Matrix metalloproteinase 9 expression: new regulatory elements

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Abstract Retinal ganglion cells apoptosis is linked to matrix metalloproteinase 9 (MMP-9) controlled changes of extracellular matrix. Abnormal expression of MMP-9 is associated with glaucomatous alterations. Thus, the knowledge of MMP-9 regulation is important for the understanding the pathogenesis of glaucoma. Here, we investigated the role of 3'-untranslated regions (3'-UTR) and microRNAs in MMP-9 regulation. We used in vitro mutagenesis and Luc reporter system to identify regulatory elements in the 3'-UTR of MMP-9. microRNAs were analyzed by qRT-PCR, and their role was investigated with inhibitors and mimics. We identified targets for miRNAs in 3'-UTR of MMP-9 involved in the regulation of MMP-9 expression. We then isolated miRNAs from the optic nerve A7 astrocytes and 293 T cells and confirmed the role of mi340 in the regulation using specific inhibitors and mimics. The results obtained show a new miRNA-mediated mechanism of MMP-9 expression regulation.

Keywords Retinal ganglion cells ·
Matrix metalloproteinases · Extracellular matrix ·
MicroRNA · Untranslated region

Introduction

Vision loss in glaucoma is attributed to retinal ganglion cell (RGC) death, and intraocular pressure (IOP) is the major modifiable risk factor [1]. Presumably IOP disrupts the function of RGC axons by increasing mechanical forces on the lamina cribrosa of the optic nerve head (ONH). Although exact underlying mechanisms that link elevated IOP to glaucomatous RGC death are not completely understood, RGC apoptosis is considered an important step leading to glaucoma development [2–4]. Whatever are the primary and secondary factors inducing apoptosis, the end result in glaucomatous eyes is the dysfunction and death of RGCs. This leads to an irreversible visual loss, as a result of a complex interplay of multiple factors acting both on the RGC bodies and on their axons in the optic nerve. Understanding the mechanism of neuronal cell death in retinal diseases like glaucoma is important for devising new treatments. Investigation by several teams demonstrated an important role of matrix metalloproteinases (MMPs)—the major extracellular matrix (ECM) degrading enzymes in glaucomatous alterations both in the retina and in the ONH. Molecular substrates for the MMPs include all classes of ECM proteins, as well as a variety of other molecules involved in determining tissue structure and controlling tissue remodeling [5].

The studies of molecular and cellular alterations in glaucomatous ONH have shown extensive remodeling of the ECM, in which MMPs play a key role [6, 7]. Possible links between elevated IOP and glaucomatous alterations in the optic nerve was revealed by several research teams. Elevated IOP activates optic nerve astrocytes [8], MMPs expression is upregulated in activated astrocytes [9] causing matrix remodeling [6, 7].

The role of MMPs in glaucomatous alterations in retinal cells has been shown in several studies, including results

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supporting an association between RGC apoptosis and specific ECM-related changes of MMP-9 expression [10, 11]. Manabe and coauthors [12] demonstrated that an important factor contributing to the RGC death was abnormal activation of MMP-9 which triggers an extracellular signaling cascade leading to apoptosis. Genetic data also support the involvement of MMP-9 in glaucoma. Population studies revealed an association of single nucleotide polymorphisms in MMP-9 gene with primary angle closure glaucoma [13].

In another study, an enhanced MMP-9 activity was detected in apoptotic RGCs along with decreased deposition of laminin in the RGC layer suggesting a reduced degradation of the ECM at the retinal site in response to exposure to elevated IOP. The correlation between the level of RGC apoptosis and MMP-9 activity suggests the existence of a mechanism mediating glaucomatous RGC loss via MMP-9 [14]. In agreement with this data are results showing that the inhibition of MMP-9 reduces RGC apoptosis and tissue remodeling [15]. An interesting although not completely understood link between MMP-9 and glaucoma is the finding of upregulation of MMP-9 expression in circulating leucocytes in patients with vasospastic normal-tension glaucoma [16].

These results received in the studies of ocular cells and tissues are in a good agreement with the data about the association of neuronal apoptosis with increased MMP-9 activity in the central nervous system [17, 18] suggesting a more general function of MMP-9 modulating apoptosis in neuronal tissues and in glia-neurons interaction. MMP-9 is considered one of the pharmacological targets in the treatment of glaucoma [15] and other diseases [19–22]. Thus, the knowledge of the mechanisms regulating MMP-9 is important for the understanding of the neuron survival—death pathways and for deciphering the glaucomatous alterations in the retina and optic nerve.

Here we depict new *cis*-elements in the 3′-untranslated region (3′-UTR) of the MMP-9 gene and identify miRNAs which may regulate MMP-9 expression. The regulatory role of one of them, miR340, was confirmed using inhibitors and mimic both in the reporter assay and by their effect on MMP-9 expression.

Materials and methods

Cell cultures

Culture of A7 astrocytes from the rat optic nerve The culture of immortalized optic nerve astrocytes from newborn rats was a generous gift of Dr. Herbert Geller (NHLBI, NIH, Bethesda, MD). The cells were grown as described previously [23] in DMEM with 2 mM glutamine, 10% FBS, 4.5 g/l glucose in the presence of penicillin/streptomycin (final concentration of penicillin 100 IU/ml, streptomycin 100 µg/ml). The cells

expressed an astrocyte-specific marker, glial fibrillary acidic protein.

Human embryonic kidney 293 T cells (HEK 293T) were purchased from American Type Culture Collection (ATCC), cultivated in ATCC-formulated Dulbecco's modified Eagle's medium (30-2002) supplemented with fetal bovine serum to a final concentration of 10% as recommended by the manufacturer.

Isolation of RNA and reverse transcription Total RNA was isolated from A7 or 293 T cells and converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, CA). cDNA was used as a template for amplification in GC-Rich polymerase chain reaction (PCR) System (Roche Applied Science, Indianapolis, IN). Amplification products were digested by corresponding restriction enzymes and purified using Microcon PCR system (Millipore, Bedford, MA).

Generation of expression vector and transfection pGL3-control vectors were digested by the same restriction enzymes as PCR products and ligated using Rapid DNA Ligation Kit (Roche Applied Science). Transformation in XL1-Blue Gold cells from Stratagene was done according to the manufacturer's recommendations. DNA was isolated from individual colonies by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the sequences of inserted fragments were confirmed by sequencing analysis. For A7 and 293 T cells FuGENE-HD (Roche Applied Science, Indianapolis, IN) was used as a transfection agent; 500 ng of a target pGL3 vector with different inserts and 14 ng of pVL-SV40 vector were used for transfection of cells split into 24-well plate. After 48 h, the cells were washed by PBS and suspended into 100 µl of 1×PBL buffer, supplied by Dual-Luciferase Reporter Assay (Promega, Madison, WI). The Synergy HT Multi-Detection Microplate Reader was used for the detection of luciferase activity.

Site-directed mutagenesis and dual-luciferase assay A 446 bp fragment of 3′-untranslated region of MMP-9 (UTR446) generated by PCR was inserted into the proximal or distal site of vector pGL3 (Fig. 1b) using corresponding primers (Table 1, I). Truncated versions of this fragment were generated using “Site-directed mutagenesis kit” (Stratagene, La Jolla, CA). The sequences of primers used for mutagenesis are shown in Table 1, IV. The effect of this fragment on the efficiency of the luciferase gene transcription was analyzed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as described elsewhere [24]. To normalize the firefly luciferase reporter gene pGL3 the cells were cotransfected with a reporter pRL-SV40 vector used as

Fig. 1 a Nucleotide sequence of the rat 3'-UTR of MMP-9 gene downstream of the stop codon (3'-UTR446). Polyadenylation signal AATAAA (arrow) and AT-rich regulatory elements ATTTA (ARE1 and ARE2) are *underlined*. Putative miRNA targets for miR328 and miR132 are shown in *small letters*. Putative miRNA targets for miR340, miR130b, and miR540 are *underlined*. **b** pGL3-Control Vector Map showing the sites where 3'-UTR446 was inserted in distal (dist) and proximal (prox) position. MCS multiple cloning site. **c** Alignment of nucleotide sequences of MMP-9 3'-UTR corresponding to the targets for mi340 from several mammalian species. A high level of conservation suggests a functional role for these sequences

an internal control. This vector contains the cDNA encoding Renilla luciferase (Rluc) and provides constitutive expression of this protein. The ratio of firefly to Renilla luminescent (F/R) was calculated and expressed in percentage of control values.

MMP-9 promoter driven expression vector pGL3-basic vector in which SV-40 promoter was replaced for human 5'-MMP-9 promoter was a kind gift of Dr. D. Boyd (M.D. Anderson Cancer Center, Houston, TX). Using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) we deleted XbaI site present in the promoter area at position -1502

and used such DNA as a template for the amplification with primers shown in Table 1, V.

Quantification of firefly mRNA by quantitative reverse transcriptase PCR (qRT-PCR) using TaqMan assay

RNA isolation RNA was isolated from 4×10^6 A7 astrocytes and 293 T cells, using the spin column method of an RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and stored at -80°C . The isolated RNA was quantified by spectrophotometry

Table 1 Nucleotide sequences of putative regulatory elements, their modified forms and primers used for their generation**I. Primers used for generation of UTR446**

For insertion into the proximal position of pGL3 vector

F: 5'- AACTAGTACTAAGGCTCCTCTTTT- 3'

R: 5'- TACTAGTACGCACAGTAAGCATTCTAT -3'

For the insertion in the distal position of pGL3

F: 5'- TGATATCACAAGGCCTATTCTGCCAT- 3'

R: 5'- TGTCGACACGCACAGTAAGCATTCTAT -3'

II. Sequences of t340p, its fragments and primers used to generate them

Sequence of t340

ACTAGGAAGGAGTGGAGGCGGG

Primers used for t340 ligation into pGL-3 control vector

F : 5'- CGCCGTGTAATTCTACTAGGAAGGAGTGGAGGCGGGAGAGTCGGGGCGGC-3'

C: 5'- GCCGCCCCGACTCTCCCGCTCCACTCCTTCCTAGTAGAATTACACGGCG -3'

III. Sequences of the truncated forms of t340 and primers used for their generation

Sequence of UTR-6: AGGAAGGAGTGGAGGC

F: 5'-TGATCTCTTCTAGAGACTGGGCAGGGCCCTCTC-3'

C: 5'-GAGAGGGCCCTGCCAGTCTCTAGAAGAGATCA-3'

Sequence of UTR-12: 5'-AAGGAGTGA-3'

F: 5'-CTCTTCTAGAGACTAGGGGCGGGCAGGGCCCT-3'

C: 5'-AGGGCCCTGCCCGCCCTAGTCTCTAGAAGAGA-3'

Sequence of UTR-19: 5'-GAG-3'

F: 5'-TTCTAGAGACTAGGAAGTGGAGGCGGGCAGGGC-3'

C:5'-GCCCTGCCCGCCTCCACTTCCTAGTCTCTAGAA-3'

IV. Primers used for in vitro mutagenesis of the 3'-UTR

Primers used to delete ARE1

F : 5'- CCTTACCGGCCCTTTTTATGTATGTGGTCATGTTACACAC -3'

C: 5'- GTGTGAACATGACCACATACATAAAAAAGGGCCGTAAGG-3'

Primers used to delete ARE2

F: 5'- TGGTCATGTTACACACATGTACCTATAGAATGCTTAC -3'

C: 5'- GTAAGCATTCTATAGGTACATGTGTGTGAACATGACCA -3'

V. Primers used to alter a XbaI site to BspH1 site in MMP-9 promoter

F: 5'- CATGTCTGCTGTTTTTCATGAGGCTGCTACTGTC-3'

C: 5'- GACAGTAGCAGCCTCATGAAAACAGCAGACATG-3'

F forward primer, *R* reversed primer, *C* complement primer used for mutagenesis

metric absorbance assuming that 1 absorbance unit at 260 nm in 10 mM Tris-HCl, pH 7.5 corresponds to an RNA concentration 44 µg/ml. Only samples with an A260/A280 ratio higher than 1.9 were used. The integrity of RNA was checked by denaturing 1.2% agarose gel electrophoresis in the presence of formaldehyde. rRNAs appeared as sharp bands, and the ratio of 28S rRNA to 18S rRNA quantified under UV light with Kodak Image Station 440CF (Eastman Kodak Co., Rochester, NY) was 2:1. The RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, CA).

Relative quantification of luciferase RNA by qRT-PCR Relative quantification was conducted in a two step qRT-PCR protocol using TaqMan starter kit reagents, Quantitative

Real-Time PCR kits, and a 7300 series instrument from Applied Biosystems. The probe for a junction between exon 6 and exon 7 of the firefly luciferase gene TACGTCGCCAGTCAAGTAAC was used for an assay developed by Applied Biosystems. As an endogenous control we used Rn00667869-m1 for β-actin (Applied Biosystems). Reverse transcription was carried out as described above. A minus RT control containing all the reaction components except the reverse transcriptase was included in all qRT-PCR experiments to test genomic DNA contamination. Amplification was performed using TaqMan Universal PCR master mix (Applied Biosystems) under the following conditions: 2 min at 50°C (hold), 10 min at 95°C (hold); 15 s at 95°C, 45 cycles; 1 min 60°C (hold).

Bioinformatic analysis of the 3'-UTR Putative targets for miRNA in UTR446 were searched using microRNA. sanger.ac.uk website.

Relative quantification of the microRNA expression RNA samples were digested with RNase-Free DNase I for 30 min at 37°C. Low molecular weight (LMW) RNA (~0–200 nucleotides) was purified from total RNA by size fractionation on YM-100 ultra filtration columns (Millipore) and further purified on RNeasy MinElute (Qiagen, Valencia, CA) columns using a small RNA Protocol. A pool of RNA from twenty different human tissues – Human total RNA pool (Ambion, Austin, TX) or LMW RNA (Rat LMW RNA pool) prepared from a pool of ten different rat tissue RNA samples (Ambion, Austin, TX) was used as a positive control for validation of the TaqMan® miRNA assays. All TaqMan® miRNA assays selected for this study were expected to work in rat samples. TaqMan® miRNA assays for microRNAs, miR-298, miR-384-3p, miR-483, and miR-672 were not expected to work in human samples based on the target microRNA sequence. The LMW RNA samples were reverse transcribed with miRNA-specific RT primers for miR-130b, miR-132, miR-185, miR-212, miR-296, miR-298, miR-328, miR-340, miR-384-3p, miR-483, miR-672, and miR-877 individually using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). The cDNA was amplified by qRT-PCR using TaqMan® Universal PCR Master Mix (Part Number: 4304437) and miRNA-specific TaqMan® probes (Applied Biosystems) according to the manufacturer's protocol on StepOnePlus™ Real-Time PCR System (Applied Biosystems). All reactions were analyzed by computing the ΔC_t (C_t for all samples (sample C_t)). Relative quantity of the microRNA expression was computed using the formula, Relative Quantity (RQ) = $2^{-\Delta C_t}$ and plotted for the rat A7 astrocytes and Human 293 T cells.

miRNA inhibitors and mimics

Targeting and negative control miRIDIAN miRNA inhibitors and mimics were designed and synthesized by Thermo Fisher Scientific (Dharmacon Products, Lafayette, CO). The following miRNA miRIDIAN hairpin inhibitors containing flanking hairpin structures were used. For human miRNAs: for hsa-miR-130B—MIMAT0004680, for hsa-miR-340—MIMAT0000750; for hsa-miR-132—MIMAT0004594. We used MIMAT0000564 as inhibitor for rno-miR-328. MiRIDIAN microRNA inhibitor positive control—IP-004000-01-05. MiRIDIAN microRNA hairpin inhibitor negative control—IN-001005-01-05.

MiRIDIAN miRNA mimics are synthetic duplexes representing mature miRNAs (<http://microrna.sanger.ac.uk>). The

following miRIDIAN mimics were used (Thermo Scientific Dharmacon). Mimics for rat miRNAs: miRIDIAN rno-miR-340-p—MI0000622; miRIDIAN microRNA mimic housekeeping positive control (GAPD)—CP-003000-02-05; miRIDIAN microRNA mimic negative control—CV-001000-01-05.

For transfection of A7 astrocytes the cells were split in 96-well plate and cotransfected with 120 ng DNA/well together with inhibitors (0.1–10 nM) and mimics (20–400 nM). DharmaFECT (0.25 μ l/well) Dual Transfection Reagent T-2010-01 was used as recommended by manufacturer (Thermo Scientific Dharmacon). Cells were grown for 72 h at 37°C and harvested for luciferase assay.

Statistical analysis

At least three sets of transfection were performed for each experiment. A paired Student's *t* test was used to assess significant differences between groups.

Western blotting and zymography

A7 cells were split into 24×well plate and incubated overnight. After this the cells were treated by different concentrations of mimic rno-miR-340-3p in the presence of DharmaFECT in serum-free media. After 72 h the media was withdrawn and concentrated ten times using the Microcon centrifugal Filter Devices (Amicon). The cells were harvested in BD-lysis buffer (BD-Transduction Lab) containing protease cocktail (Roche Applied Science, Indianapolis, IN). To all samples SB containing β -mercaptoethanol was added, the samples were boiled and loaded on 8% PAGE. Polyclonal antirabbit antibody against human MMP-9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-6841-R) was diluted 1:250. Antirabbit HRP conjugated at 1:40,000 was used as a secondary antibody with dilution 1:40,000. The proteins were visualized on the film and scanned using Kodak Imaging Systems (Rochester, NY). The relative quantification of the bands was carried out using Kodak Molecular Imaging Software. MMP-9 activity was determined after electrophoresis of cell extracts in 10% SDS-PAGE with 0.1% gelatin.

Results

3'-UTR of MMP-9 contains regulatory elements inhibiting gene expression

To identify regulatory elements in a 3'-UTR of MMP-9 involved in the modulation of its expression we used pGL3 Luciferase Reporter Vectors which provide a basis for the quantitative analysis of factors regulating mammalian gene

expression. We inserted the 446 bp long 3'-UTR fragment (UTR446) (Fig. 1a) located directly downstream of the stop codon into two sites of pGL3 vector (Fig. 1b). One of them, proximal site is located immediately downstream of Luc gene (Fig. 1b, prox), the other is the distal site located at the border of SV40 enhancer (Fig. 1b, dist). The insertion of the UTR446 in the proximal site significantly reduced SV40 promoter driven luciferase activity to $40 \pm 6\%$ and $28.1 \pm 6\%$ in A7 and 293 T cells, respectively (Fig. 2a). The UTR446 fragment inserted into distal region of the expression vector pGL3 downstream of the SV-40 enhancer (Fig. 1B) decreased Luc expression in a lesser extent, down to 60.8 ± 8 and $56 \pm 6\%$ in A7 and 293 T cells, respectively (Fig. 2a, distal). The insertion of UTR446 in both proximal and distal sites of the vector did not increase the inhibitory effect (Fig. 2a, both). Since the insertion of UTR446 fragment in a

proximal position possesses a stronger effect on the expression level than the insertion in a distal site, we used in future experiments only the construct with the putative regulatory element in the proximal site. The UTR446 fragment contains previously described AU-rich *cis*-regulatory elements (ARE, Fig. 1a) which target host mRNAs towards rapid degradation and therefore may affect the efficiency of MMP-9 expression [25, 26]. We then asked whether UTR446 inhibitory properties that we observed could be explained exclusively by ARE inhibitory activity.

Effect of ARE1 and ARE2 deletion on the inhibitory activity of UTR446

Figure 2b shows that the deletion of ARE1 or ARE2 (-ARE1 and -ARE2, respectively) only slightly changes the

Fig. 2 Effect of the 3'-UTR fragment of the MMP-9 gene and its fragments on the efficiency of the reporter luciferase gene expression. The UTR446 shown in Fig. 1a was amplified using cDNA as a template. The forward primer was directly upstream of the stop codon and the reverse primer 446 bp downstream of the stop codon (primer's sequences are presented in Table 1, I). All constructions were inserted in *E. coli*, and transfected into A7 (gray bars) or 293 T cells (black bars). Luc activity was determined using Dual-Luciferase Reporter Assay System (Promega). Y axis ratio of Firefly to Renilla luminescence was first calculated in arbitrary units and then expressed in percent. The expression in a–c is driven by SV40 promoter, in d by MMP-9 promoter. In b–d the putative regulatory sequences were inserted in a proximal position. Columns represent the mean of at least three individual experiments; bars are SD. a The UTR446 was inserted into proximal, distal and in both positions of pGL3 Control vector. b The effect of deletion of ARE1 (-ARE1) and ARE2 (-ARE2)

fragments from UTR446 on its inhibitory activity. All fragments were inserted into proximal position. pGL3 control; +UTR indicates that the UTR446 is inserted; -ARE1 signifies that the UTR446 with deleted ARE1 is inserted; -ARE2 indicates that UTR446 with deleted ARE2 is inserted. c The effect of truncated forms of UTR446 on Luc expression. The following fragments have been inserted using primers shown in Table 1, I. +UTR indicates that 3'-UTR446 has been inserted; +t340 signifies that putative target for mi340 has been inserted; UTR-6 means that UTR446 with deleted six nucleotides has been inserted; UTR-12 means that UTR446 with deleted 12 nucleotides has been inserted; UTR-19 means that UTR446 with deleted 19 nucleotides has been used. d Luc expression driven by the basic pGL3 vector with human MMP-9 promoter 2.2 kb fragment. pGL3 control value of Luc expression; +t340 putative target for miR340 has been inserted in a proximal position

inhibitory effect of UTR446 in a cell-specific manner. Deletion of the ARE1 (-ARE1, Fig. 2b) reduced inhibitory effect of UTR446 in 293 T cells (control UTR446 inhibits Luc activity to $28.1 \pm 6\%$, UTR with deleted ARE1 inhibits to $58.3 \pm 7\%$ activity), but did not change it in A7 astrocytes. At the same time, deletion of ARE2 element (-ARE2, Fig. 2b) reduced the inhibitory effect in A7 astrocytes ($40.0 \pm 6\%$ Luc activity with intact UTR446, and $66.1 \pm 5\%$ activity with UTR446 after deletion of ARE2). To the contrary, deletion of ARE2 almost did not change it in 293 T cells (Fig. 2b). Thus, deletion of ARE1 and ARE2 either does not change the inhibitory effect of UTR446 or even reduces it. These data suggest that in addition to these ARE elements other *cis*-regulatory elements are present in UTR446 which may be responsible for its inhibitory effect.

Analysis of regulatory elements in UTR446 by bioinformatics methods

Bioinformatics analysis of the rat MMP-9 3'-UTR by prediction algorithms [27] miRbase (<http://microrna.sanger.ac.uk>) and miRanda <http://www.microrna.org/microRNA/home.do>) revealed regions containing putative targets for microRNA (miRNA) that could control the level of its expression. Putative

targets for miRNAs (t340, t328, t130b, and t132) are underlined or shown in small letters in Fig. 1a and their predicted binding is shown in Fig. 3. Alignments of putative targets for mi340 (t340) from several mammalian species showed a high conservation of these sequences (Fig. 1c). [28, 29]

To reveal the effect of these putative miRNA targets on the gene expression we used the pGL3 Luciferase Reporter Vectors. The effect of these putative miRNA targets on the efficiency of gene expression was monitored by determining how they affect the luciferase activity after transfection of pGL3 based constructs in astrocyte A7 culture and HEK293T cells (Fig. 2c, d).

Effect of the putative mi340 target (t340) on the gene expression in SV-40 driven Luc reporter system

Insertion of the target for miRNA340 (t340) in the proximal site of the reporter vector caused a strong inhibition of the Luc expression in a reporter system. In A7 cells the Luc activity was reduced to $17.8 \pm 6.1\%$, in 293 T cells—to $40.6 \pm 5.9\%$ compared with control values (Fig. 2 C, +t340). Thus, insertion of the t340 inhibited Luc expression in A7 cells even more efficiently, than the insertion of the whole UTR-446, while in 293 T cells t340 also caused significant inhibition. So next we investigated what part of the t340 fragment is responsible for the inhibitory effect of UTR-446. The sequences of the truncated forms of t340 (UTR-6, UTR-12, and UTR-19) used in further experiments are shown in Table 1, III. The data presented in Fig. 2c show that the deletion of 19 bp (UTR-19) and 6 bp (UTR-6) caused almost complete inhibition of Luc expression, whereas after deletion of 12 bp (UTR-12) the expression was decreased to $39.6 \pm 5\%$ and $35.0 \pm 6.9\%$ in A7 and 293 T cells, respectively. Thus the deletion of 12 bp practically did not change the level of inhibition of the UTR446 (compare bars for +UTR and UTR-12 in Fig. 2c).

Effect of t340 on the MMP-9 promoter driven Luc expression

We then analyzed the effect of t340 on Luc expression driven by human MMP-9 promoter. Luc expression under this promoter in rat A7 astrocytes was undetectable, presumably because of the species specificity of the transcription machinery (Fig. 2d, gray bars). In human 293 T cells we observed inhibition of Luc activity by t340 to $63.1 \pm 5.2\%$ compared to control (Fig. 2d, black bars). Thus, t340 inhibited MMP-9 promoter driven Luc expression similar to SV40 promoter driven expression, although the inhibitory effect with MMP-9 promoter was lower than that found with SV-40 promoter (63.1 ± 5.2 and $40.6 \pm 5.9\%$, respectively) (Fig. 2c, +t340 and Fig. 2d, +t340, black bars).

Fig. 3 The predicted binding sites of rat miRNAs (blue) 340-3p, 328, 130b, and 132 in the 3'-UTR of MMP-9 mRNA identified with miRanda algorithm [28, 29]. The number below the green bar indicates the position relative to the UGA stop codon of the MMP-9 mRNA (accession number 031055)

Quantification of firefly mRNA by qRT-PCR using TaqMan assay

miRNA binding to targets in 3'UTR of the genes regulates gene expression through either translational attenuation or mRNA degradation. On the next step we asked whether the alterations of luciferase activity that we have observed are associated with the changes in mRNA level. For this purpose we isolated total RNA from transfected cell cultures and quantified luciferase mRNA using qRT-PCR with firefly luciferase gene-specific probes. The changes of luciferase mRNA level measured by this method are in a good agreement with the alterations of the luciferase activity that we have observed in response to the insertion of different regulatory elements (Fig. 2c, d). UTR reduced the level of luciferase mRNA to $42.7 \pm 4.05\%$, t340—to $20.3 \pm 3.1\%$ when SV-40 promoter driven expression was measured and to $62.1 \pm 4.2\%$ in MMP-9 promoter driven expression (Fig. 4). These results confirm that changes of luciferase activity are associated with the alterations of the corresponding mRNA level.

Identification of miRNA in cell extracts by TaqMan miRNA assays

The results of analysis of relative miRNA expression are presented in Fig. 5 in duplicate for each cell line. This analysis shows that the relative expression of miRNA differs significantly in two cell cultures. Differential miRNA expression is seen between A7 rat cells and 293 T human cells for miR-130b, miR-132, miR-212, miR-298, miR-328, miR-340, and miR-877. miR-483 was not detected in either of the cell lines, whereas miR-298, miR-384-3p, and miR-672 were undetectable in human 293 T cells as expected due to species specificity

Fig. 4 Quantification of luciferase firefly mRNA by qRT-PCR using Taqman assay. The quantity of luciferase mRNA in A7 cells transfected by pGL3 control vector with inserted UTR or t340 fragments was compared with the quantity in cells transfected with intact pGL3 vector which was considered to be 100%. Y axis as in Fig. 1. Columns represent the mean of at least three individual experiments; bars are SD

of the TaqMan miRNA assays. Functional validity of all TaqMan miRNA assays were confirmed using Human total RNA pool or Rat tissue LMW RNA pool as positive control (not shown).

Effect of inhibitors and mimics

To further characterize the functional properties of miRNAs identified in these cell cultures we have selected miR340 and used for this purposes corresponding miRNA mimics and inhibitors. Since mimics behave like endogenous miRNAs they can potentially increase the activity of corresponding miRNAs, whereas inhibitors of miRNAs usually possess an opposite effect.

To determine the effects of miRNA mimics and inhibitors on gene expression we used the pGL3 control plasmid with inserted 3'-UTR446 together with pRL-SV40 plasmid Luc reporter construct. The reporter plasmids bearing the UTR446 were cotransfected with different concentration of mimic-mir340p or inhibitor miR340 and the luciferase activity was measured using the Dual-Light luciferase and Renila reporter gene assay system at 72 h after transfection. Transfection of A7 cells with mimic rno-340-3p caused a dose-dependent reduction of the luciferase activity (Fig. 6a, left part). The highest level of inhibition by rno-340-3p was observed at concentration 400 nM (Fig. 6a, bar 5). On the other hand, inhibitor of mi340 increased luciferase activity compared to the cells transfected by the negative control (Fig. 6 right panel, bar 1).

Importantly, the level of MMP-9 measured by Western blotting in A7 cells transfected with different concentration of mimic rno-340-3p is significantly decreased (Fig. 6b). In gel activity, determination with gelatin (zymography) showed similar reduction of MMP-9 band corresponding to the molecular weight 92 kDa in the presence of mimic rno-340-3p (not shown). These results demonstrate that this mimic is an effective inhibitor not only in a model system, but in cell expressing MMP-9.

Discussion

In this study we describe new regulatory elements implicated in MMP-9 expression. We found targets for miRNA in the 3'-UTR of MMP-9 and identified miRNAs which can modulate its expression.

MMP-9 is secreted in the body in a latent form and upon activation acts on many inflammatory substrates, contributing to the progression of many human disorders, including glaucoma and other retinal disorders [14, 30, 31]. In the earlier studies regulation of MMP-9 protein levels has been largely ascribed to transcriptional activation of the gene [32].

Fig. 5 Relative expression of microRNAs in A7 astrocytes and T293 cells are shown in duplicate for each point. Functional validity of all Taqman miRNA assays were confirmed using Human total RNA pool or Rat tissue LMW RNA pool as positive control (not shown). Differential microRNA expression is seen between A7 cells and 293 T cells for miR-130b, miR-132, miR-212, miR-296, miR-328, miR-340, and miR-877

Recent studies demonstrated that MMP-9 regulation is not limited by transcriptional level. In addition to transcriptional events, MMP-9 gene expression is regulated by post-transcriptional events, such as those controlling mRNA turnover and translation [32–35]. However, detailed molecular mechanisms regulating MMP-9 expression via 3'-UTR needs to be elucidated.

We demonstrate here that 3'-UTR of MMP-9 possesses new regulatory properties and contains targets for miRNA. Eberhardt and coauthors were the first who revealed the role of *cis*-regulatory AU-rich elements (ARE) in the 3'-untranslated region of MMP-9 mRNA in the regulation of mRNA stability. Their studies showed that mRNA-stabilizing factor HuR and the presence of NO regulated

Fig. 6 Effect of mi340 mimics and inhibitors on the efficiency of expression. **a** Target validation of miRNA340 by Luc assay using mimics and inhibitors. The A7 astrocytes cell line was transfected with 20–400 nM of mimic rno-340-3p (*left panel*, mimics) or 1 nM of anti-miR340 (*right panel*, inhibitors). The cells were cotransfected for 72 h with Luc reporter constructs bearing a 3'-UTR446 target sequence and assayed for luciferase activity. *Left panel* 1 pGL3 with UTR446; 2 the same as 1 +20 nM mimic-340; 3 the same as 1 + 100 nM mimic-340; 4 the same as 1 + 200 nM mimic-340; 5 the same as 1 + 400 nM mimic-340; 6 mimic negative control. *Right panel* 1

pGL3 with UTR446 + 1 nM of the mi340 inhibitor; 2 inhibitor negative control. The use of mimics and inhibitors of mi340 showed that the luciferase activity of cells transfected with mimic was decreased and that transfected with anti-miRNA340 was increased. *Y* axis is as in Fig. 2. Columns represent the mean of at least three individual experiments; *bars* are SD. These results confirm that mi340 is able to bind to its target in the 3'-UTR446. **B.** Western blotting of A7 cells transfected by mimic miR340. 1 Control sample, 2 200 nM of mimic-340; 3 400 nM of mimic-340. *Arrow* corresponds to 92 kDa protein. **c** Scan of the Western blot shown on **b**

MMP-9 mRNA stability [26, 35]. In addition to AREs, we found other types of regulatory elements in the 3'-UTR, i.e., targets for miRNAs affecting efficiency of gene expression. Insertions of UTR446 or only a target for mi340 (t340) in reporter constructs were associated with a significantly decreased expression of luciferase in A7 astrocytes and 293 T cells. An important role in the MMP-9 regulation by 3'-UTR region is played by the elements located upstream of the AREs. A 22 nucleotides long regulatory element t340 located 109 nucleotides downstream of the stop codon and 44 nucleotides upstream of the polyadenylation signal plays a significant role in the regulation of MMP-9 expression. This fragment is a binding site for miRNA 340p, which is expressed in the optic nerve astrocytes A7 culture. The insertion of t340 in the proximal site of the reporter vector pGL3 causes significant inhibition of Luc expression driven by SV-40 or MMP-9 promoter. Deletion mutagenesis showed that elimination of several base pairs from the UTR may significantly increase the inhibitory potential of the fragment. Surprisingly, UTR-12 has less effect on Luc expression than either UTR-6 or UTR-19 (Fig. 2c). This result most probably can be explained by the changes in the stem-loop structure of the mRNA 3'-end affecting exposed nucleotide sequences which serve as a target for miRNAs.

The results of qRT-PCR with probe specific for firefly luciferase demonstrated a good correlation between the changes in the luciferase activity observed in a Luc reporter system and the level of corresponding mRNA. This suggests that the regulatory element t340 affects gene expression via binding with corresponding miRNAs which changes mRNA level (most probably by the destabilization of the target mRNA), but not translational inhibition. The experiments with inhibitors and mimics confirmed the ability of mi340p to bind to 3'-UTR and regulate gene expression. MiRIDIAN mimics contain chemical modifications incorporated into a given strand of the molecule to ensure that the mature (targeting) miRNA strand is preferentially loaded into RNA-induced silencing complex (RISC). Mimic340 caused a dose-dependent inhibition of gene expression, while mi340-specific inhibitor increases the level of gene expression validating the role of mi340 as a specific regulator which acts 3'-UTR area.

According to the qRT-PCR analysis, A7 astrocytes contain lower amount of mir340, than 293 cells (Fig. 5), however, the inhibition by t340 is greater in astrocytes compared with 293 cells (Fig. 2c). This may be explained by the difference in the concentration of target mRNAs in these cells, changing the ratio between t340 and target or the existence of other target mRNAs with higher affinity to t340 in 293 cells which preferentially bind this miRNA. Alternatively, this discrepancy may be explained by the existence of miRNA modifiers in one of these cells.

MMP-9 degrades ECM, thus contributing to neuronal degeneration by a form of apoptosis known as anoikis. Therefore this enzyme is considered one of the pharmacological targets in the treatment of glaucoma [15, 36, 37] and other diseases. Activation of MMP-9 contributes to RGC death [12], whereas downregulation or inhibition of MMP-9 increases neuronal survival under pathological conditions [10, 38]. The finding that MMP-9 mRNA contains targets for miRNA binding and the identifications of miRNAs which are able to regulate MMP-9 expression open new approaches for the fine tuning of this protein expression.

Among non-coding RNAs, microRNAs belong to one of the best known subgroups, due to their unique function of negatively controlling gene expression, by either degrading target messages or binding to their 3'-untranslated region to inhibit translation. The gene expression can be repressed through post-transcriptional regulation, implemented as a "dimmer switch", in contrast to the all-or-none mode of suppression. Importantly, each miRNA usually binds to multiple targets because the binding of miRNA to the 3'-UTR does not require perfect complementarity. This ambiguity makes it possible for a miRNA to regulate several genes in a pathway [39]. A coordinated action on multiple target genes could ensure a powerful mechanism for a single miRNA to have a strong impact on a regulatory network. Coordinated effect of a miRNA on several ECM genes was recently reported [40].

miRNA target prediction database (<http://www.targetscan.org>) shows that mi-340 binding sites in addition to MMP-9 are present in other genes, including: dystrophin (DMD, cytoskeletal protein which bridges F-actin and the ECM), ephrin-A4 (EFNA4, a member of receptor protein-tyrosine kinases) and catalytic subunit of protein phosphatase 1, β -isoform (PPP1CB, catalytic subunit of serine/threonine protein phosphatase involved in the regulation of a variety of cellular processes). Thus it is conceivable that mi340 is involved in the regulation of ECM structure via its effect on MMP-9, cytoskeletal proteins and biochemical pathways controlled by phosphorylation-dephosphorylation. In a recent study, the role of miRNA340 was associated with ovarian cancer response to cytotoxic agents and inherent resistance to platinum [41].

Application of miRNA in ocular cells and tissue is an emerging area of investigation. Recent evidence shows that AAV-mediated miRNA delivery can efficiently silence specific gene expression in the retina [42]. In another study the transfection of trabecular HTM cells with miR-29b mimic resulted in downregulation of multiple ECM components [43]. Strategies to increase miRNA-340 expression may be beneficial to limit MMP-9 activity, prevent ECM degradation and increase RGC survival.

Conclusion

The mechanism of MMP-9 regulation based on the interaction of miRNAs with 3'-UTR targets may be used as a new tool for modulating MMP-9 activity. Such approach can protect ECM and prevent the development of glaucoma and other neurodegenerative diseases.

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References

- Sommer A. Intraocular pressure and glaucoma. *Am J Ophthalmol*. 1989;107:186–8.
- Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res*. 1995;61:33–44.
- Kerrigan L, Zack D, Quigley H, et al. TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch Ophthalmol*. 1997;115:1031–5.
- Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Investig Ophthalmol Vis Sci*. 1995;36:774–86.
- Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev*. 2000;14:2123–33.
- Agapova OA, Ricard CS, Salvador-Silva M, Hernandez MR. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human optic nerve head astrocytes. *Glia*. 2001;33:205–16.
- Yan X, Tezel G, Wax MB, Edward DP. Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. *Arch Ophthalmol*. 2000;18:666–73.
- Morgan JE. Optic nerve head structure in glaucoma: astrocytes as mediators of axonal damage. *Eye (Lond)*. 2000;14:437–44.
- Hernandez MR. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. *Prog Retin Eye Res*. 2000;19:297–321.
- Chintala SK, Zhang X, Austin JS, Fini ME. Deficiency in matrix metalloproteinase gelatinase B (MMP-9) protects against retinal ganglion cell death after optic nerve ligation. *J Biol Chem*. 2002;277:47461–8.
- Zhang X, Chintala SK. Influence of interleukin-1 beta induction and mitogen-activated protein kinase phosphorylation on optic nerve ligation-induced matrix metalloproteinase-9 activation in the retina. *Exp Eye Res*. 2004;78:849–60.
- Manabe S, Gu Z, Lipton S. Activation of matrix metalloproteinase-9 via neuronal nitric oxide synthase contributes to NMDA-induced retinal ganglion cell death. *Investig Ophthalmol Vis Sci*. 2005;46:4747–53.
- Cong Y, Guo X, Liu X, Cao D, Jia X, Xiao X, et al. Association of the single nucleotide polymorphisms in the extracellular matrix metalloproteinase-9 gene with PACG in southern China. *Mol Vis*. 2009;15:1412–7.
- Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF. Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and iop-induced effects on extracellular matrix. *Investig Ophthalmol Vis Sci*. 2005;46:175–82.
- Mozaffarieh M, Flammer J. Is there more to glaucoma treatment than lowering IOP? *Surv Ophthalmol*. 2007;52:S174–9.
- Golubnitschaja-Labudova O, Liu R, Decker C, Zhu P, Haeffliger IO, Flammer J. Altered gene expression in lymphocytes of patients with normal-tension glaucoma. *Curr Eye Res*. 2000;21:867–76.
- Rivera S, Ogier C, Jourquin J, et al. Gelatinase B and TIMP-1 are regulated in a cell- and time-dependent manner in association with neuronal death and glial reactivity after global forebrain ischemia. *Eur J Neurosci*. 2002;15:19–32.
- Vaillant C, Meissirel C, Mutin M, Belin MF, Lund LR, Thomasset N. MMP-9 deficiency affects axonal outgrowth, migration, and apoptosis in the developing cerebellum. *Mol Cell Neurosci*. 2003;24:395–408.
- Chow AK, Cena J, Schulz R. Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. *Br J Pharmacol*. 2007;152:189–205.
- Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm. doxycycline selectively depletes aortic wall neutrophils and cytotoxic t cells. *Circulation*. 2009;119:2209–16.
- Murosik ME, Roycik MD, Newcomer RG, Van den Steen PE, Opendakker G, Monroe HR, et al. Matrix metalloproteinase-9/gelatinase B is a putative therapeutic target of chronic obstructive pulmonary disease and multiple sclerosis. *Curr Pharm Biotechnol*. 2008;9:34–46.
- Nothnick WB. Novel targets for the treatment of endometriosis. *Expert Opin Ther Targets*. 2004;8:459–71.
- Geller HM, Dubois-Dalcq M. Antigenic and functional characterization of a rat central nervous system-derived cell line immortalized by a retroviral vector. *J Cell Biol*. 1988;107:1977–86.
- Surgucheva I, Surguchov. γ -Synuclein: cell-type specific promoter activity and binding to transcription factors. *J Mol Neurosci*. 2008;35:267–71.
- Barreau C, Paillard L, Osborne HB. AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res*. 2006;33:7138–50.
- Akool el S, Kleinert H, Hamada FM, Abdelwahab MH, Förstermann U, Pfeilschifter J, et al. Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR. *Mol Cell Biol*. 2003;23:4901–16.
- Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005;37:495–500.
- Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res*. 2008;36:D149–53.
- Brennecke J, Stark A, Russell RB, Cohen SM. Principles of micro-RNA-target recognition. *PLoS Biol*. 2005;3:e85.
- Mali RS, Cheng M, Chintala SK. Intravitreal injection of a membrane depolarization agent causes retinal degeneration via matrix metalloproteinase-9. *Investig Ophthalmol Vis Sci*. 2005;46:2125–32.
- Ganea E, Trifan M, Laslo AC, Putina G, Cristescu C. Matrix metalloproteinases: useful and deleterious. *Biochem Soc Trans*. 2007;35:689–91.
- Sivak JM, West-Mays JA, Yee A, Williams T, Fini ME. Transcription factors Pax6 and AP-2alpha interact to coordinate corneal epithelial repair by controlling expression of matrix metalloproteinase gelatinase B. *Mol Cell Biol*. 2004;24:245–57.
- Eberhardt W, Doller A, Akool el S, Pfeilschifter J. Modulation of mRNA stability as a novel therapeutic approach. *Pharmacol Ther*. 2007;114:56–73.
- Fähling M, Steege A, Perlewitz A, Nafz B, Mrowka R, Persson PB, et al. Role of nucleolin in posttranscriptional control of MMP-9 expression. *Biochim Biophys Acta*. 2005;1731:32–40.
- Jespersen C, Doller A, Akool el-S, Bachmann M, Müller R, Gutwein P, et al. Molecular mechanisms of nitric oxide-dependent

- inhibition of TPA-induced matrix metalloproteinase-9 (MMP-9) in MCF-7 cells. *J Cell Physiol.* 2009;219:276–87.
36. Fingleton B. Matrix metalloproteinases as valid clinical targets. *Curr Pharm Des.* 2007;13:333–46.
 37. Seki M, Soussou W, Manabe S, Lipton SA. Protection of retinal ganglion cells by caspase substrate-binding peptide IQACRG from *N-methyl-D-aspartate* receptor-mediated excitotoxicity. *Investig Ophthalmol Vis Sci.* 2010;51:1198–207.
 38. Gu Z, Cui J, Brown S, Fridman R, Mobashery S, Strongin AY, et al. A highly specific inhibitor of matrix metalloproteinase-9 rescues laminin from proteolysis and neurons from apoptosis in transient focal cerebral ischemia. *J Neurosci.* 2005;25:6401–8.
 39. Makeyev EV, Maniatis T. Multilevel regulation of gene expression by microRNAs. *Science.* 2008;319:1789–90.
 40. Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, et al. MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. *Proc Natl Acad Sci USA.* 2008;105:5874–8.
 41. Boren T, Xiong Y, Hakam A, Wenham R, Apte S, Chan G, et al. MicroRNAs and their target messenger RNAs associated with ovarian cancer response to chemotherapy. *Gynecol Oncol.* 2009;113:249–55.
 42. Georgiadis A, Tschernutter M, Bainbridge JW, Robbie SJ, McIntosh J, Nathwani AC, et al. AAV-mediated knockdown of Peripherin-2 in vivo using miRNA-based hairpins. *Gene Ther.* 2010;17:486–93.
 43. 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.