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Computational prediction of MicroRNAs targeting GABA receptors and experimental verification of miR-181, miR-216 and miR-203 targets in GABA-A receptor

Chunling Zhao^{1,2}, Chaoqun Huang¹, Tingting Weng¹, Xiao Xiao¹, Hong Ma¹ and Lin Liu^{1,3*}

Abstract

Background: GABA receptors are well known as the inhibitory receptors in the central nervous system and are also found in peripheral tissues. We have previously shown that GABA receptors are involved in lung development and fluid homeostasis. However, the microRNAs that regulate GABA receptors have not yet been identified.

Results: In this study, we used the online software, TargetScan and miRanda, to query the microRNAs that directly target GABA receptors and then selected some of them to verify experimentally using 3'-UTR reporter assays. Computational approaches predict many microRNA binding sites on the 3'-UTR of GABA_A receptors, but not on GABA_C receptors. 3'-UTR reporter assays only verified miR-181, miR-216, and miR-203 as the microRNAs that target GABA receptor α 1-subunit among 10 microRNAs tested.

Conclusions: Our studies reinforce that microRNA target prediction needs to be verified experimentally. The identification of microRNAs that target GABA receptors provides a basis for further studies of post-transcriptional regulation of GABA receptors.

Background

GABA receptors are well known as the inhibitory receptors in the central nervous system [1,2]. However, GABA receptors are also found in several peripheral tissues [3-6]. The functions of GABA receptors in peripheral tissues are less studied. They may be involved in ion homeostasis [7], cell proliferation and differentiation [8], development [1], and hormone secretion [5,9].

We have initially identified GABA receptor π -subunit as a specific alveolar epithelial type II cell marker through DNA microarray analysis [10]. The expression pattern of the GABA receptor π -subunit is regulated by various culture conditions and is consistent with the type II cell phenotypes [11]. We have further identified 19 subunits of the ionotropic GABA receptors in alveolar epithelial cells [6]. Their expression is dynamically

changed during lung development [12]. Functionally, GABA receptors play important roles in fluid homeostasis in the adult lung and fetal lung development [6,13].

GABA receptors can be classified into two major types: GABA_A and GABA_C as ligand-gated Cl⁻ channels, and GABA_B receptor as a metabotropic receptor coupled to a heterotrimeric G-protein. GABA_A and GABA_C receptors share a conserved structure that contains a long extracellular N-terminal region, 4 transmembrane domains (TM1-TM4), a large intracellular loop between TM3 and TM4, and a short extracellular C-terminus [1,2,14-16]. The N-terminal segment is responsible for ligand binding and subunit assembly. The TM2 domain forms the lining of the ion pore. The intracellular loop is the site for post-translational modifications and binding with other proteins. This loop harbors a number of consensus phosphorylation sites for protein kinase A and C (PKA and PKC) and tyrosine kinases [17].

MicroRNAs are small non-coding RNAs. They form a ribonucleoprotein complex, termed RISC that cleaves

* Correspondence: lin.liu@okstate.edu

¹The Lundberg-Kienlen Lung Biology and Toxicology Laboratory, Department of Physiological Sciences, Oklahoma State University, Stillwater, Oklahoma 74078, USA

Full list of author information is available at the end of the article

mRNA or represses protein translation. MicroRNAs regulate various biological processes [18,19]. Several microRNAs such as miR-17-92 cluster and miR-127 are involved in lung development [20-22]. MicroRNAs have also been implicated in many lung diseases including lung inflammation, Chronic Obstructive Pulmonary Disease, Asthma and Idiopathic Pulmonary Fibrosis [23-30]. Nevertheless, microRNAs that regulate GABA receptors have not yet been reported. In this study, we used online software, TargetScan (<http://www.targetscan.org>) [31] and miRanda (<http://www.microrna.org>) [32] to predict the microRNAs that possibly target to GABA receptors and then selected some of them to verify experimentally using 3'-UTR reporter assays. We found that miR-181, miR-23 and miR-216 target the GABA receptor $\alpha 1$ -subunit.

Methods

Construction of microRNA expression vectors

Human microRNA expression vectors were constructed as previously described [22]. Mature microRNAs with the flanking sequences (~200 base pairs at each end) were PCR-amplified from human genomic DNA. The primers used for PCR amplification are listed in Table 1. The PCR products were inserted into a modified pLVX-Puro lenti-viral vector (Clontech) between CMV-driven enhanced green fluorescent protein (EGFP) and SV40 polyA terminal sequences.

Construction of 3'-UTR reporter vectors

The full length 3'-UTR or the microRNA binding sites in the 3'-UTR of rat GABA receptors were PCR-amplified and inserted into the pRL-TK vector containing a *Renilla* luciferase (Promega). The primers used for PCR amplification are listed in Table 2.

3'-UTR reporter assay

HEK 293T cells (2×10^4 /well) were seeded in each well of a 96-well plate. After one day of culture, the cells were transfected with 100 ng microRNA expression vector or control vector without miRNA insert, 2.5 ng 3'-UTR *Renilla* luciferase reporter vector and 15 ng pGL3 control vector (firefly luciferase reporter) using Lipofectamine. After a 2 day transfection, the cells were lysed and dual luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The *Renilla* luciferase activities were normalized with firefly luciferase activity. Data was expressed as a ratio to the control vector without miRNA insert.

Results and discussion

Both GABA_A and GABA_C receptors are ligand-gated Cl⁻ channels. However GABA_C receptors have very unique ligand binding characteristics in comparison with GABA_A and GABA_B receptors, including a high sensitivity to the physiological ligand, GABA, insensitivity to bicuculline, barbiturates, and baclofen, very weak de-

Table 1 Primers for miRNA expression vectors

	Forward	Reverse
miR-15a	CCGCTCGAGTATTCTTTGTGTTTCTAACCTAT	GCGGAATTCTCAATAAATACTGAAAAGACTATC
miR-15b	CACCTCGAGCGGCTGCAGAGATAAATACTTC	GAGAATTCGTTGCTGTATCCCTGTCCACT
miR-16-1	GGGCTCGAGAAACATAGATTTTTTACATGC	CCCGAATTCGAATTCCTCTAATGCTGCATAAG
miR-16-2	CACCTCGAGCCTTAAAGTACTGTAGCAGCACAT	GAGAATTCGGTAAATCAAACACCAAGTGTACAG
miR-103-1	CCGCTCGAGTACTTCCCAATCCATTTAAAGTAT	GCGGAATTCGAAGTAGCAGATAAATTCAAATTG
miR-103-2	CGCCTCGAGTTCCAACAAATGTTAATTAATTCTG	GGCGAATTCGAAGTAGAGGAAGAGTGGAAAGGT
miR-107	CACCTCGAGGATACAATTACAACCCATGTC	GAGAATTCCTGTCTTTCTAATATACCTCAGTG
miR-137	CACCTCGAGTTATGGATTTATGGTCCCGGTCAAG	GAGAATTCCTCAGTCTGGTACCAGAGGCC [^]
miR-146a	CACCTCGAGATCCACCCACATCAGCCTTC	GGTTGAAGACTGAATTCGAGTAGCAGCAGCAAGAGAG
miR-146b	CACCTCGAGTCAGCTCCAAGCTCAGACCCTC	GGTTGGTCTCGAATTCAGCACTGAGGAAGGACCAGCAT
miR-181a-2	CACCTCGAGTTCTCAGACATTCATTGAGTTC	CACGAATTCCTCATCATGGACTGCTCCTTAC
miR-181b-1	GTTCTCGAGTATGACTAAAGGTAAGTGTGTTTC	GCTGGTCTCAATTTGATACTGTACGTTTGTAGGAC
miR-181c	CACCTCGAGCTGCACTGCTACATCTCCATCC	GAGAATTCACACCAGCTCTCCTCTCCAAG
miR-181d	ATTCTCGAGCTCTCCTCTCCCTCTTCATGCTC	ATAGAATTCAGATTTGGGCCACTGCACTCCAGC
miR-203	ATTCTCGAGGCGGGCTGGGCTTGGCGGCTG	TTTGAATTCGCGCGCCCCACCCAGCGGTTTC
miR-216b	CCGCTCGAGTTTTCTTCAATCTCTATGGAGAT	GGCGAATTCGATGCTAGTCTGGGAATCAAAC
miR-221	CACCTCGAGCGACCTTCCATCCAGCTTT	GAGAATTCCTTGACAGTTGAGCAGGGAGAAG
miR-424	CACCTCGAGCAGCTCTGAAATCAAATGGTG	GAGAATTCCTTACCCTGGCAGCGGAAACAATAC
miR-497	CACCTCGAGATGGTCCGCTTCCAGTTG	GAGAATTCGGGTGTGGGCAACAAGACTC

Table 2 Primers used for constructing 3'-UTR reporter vectors

Names	3'-UTR or binding Sites	Sequences
GABRA1-F-Spe I	9-1962	<u>ggtactaGTCGTATTCTGTTGTTTCAGTC</u>
GABRA1-R-PspOMI		<u>gttggggcccTCTATACATGAAATGTCCTTGG</u>
GABRG2-F	2095-2102 (miR-103/107)	<u>CTAGTATGGACTTTTACAATAAAAATGCTGCATTCTA</u>
GABRG2-R		<u>GCCTAGAATGCAGCATTTTTATTGTAAAAGTCCATA</u>

F: forward primers; R: reverse primers; microRNA binding sites are underlined

sensitization, a smaller single-channel conductance, and a longer open time [14-16]. Eight different subunits of GABA_A and GABA_C receptors (α 1-6, β 1-3, γ 1-3, δ , θ , ϵ , π , and ρ 1-3) have been identified. The assembly of a heteropentamer, with at least one α -, one β -, and one γ -subunit, forms functional GABA_A receptor channels. δ -, θ -, ϵ -, and π -subunits can substitute for the γ -subunit. However, GABA_C receptors are exclusively composed of ρ subunits in the form of homo- or hetero-pentamers.

To identify the microRNA that may potentially regulate GABA_A and GABA_C receptors, we used the online computer software, TargetScan (v 5.2) to predict the binding sites of microRNAs on the 3'UTR of rat GABA receptors. We chose rat GABA receptors because we use rats as most of our animal or cell models. Since our microRNA expression vectors use human sequences, we only queried conserved microRNA target sites among mammals based on conserved 8 mer and 7 mer sites that match the seed sequence of a microRNA. The results are listed in Table 3. Among α -subunits, we found that α 1 had most of the microRNA binding sites. There were 6 binding sites for 6 microRNAs on α 1 3'-UTR. For other α -subunits, we found miR-128, miR-27ab, let-7/miR-98 for α 6. We did not find any microRNAs for α 2, α 4, and α 5. There was no information available for α 3.

For β -subunits, we found two binding sites for miR-30a/30a-5p/30b/30b-5p/30/384-5p and one binding site for miR-103/107. There were 15 binding sites for 15 microRNAs on β 2 and 5 binding sites for 7 microRNAs on β 3. For γ -subunits, we found two binding sites on γ 2 and no binding sites on γ 1 and γ 3, There was only one binding site for ϵ and no binding sites on π -, δ -, θ -, ρ 1- and ρ 2-subunits. In general, the "common" subunits (α , β , and γ) had more miRNA target sites than the "rare" subunits (δ , θ , ϵ , π , and ρ). This is probably because these subunits had shorter 3'-UTRs, in particular for ρ -subunits.

We also used another software, miRanda to predict the microRNA that target to GABA receptors (Table 3). In general, miRanda predicted more microRNAs than TargetScan. There were some common microRNAs that were predicted by both software. For example, miR-137, miR-181, miR-203, and miR-216a for α 1; miR-103, miR-107, miR-30, and miR-384-5p for β 1; and miR-204, miR-211, miR-23, and miR-26 for β 3.

We further utilized a recently developed software, miRWalk [33], to predict the miRNAs targeting 3'-UTR and open reading frame (ORF) of GABA receptors. The results are presented in Table 4. Obviously, this method is less stringent compared to TargetScan and miRanda, since the miRWalk query yielded 79 miRNAs for GABA receptor α 1 subunit in comparison with only 6 by TargetScan and 29 by miRanda.

We selected two subunits, α 1 and γ 2 for experimental verification of the predictions. For α 1-subunit, we constructed a 3'-UTR reporter vector, in which the 3'-UTR of α 1-subunit was placed after a *Renilla* luciferase reporter gene (Table 2). For γ 2-subunit, we cloned the predicted binding site of miR-103/107 into the downstream of a *Renilla* luciferase reporter gene. We then co-transfected a microRNA expression vector with the reporter into HEK293T cells to see whether the microRNA depressed the reporter activity. The firefly luciferase pGL3 vector was used for normalization. As shown in Figure 1, among the predicted microRNAs tested, miR-181, miR-216, and miR-203 inhibited the reporter activity. Four miR-181 isoforms, a-2, b-1, c and d-1 generated the same mature miR-181 and all of them depressed the reporter activity. All other microRNAs tested had no effects. The results suggest that miR-181, miR-216, and miR-203 are the microRNAs that regulates GABA receptor α 1-subunit. It is noted that miR-15, miR-16, miR-146, miR-221, miR-424 and miR-497 were predicted to target the GABA receptor α 1-subunit by an earlier version (v.4.2) of TargetScan. Some of the miRNAs such as miR-181 are expressed in astrocytes naturally expressing GABA receptors [34]. For γ 2-subunit, we did not find major effects of miR-103-1, miR-103-2, and miR-107 on the reporter activity (Figure 2).

It should be noted that we did not measure miRNA levels in the miRNA-overexpressed cells. Thus, there are possibilities that some of miRNAs may not be over-expressed in the experimental set-up; particularly for these miRNAs that had no effect on 3'-UTR reporter activity. However, the transfection efficiency is 90-100% under our experimental conditions based on the GFP reporter expression encoded in the same miRNA expression vector. Additionally, the effect of a miRNA on the luciferase activity does not necessarily mean that it was a direct effect on the binding of a miRNA to the

Table 3 Predicted microRNAs targeting rat GABA receptor subunits by TargetScan and miRanda

GABA receptor subunits	Entrez Gene symbol	Lengths of 3'-UTR in TargetScan (v5.2)	Conserved microRNAs targeting to GABA receptors predicted by TargetScan (v5.2)	Lengths of 3'-UTR in miRanda	Conserved microRNAs targeting to GABA receptors predicted by miRanda
$\alpha 1$	GABRA1	2017	miR-208/208ab, miR-499/499-5p, miR-181, miR-216/216a, miR-137, miR-203	2018	miR-129 (2), miR-130b, miR-136, miR-137, miR-148b-3p, miR-150, miR-152, miR-181a (2) bc (3) d, miR-182, miR-186, miR-203 (2), miR-210, miR-216a, miR-26ab, miR-30acde, miR-30b-5p, miR-320, miR-340-5p, miR-361, miR-374, miR-375, miR-376c, miR-377, miR-384-5p, miR-410, miR-433, miR-488 (2), miR-539, miR-874
$\alpha 2$	GABRA2	747	0	NA	NA
$\alpha 3$	GABRA3	0	0	NA	NA
$\alpha 4$	GABRA4	7478	0	88	miR-186, miR-200bc, miR-203, miR-429, miR-495
$\alpha 5$	GABRA5	586	0	880	miR-124, miR-132, miR-133ab, miR-195, miR-212, miR-223, miR-30acde, miR-30b-5p, miR-322, miR-346, miR-376c, miR-378, miR-384-5p, miR-494 (2), miR-495, miR-539
$\alpha 6$	GABRA6	809	miR-128, miR-27ab, let7/miR-98	NA	N/A
$\beta 1$	GABRB1	407	miR-30a/30a-5p/30b/30b-5p/ 30/384-5p (2), miR-103/107	428	miR-103, miR-107, miR-128, miR-143, miR-148b-3p, miR-152, miR-30a (2) c (2) d (2) e (2), miR-30b-5p (2), miR-384-5p (2), miR-411
$\beta 2$	GABRB2	5618	miR-203, miR-135, miR-218, miR-21/590-5p, miR-10, miR-101, miR-19, miR-144, miR-9 (2), miR-455/455-5p, miR-33/33ab	499	miR-128, miR-199a-5p, miR-203, miR-33, miR-411, miR-485
$\beta 3$	GABRB3	4060	miR-26ab/1297, miR-204/211, miR-23ab, miR-27ab, miR-218	508	miR-122, miR-186, miR-199a-5p, miR-204, miR-210, miR-211, miR-23ab, miR-26ab, miR-320, miR-324-5p, miR-329, miR-381, miR-539
$\gamma 1$	GABRG1	3428	0	240	miR-203, miR-218, miR-379, miR-410, miR-455, miR-488
$\gamma 2$	GABRG2	2106	miR-150, miR-103/107	NA	N/A
$\gamma 3$	GABRG3	96	0	114	miR-15b, miR-16, miR-195, miR-26ab, miR-322, miR-497
π	GABRP	1178	0	NA	N/A
δ	GABRD	433	0	393	miR-145, miR-19ab, miR-24, miR-328, miR-365
ϵ	GABRE	1485	miR-22	NA	N/A
θ	GABRQ	80	0	NA	N/A
$\rho 1$	GABRR1	464	0	NA	N/A
$\rho 2$	GABRR2	113	0	191	0
$\rho 3$	GABRR3	N/A	N/A	271	miR-191

The conserved microRNAs targeting GABA receptors are predicted by Targetscan 5.2 <http://www.targetscan.org/> and miRanda software (<http://www.microrna.org>). The number in parenthesis is the number of the binding sites and none means one binding site. N/A means no information is available in TargetScan or miRanda

Table 4 Predicted microRNAs targeting 5'-UTR, ORF and 3'-UTR region using miWalk software

GABA receptor subunits	Entrez Gene symbol	MicroRNAs targeting 5'-UTR	MicroRNAs targeting ORF	Numbers of microRNA targeting 3'-UTR with p -value < 0.05
$\alpha 1$	GABRA1	miR-326, miR-28*, miR-29b-1*, miR-539, miR-542-5p, miR-147, miR-423, miR-598-5p	miR-341, miR-503, miR-150, miR-378	79
$\alpha 2$	GABRA2	N/A	N/A	N/A
$\alpha 3$	GABRA3	miR-27b, miR-27a, miR-185, miR-343, miR-346, miR-17-5p, miR-93, miR-128, miR-143, miR-291a-5p, miR-20b-5p	miR-350, miR-431, miR-542-3p, miR-322, miR-323*, miR-140, miR-148b-3p, miR-29a*, miR-152, miR-497	0
$\alpha 4$	GABRA4	N/A	N/A	N/A
$\alpha 5$	GABRA5	miR-345, miR-22, miR-451, miR-541, miR-369	miR-24-1*, miR-24-2*, let-7d, miR-346, miR-153, miR-296, miR-376c, miR-466c	37
$\alpha 6$	GABRA6	0	miR-126*, miR-743b, miR-323*, miR-330*, miR-21*, miR-153, miR-880	0
$\beta 1$	GABRB1	miR-323, miR-219-2	miR-140, miR-351, miR-324-5p, miR-325-3p, miR-7a*, miR-10a-5p, miR-125a-5p, miR-125b-5p, miR-376b-5p, miR-384-3p	26
$\beta 2$	GABRB2	0	miR-20a*, miR-150, miR-297, miR-541	0
$\beta 3$	GABRB3	miR-188	miR-300-5p, miR-350, miR-433, miR-881, miR-672,	17
$\gamma 1$	GABRG1	miR-497, miR-322, miR-103-2, miR-103-1, miR-107	miR-182, miR-216a, miR-483, miR-327, miR-338, miR-205, miR-296, miR-320, miR-880	miR-126*, miR-379, miR-742, miR-871
$\gamma 2$	GABRG2	0	miR-182, miR-483, miR-382, miR-505	0
$\gamma 3$	GABRG3	miR-151*, miR-125a-3p	miR-142-3p, miR-298, miR-15b, miR-16, miR-28, miR-34a, miR-195, miR-214, miR-290, miR-449a, miR-880, miR-708	14
π	GABRP	miR-28, miR-708	miR-345-5p, miR-199a-3p, miR-873	0
δ	GABRD	miR-210	miR-322, miR-338, miR-193, miR-370, miR-497, miR-873	29
ϵ	GABRE	0	miR-485, miR-484, miR-342-3p, miR-344-5p, miR-223, miR-671, miR-322, miR-24, miR-139-3p, miR-199a-5p, miR-298, miR-483, miR-497, miR-743b, miR-672	0
θ	GABRQ	0	miR-350, miR-34c, miR-92a, miR-92a, miR-300-5p, miR-92b, miR-7a*, miR-32	0
$\rho 1$	GABRR1	miR-29b-1*, miR-26c	miR-338, let-7d, miR-204*, miR-421, miR-672, miR-674-3p	92

Table 4 Predicted microRNAs targeting 5'UTR, ORF and 3'UTR region using miRWalk software (Continued)

p2	GABRR2	miR-873, miR-134, miR-210, miR-207, miR-380,	miR-218*, let-7d, miR-34a, miR-204*, miR-421, miR-449a, miR-431, miR-381, miR-674-3p	miR-191, miR-872*
p3	GABRR3	miR-350, miR-30c-1*, miR-30c-2*, miR-148b-3p, miR-152, miR-872*, miR-874, miR-672	miR-338, miR-341, miR-23a*, miR-143, miR-384-5p, miR-324-3p, miR-30c, miR-30e, miR-30b-5p, miR-30d, miR-30a, miR-204*, miR-539, miR-742, miR-873	miR-191, miR-344-1, miR-217, miR-883, miR-484

MicroRNAs which target 5'-UTR, open reading frame (ORF) and 3'-UTR of GABA receptors were predicted by miRWalk software <http://www.ma.uni-heidelberg.de>. N/A means that no information is available in miRWalk

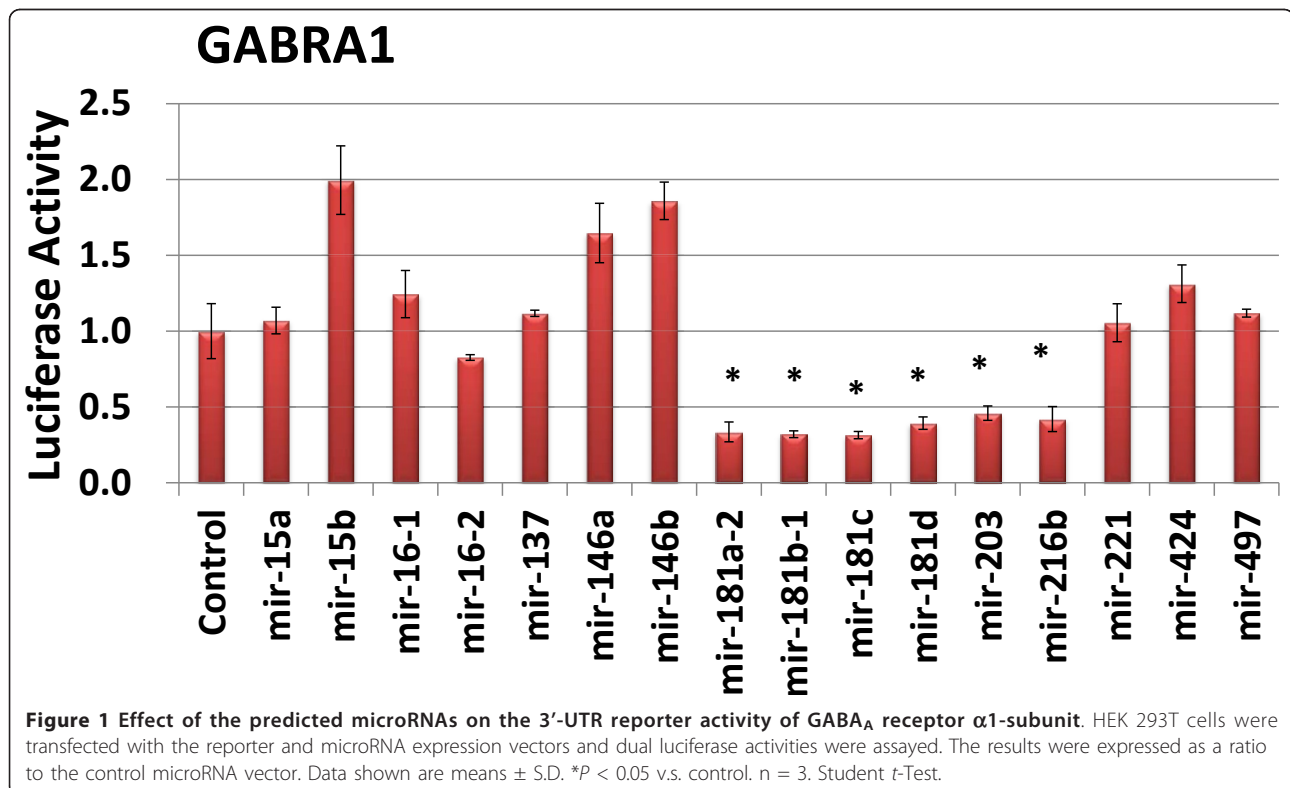
3'-UTR reporter construct. A miRNA could have indirect effects. The mutations of seed sequences in the miRNA binding sites are needed to exclude indirect effects. Further studies are also needed to see whether the overexpression of miR-181, miR-203, and miR-216 in a physiologically relevant cell type modifies GABA receptor expression, and whether these miRNAs are differentially regulated in diseased states.

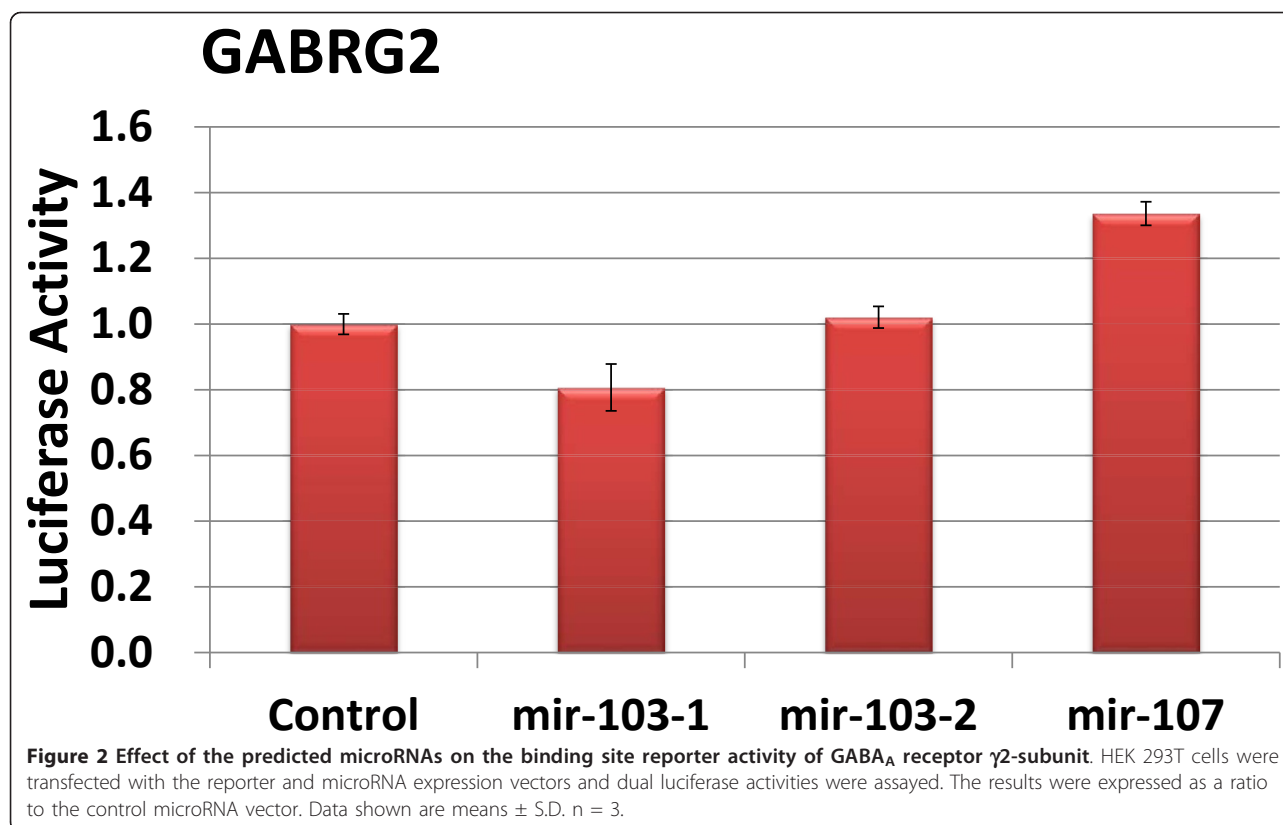
It is also interesting to note that miR-15b and miR-146a/b actually increased the 3'-UTR reporter activity. It has been reported that miRNA increases translation [35]. However, it is also possible that this is a result of indirect effects.

We have previously shown that the activation of GABA receptors promotes fetal lung development [13]. The inhibition of miRNAs that target GABA receptors may increase receptor density and thus sensitivity of GABA receptors, which may benefit the development of therapy in treating diseases related to developmental anomalies.

Conclusions

In summary, computational approaches predict many microRNA binding sites on the 3'-UTR of GABA_A receptors, but not on these of GABA_C receptors. 3'-UTR reporter assays only verified miR-181, miR-216,





and miR-203 as the microRNAs that target GABA receptor α 1-subunit among 10 microRNAs tested. These studies reinforce that microRNA target prediction needs to be verified experimentally. The identification of microRNAs that target to GABA receptors provides a basis for further studies of post-transcriptional regulation of GABA receptors.

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Author details

¹The Lundberg-Kienlen Lung Biology and Toxicology Laboratory, Department of Physiological Sciences, Oklahoma State University, Stillwater, Oklahoma 74078, USA. ²Department of Physiology, Lu Zhou Medical College, Lu Zhou, Sichuan Province 64600, People's Republic of China. ³Department of Physiological Sciences, Oklahoma State University, 264 McElroy Hall, Stillwater, Oklahoma 74078, USA.

Authors' contributions

CZ, TW and HM carried out experiments. CZ, CH and XX analyzed data and performed target predictions. LL conceived of the study, and participated in its design and coordination. LL and CZ drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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