, Takeki M, Klisovic RB, Baiocchi RA, Blum W, Porcu P, Garzon R, Byrd JC, Perrotti D, Caligiuri MA, Chan KK, Wu LC, Marcucci G. Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF-kappaB-dependent DNA methyltransferase activity in acute myeloid leukemia. Blood 2008; 111:2364-73. [PMID: 18083845]

47. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CEA, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene re-expression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 2009; 113:6411-8. [PMID: 19211935]

48. Fasciano S, Kaufman A, Patel RC. Expression of PACT is regulated by Sp1 transcription factor. Gene 2007; 388:74-82. [PMID: 17125937]

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