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MicroRNA-29b over-expression decreases extracellular matrix mRNA and protein production in human corneal endothelial cells

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Abstract

Purpose—MicroRNAs are small non-coding RNAs which regulate gene expression at the post-transcriptional level. We reported that levels of microRNA (miR)-29 family are decreased in Fuchs endothelial corneal dystrophy (FECD) patient corneas. The miR-29 family regulates production of extracellular matrix (ECM) proteins. Accumulation of ECM proteins in Descemet's membrane is an important pathologic change in FECD. In this study, we transfected miR-29b into human corneal endothelial cells and tissues and evaluated ECM protein expression levels.

Methods—Immortalized Fuchs human corneal endothelial cell line (iFECD) was established by infection of FECD patient's corneal endothelial cells with hTERT lentivirus. MiR-29b was transfected into iFECD and expression levels of ECMs (COL1A1, COL4A1, LAMC1) were evaluated with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and Western blot. Expression level of LAMC1 protein in miR-29b transfected donor corneal endothelium was also evaluated by Western blot.

Results—Compared with control, miR-29b expression level after transfection of iFECD was increased to 335.6 (±91.0)% and ECM expression levels were significantly decreased. Compared with control, qRT-PCR demonstrated reduction of ECM to the following levels: COL1A1: 1.9 (±0.4)%; COL4A1: 7.1 (±1.7)%; LAMC1: 21.5 (±2.7)%. Western blot showed reduced protein expression: COL1A1: 4.8 (±3.2)%; COL4A1: 42.5 (±25.0)%; and LAMC1: 44.8 (±3.1)%. In miR-29b transfected corneal tissue, LAMC1 protein expression level was decreased to 14.4 (±20.5)%.

Conclusions—Over-expression of miR-29b decreased ECM protein production in human corneal endothelial cells. Thus, miR-29 replacement therapy might be a new treatment strategy for FECD aimed at reducing pathologic production of ECM proteins in Descemet's membrane.

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Keywords

Fuchs endothelial corneal dystrophy; corneal endothelium; miR-29b; extracellular matrix; microRNA

Introduction

Fuchs endothelial corneal dystrophy (FECD) is a leading cause for corneal transplantation in the United States and world-wide[1–4]. Corneal transplantation has been the only definitive treatment for FECD although newer, non-surgical treatments appear promising[5]. Recent studies have indicated important roles for a variety of pathophysiologic processes in FECD, including oxidative stress, unfolded protein response activation, endoplasmic reticulum stress, autophagy, and apoptosis[6–12].

MicroRNAs are small (~22 nucleotides), single-stranded RNAs regulating eukaryotic gene expression at the post-transcriptional level[13,14]. They cause mRNA destabilization and cleavage or direct translational repression[13]. These short RNAs are predicted to regulate as many as 60% of human protein-coding genes with a given microRNA potentially binding up to 4500 human gene targets within 3' untranslated regions[13].

In non-ocular tissue, a role for miR-29b as a regulator of fibrosis including ECM expression control[15–20] has been proposed. It also has been shown that miR-29 plays a critical role in the pathology of eye diseases, including pterygium[21], cataract[22], glaucoma[23–25] and FECD[26]. In trabecular meshwork cells, miR-29 is a critical regulator of ECM expression [23–25]. We have previously described a comparison of miRNA profiles between FECD and normal corneal endothelial cells and demonstrated widespread miRNA downregulation in FECD[26]. In particular, endothelial expression of miR-29 family members was decreased, which might contribute to increased sub-endothelial ECM accumulation in FECD[26]. In FECD patient corneas, it is known that components of abnormal ECM excrescences (guttae) include: collagen type 1 alpha 1 (COL1A1), collagen type 4 alpha 1 (COL4A1) and laminin gamma 1 (LAMC1)[27,28].

Thus, we hypothesized that miR-29 replacement could be a potential target of future conservative therapeutic approaches in FECD. To this end, we investigated the effects of over-expressing microRNA-29b on extracellular matrix protein expression levels in immortalized cells and normal human corneal endothelial tissue.

Materials and Methods

Cell Culture

An immortalized FECD human corneal endothelial cell line (iFECD) was established by infection of an FECD patient's corneal endothelial cells with hTERT lentivirus. Culture and use of human FECD cells was conducted after patient informed consent via a Johns Hopkins Medicine Institutional Review Board approved protocol (NA_00023119). iFECD was seeded in culture dishes with OptiMEM-1 (ThermoFisher Scientific, Waltham, MA) supplemented with 8% fetal calf serum, antibiotic/antimycotic solution (10,000 units

penicillin (base), 10,000 μg streptomycin (base), and 25 μg of amphotericin B/ml) (Invitrogen, Grand island, NY), 50 μg/ml gentamicin (Invitrogen), 5ng/ml EGF, 20ng/ml NGF, 100μg bovine pituitary extract, 20μg/ml ascorbic acid, 200mg/l calcium chloride and 0.08% chondroitin sulfate. The medium was changed every two days until the cells reached confluence.

Transfection of miR-29b Psh to iFECD

MiR-29b mimic containing a short hairpin structure with a stem loop (miR-29b Psh, Bonac, Fukuoka, Japan) was used in this study. The stem loop contained proline derivatives similar to PnkRNA, as previously described[29]. MiR-29b was transfected into iFECD using miR-29b Psh with lipofectamine RNAiMAX (Invitrogen, San Diego, CA) using standard protocols. The iFECD cells were seeded in 6-well plates (5×10^5 cells/well) at 50% confluency and were used at 70% for transfection the next day. The miR-29b Psh (150μl) and lipofectamine (150μl) were mixed and added to 3ml culture media in each well, followed by incubation at 37°C. Final concentration of miR-29b Psh was 100nM. After 48 hours incubation, total RNA and protein were extracted as described below.

Transfection of miR-29b Psh to human corneal tissue

Six human corneas from three donors were obtained from Lions VisionGift (Portland, OR). Corneas were maintained in preservation media (Optisol-GS; Bausch & Lomb, Rochester, NY) at 4°C. Standard eye bank protocol for informed consent and for protection of donor confidentiality was used. The donor corneas were unsuitable for transplantation. Each cornea was divided into halves and incubated at 37°C for transfection. As with the case of iFECD transfection, cell culture medium containing Psh-lipofectamine complex (Psh final concentration was 100nM) was used. After 48 hours incubation, Descemet's membrane with endothelial cells were stripped and proteins were extracted for Western blot (see below).

RT-PCR

Total RNA samples were extracted from cultured cells using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). Complementary DNA samples were synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), and Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed using TaqMan Universal Master Mix II, no UNG (Applied Biosystems), preamplified cDNA products (diluted 1:20; 5 ul), nuclease free water, and human specific primers (Applied Biosystems, Table 1) in a 20 μL reaction volume. A no-template control was included in each experiment to confirm the absence of DNA contamination. All assays used similar amplification efficiency, and a ΔC_T experimental design was used for relative quantification. Data analysis was performed using StepOne™ software (Version 2.2, Applied Biosystems). COL1A1, COL4A1, and LAMC1 were normalized to GAPDH whereas miR-29b was normalized to RNU48.

Western blot

After miR-29b transfection, cultured cells and endothelial cells from corneal tissue were lysed with ice-cold Tissue Protein Extraction Reagent (Thermo Fisher Scientific)

supplemented with protease inhibitor (1%) and EDTA (1%). Total protein concentration was measured using a protein assay kit (Thermo Fisher Scientific), and each sample was adjusted to 20 mg/ml. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Mini-PROTEAN TGX Gels: Bio-Rad, Hercules, CA) and transferred to PVDF membranes (The PerfectMembrane: GenHunter Corporation, Nashville, TN) that had been soaked in methanol for 1 minute. After blocking with 5% milk for 1 hour, the membranes were then incubated overnight with rabbit anti-COL1A1 antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA), rabbit anti-COL4A1 antibody (1:1000; Cell Signaling Technology), or rabbit anti-LAMC1 antibody (1:1000; Cell Signaling Technology). The membranes were washed and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:10000 dilution; Cell Signaling Technology) for 30 minutes. The membrane was washed with TBST and antigen was detected using ECL solution (Pierce Biotechnology, Rockford, IL). The membrane then was stripped and restained for GAPDH using rabbit anti-GAPDH antibody (1:10000; Cell Signaling Technology) with 1 hour incubation at room temperature.

Statistical analysis

Statistical analysis was performed using JMP (version 12.1.0, SAS, Cary, NC). RT-PCR and Western blot results were compared using the two-tailed Student's t-test. P values less than 0.05 were considered significant.

Results

Psh miR-29b induced miR-29b over-expression in iFECD

Control Psh or miR-29b Psh was transfected into iFECD with lipofectamine. Forty-eight hours later, transfected cells were collected and miR-29b expression was measured using RT-PCR. Compared with the control group, miR-29b expression level in miR-29b Psh transfected cells was increased 328.7 (±45.7)% (p=0.004) (Figure 1).

ECM mRNA and protein expression was decreased in iFECD over-expressing miR-29b

ECM gene expression levels by RT-PCR in immortalized Fuchs corneal endothelial cells transfected with miR-29b Psh were significantly decreased to the following percent of expression levels relative to control Psh transfected cells (mean ± standard deviation): COL1A1: 1.9 (±0.4)% (p=0.021); COL4A1: 7.1 (±1.7)% (p=0.035); LAMC1: 21.5 (±2.7)% (p=0.007) (Figure 2A). ECM protein expression levels in iFECD transfected with miR-29b Psh were significantly decreased to the following percent of expression levels in control Psh transfected cells (mean ± standard deviation): COL1A1: 4.8 (±3.2)% (p=0.001); COL4A1: 42.5 (±25.0)% (p=0.038); LAMC1: 44.8 (±3.1)% (p=0.011) (Figure 2B, 2C, 2D).

LAMC1 protein expression in human corneal endothelium *in situ* was decreased after miR-29b Psh transfection

We sought to determine the effects of miR-29b Psh transfection into human corneal endothelium *in situ*. Human corneoscleral tissue was transfected with miR-29b Psh for 48 hours, and stripped Descemet's membrane/endothelium was used for Western blot analysis. LAMC1 protein expression level in corneal endothelium transfected with miR-29b Psh was

significantly decreased to 14.4 (±20.5)% of control Psh transfected corneal endothelium (p=0.017, Figure 3A, 3B).

Discussion

MicroRNAs regulate eukaryotic gene expression through mRNA destabilization and cleavage or direct translational repression[13,14]. These short RNAs are considered to be important regulators of gene expression in normal and some disease states. Thus, they could represent potentially attractive therapeutic targets.

The present study investigated the effects of over-expression of miR-29b on ECM mRNA and protein levels in iFECD and human corneal endothelium. First, miR-29b expression was increased using miR-29b Psh transfection in iFECD. Over-expressed miR-29b in iFECD down-regulated ECM expression. Further, using *ex vivo* human corneal tissue, over-expression of miR-29b also down-regulated ECM expression in corneal endothelium. The latter finding supports the potential feasibility of modifying miR-29b levels *in vivo* to alter ECM protein production.

Our previous study profiling miRNAs between FECD and normal endothelial cells demonstrated widespread miRNA downregulation in FECD including significant effects on the miR-29 family[26]. This finding was associated in FECD corneas with increased sub-endothelial accumulation of ECM proteins which are targets of miR-29[26]. Furthermore, decreased endothelial expression of DICER1, an important component of miRNA biogenesis, was demonstrated. These results raise the interesting possibility that altered miRNA biogenesis caused by a decrease in DICER1 may contribute to reduction of miRNA abundance. Recent studies demonstrate cytotoxicity of DICER1 depletion in various tissues, and this down-regulation may be triggered by oxidative stress[30–32]. These findings suggest the interesting possibility that oxidative stress in FECD may contribute to DICER1 depletion and a subsequent decrease in miRNA levels with further consequences in FECD pathogenesis[9].

In the early-onset form of FECD, it has been shown that missense mutations Q455K and L450W in the gene encoding the α2 subunit of collagen VIII (COL8A2) are associated with disease pathogenesis[27,33], and collagen type VIII is a component of guttae[34]. Although the α1 subunit of collagen VIII has been reported to be regulated by miR-29b[35], to our knowledge, COL8A2 has not described as a miR-29b target.

In this study, we successfully transfected miR-29b into human corneal endothelial cells by incubation with miR-29b Psh. This result suggests that miR-29b could form the basis of an efficient treatment of FECD in the future. Presently miRNA-based therapies for a variety of diseases are in preclinical or clinical trial stages[36]. Potential approaches for FECD treatment could include delivery of oligonucleotides, plasmids, or adeno-associated virus encoding miR-29b via topical or intracameral route[36].

Increased ECM protein expression is a hallmark feature of FECD. In the present study, miR-29b Psh was used to transfect iFECD and human corneal tissue, resulting in reduced ECM protein expression. Since the Psh platform is DICER independent, we hypothesize that

any potential decrease in DICER levels in these cells would not affect the ability to over-express miR-29b. Thus, miR-29b Psh therapy could be a feasible approach for future FECD treatment and warrants further study.

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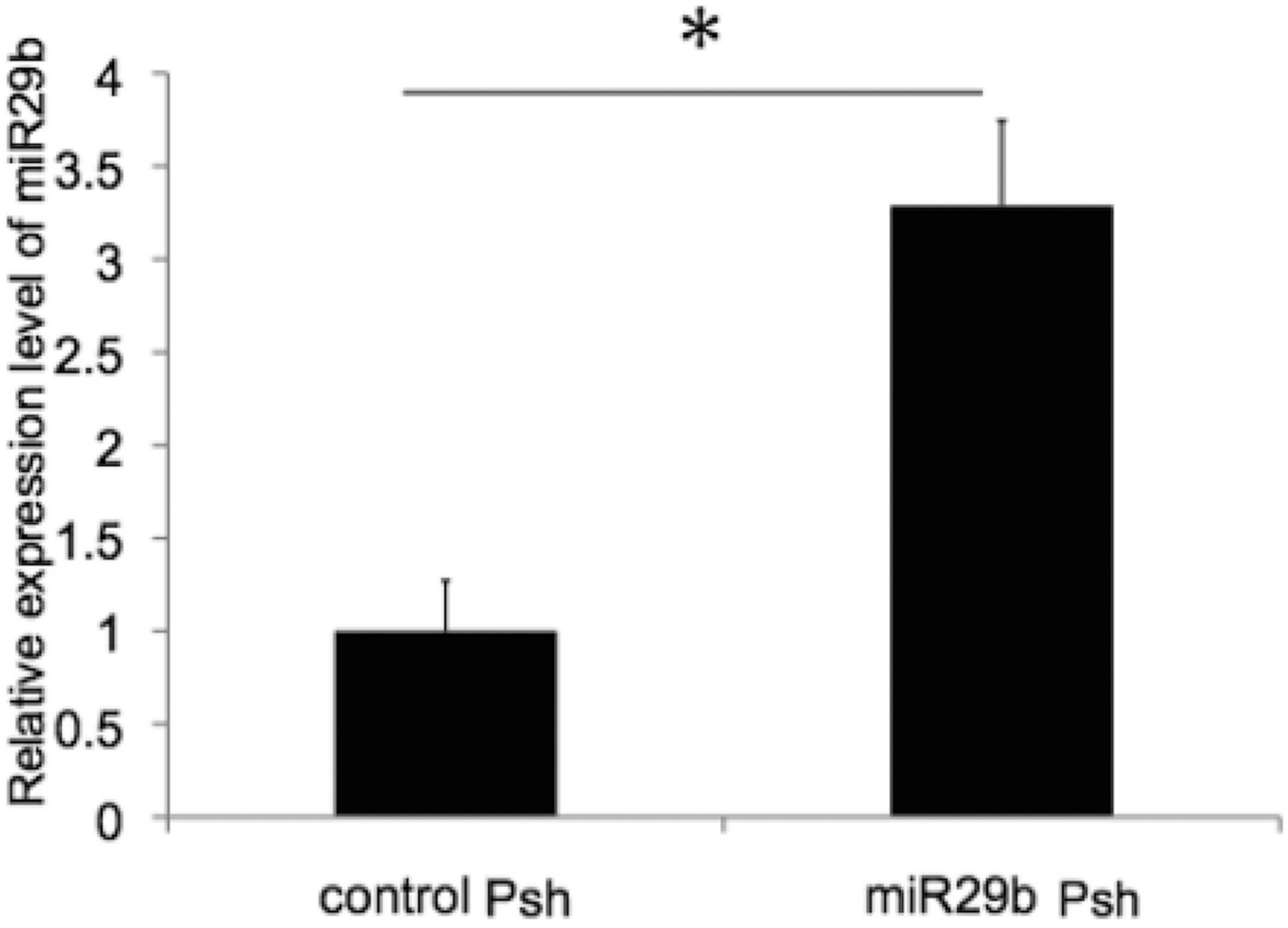

Figure 1. Over-expression of miR-29b in immortalized Fuchs dystrophy corneal endothelial cells

Control Psh and miR-29b Psh were transfected into iFECD. Compared to control, miR-29b expression level in the miR-29b Psh group was increased 3.3 fold (*$p < 0.05$, n = 3 per each condition, bar = standard deviation).

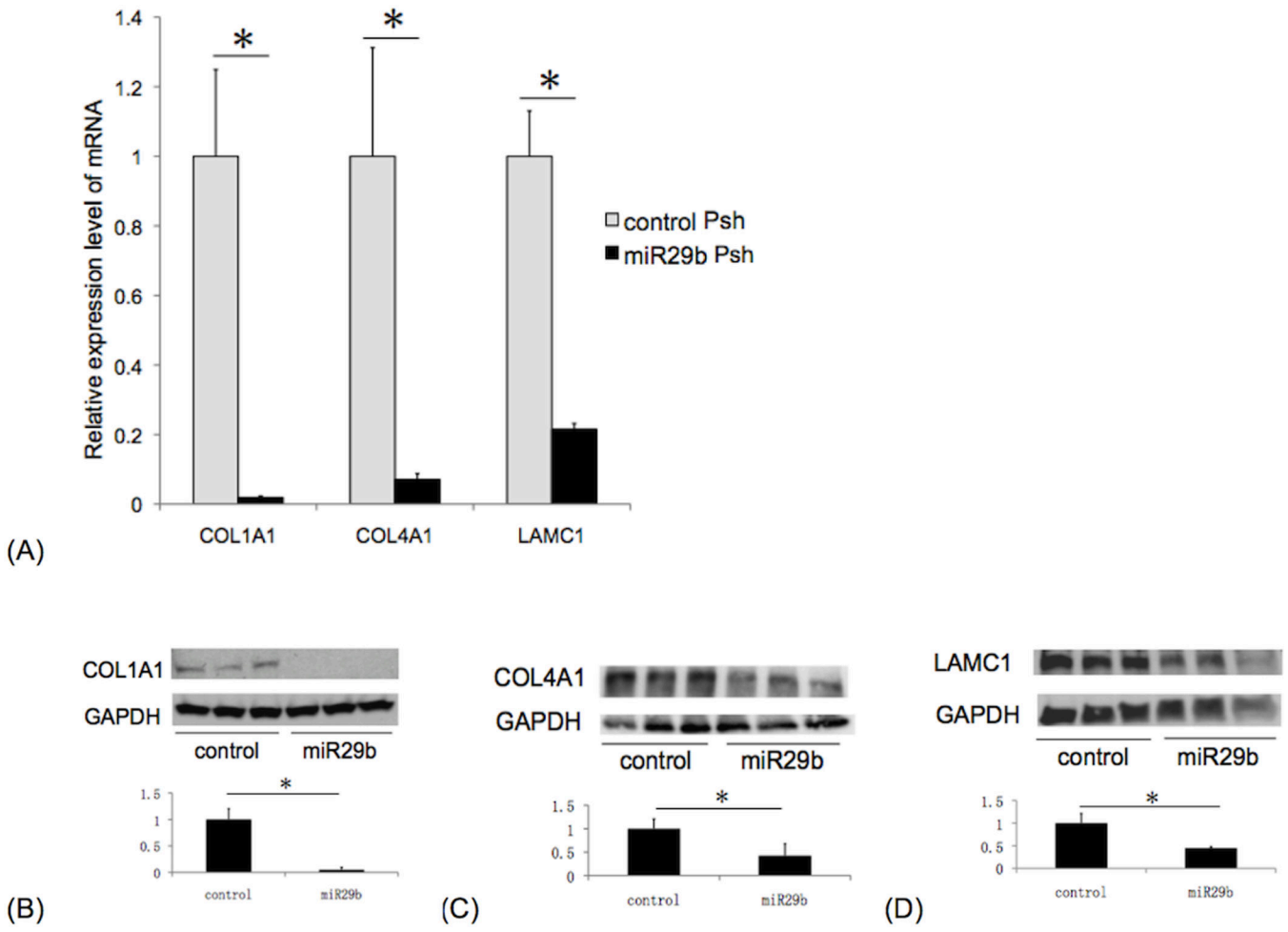

Figure 2. ECM mRNA and protein expression in miR-29b Psh transfected immortalized Fuchs dystrophy corneal endothelial cells

RT-PCR (A): Compared to control Psh transfected cells, miR-29b Psh transfected cells showed the following levels of ECM gene expression (mean ± standard deviation): COL1A1: 1.9 (±0.4)%; COL4A1: 7.1 (±1.7)%; LAMC1: 21.5 (±2.7)% (*$p < 0.05$, n=3 per each condition, bar: standard deviation). Western blot: compared to control Psh transfected cells, miR-29b Psh transfected cells showed the following levels of ECM protein expression (mean ± standard deviation): (B) COL1A1: 4.8 (±3.2)% , (C) COL4A1: 42.5 (±25.0)% , and (D) LAMC1: 44.8 (±3.1)% (*$p < 0.05$, n = 3 per each condition, bar: standard deviation).

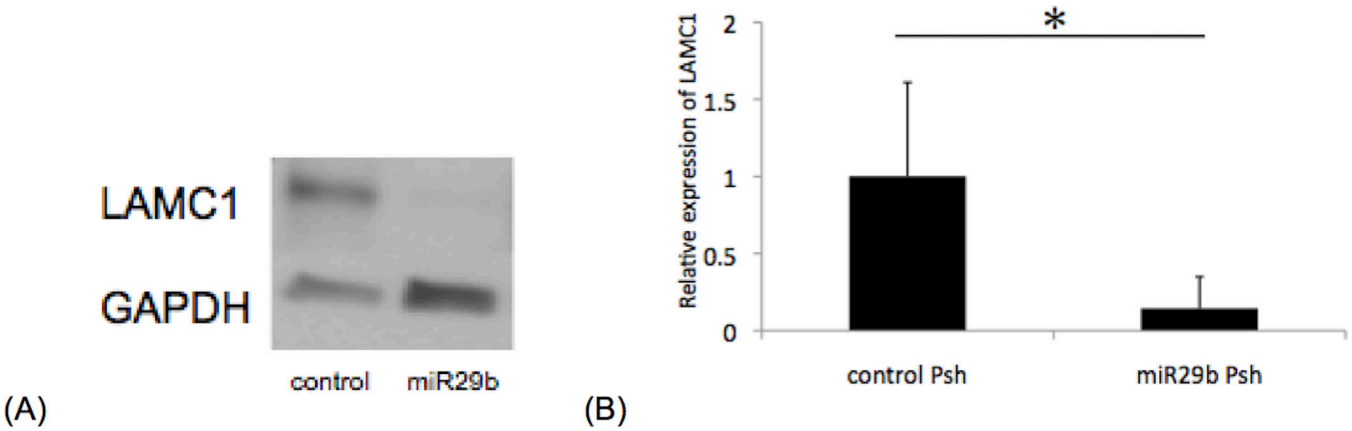

Figure 3. LAMC1 protein expression in miR-29b Psh transfected human corneal endothelium *in situ*

(A) Representative immunoblot demonstrating decreased LAMC1 protein expression in miR-29b Psh transfected corneal endothelium. (B) LAMC1 protein expression level in miR-29b Psh transfected corneal endothelium is 14.4 (±20.5)% of control Psh transfected corneal endothelium level (*$p < 0.05$, $n = 6$ per each condition, bar: standard deviation).

Table 1

Primer sets used for quantitative reverse transcriptase polymerase chain reaction.

Name	Symbol	Reference Sequence	Assay number
Collagen, type I, alpha 1	COL1A1	NM_000088.3	Hs00164004_m1
Collagen, type IV, alpha 1	COL4A1	NM_001845.4	Hs00266237_m1
Laminin, gamma 1	LAMC1	NM_002293.3	Hs00267056_m1
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	NM_173979.3	Hs02758991_g1
microRNA-29b	miR-29b	MIMAT0004515	hsa-miR-29b: 000413
small nucleolar RNA, C/D box 48	RNU48	NR_002745	RNU48: 001006