Exosomal miR-29b of Aqueous Humour Mediated Variation of Ca2+ in Diabetes and Cataract Patients

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Abstract

Background: Ca^{2+} was supposed to play an important role in the formation of cataract. Considering our increasing knowledge of exosomes, exosomal miRNAs isolated from aqueous humour may play an important role in the mechanism of diabetes and cataracts. In this study, we aimed to investigate the role of exosomal miR-29b and Ca^{2+} in regulating the function of human lens epithelial cells.

Methods: Exosomes were isolated from human aqueous humour by ultracentrifugation, and visualized by nanoparticle tracking and transmission electron microscopy. Exosomal miRNA sequencing was performed to identify differentially expressed miRNAs between diabetes and cataracts group (DMC) and age-related cataracts group (ARC). TargetScan was used to predict potential target of certain miRNA. The expression of CACNA1C mRNA was determined by quantitative real-time PCR and CACNA1C protein was determined by Western blotting. Concentration of Ca^{2+} of human aqueous humour and cell culture supernatant was detected by the Calcium Assay Kit. Cell Counting Kit-8 was used to determine cell viability.

Results: Exosomes could be isolated from human aqueous humour, which had a typical cup-shaped phenotype and a particle size distribution in accordance with micro extracellular vesicles. Exosomal miRNA sequencing revealed that miR-29b was significantly downregulated in diabetes and cataracts group (DMC) compared with age-related cataracts group (ARC). Ca^{2+} concentration of human aqueous humour in DMC was higher than that in ARC. Cell culture supernatant transfected with miR-29b inhibitors had a higher concentration of Ca^{2+} than that transfected with miR-29b mimics. miR-29b reduced the viability of human lens epithelium cells (HLECs) by up-regulating CACNA1C expression.

Conclusions: Exosomes isolated from human aqueous humour contained abundant miRNAs. A significantly expressed miRNA, miR-29b, could affect the concentration of Ca^{2+} and regulate HLEC processes by up-regulating CACNA1C.

Background

Opacification of crystalline lens is a disease that can be caused by many factors, which is called cataract. Diabetes is a complex metabolic disorder that also involves the small blood vessels, often causing widespread damage to tissues, including eyes. Cataract is a common ocular complication caused by diabetes. Bilateral cataracts occasionally occur with rapid onset in severe juvenile diabetes, which is called true diabetic cataract. However, true diabetic cataract is rare. What's more common is senile cataract in people with diabetes, which is called diabetes and cataracts in our study. The potential mechanisms for the pathogenesis of diabetes and cataracts are complicated and include the p38-MAPK signalling pathway[1, 2], polyol pathway[3], and changes in inflammatory cytokines[4–10], and others. However, the precise mechanism of diabetes and cataracts remains unclear.

Ca^{2+} existed in the endocytoplasmic reticulum and played an important role in the processing of polypeptide chain in protein translation and post-translation. It was reported that abnormal distribution of Ca^{2+} could lead to dysfunction of endocytoplasmic reticulum and mitochondria, thus causing some metabolic diseases like diabetes[11]. Previous study has reported that Ca^{2+}-CaM abnormality existed in cataracts and it was found that L-type calcium channels were extensively distributed in lens epithelial cells, and the inhibition of L-type calcium channels could lead to formation of cortical cataract[12]. L-type calcium channels were partially translated from gene CACNA1C, which was also found to mutate in diabetes and cataracts[13]. Given the critical role of Ca^{2+} in the lens epithelial cells, it is required to further investigation on the role of Ca^{2+} in diabetes and cataracts.

Exosomes are a kind of micro extracellular vesicles (30–150 nm), which could be secreted by almost all types of cells and contained nucleus acids, proteins and lipids[14]. Exosomes have been widely studied in diabetes, neural diseases, tumours, cardiovascular diseases, etc[15–25]. Previous study has reported that there are abundant exosomes in human aqueous humour[26]. Exosomal microRNAs (miRNAs) played important roles in the mechanisms of diabetes[27–29] and diabetic retinopathy[30–32]. However, the function and characterization of exosomes and miRNAs still remains unknown in diabetes and cataracts.

In this study, we deemed aqueous humour as a microenvironment on lens epithelial cells and focused on the influence of aqueous humour on the crystalline lens, and sequenced exosomal miRNAs between DMC and ARC. We found that decreased expression of miR-29b could upregulate the expression of CACNA1C, increase the concentration of Ca^{2+}, and affect the apoptosis of human lens epithelial cells (HLECs) in patients with diabetes and cataracts suggesting a potential role of exosomal miRNAs in the pathogenesis of diabetes and cataracts.

Methods

1 Ethics statement

The use of human aqueous humour (AH) samples from cataract eyes during surgery was approved by the Institutional Review Board of Eye and ENT hospital of Fudan University. This study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. Written informed consent was obtained from every enrolled participant.

2 Collection of AH and human lens epithelium

We collected AH and human lens epithelium from 36 patients with diabetes and cataracts (age from 45-76 years old, free of other ocular diseases, and lenticular opacity ranging from C3-4, NO2-3, NC2-3, and P1-3 by LOCSIII) and 43 patients with age-related cataracts (age from 62-89 years old, free of other ocular diseases, C3-4, NO2-3, NC2-3, and P1-2) before cataract surgery at Eye and ENT Hospital of Fudan University. AH samples were obtained before the collection of lens epithelium samples. The lens epithelium samples were acquired by intact continuous curvilinear capsulorhexis during cataract surgery

for ARC patients by the same experienced surgeon (Yi Luo). All AH and human lens epithelium samples were stored in a freezer at -80℃ until the next step.

3 Isolation of exosomes

The AH samples from 36 patients with diabetes and cataracts were pooled together as the DMC group, and the AH samples from 43 patients with age-related cataracts were pooled together as the ARC group. Exosomes were isolated using ultracentrifugation. Procedures were as followed: a) Take AH samples out and thaw in 27℃ water bath. b) 4℃, 2000 g, 10 min, and remove supernatant. c) 4℃, 10000 g, 30 min, and take supernatant. d) 4℃, 110000 g, 75 min, and discard supernatant. e) Resuspend pellet and filter with 0.22 um membrane. f) 4℃, 110000 g, 75 min, and abandon supernatant.

4 Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) of AH exosomes

Purified exosomes were diluted in PBS. 5 μL samples were absorbed onto copper grids and dried for 5 minutes at room temperature. After that, a drop of 2% uranyl acetate solution was added for 20 minutes, and the sample was air-dried and examined by TEM (Tecnai G2 Spirit BioTwin, FEI, USA). Particle size, concentration, and distribution of exosomes were determined by NTA (ZetaView, Particle Matrix, Germany).

5 Exosomal RNA extraction and miRNA sequencing analysis

Exosomal RNA was extracted from the DMC group and the ARC group using the miRNeasy Micro Kit (217084, QIAGEN, Germany) according to the manufacturer's guidelines. RNA libraries were prepared and sequenced on an Illumina HiSeq 2500 platform. Read counts were obtained by FeatureCounts software. FastQC software was used for quality control. Additionally, we used Cutadapt software to remove low-quality reads and high-quality reads were used to analyse miRNAs by mapping to the human reference genome using Bowtie software. A fold change>1.2 or <0.83 was considered to indicate differentially expressed miRNAs by DESeq2.

6 RNA extraction of epithelium samples and quantitative real-time PCR (qRT-PCR)

Epithelium samples from 43 patients with age-related cataracts were classified as ARC group. Epithelium samples from 36 patients with diabetes and cataracts were classified as DMC group. In each group, 4 to 5 epithelium samples were pooled together to obtain enough RNA. Total RNA from all epithelium samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with the RT reagent Kit (Takara Bio, Inc, Japan) according to the manufacturer's protocol. Expression of mRNAs was detected using the SYBR Green detection kit (Takara, Japan) on the LightCycler 480II Real-Time PCR System (Roche, Switzerland). GAPDH was detected as the internal control. RNA expression was determined by the $2^{-\Delta\Delta CT}$ method.

7 Human lens epithelial cell culture and transfection

HLECs were cultured in 35-mm culture Petri dishes with growth medium containing DMEM (Gibco, USA) with 10% foetal bovine serum (FBS, Gibco, USA). MiR-29b mimics (50 nM) and inhibitors (100 nM) were transfected into HLECs when the cells covered 70-80% of the entire dish.

8 Examination of Ca^{2+} of AH samples and cell culture supernatant

Concentration of Ca^{2+} of AH samples and cell culture supernatant was detected by using Calcium Assay Kit (Colorimetric) from Abcam (Cambridge, MA, USA) according to the manufacturer's protocol. We added 50 μL of AH samples and cell culture supernatant to each well of the 96-well plates. Then 90 μL of the Chromogenic Reagent and 60 μL of Calcium Assay Buffer were added into each well for 10 minutes at room temperature protected from light. Absorbance was measured at a wavelength of 575 nm using an automatic microplate reader (Tecan, Switzerland). Concentration of Ca^{2+} was equivalent to Abs/Vol (ug/μL). (Abs referred to absorbance of AH samples and cell culture supernatant, Vol referred to the volume of AH samples and cell culture supernatant added to each well)

9 Cell Counting Kit-8 (CCK-8) cell proliferation and cytotoxicity assay

Cell viability was determined by using CCK-8 kit (Dojindo, Japan) according to the manufacturer's protocol. Transfected cells were plated onto 96-well plates and cultured for 24 hours. CCK-8 (10 μL) was then added for 1 hour at 37°C. We used Tert-butyl hydroperoxide solution (TBHP) as an oxidative stimulus. Absorbance was measured at a wavelength of 450 nm using an automatic microplate reader (Tecan, Switzerland). The cell viability was equivalent to (At-Ab)/(Ac-Ab). (At referred to absorbance of transfected cell groups, Ac referred to absorbance of controlled groups, Ab referred to absorbance of blank groups)

10 Western blot

Protein was extracted by RIPA lysis buffer (Biotech Well, Shanghai, China). Equal amounts of proteins were resolved by SDS-PAGE using 5% acrylamide-containing gels, followed by electrophoretic transfer to PVDF membranes. The membranes were blocked with transfer buffer (Biotech Well, Shanghai, China) and incubated overnight with the monoclonal primary antibodies at a 1:200 dilution, followed by secondary antibodies at a 1:2000 dilution. The signaling of western blotting was then observed using ECL prime reagents (Biotech Well, Shanghai, China) and scanned using a Peiqing automatic gel imaging analysis system (Shanghai, China). The L-VOCC polyclonal antibody (21774-1-AP) was purchased from Proteintech Group (USA). The anti-GAPDH antibody and the goat anti-rabbit IgG (H+L) secondary antibody were purchased from Biotech Well (Shanghai, China)

12 MicroRNA target prediction

Potential targets of miR-29b were predicted by the Targetscan database (http://www.targetscan.org)[33].

13 Statistical analysis

All data are shown as the mean±SD, and experiments were repeated three times. Statistical significance was determined by two-tailed Student's *t*-test, One-way ANOVA or chi-square test using IBM SPSS 21.0(USA). *P*-value<0.05 was considered statistically significant.

Results

1 Cup-shaped phenotype observed using TEM and particle size distribution of exosomes using NTA

To identify the characteristics of exosomes, we used TEM to observe the morphology of exosomes isolated from aqueous humour, and we observed a typical cup-shaped phenotype in both groups (Fig. 1a).

The particle diameter was 129.8 nm in the ARC group and 116.3 nm in the DMC group. Particle sizes were mainly distributed at 80–120 nm, and the concentration reached more than E + 10 particles/ml in both groups (Fig. 1b).

2 Differential expression of miRNAs in AH samples of the DMC and ARC groups

miRNA sequencing was used to examine the expression of miRNAs in AH samples of the DMC and ARC groups. A total of 552 miRNAs were obtained from all samples. Of them, 119 miRNAs were unchanged; 295 miRNAs were upregulated and 138 miRNAs were downregulated in AH samples of the DMC group compared with the ARC group (fold change > 1.2 in upregulated miRNAs, and fold change < 0.833 in downregulated miRNAs) (Table S1). Among the 138 downregulated miRNAs, miR-29b was highly expressed in the DMC group compared with the ARC group (with fold change = 0.286) (Fig. 2).In addition, CACNA1C was a potential target of miR-29b through the TargetScan website. We thus investigated the role of miR-29b in the regulation of HLECs function. Part of differentially expressed miRNAs were shown in Fig. 3.

3 Differential CACNA1C expression between DMC and ARC epithelium samples

CACNA1C mRNA expression was upregulated in DMC epithelium samples compared with that in ARC epithelium samples by qRT-PCR, and the difference was statistically significant (Fig. 3).

4 Different concentration of Ca^{2+} of AH samples in the DMC and ARC groups

To determine the effect affected by the different expression of CACNA1C, we detected the concentration of Ca^{2+} of AH samples in two groups. Mean concentration of Ca^{2+} of AH samples was 0.075 ± 0.005 ug/uL in the DMC group and 0.06 ± 0.004 ug/uL in the ARC group. The concentration of Ca^{2+} of AH samples was higher in the DMC group than that in the ARC group (25% more), and the difference was statistically significant (Fig. 4).

5 MiR-29b mimics and inhibitors significantly changed CACNA1C expression in HELCs

To determine whether exosomal miR-29b influenced the expression of CACNA1C in HELCs, we used miR-29b mimics and inhibitors to transfect HLECs and then detect the expression of CACNA1C mRNA of HLECs. miR-29b mimics significantly downregulated CACNA1C expression in HELCs, while miR-29b inhibitors significantly upregulated CACNA1C expression in HELCs (Fig. 5a). Additionally, western blot analysis showed that transfection of miR-29b mimics led to a reduction in CACNA1C expression. By contrast, transfection of miR-29b inhibitors led to increased CACNA1C expression (Fig. 5b). The results were consistent with the results observed in epithelium samples.

6 Different concentration of Ca^{2+} of cell culture supernatant transfected by miR−29b mimics and inhibitors

To determine whether different expression of CACNA1C regulated by miR-29b affected the concentration of Ca^{2+}, we then detect the concentration of Ca^{2+} of cell culture supernatant transfected by miR−29b mimics and inhibitors. Mean concentration of Ca^{2+} of cell culture supernatant was 0.09 ± 0.005 ug/μL in the control group, and 0.078 ± 0.01 ug/μL transfected by miR−29b mimics, and 0.106 ± 0.002 ug/μL transfected by miR−29b inhibitors. The concentration of Ca^{2+} was higher in the cell culture supernatant transfected by miR−29b inhibitors than that in normal cell culture supernatant. The concentration of Ca^{2+} was 26.4% higher in the cell culture supernatant transfected by miR−29b inhibitors than that in the cell culture supernatant transfected by miR−29b mimics (Fig. 6). The result was similar to that of AH samples.

7 The role of miR-29b in the regulation of cell viability of HLECs

We further investigated the effect of miR-29b on the viability of HLECs. Both under normal conditions and under oxidative stress, miR-29b inhibitors significantly reduced the viability of HLECs compared with miR-29b mimics, and the difference was statistically significant (Fig. 7a, b). Under oxidative stress, the viability of HLECs transfected with miR-29b mimics was slightly reduced, but the difference was not statistically significant (Fig. 7c). However, the viability of HLECs transfected with miR-29b inhibitors was significantly reduced, and the difference was statistically significant (Fig. 7d).

Discussion

Cataracts are more likely to be formed in patients with diabetes, the symptoms of which are similar to those of age-related cataracts, while the progression is more rapid. Recently, exosomes have been widely studied in many fields, especially in diabetes. To seek a possible mechanism of diabetes and cataracts, we investigate the role of exosomal miR-29b and Ca^{2+} in human lens epithelial cells.

Ca^{2+} is an important factor in life activity. Ca^{2+} is potentially involved in the formation and the regulation of diabetes and cataracts. Abnormal distribution of Ca^{2+} affects the function of endocytoplasmic reticulum and mitochondria, thus causing some metabolic diseases like diabetes[11]. Besides, Ca^{2+}-CaM abnormality exists in cataracts. L-type calcium channels are extensively distributed in lens epithelial lens

cells, and the inhibition of L-type calcium channels could lead to formation of cortical cataract[12]. In addition, L-type calcium channels are partially translated from gene CACNA1C, which is mutated in diabetes and cataracts[13]. More importantly, calcium might induce the conformational damages of A-crytallin and accelerate the development of the cataract[34]. However, the precise mechanism of how Ca^{2+} plays its role still remained unclear. In our study, we detected the concentration of Ca^{2+} of AH samples of DMC and ARC, and found that the concentration of Ca^{2+} of AH samples is higher in the DMC than that in the ARC, showing a potential relationship among Ca^{2+} and diabetes and cataracts. Besides, CACNA1C mRNA expression is upregulated in DMC epithelium samples compared with that in ARC epithelium samples, which partially translates L-type calcium channels that might lead to the different concentrations of Ca^{2+} in two groups.

Exosomes have been widely studied in diabetes, neural diseases, tumors, cardiovascular diseases, etc[15–25]. Abundant exosomal miRNAs were found in human aqueous humour, containing miR-486, miR-204, miR-184, etc[26]. Exosomal miRNAs played important roles in the mechanisms of diabetes[27–29]. In diabetes-associated ocular diseases (such as diabetic retinopathy), exosomal miRNAs play an important role by affecting the integrity of vascular endothelia[31]. It was also reported that pancreatic β cell derived exosomal miR-15a might damage the retina by targeting Akt3 and causing diabetic retinopathy[30]. In addition, exosomes of plasma carrying IgG can damage retinal vessels[32], and retinal pigment epithelium (RPE) cell-derived exosomes can participate in immunoregulation by killing targeted monocytes[35, 36], thus causing diabetic retinopathy. In addition, miRNAs play important roles in regulating the function of coding genes. Down-regulation of miR-2113 inhibits high glucose-induced mesenchymal activation and fibrosis[37]. MiR-30a combined with circHIPK3 could regulate the expression of VEGFC, FZD4 and WNT2, thus changing the viability and apoptosis of retinal pigmented epithelial cells[38]. More importantly, our previous study showed up-regulation of miR-193a caused by down-regulation of circHIPK3 could regulate the formation of cataract by targeting CRAYY[39]. However, there are few studies reporting the function and characterization of exosomes and miRNAs in diabetes and cataracts.

In our study, we deemed exosomes of aqueous humour as the micro environment that could influence the viability of human lens epithelium cells. Therefore, we isolated exosomes of aqueous humour by ultracentrifugation and identified the exosomes through NTA and TEM. We then sequenced exosomal miRNA and revealed that miR-29b is differentially downregulated in patients with diabetes and cataracts and compared the data with that from age-related cataracts. MiR-29b was previously reported to be significantly changed across the diabetes spectrum and associated with measures of pancreatic islet β cell function and glycemic control[40]. In culturing trancfected cells, the concentration of Ca^{2+} was 26.4% more in the cell culture supernatant transfected by miR−29b inhibitors than that in the cell culture supernatant transfected by miR−29b mimics, which almost equivalent to that of AH samples (25%). The results in our study revealed that the downregulation of miR-29b could cause the upregulation of CACNA1C expression and an increase of concentration of Ca^{2+}, which results in a lower cell viability of human lens epithelium cells. Besides, HELCs transfected with miR−29b inhibitors are more sensitive to

oxidative stress. Here, we provide a novel mechanism that the function of HLECs in diabetes and cataracts might be regulated through exosomal miR−29b/CACNA1C/Ca^{2+} (Fig. 8).

Conclusions

Exosomes isolated from human aqueous humour contained abundant miRNAs. A significantly expressed miRNA, miR-29b, could affect the concentration of Ca^{2+} and regulate HLEC processes by up-regulating CACNA1C.

Abbreviations

DMC	Diabetes and cataracts group
ARC	Age-related cataracts group
HLECs	Human lens epithelial cells
AH	Aqueous humour
TEM	Transmission electron microscopy
NTA	Nanoparticle tracking analysis
CCK-8	Cell Counting Kit-8
qRT-PCR	quantitative real-time PCR

Declarations

Ethics approval and consent to participate

The use of human aqueous humour (AH) samples from cataract eyes during surgery was approved by the Institutional Review Board of Eye and ENT hospital of Fudan University. This study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. Written informed consent was obtained from every enrolled participant.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary files.

Competing interests

There is no competing interests in this study.

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Authors' contributions

CG designed the study, conducted part of experiments, analyzed part of data, and was a major contributor in writing the article.

XL designed the study, conducted part of experiments, collected samples, analyze part of data, and write part of the article.

FF conducted part of experiments, collected samples, and write part of the article.

JY conducted part of experiments, helped collected samples, and proofread part of the article.

XZ conducted part of experiments, helped collected samples, and proofread part of the article.

HM conducted part of experiments, helped collected samples, and proofread part of the article.

XL conducted part of experiments, helped collected samples, and proofread part of the article.

YL was the corresponding author, designed the study, proofread and approved the final version of the article.

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Figures

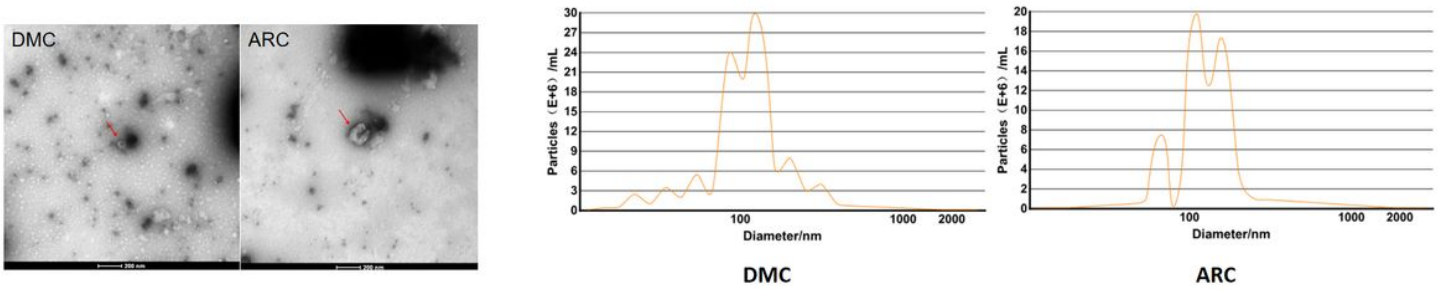

Figure 1

Identification of exosomes using TEM and NTA. a) Typical cup-shaped phenotype under TEM in the DMC and the ARC group. b) Particle size distribution in DMC and ARC group by NTA.

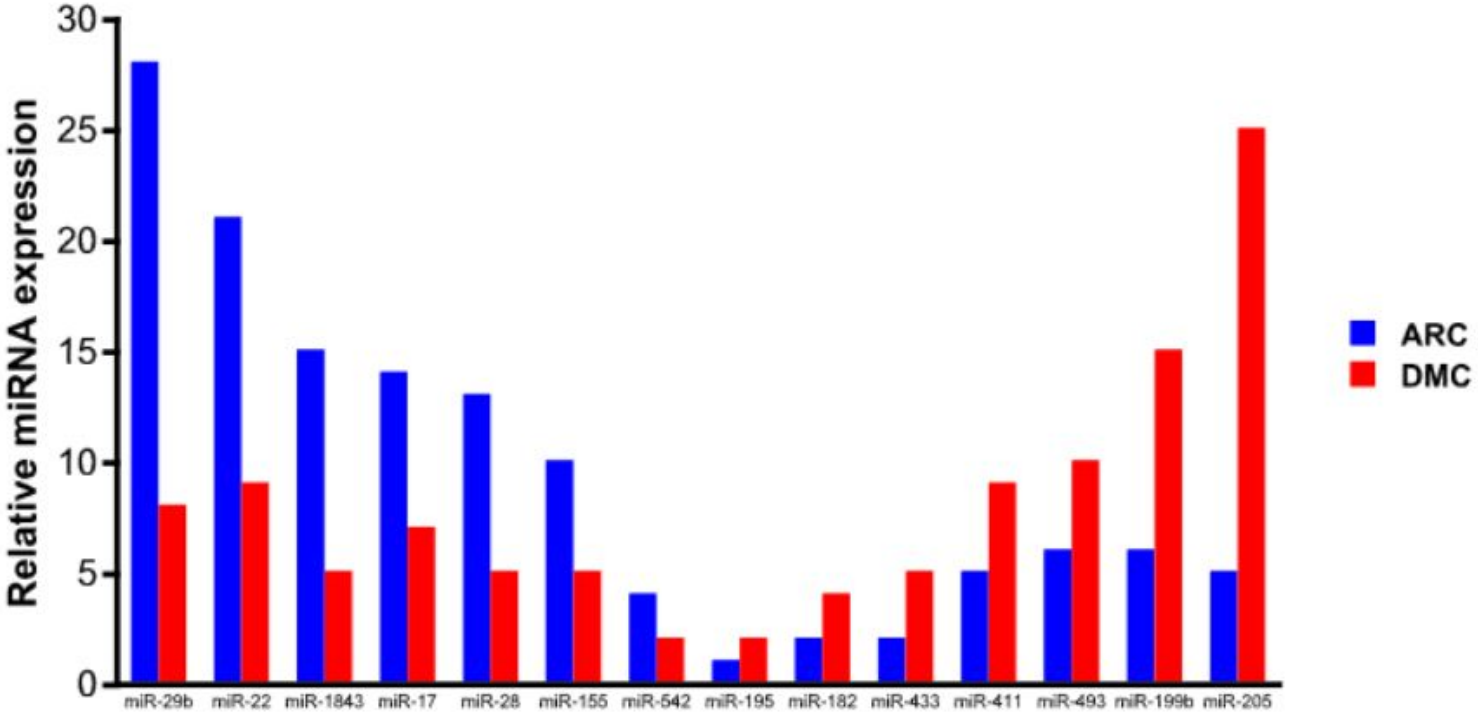

Figure 2

Part of downregulated and upregulated miRNA expression in the DMC group compared with the ARC group

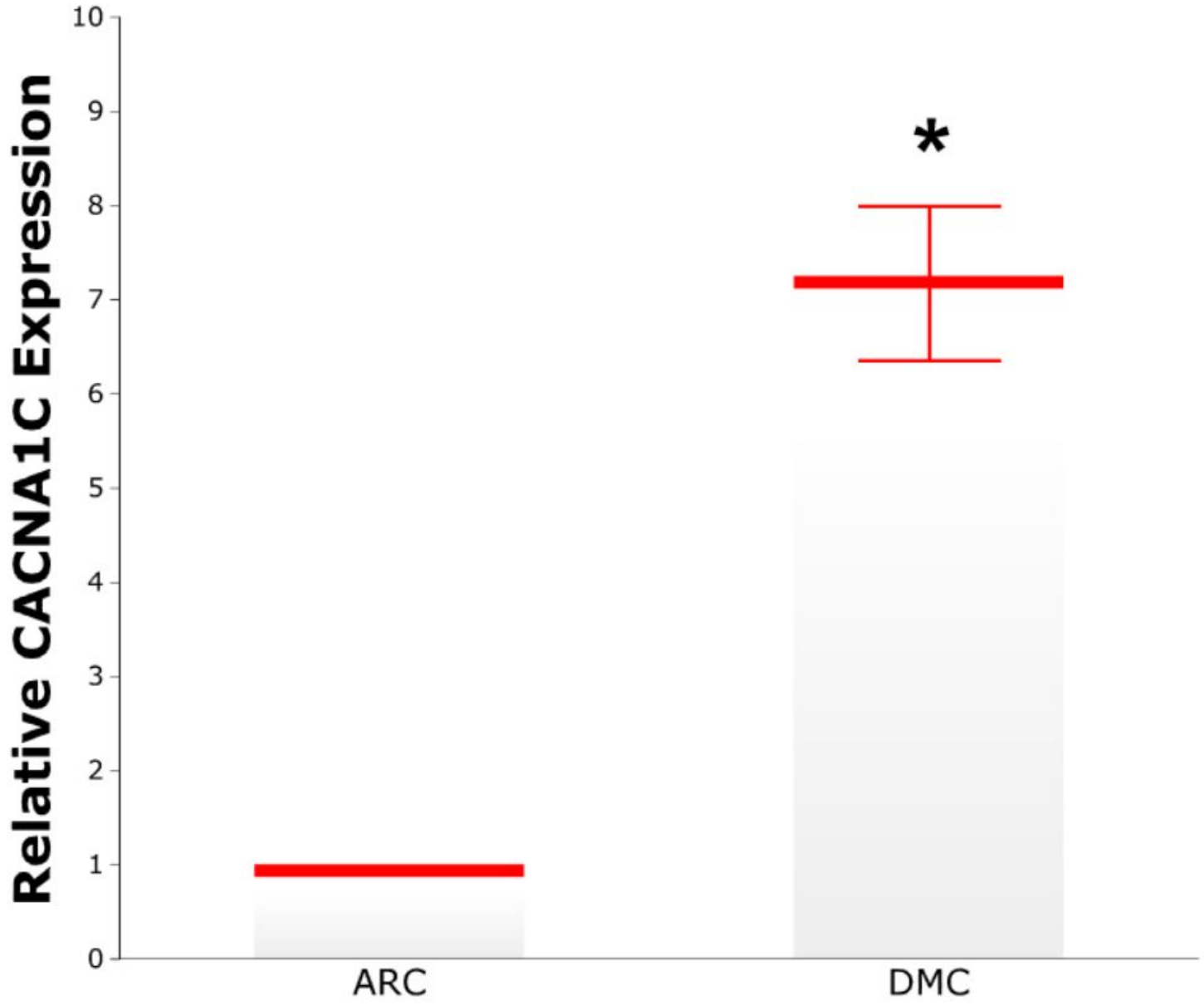

Figure 3

Expression of CACNA1C mRNA was detected by qRT-PCR.

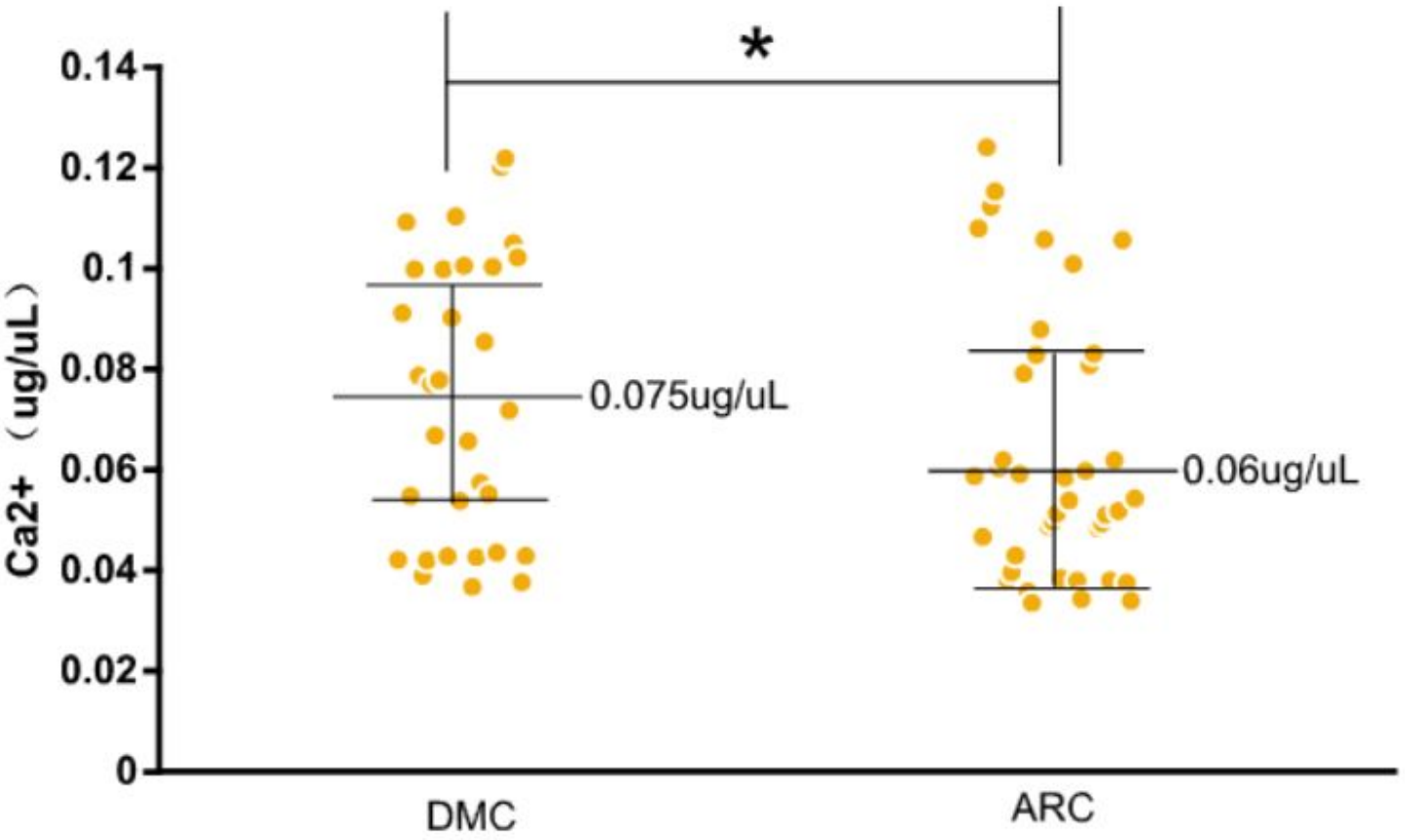

Figure 4

Concentration of Ca2+ of AH samples in the DMC and ARC groups

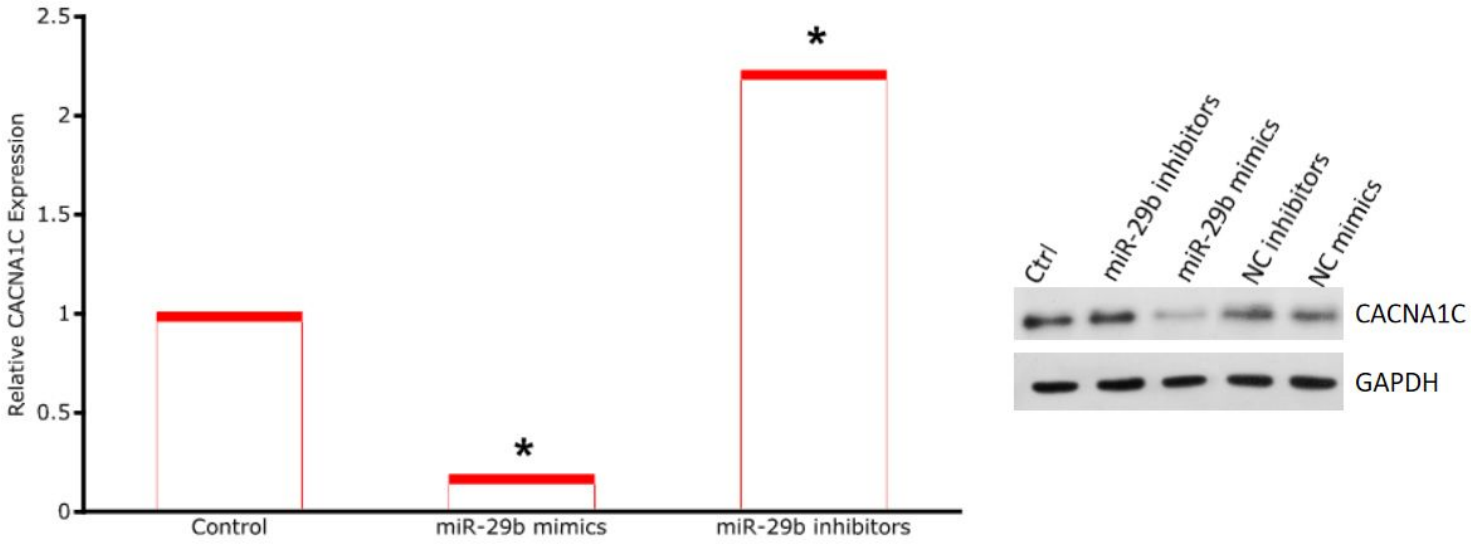

Figure 5

MiR-29b mimics and inhibitors changed CACNA1C expression in HELCs. (a) Expression of CACNA1C mRNA was affected by miR-29b mimics and inhibitors as demonstrated using qRT-PCR. (b) Protein levels of CACNA1C were affected by miR-29b mimics and inhibitors as shown using Western blotting.

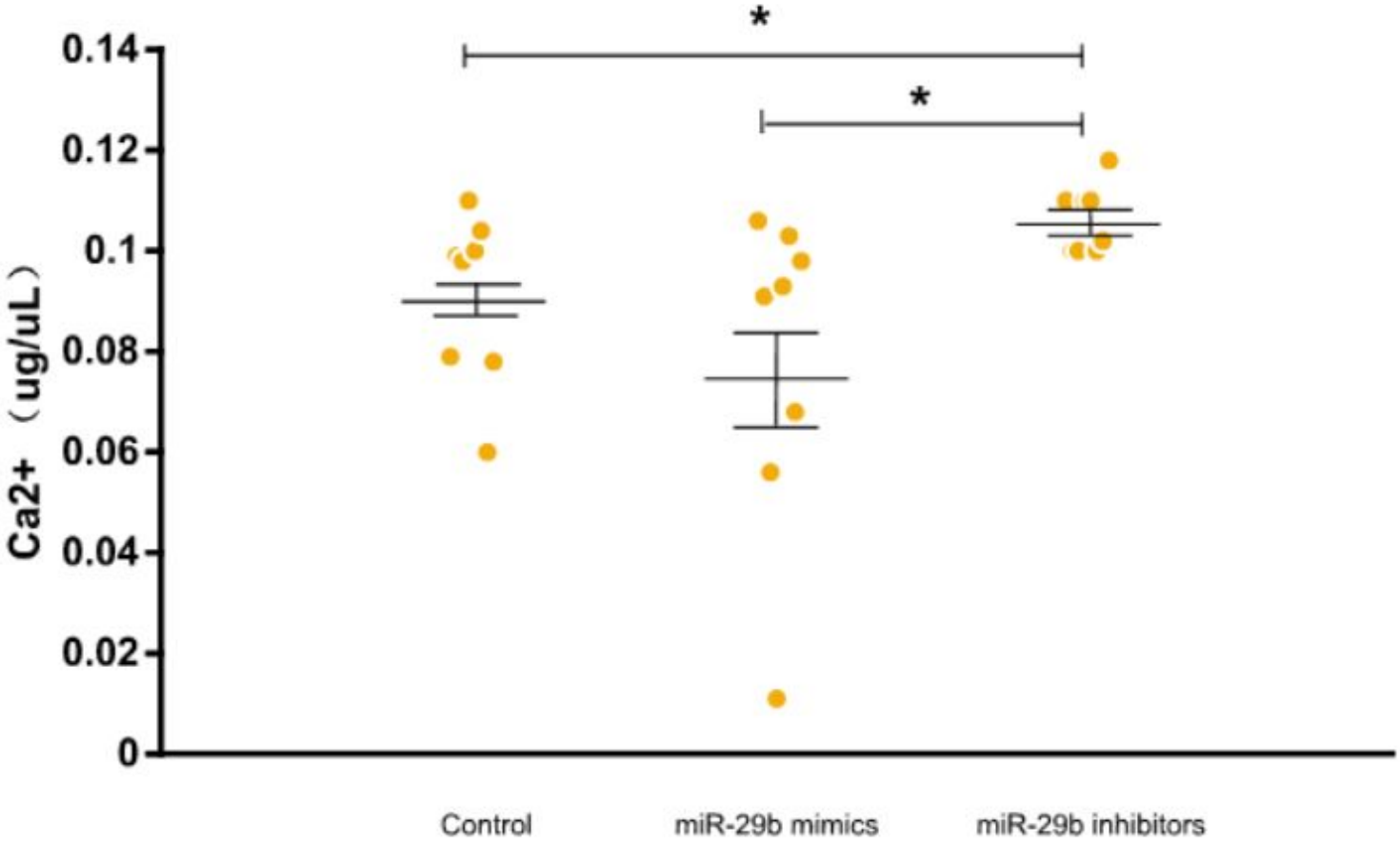

Figure 6

Concentration of Ca2+ in normal cell culture supernatant and in cell culture supernatant transfected by miR-29b mimics and inhibitors

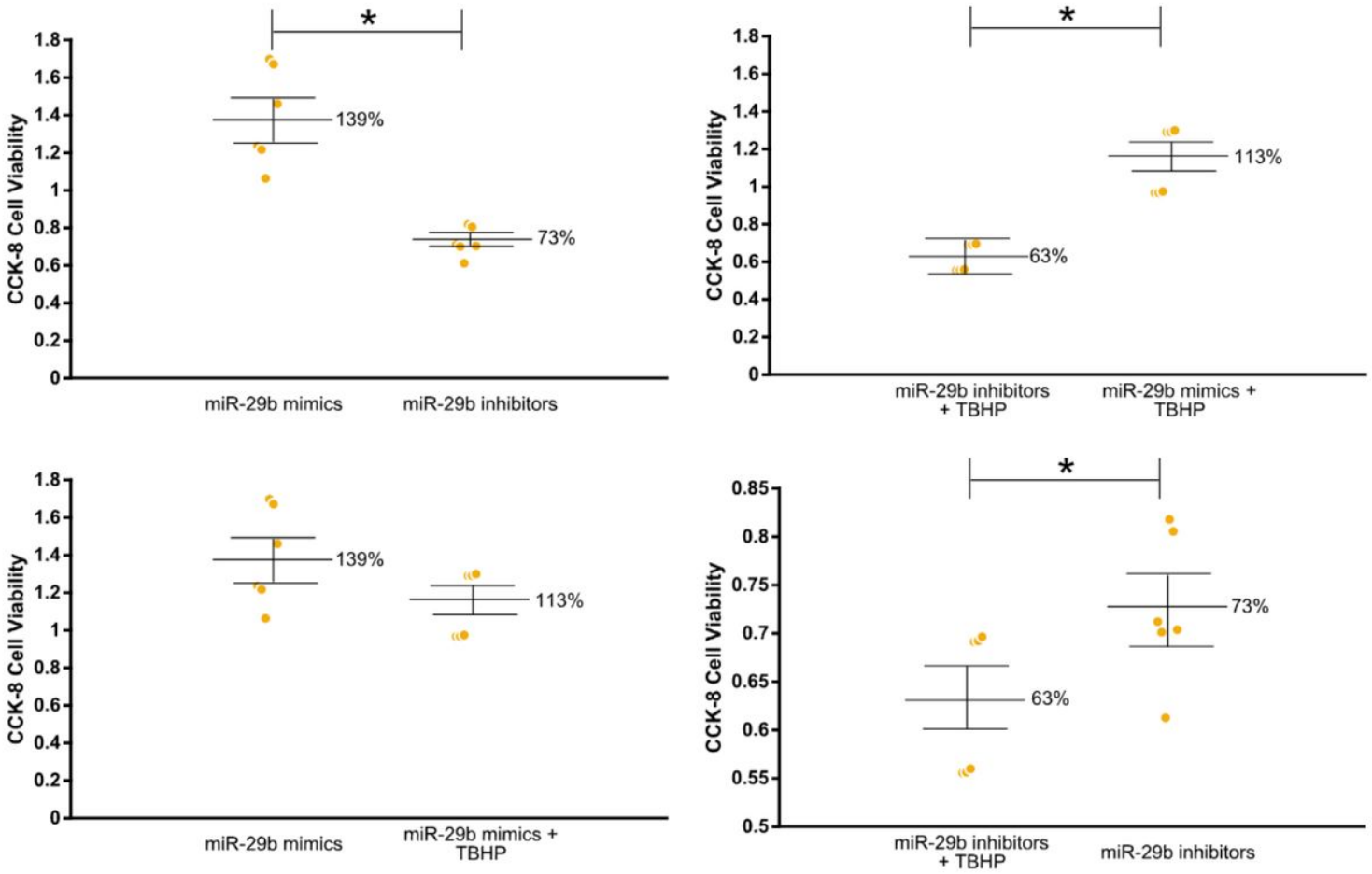

Figure 7

Cell viability was measured by CCK-8. (a) The viability of HLECs trancfected with miR-29b mimics and inhibitors in normal conditions. (b) The viability of HLECs trancfected with miR-29b mimics and inhibitors under oxidative stress. (c) The viability of HLECs trancfected with miR-29b mimics in normal conditions and under oxidative stress. (d) The viability of HLECs trancfected with miR-29b inhibitors in normal conditions and under oxidative stress.

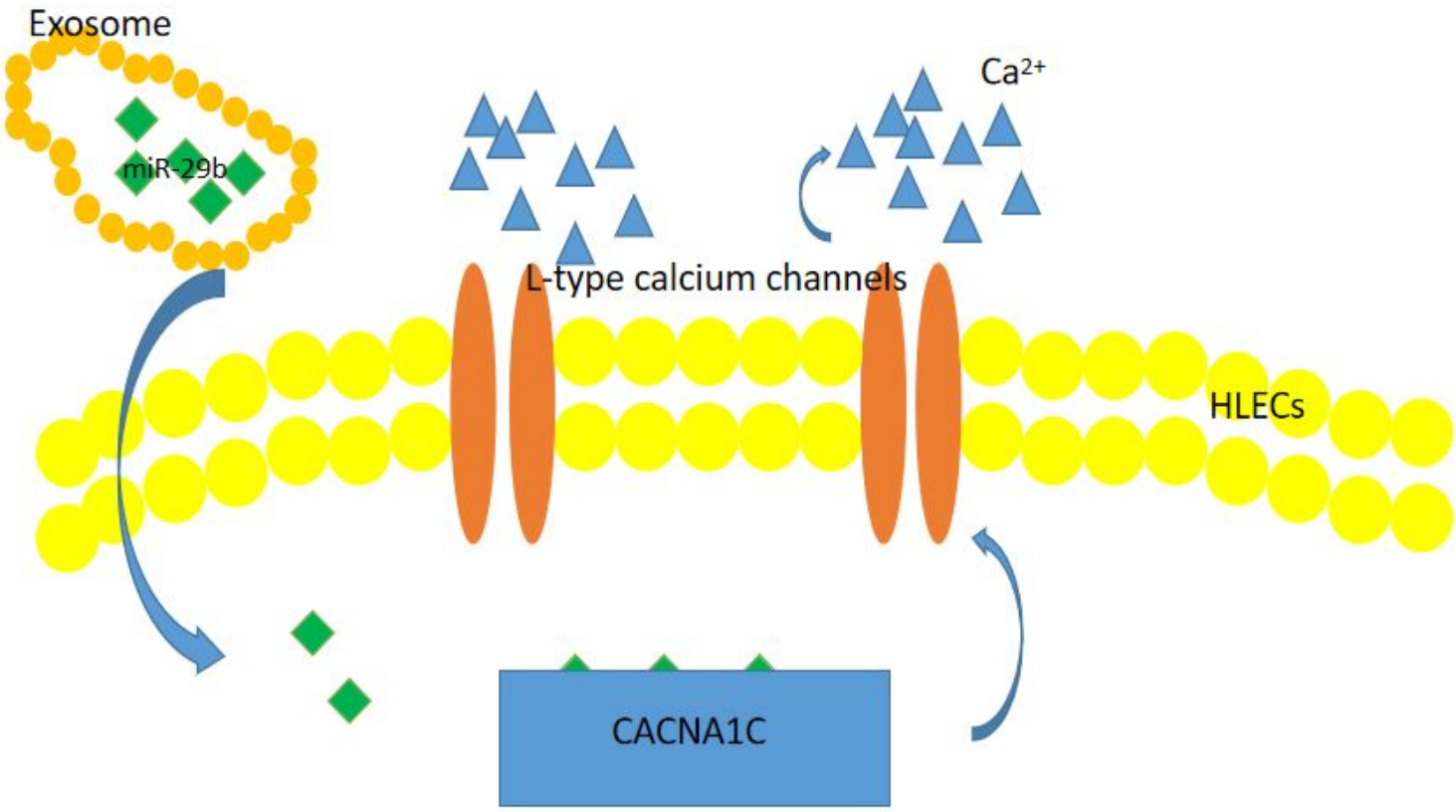

Figure 8

Possible regulation of function of HLECs through exosomal miR-29b/CACNA1C/Ca2+

Supplementary Files

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- supplement17.jpg
- supplement18.jpg
- supplement19.xlsx
- supplement20.xlsx
- supplement21.xlsx
- supplement22.xlsx
- supplement23.xlsx