Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

# MicroRNA-626 inhibits mTOR pathways activity of retinal pigment epithelial cells by targeting SLC7A5 in human ARPE-19 Cells

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**ARTICLE INFO**

***Original paper***

***Article history:***
*Received: March 12, 2023*
*Accepted: July 05, 2023*
*Published: October 31, 2023*

***Keywords:***

*AMD, ARPE-19, mir-626, mTOR, RPE, SLC7A5*

**ABSTRACT**

Recent studies have shown that miRNAs are associated with the pathological process involved in age-related macular degeneration (AMD). However, the microRNA-mediated post-transcriptional regulation in human retinal pigment epithelium (RPE) cells has not been adequately investigated. We investigated how miR-626 inhibits mTOR activity pathways and pathway-related genes in retinal pigment epithelial cells by targeting the solute carrier family seven-member 5 (SLC7A5) in ARPE19 cells. We transfected mir-626 mimic, mir-626 inhibitör and siRNA in human retinal pigment epithelial cell line was examined using RT-PCR and western blot, respectively. We knocked down mir-626 levels and overexpression by mir-626-siRNA transfection of human RPE cell lines, and using an MTT assay, we assessed the role of SLC7A5 on RPE cell proliferation. We additionally measured the expression of mTOR, Akt1, caspase 3, Bax, SLC17A7, SLC17A8, Creb1, Pten, HIF1A, HIF2A. The findings demonstrate that mir-626 inhibits SLC7A5 gene expression and proliferation of ARPE-19 cells. Short interfering RNA (siRNA) mediated suppression of SLC7A5, a predicted target of mir-626, has the same effect on ARPE-19 cells. We identified how miR-626 causes apoptosis and macula degeneration in RPE cells by targeting SLC7A5 through the mTOR signaling pathway. miR-626 was an essential regulator of the expression of the Slc7a5 gene. Importantly, we determined that miR-626 is essential to play a role in AMD. This research project shows that SLC7A5 is a direct target of mir-626 in ARPE-19 cells for the first time.

**Doi:** http://dx.doi.org/10.14715/cmb/2023.69.10.3 

## Introduction

Age-related macular degeneration is a multifactorial disease associated with a complex interaction of genetic and environmental factors. The early stage of AMD is characterized by dysfunction of RPE cells, with pigment changes and drusen deposition, which is essential for the function of the photoreceptors. In contrast, late-stage disease develops neovascularization and/or geographic atrophy with significant vision loss (1,2). Emerging evidence suggests that besides environmental and genetic factors, epigenetic mechanisms, such as microRNA (miRNA) regulation of gene expression, are relevant to AMD, providing an exciting new avenue for research and therapy (3). Some in vivo and in vitro studies suggested that numerous miRNAs are associated with the pathological process involved in AMD and diabetic retinopathy, such as pathological angiogenesis, oxidative stress, and inflammation, which indicates that these miRNAs might be potential therapeutic targets (4-6). The function and effects of miRNAs are reasonably well understood in the cell by recent studies and are promising for future miRNA-based studies. Furthermore, dysregulated miRNAs could be a treatment option by using miRNA mimics or antagomirs to modulate miRNA levels in the cell (7,8).

Our previous study showed that circulating miR-626 was significantly higher expression of serum miRNAs in patients with AMD (9). SLC7A5 is a molecular target of mir-626 demonstrated according to the MirTarbase.

SLC7A5 alias L type amino acid transporter (LAT1) (6) is a sodium-independent high-affinity amino acid transporter and mediates cellular uptake of the large neutral amino acids such as phenylalanine, tyrosine, leucine, and tryptophan (10). Leucine is an anabolic amino acid that stimulates the protein kinase, mammalian rapamycin (mTOR) target, protein translation, and cell growth (11-18). mTOR is the catalytic component of two complexes, the mammalian target of rapamycin complex 1 (mTORC1) and the mammalian target of rapamycin complex 2 (mTORC2). Also, Xu et al (19) have shown that SLC7A5 knockdown also decreased mTOR pathway activity (20).

The serine-threonine protein kinase Akt is a common mediator of cell survival signals. Akt signalling targets mTOR, which promotes angiogenesis. Failure of Akt-mediated signalling can cause apoptosis, leading to photoreceptor degeneration, brunch membrane thickening,

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extracellular deposits, and decreased permeability leading to RPE damage, causing AMD.

Jomarv et al. demonstrated that in rd mice, inactivation of the Akt survival pathway results in photoreceptor cell death (21). Zhao et al. showed that mTOR-mediated dedifferentiation of the RPE indicates photoreceptor degeneration in mice (22). Therefore, we decided to examine mir 626 inhibiting mTOR pathway-related activity of retinal pigment epithelial cells by targeting SLC7A5 to understand the mTOR pathway-related activity molecular mechanism in the RPE of AMD. These findings were also confirmed by looking at mTOR, Akt1, caspase 3, Bax, SLC17A7, SLC17A8, Creb1, Pten, HIF1A, HIF2A, which are genes involved in pathways associated with macular degeneration.

## Materials and Methods

### Pathway Analyses

Target prediction and functional annotation were carried out as described in detail previously (9) that were performed using the miRSystem database (version 20160513) to evaluate the functions of candidate miRNAs of AMD patients, an integrated system for characterizing enriched processes.

### Cell Culture and Transfection

The human ARPE-19 cell line was purchased from ATCC (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) supplemented with 10% FBS (Hyclone, Logan, Utah), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Invitrogen, Gibco, Carlsbad, CA) at 37 °C under a humidified 5% $CO_2$: 95% air atmosphere. The media has been changed once every three days. Synthesized RNA duplexes of miR-626 mimic, miR-626 inhibitor and short interfering RNAs (siRNAs) targeting SLC7A5 (AM1620) were purchased from Ambion (Austin, Texas). ARPE19 cells were seeded in 12-well plates at 1.5610 5 cells/well and cultured for 48 h and then transfected with scrambled miR-626 mimics, miR-626 inhibitor, short interfering RNAs (siRNAs) targeting SLC7A5 (AM1620) and controls at a final concentration of 50 nM in all experiments using Lipofectamine 2000 and OPTI-MEM I (Invitrogen Life, Technologies, Carlsbad, CA) and further incubated for 72 h before harvesting for RNA and protein analyses according to the manufacturer's protocol. Cells were incubated with the transfection complexes for 6h before replacing the medium. Cells were replaced with fresh growth medium daily (23).

### Total RNA Extraction and qRT-PCR

Total RNA was isolated using a Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, U.S.A) in accordance with the manufacturer's protocol. The qRT-PCR was performed following our previous descriptions (9). For quantification of mRNAs, mature miR-626 and RNU6, reverse transcription was performed using cDNA synthesis using the qScript cDNA Synthesis kit (Quanta Biosciences, Canada).

RT primers for mature miR-626 and RNU38B were supplied by SYBR Green reverse-transcription quantitative PCR performed with CFX Bio-Rad connect TM Real-Time Detection System ( California, USA). Both groups' samples were analyzed in duplicate. The PCR program used for amplification was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 45 s. GAPDH was used for normalization. PCR primer sequences are in Table 1. The results of mRNA and miRNAs were expressed as ΔCT, and fold changes in relati-

**Table 1.** PCR Primer Sequences.

| Gene Names | Primer Sequences |
|---|---|
| SLC7A5 Forward | 5' ACGGCCGTGAACTGCTAC 3' |
| SLC7A5 Reverse | 5' GGATCTAGATTGCGCAGAGGCCAGAGTT 3' |
| Akt1 Forward | 5' CATCACACCACCTGACCAAT 3' |
| Akt1 Reverse | 5' CTCTAAAATGCACCCGAGAAAAT 3' |
| Bax Forward | 5' ATCCAGGATCGAGCAGGGCG 3' |
| Bax Reverse | 5' GGTTCTGATCAGTTCCGGGCA 3' |
| Pten Forward | 5' TGAGTTCCCTCAGCCGTTACCT 3' |
| Pten Reverse | 5' GAGGTTTCCTCTGGTCCTGGTA 3' |
| Caspase 3 Forward | 5' TGCCTGTAACTTGAGTAGATGG 3' |
| Caspase 3 Reverse | 5' CTTCACTTTCTTACTTGGCGATGG 3' |
| HIF1A Forward | 5' GAAACCACCTATGACCTGC 3' |
| HIF1A Reverse | 5' CTGTTTGTTGAAGGGAGAA 3' |
| HIF1A Forward | 5' CCTGGCCATCAGCTTCCTT 3' |
| HIF2A Reverse | 5' GGTCGGCCTCAGCTTCAG 3' |
| Mtor Forward | 5' ACCAGTGTGAGACCGTTTCC 3' |
| Mtor Reverse | 5' AGGCAGGACTGGTGTATTGG 3' |
| Creb 1 Forward | 5' GACCACTGATGGACAGCAGATC 3' |
| Creb 1 Reverse | 5' GAGGATGCCATAACAACTCCAGG 3' |
| Antisense SLC7A5 | 5' AACGGCGTGGCCATCATCGTGCCTGTCTC 3' |
| Sense SLC7A5 | 5' AACACGATGATGATGGCCACGCCGCCTGTCTC 3' |
| GAPDH Forward | 5’ ACT CCA CTC ACG GCA AAT TC 3’ |
| GAPDH Reverse | 5’ CAGTAGACTCCACGACATACT C 3’ |
| hsa-mir-626 | AGCUGUCUGAAAAUGUCUU |
| SLC17A7 Forward | 5’ GCAAGTACATCGAGGACGCCAT 3' |
| SLC17A7 Reverse | 5’ GCCACGATGATGGCATAGACTG 3' |
| SLC17A8 Forward | 5’ ACCACCTTTGGAGAGAAGCCGA3 ' |
| SLC17A8 Reverse | 5’ GGACCATCCAATGTACTGCACC 3' |

ve mRNA, and miRNA expression levels were calculated using 2-ΔΔCt (22).

### Western Blotting Analysis

Protein samples were isolated from the confluent ARPE-19 cells growing on 6-well plates by washing them in ice-cold PBS. Cells were lysed using RIPA lysis buffer containing Protease Inhibitör Cocktail (Pierce, USA). The total protein concentration with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) by a Multiskan™ GO Microplate spectrophotometer. Proteins separated ON 10% SDS polyacrylamide gels and transferred to PVDF membrane by electroblotting. Membranes were blocked with 5% milk solution prepared in TBST (Tris-buffered saline, 0.1% Tween 20) buffer and incubated with one of the following primary antibodies against human SLC7A5 (3157-1, 1:1000, Epitomics), Lamin B1 (G-1 sc-373918, 1:100, Santa CruzBiotechnology). The membranes were washed three times in TBST and then incubated with ECL anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (1:5000, GE Healthcare Life Sciences, Amersham, UK), and proteins were developed by the luminol-based substrate (Advansta, San Jose, CA, USA).

### Statistical Analysis

Data were expressed as a mean six standard error of the mean (SEM). Numerical data were expressed as means ± standard deviations. A student's t-test was used to compare the numerical variables. Statical significance was established based on a p-value ≤ 0.05.

## Results

### mir-626 interacts with the 3'UTR of the human SLC7A5 mRNA

Our previous study demonstrated that circulating miR-626 revealed significantly higher expression in AMD patients' serum than in the control group (9). To identify potential targets for miR-626, the human mir-626 was predicted to be the putative target of SLC7A5 by using miRSystem and mirTARBASE algorithms. In silico analysis of these databases demonstrated that the human SLC7A5 3'UTR has three binding sites for the mir-626; therefore, in this investigation, mir-626 and molecular pathways with SLC7A5 were our main focus. The bioinformatic analysis for the target site of mir-626 in SLC7A5 3'UTR is shown in Figure 1.

### Overexpression of miR-626 inhibits the proliferation of ARPE-19 cells by targeting SLC7A5

The mir-626 might play a role in regulating the proliferation of ARPE-19 cells to identify potential targets. In silico analysis of mir-626 using the miRNA target prediction databases TargetScan and PicTar was performed (9). However, there is no study of the SLC7A5 gene inhibited by mir-626, which is overexpressed in ARPE-19. To identify potential targets for miR-626 that ARPE-19 cells were transiently transfected with miR-626 mimics or mir-626 inhibitor or siRNAs specific to SLC7A5 (siSLC7A5) to understand the role of miR-626 by the proliferation of cells with cultured for 72h. (supplementary data 1).

Overexpression of miR-626 resulted in a significantly decreased proliferation of ARPE-19 cells, evident from 72 h post-transfection. According to the literature, the transfection of miR-626 mimics into ARPE-19 cells showed an increased miR-626 expression level compared to mir-626 inhibitor control-transfected cells (Figure 2A), as expected.

### Overexpression of miR-626 suppresses SLC7A5 expression at both mRNA and the protein levels

We next investigated the effect of miR-626 overexpression on SLC7A5 expression. The miR-626 overexpression was observed in ARPE-19 cells as determined by qRT-PCR. The expression of SLC7A5 at the mRNA level was significantly down-regulated ($p < 0.0001$) in mir-626

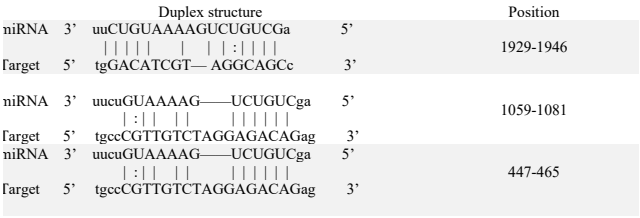


**Figure 1. The human SLC7A5 3'UTR contains three putative miRNA binding sites.** omplementarity between the miR-626 and the putative human SLC7A5 3'-UTR site targeted.

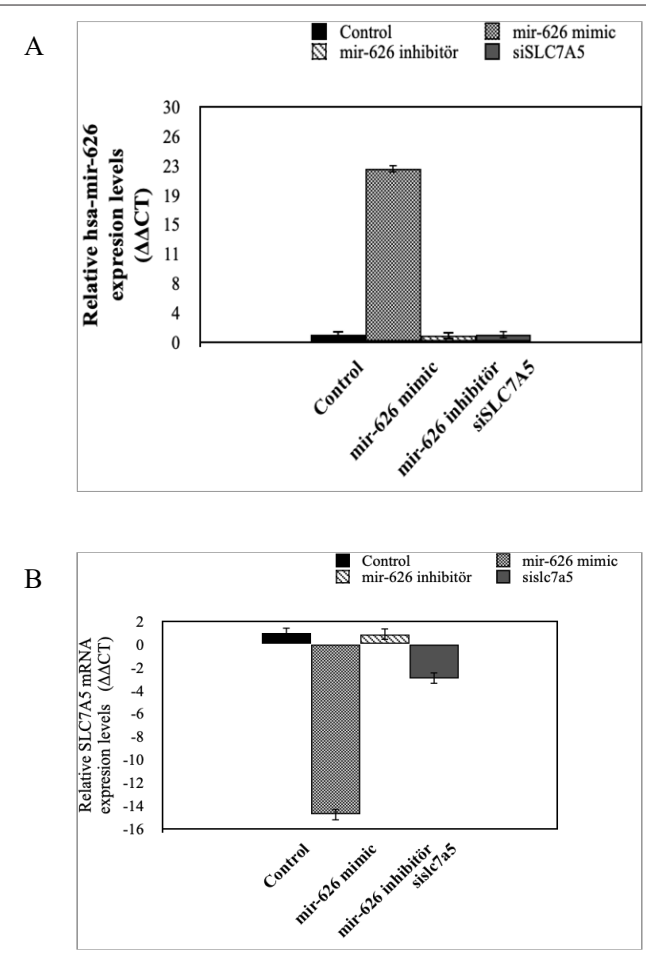


**Figure 2. A:** The mir-626 expressed as fold change ±SD compared to control. Experimental points with significance indicated as *P ≤ 0,0001 respectively. **B:** Relative SLC7A5 gene mRNA expression levels in ARPE-19 cells was detected by qRT-PCR. 1–4 represented control group, miR-626 mimics, miR-626 inhibitors group and siSLC7A5 group respectively. Each experiment was repeated three times. Data are presented as the mean ± SD. Multiple-group comparisons were analyzed by T-test. *p < 0.05 compared with the control group.

mimics (20 nM) from ARPE-19 cells as compared to mir-626 inhibitor (20 nM) from ARPE-19 cells ($p<0.005$) (Figure 2B ). The mRNA expression levels of SLC7A5 were down-regulated in the siSLC7A5 group compared with the control group ($p < 0.05$).

The western blot analyses further confirmed the qRT-PCR assay results. Our result showed that the protein expression levels decreased in the mir-626 mimics group compared with the control group ($p < .05$), as you see in Figure 3A-3B. Transfection with the inhibitör of miR-626 resulted in no significant change in SLC7A5 protein expression ($p > .05$) compared with the control, whereas the inhibitör of miR-626 protected the miR-626 targeting of SLC7A5. Also, the siSLC7A5 group was shown the same result as the mir-626 mimics group ($p < .05$).

### Akt-mediated signalling mTOR pathway, angiogenesis and apoptosis pathways genes mRNA levels

According to the results of Rt-PCR (Figure 4), with the control group, significant down-regulated was observed in the mRNA levels of mTOR, Akt, SLC17A7 in the mir-626 mimics group ($p > .05$). The mir-626 mimics group, compared with the control group, significantly upregulated Bax, PTEN, Caspase-3, HIFIa, HIFIIa and SLC17A8 mRNA levels ($P < .05$). There were no significant differences in the mRNA levels of Creb1 in the mir-626 mimic group compared with the control group ($p > .05$).

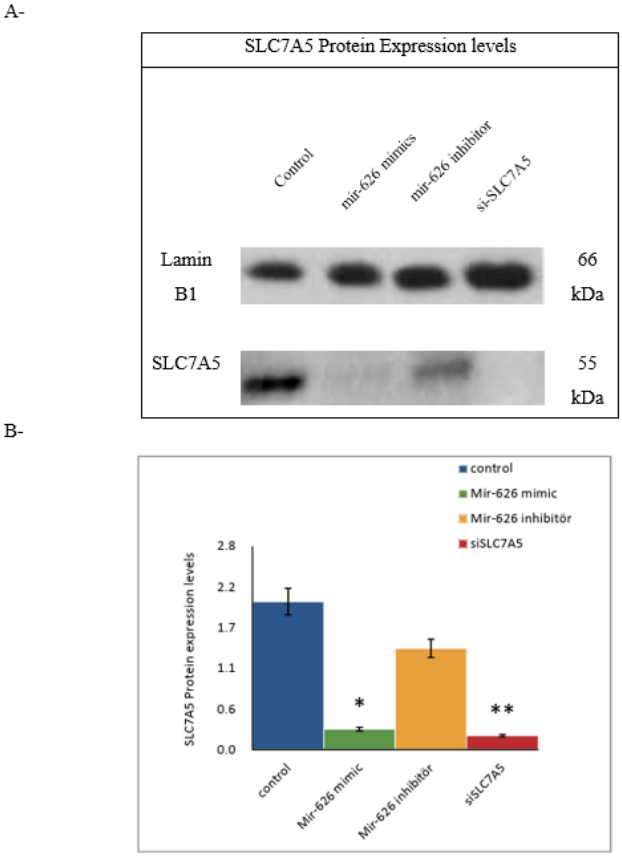


**Figure 3. Effects of miR-626 mimics and inhibitors on SLC7A5 protein expression in ARPE-19 cells. A-** Western Blot images of SLC7A5 protein levels in ARPE-19 cells of different groups: control, mir-626 mimics, mir-626 inhibitors, siSLC7A5. Values are presented as mean SEM; n = 3. *p,0.05 vs. control. **B-** Quantification of relative SLC7A5 protein levels. The band intensity was measured by using ImageJ software (see 'Materials and Methods'). The data reported here are representative of the experiment performed in triplicate. Values are presented as mean SEM; n = 3. *p,0.05 vs. control.

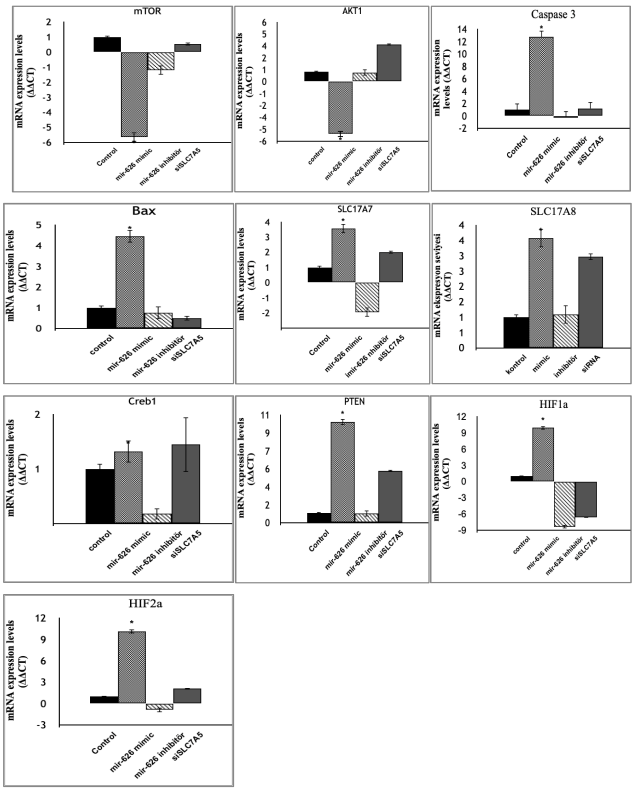


**Figure 4. miR-626 targets SLC7A5 to affect the Akt-mTOR pathway and Angiogenesis pathway in ARPE-19 cells.** mTOR, Akt1, caspase 3, Bax, SLC17A7, SLC17A8, Creb1, Pten, HIF1A, and HIFI2A mRNA expression in ARPE-19 cells was detected by qRT-PCR.1–4 represented control group, miR-626 mimics, miR-626 inhibitors group and siSLC7A5 group respectively. Each experiment was repeated three times. Data are presented as the mean ± SD. Multiple-group comparisons were analyzed by T-test. *p < 0.05 compared with the control group and the miR-626 mimic group or compared with the miR-626 inhibitor group or compared with the siSLC7A5 group.

## Discussion

miRNAs are key regulators of several biological processes, such as pathological angiogenesis and the response to oxidative stress, and miRNA dysregulation has been linked with numerous diseases. miRNAs are involved in AMD pathology, and several miRNAs target genes and signaling pathways were identified concerning AMD pathogenesis and progression (24). Concerning AMD, recent miRNA studies focused on therapeutic research but have not shown any specific miRNAs directly linked with the pathology of AMD. For example, miR-126 can control vascular integrity and angiogenesis, which may provide a novel target for neovascular AMD (4). Our previous study showed that circulating mir-626, highly regulated and validated in sera from AMD patients, maybe a potential biomarker. Therefore, the bioinformatic database using mirTarbase showed that the human SLC7A5 3'UTR has three binding sites for the mir-626 (9). On the other hand, no studies were focusing on the mechanism of miR-626 angiogenesis and apoptosis in AMD by targeting specific genes.

To investigate the function of overexpressing the mir-626 in RPE cells, we focused on the SLC7A5 and hypothesized that the mir-626 target of SLC7A5 may modulate

via mTOR pathways activity to cause macula degeneration. Our data shows that mir-626 mimics and siRNA (siSLC7A5) suppressed the mRNA expression level and the protein level of SLC7A5 significantly.

In addition, recent studies have shown that the SLC7A5 gene is directly related to the mTOR pathway. Sokolov et al. demonstrated that Slc7a5 is required for mTOR pathway activity, maturation and survival, which may help explain why Slc7a5 mutations prevent normal brain development and function (25). Also, another recent report showed overexpressed SLC7A5 promotes mTOR-P70S6K signals and enhances the expression of MMP3 and MMP13 at the protein level in rheumatoid arthritis synoviocytes (20).

Akt signalling targets mTOR, which promotes angiogenesis (4). Jomarv et al. demonstrated that in mice, inactivation of the Akt survival pathway results in photoreceptor cell death (21). Our results showed that AKT1 mRNA expression levels were significantly decreased, and accordingly, mTOR mRNA expression levels were also significantly low expression in mir-626 mimics ARPE-19 cells compared to control cells.

The phosphatidylinositol 3- kinase (PI3K)/AKT pathway is directed downstream by the target of PTEN, PI3K signalling is suppressed, and pathway signalling does not occur. PI3K is involved in several cellular processes, including cell proliferation, apoptosis, and differentiation (26). Also, our data showed that Pten mRNA expression is significantly increased in mir-626 mimic groups and siSLC7A5 groups.

Elorza et al. showed that activation of the HIF2A pathway increases mTORC1 activity by upregulating the expression of the amino acid carrier SLC7A5 (27). Also, a recent study by Sokolov et al. reported that Slc7a5 is required for mTORC1 pathway activity, dendrite maturation, and survival (25). However, our result showed HIF1A and HIF2A mRNA expression levels were significantly upregulated in mir-626 mimics ARPE-19 cells compared to control cells. This study provides evidence that despite the increased expression of HIF1A and HIF2A genes, SLC7A5 gene expression and mTOR pathway activity in the cell ceased due to suppression of SLC7A5 by mir-626.

Another interesting fact is that many of these proteins are associated with the HIF-1 signaling pathway. HIF-1 is a transcriptional regulator that mediates the cellular responses to reduced oxygen levels through changes in gene expression (28).

Santos and colleagues came to the following conclusion in a study they conducted; glycolytic enzymes and glucose transporters appear to be upregulated in response to HIF-1 (29). HIF-1a regulates SLC17A7 (Glut 1) and SLC17A8 (Glut 3). These data confirm our high expression levels of SLC17A7 and SLC17A8 mRNA.

The transcription factor CREB plays an important role in regulating cellular responses, like proliferation and survival, across multiple cell types exposed to oxidative stress (30). Creb anti-apoptotic signalling might inhibit caspase 3 activity and other cell death reactions (31). Our result demonstrated that Creb mRNA levels showed no significant change.

Some reports have revealed that H2O2-induced ARPE-19 cells apoptosis is related to the mitochondrial apoptotic signalling, which involves the proapoptotic protein Bax and the downstream protein caspase-3 (32-34). Bax and caspase3 mRNA expression levels were examined to understand the mir-626 role in the apoptosis pathway.

SLC7A5 knockdown by mir-626 significantly increases the Bax and caspase-3 mRNA expression levels in the apoptosis pathway in the mir-626 mimics group. In summary, our work has identified miR-626 as an essential regulator of the expression of the SLC7A5 gene. Thus, we identified how miR-626 causes apoptosis and macula degeneration in RPE cells by targeting SLC7A5 through the mTOR signaling pathway. Importantly, we determined that miR-626 is essential to play a role in AMD. However, more research is needed to understand the mechanism of miR-626 targeting SLC7A5 in RPE cells through the AKT- mTOR signaling pathway.

**Acknowledgement**
The authors would like to acknowledge that this paper is submitted in partial fulfilment of the requirements for the Ph.D. degree at Yildiz Technical University.

**Funding**
Founded by Bezmialem Vakıf University Scientific Research Project Office.

**Disclosure of potential interest conflicts**
None of the authors has any potential conflict of interest.

**Contribution**
Involved in the design and conduct of the study (CE, AE, NOO); data collection (CE, AE); analyse of the data (CE, FA, NOO); writing the article (CE, ESA, NOO); review of the study (CE, ESA, NOO, HA).

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