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MicroRNAs that target *RGS5*

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ABSTRACT

Objective(s): An earlier meta-analysis on gene expression data derived from four microarray, two cDNA library, and one SAGE experiment had identified *RGS5* as one of only ten non-housekeeping genes that were reported to be expressed in human trabecular meshwork (TM) cells by all studies. *RGS5* encodes regulator of G-protein signaling-5. The TM tissue is the route of aqueous fluid outflow, and is relevant to the pathology of glaucoma. MicroRNAs constitute the most recently identified components of the cellular machinery for gene regulation in eukaryotic cells. Given our long standing interest in glaucoma, we aimed to identify miRNAs that may target *RGS5*.

Materials and Methods: Eight miRNAs were selected for study using bioinformatics tools and available data on miRNAs expressed in the eye. Their effects were assessed using the dual luciferase assay. 3'-UTR segments of *RGS5* mRNA were cloned downstream of a luciferase coding gene in psiCHECK2 vectors, and these were co-transfected with each of the miRNAs into HEK293 cells.

Results: The outcomes evidenced that one or more of the segments are in fact targeted by miR-7, miR-9, miR-96, miR-23a, miR-23b, miR-204, and miR-211. Down regulations by the miRNAs were statistically significant. The effect of miR-204 is considered particularly important as this miRNA is well known to regulate eye development and to affect multiple ocular functions.

Conclusion: Our results justify further studies on regulation of *RGS5* expression and *RGS5* downstream functions by these miRNAs.

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Introduction

RGS5 encodes regulator of G-protein signaling-5. There are more than 20 regulator of G-protein signaling (RGS) proteins, and *RGS5* is a member of the R4/B subfamily (1-4). RGS proteins are components of G-protein (guanine nucleotide binding protein) coupled receptor (GPCR) complexes, and they act to shorten the duration of signaling resulting from ligand binding to GPCRs by acting as activators of the GTPase activity of the α subunit of G proteins (5). GTPase activation of *RGS5* is apparently specific for the Gαi and Gαq G protein subunits (6, 7). Gαi and Gαq, respectively, can inhibit adenylyl cyclase and activate phospholipase C (8, 9). In addition to G proteins, there is emerging evidence that RGS proteins also interact with other proteins downstream of GPCRs (10).

There is abundant evidence for roles of *RGS5* in vascular and cardiac remodeling and blood pressure homeostasis (11-13). It has been suggested that it is a stimulator of apoptosis of endothelial cells (14). A role for *RGS5* in various cancers has also been reported (15-19). With respect to ocular functions, it was first found that *RGS5* mRNA is up-regulated in hypertensive monkey eyes (20). Subsequently, high expression of *RGS5* mRNA was reported in human ciliary body and trabecular meshwork (TM) tissues and a novel alternative spliced variant of *RGS5* mRNA was identified in human ocular tissues (21). The TM is positioned at the angle formed by the cornea and iris. It is a major component of the conventional outflow pathway of aqueous humor and significantly modulates outflow of this fluid from the anterior chamber to venous blood via Schlemm's canal (22). Increased TM resistance causes decreased outflow and increased intraocular pressure, the latter being a major risk factor for glaucoma (23). Our long standing interest in glaucoma prompted a study aimed at identification of non-housekeeping genes expressed in human TM cells (24). A meta-analysis on TM gene expression data derived from four microarray, two cDNA library, and one SAGE (serial analysis of gene expression) experiment identified only ten non-housekeeping genes that

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were reported to be expressed in the human TM by all studies (24-30). Their identification in all studies was taken to mean that these are highly and consistently expressed non-housekeeping genes in the human TM (24). *RGS5* was one of these ten genes.

MicroRNAs (miRNAs), which are small (~22 nucleotides) single stranded non-coding RNAs, are now considered major components of the cellular machinery for gene expression regulation in multicellular eukaryotes (31, 32). They have important roles in various biological processes, including development, cell growth, cell signaling, and apoptosis (33). MiRNAs have been implicated in the etiology of various diseases (34-36). They act at the post-transcriptional level, by promoting mRNA degradation or by having inhibitory effects on translation, and they usually exert fine tuning regulatory effects; on average, they affect a two-fold decrease in production of target proteins (37-39). Each miRNA can have multiple mRNA targets, and the expression of up to 60% of human genes is thought to be affected by miRNAs (40, 41). The most critical factor in miRNA targeting is complementary pairing of sequences within the 3' untranslated (3'-UTR) regions of mRNAs with a seven nucleotide seed region within the miRNAs (41, 42). Various bioinformatics tools use this feature and more subtle criteria to predict miRNAs that may target specific genes and to predict genes that an miRNA of interest may target (43, 44). MiRNA expression and the genes and pathways they affect in ocular tissues have now been investigated in several studies (45-49). We recently reported the effect of miR-204 on the expression of *FOXC1* and other genes in human TM cells (50). In a preliminary effort to gain a more complete understanding of aspects of *RGS5* expression in ocular tissues, we here aimed to identify miRNAs which may target *RGS5* mRNAs.

Materials and Methods

In silico analysis for miRNA selection

Candidate miRNAs to be tested for targeting *RGS5* mRNA were selected in three steps. Initially, MiRWalk was used to identify miRNAs with predicted binding sites within the mRNA's 3'-UTR segment ((51); http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/). This *in silico* bioinformatics tool relies on information accrued from predictions made by nine (RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PITA, PICTAR4, PICTAR5, and Diana-microT) established programs for binding of miRNAs to 3'-UTRs of all known human genes. MiRWalk ultimately reports predictions made by ten algorithms, including its own algorithm and those of the nine programs specified above. MiRNAs reported by at least four programs were further considered. Among the miRNAs thus identified, those whose expression in the eye had been experimentally validated were chosen at the second level of selection. Expression in the eye was assessed on the basis of information available at MiRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and review of the literature (47). Finally, seven miRNAs with high prediction scores were chosen to be tested for experimental verification of targeting the 3'-UTR segment of *RGS5* mRNA by the dual luciferase assay. An eighth miRNA that is a family member of the seven selected miRNAs was also tested. MiRNAs were purchased either as cloned sequences within pCMV-MIR vectors (OriGene, Rockville, MD, USA) or as un-cloned miRs (Ambion, Austin, TX, USA). The miRNAs in the pCMV vector are expressed under the constitutive cytomegalovirus promoter. Empty pCMV vector and a scrambled mimic miRNA were used as negative controls for assessing the effects, respectively, of the cloned and un-cloned miRNAs that were tested. Scrambled mimic miRNA does not produce identifiable effects on known human mRNA functions (reference for SNORD; http://www.invitrogen.com/1/1/11216-mir-vana-mirna-mimic-negative-control-1.html).

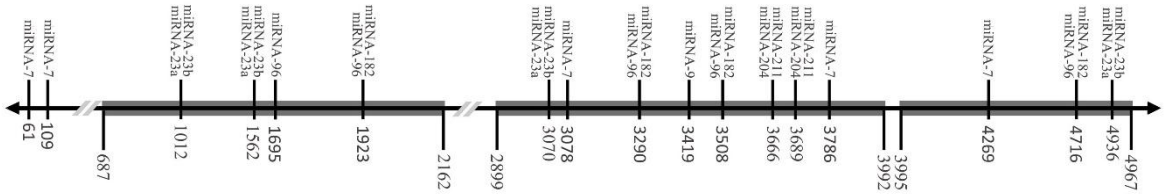
Preparation of psiCHECK2 constructs containing 3'-UTR sequences

The 3'-UTR segment of *RGS5* mRNA consists of 5038 nucleotides (NM_003617). To facilitate cloning, genomic DNA encoding the 3'-UTR segment was amplified by PCR in three shorter amplicons, *RGS5*-1 (nucleotides 687-2162), *RGS5*-2 (2899-3992), and *RGS5*-3 (3995-4967). Forward and reverse primers for all amplicons were designed to include, respectively, Xho1 and Not1 restriction enzyme recognition sites. The three fragments collectively contained almost all recognition sites predicted by the MiRWalk analysis (Figure 1A). Each fragment was cloned downstream of the renilla luciferase reporter gene, directly 3' to its amino acid coding region, in psiCHECK2 to create vectors psiCHECK2-*RGS5*-1, psiCHECK2-*RGS5*-2, and psiCHECK2-*RGS5*-3 (Promega Corporation, Madison, WI, USA). The psiCHECK2 vector, in addition to the renilla gene which is expressed under the T7 promoter, contains the firefly luciferase gene adjacent to the HSV-TK promoter. For verification, the cloned fragments were sequenced by the dideoxynucleotide termination protocol. The sequences of primers used for the cloning reactions are presented in Table 1.

Plasmid transfections and dual luciferase assays

The effects of the miRNAs were assessed in Human Embryonic Kidney (Hek)-293 cells (National Institute of Genetic Engineering, Tehran, Iran). Empty or recombinant psiCHECK2 vectors (250 ng) were co-transfected in 24-well plates (2 x 10^5 cells/well) with cloned miRNAs (250 ng pCMV-MIR DNA (OriGene; miR-7: Cat # SC400648, miR-9: Z Cat

A:



B:

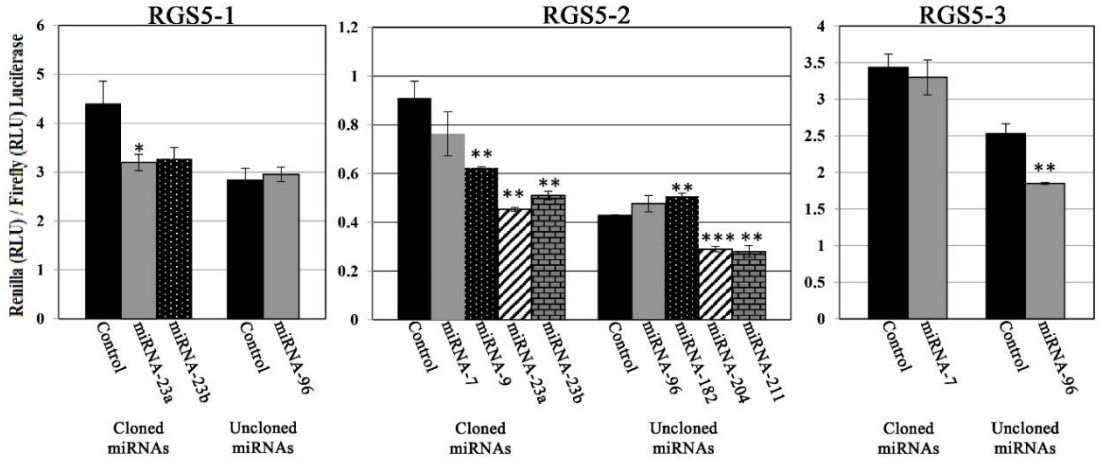


Figure 1. Dual luciferase assay. A- Distribution of predicted target sites of miRNAs on 3'-UTR of GRS5. Nucleotide positions are with reference to the translation termination codon, and the position of the first nucleotide after that codon is considered +1. The thick lines represent *RGS5*-1, *RGS5*-2, and *RGS5*-3 that were cloned; the nucleotide positions at the termini of the fragments are shown. B- Dual luciferase assay results in HEK-293 cells. Standard deviations based on three replicative transfection experiments are shown. *indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$ for comparison of renilla/firefly luciferase ratios between control transfections and transfections with miRNAs. RLU, relative light units

SC400676, miR-23a: Cat # SC400294, or miR-23b: Cat # SC400295)) or with un-cloned miRNAs (final concentration of 25 nM (Ambion; miR-96: PM10422, miR-182: PM12369, miR-204: PM11116, or miR-211: PM10168)) using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA, USA). Each transfection reaction was performed in triplicate. Forty eight hours after transfections, renilla and firefly luciferase activities were measured using dual luciferase assays (Promega Corporation) according to the manufacturer's instructions. Expression of cloned miRNAs was verified by real time PCR using SNORD-47 miRNA as control. SNORD-47 is considered a "housekeeping" miRNA in human cells. Statistical analysis was performed using the Student's t-test.

Results

Based on MiRWalk and review of the literature, targeting of the 3'-UTR of *RGS5* mRNA has not yet been experimentally validated for any miRNA. Seven hundred seventy six miRNAs were computationally predicted to target this 3'-UTR by the algorithm used by MiRWalk or at least one of the miRNA prediction programs included therein. Four hundred thirty five of these met our threshold selection criterion of being predicted by at least four programs. Of these, the expression of at least 55 has been experimentally evidenced in the eye (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/, (47)). As empirical evaluation of all these was beyond the scope of the present study, seven of the miRNAs were chosen on the basis of predictive scores to be tested by the dual luciferase assay. The seven miRNAs were miR-7, miR-9, miR-23a, miR-23b, miR-96, miR-182, and miR-204. An eighth miRNA (miR-211) was also selected because it belongs to the same family as miR-204 which is known to be an important miRNA with respect to various ocular functions, and because miR-211 has been shown to be relevant to glaucoma related functions of the TM (45, 46, 49, 52). The distribution of the predicted recognition sites of these miRNAs on the 3'-UTR of *RGS5* mRNA and on these cloned fragments is shown in Figure 1A. The sequences of the 3'-UTR fragments cloned in

Table 1. Sequences of primers used for PCR-amplification of *RGS5*-1, *RGS5*-2, and *RGS5*-3

	forward primer	reverse primer
RGS5-1	5'-CCGCTCGAGCCTGCCCATAGTCACCAAATTC-3'	5'-ATAACCCTGCGGCCGCCTGGAGTCTTCATAACATCAACC-3'
RGS5-2	5'-CCACTCGAGTAAGATTAGCCCAGGTAGTTGCC-3'	5'-ATATGTATGCGGCCGCGGTTTCGATGCTTGGATGTTGG-3'
RGS5-3	5'-CCGCTCGAGAGGGGTAGGCAATTCTATGTAGG-3'	5'-AAGGAAAAAAGCGGCCGCCAGAAGCATGAGTCACATAGTAC-3'

*XhoI recognition sites on forward primers and NotI recognition sites on reverse primers are shaded

Table 2. Summary of dual luciferase assays

	RGS5-1			*RGS5*-2			*RGS5*-3		
	No. sites	fold decreased renilla/luciferase	*P-value*	No. sites	fold decreased renilla/luciferase	*P-value*	No. sites	fold decreased renilla/luciferase	*P-value*
mir-7	0			2	1.2x	0.21	1	1.0x	0.628
mir-9	0			1	1.5x	0.009	0		
mir-23a	2	1.4x	0.044	1	2x	0.002	1	ND	
mir-23b	2	1.4x	0.062	1	1.8x	0.003	1	ND	
mir-96	2	1.0x	0.66	2	0.9x	0.172	1	1.4x	0.004
mir-182	1	ND		2	0.8x	0.003	1	ND	
mir-204	0			2	1.5x	0.0002	0		
mir-211	0			2	1.5x	0.002	0		

ND: not done

psiCHECK2- *RGS5*-1, psiCHECK2-*RGS5*-2, and psiCHECK2-*RGS5*-3 were shown to be correct. The number of predicted target sites for each miRNA in each 3'-UTR fragment tested was either one or two.

Expression of the cloned miRNAs after transfection into HEK293 cells was confirmed by real time PCR (not shown). The results of effects of the miRNAs as assessed by dual luciferase assays are presented in Figure 1B and Table 2. Effects on fragment *RGS5*-2 were comprehensively tested, but effects of miR-182 on *RGS5*-1 and miR-23a, miR-23b, and miR-182 for *RGS5*-3 were not assessed. Results of replicate assays were impressively similar as evidenced by small standard deviations in the renilla/firefly luciferase ratios (Figure 1B). Renilla/firefly luciferase ratios were not significantly different in cells transfected with only psiCHECK2 backbone, scrambled miRNA, or any of the miRNAs being tested (not shown). This means that the differences observed in the presence of psiCHECK2 vectors containing 3'-UTR sequences are due to miRNA effects that were elicited only in the presence of those sequences. Among the thirteen assays performed with psiCHECK2 vectors containing 3'-UTRs, the tested miRNAs decreased target gene expression at a statistically significant level in seven of the reactions. This was evidenced by decreased renilla/ firefly luciferase ratio in presence of the tested miRNA as compared to control reactions (Figure 1B). Fold decrease ranged between two-fold and 1.4-fold. Unexpectedly, miR-182 caused increased renilla/ firefly luciferase ratio in the presence of psiCHECK2-*RGS5*-2. The effect of miR-204 in presence of psiCHECK2-*RGS5*-2 (1.4-fold) was notable because it reached statistical significance at the level of $P<0.0002$. MiR-211 which belongs to the same miRNA family as miR-204 had a similar effect. MiR-23a and miR-23b, that share common seed sequences, are each predicted to have four recognition sites in the 3'-UTR of *RGS5*. Effects of these miRNAs on the single site present in *RGS5*-3 were not tested; however they caused decreased renilla luciferase expression in the presence of *RGS5*-1 and *RGS5*-2 that contain the remaining recognition sites (Figure 1B, Table 2). Effects of miRN-23a and miR-23b on *RGS5*-2, respectively, were 2.0- and 1.8-fold. Having two as compared to one recognition site did not necessarily result in increased miRNA effect (Table 2). Among the five assays in which statistically significant effects of miRNAs were not observed, four had two recognition sites for the tested miRNA; among the seven reactions in which the miRNAs reduced renilla luciferase expression, four had one recognition site and three had two.

Discussion

We have shown by the luciferase assay that all miRNAs tested except miR-182 had a highly reproducible effect on down regulation of the renilla luciferase gene when the gene was associated with a partial 3'-UTR segment of the *RGS5* gene. The up-regulatory effect of miR-182 in the presence of *RGS5*-2 may have been indirect and caused by effects of this miRNA on other regulatory components active in HEK-293 cells. The observed effects of miR-7, miR-9, miR-23a, miR-23b, miR-96, miR-204, and miR-211 on *RGS5*-1, *RGS5*-2, and/or *RGS5*-3 were modest. Although we did not observe that having two rather than one binding site was correlated with increased down regulation; it is generally believed that the presence of multiple target sites does increase effects of miRNAs. In this light, it is possible that the presence of the entire 3'-UTR segment (5038 200 nucleotides) downstream of the renilla luciferase gene would have a more pronounced effect. MiRNAs miR-7, miR-9, miR-23a,and miR-23b are each predicted to have four or five target sites within the entire 3'-UTR segment, whereas there was a maximum of two target sites in the segments tested (Figure 1A).

Known ocular related functions of the miRNAs here tested, were reviewed with the consideration that the functions may shed light on their putative effects on *RGS5* expression. MiR-7 has been reported to be among the miRNAs whose levels where changed in TM cells upon treatment with mechanical stress which is a factor that potentially contributes to the pathogenesis of glaucoma (53). MiR-9 is necessary for proper eye development, and has a role in inflammatory responses of retinal microglia cells (54). MiR-23a and/or miR-23b are thought to be essential for proper eye development, to protect retinal epithelial cells from oxidative damage, and to be involved in choroidal neovascularization (54).

Both are present in human aqueous humor and may have roles in the etiology of cataract (47, 48). Among the miRNAs tested, miR-204 is likely to be the most important with respect to ocular development and ocular functions. Some of its functions are mediated through the important eye transcription factors MEIS2 and FOXC1 (46, 50). MiR-204 is known to be expressed in murine eye tissues from early developmental stages through adulthood, and has been shown to regulate multiple aspects of eye development in the medaka fish (45, 46, 55); it is expressed in human TM cells (49, 52). The miRNA may have roles in the etiology of cataract and is involved in the regulation of apoptosis, endoplasmic reticulum stress response, and inflammation in human TM cells (48, 49).

Conclusion

The above findings do not provide an exact signal for the biological significance of the effects of the miRNAs on *RGS5* expression. Nevertheless, given that *RGS5* is definitively expressed in the human TM (23, 25-27), and that it is up-regulated in hypertensive eyes (20), the results presented here justify further investigations on the regulatory functions of the miRNAs on *RGS5* expression and on genes downstream of G-protein coupled receptors that associate with Gαi and Gαq. This having been said, we emphasize that our results are preliminary, and that the effects of the miRNAs need to be studied at the protein level, and most importantly, in ocular relevant cells such as TM cells.

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