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Original

Myocardin-related transcription factor A (MRTF-A) regulates TGF-β2-induced type I collagen production in human lens epithelial cells.

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Abstract

We performed several experiments using human lens epithelium B3 (HLE-B3) cells to clarify whether myocardin-related transcription factor A (MRTF-A) affects type I collagen expression in transforming growth factor-β (TGF-β) -stimulated lens epithelial cells. To study the effect of MRTF-A, HLE-B3 cells were transfected with a small-interfering RNA (siRNA) against MRTF-A and cultured with or without TGF-β2. The effect of CCG203971, an MRTF-A inhibitor, on α-smooth muscle actin (α-SMA) and type I collagen expression was also examined. Gene expression was studied by quantitative real-time PCR, and subcellular localization of MRTF-A was studied by immunocytochemistry. TGF-β2 treatment promoted nuclear translocation of MRTF-A from the cytoplasm. TGF-β2 treatment increased α-SMA and type I collagen expression in HLE-B3 cells transfected with control siRNA, but not in MRTF-A siRNA transfectants. In addition, CCG203971 abolished TGF-β2-dependent α-SMA and type I collagen induction. Our results showed that TGF-β2 promoted α-SMA and type I collagen expression in HLE-B3 cells by stimulating the nuclear translocation of MRTF-A. These findings suggest that CCG203971, as can MRTF-A inhibitor, may prevent deterioration of visual quality by anterior subcapsular cataracts and posterior capsular opacification.

Key words: *lens epithelial cell, α-smooth muscle actin, CCG203971, epithelial-mesenchymal transition, type I collagen*

I. Introduction

Some lens epithelial cells can trans-differentiae into myofibroblast-like cells, which bring about anterior subcapsular cataracts (ASC)[1-3] and posterior capsular opacification (PCO) after cataract surgery[3,4]. These transdifferentiated lens epithelial cells not only express α-smooth muscle actin (α-SMA)[4-6], a biomarker of myofibroblasts [7],

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but also produce extracellular matrices that are mainly composed of type I collagen[5,6,8]. This phenomenon is known as the epithelial-mesenchymal transition (EMT)[3,8]. The deposition of these extracellular matrices can lead directly to decreased visual function [4,6,8,9], as well as other postoperative complications, such as contraction of the anterior capsular opening[8,10]. This contraction not only interferes with postoperative ophthalmoscopic examinations of the peripheral retina, but can also hinder retinal photocoagulation and vitreous surgery[11]. Thus, it is important to prevent EMT and decrease the deposition of these extracellular matrices.

Transforming growth factor-β (TGF-β) is a key factor in the induction of EMT in lens epithelial cells[2,3,12-16], retinal pigment epithelial cells[13,17-19], mammary epithelial cells[20-22], and renal tubular epithelial cells[23,24]. TGF-β promotes α-SMA expression and collagen production in these cells[12-24]. Recently, it was reported that myocardin-related transcription factor A (MRTF-A) plays an important role in the EMT of mammary epithelial cells[20-22] and renal tubular epithelial cells[23-25]. MRTF-A is a transcriptional co-activator that is sequestered in the cytoplasm[25,26]. After TGF-β stimulation, MRTF-A translocates into the nucleus and interacts with serum response factor (SRF) to promote gene expression, including that of α-SMA[24,25]. In kidney epithelial cells, both a dominant-negative MRTF-A variant and MRTF-A knockdown were found to prevent α-SMA mRNA expression induced by TGF-β1[24]. In an ex vivo rat lens explant model, TGF-β promoted nuclear translocation of MRTF-A and expression of the α-SMA protein[14,15].

However, it is not known whether MRTF-A affects type I collagen gene expression in TGF-β-stimulated lens epithelial cells. To reveal the effect of MRTF-A, human lens epithelial cells were transfected with small interfering RNA (siRNA) against MRTF-A and cultured with or without TGF-β2. Moreover, the effect of CCG 203971, a novel inhibitor of MRTF-A[27], on the expression of α-SMA and type I collagen was also examined.

II. Materials and Methods

1. Experimental procedures

1) HLE-B3 cell culture

Human lens epithelium B3 (HLE-B3) cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% (v/v) fetal bovine serum (FBS) (GE Healthcare Life Sciences, South Logan, UT, USA) in a humidified atmosphere with 5% CO_2.

The medium on subconfluent HLE-B3 cells was changed to DMEM containing 0.1% FBS, and the cells were treated with TGF-β2 (12.5 ng/ml; Sigma, St. Louis, MO, USA) for 24 h. CCG203971 was dissolved in DMSO and diluted in DMEM to 3 or 10 μM (final DMSO concentration: 0.1%).

2) siRNA transfection

We designed a 21-nucleotide duplex siRNA against human MRTF-A that targeted the 5'-AAGAACATCCTTCCTGTTGAG-3'seed sequence. The siRNA was synthesized by Nippon Gene Material Co., Ltd. (Toyama, Japan). The target sequence of the control Photinus pyralis luciferase siRNA was

5'-AAGCCATTCTATCCTCTAGAG-3', which has no significant homology to any mammalian gene sequence. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's protocol and cultured for 2 d before analysis.

3) RNA preparation and quantitative reverse transcription PCR (RT-PCR) analysis

Total RNA from HLE-B3 cells was prepared using the ISOGEN reagent (Nippon Gene). ARPE-19, human retinal pigment epithelial cells , were used as a positive control (ATCC). Complementary DNAs were prepared from 1 μg of total RNA using PrimeScript Reverse Transcriptase (Takara Bio, Kyoto, Japan), according to the manufacturer's instructions. PCR amplification was performed using 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72°C for 1 min. Quantitative RT-PCR was performed using Ex Taq polymerase (Takara Bio) for expression levels of α-SMA and type I collagen. Quantitative RT-PCR was performed using the EcoTM RT-PCR system (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The primers used were 5'-ATGGCCATGATTTGCAGCTG-3' (sense) and 5'-AGAGCCACGATTGGAGTAC-3' (antisense) for human MRTF-A; 5'-ACCCACAGGACCTACCGCTG-3' (sense) and 5'-AAAAACTGACCTTTTAGAATC-3' (antisense) for human MRTF-B; 5'-ATTGCCGACCGAATGCAGAAG-3' (sense) and 5'-AGAGGCCAGGATGGAGCCAC-3' (antisense) for human smooth muscle actin (ACTA2); 5'-TGGACCAGCAGACTGGCAAC-3' (sense) and 5'-TCGTGCAGCCATCGACAG TGAC-3' (antisense) for human collagen type I A; and 5'-AGGTCATCCATGACAACT TTG-3' (sense) and 5'-TTCAGCTCAGGGAT GACCTT-3'(antisense) for human GAPDH.

2. Immunocytochemistry

Immunocytochemical analysis was performed using the anti-MRTF-A antibody[28], as described above[29]. All cell-culture media were from Life Technologies, Inc (MD, USA). An Alexa 488-conjugated anti-rabbit antibody and Hoechst33342 were obtained from Molecular Probes (Oregon, USA) and used for nuclear staining. Subconfluent HLE-B3 cells were cultured in DMEM containing 0.1% FBS. Subconfluent HLE-B3 cells were cultured in DMEM containing 0.1% FBS. Then, the cells were treated with TGF-β (12.5 ng/ml; Sigma, St. Louis, MO, USA) for 24 h.

3. Statistics

Data are expressed as the mean±standard error. Statistical analysis was performed using one-way analysis of variance, followed by a post-hoc comparison with Tukey's multiple comparison test. Statistical tests were performed using KaleidaGraph software, version 4.1 (Synergy Software, Reading, PA, USA).

4. Institutional review board/ethics committee approval (IRB)

The IRB/Ethics Committee at our institution ruled that approval was not required for this study.

III. Results

Two MRTF isoforms exist, namely MRTF-A and –B[30]. ARPE-19 , human retinal pigment epithelial cells, were used as a positive control. HLE-B3 cells expressed MRTF-A mRNA, but

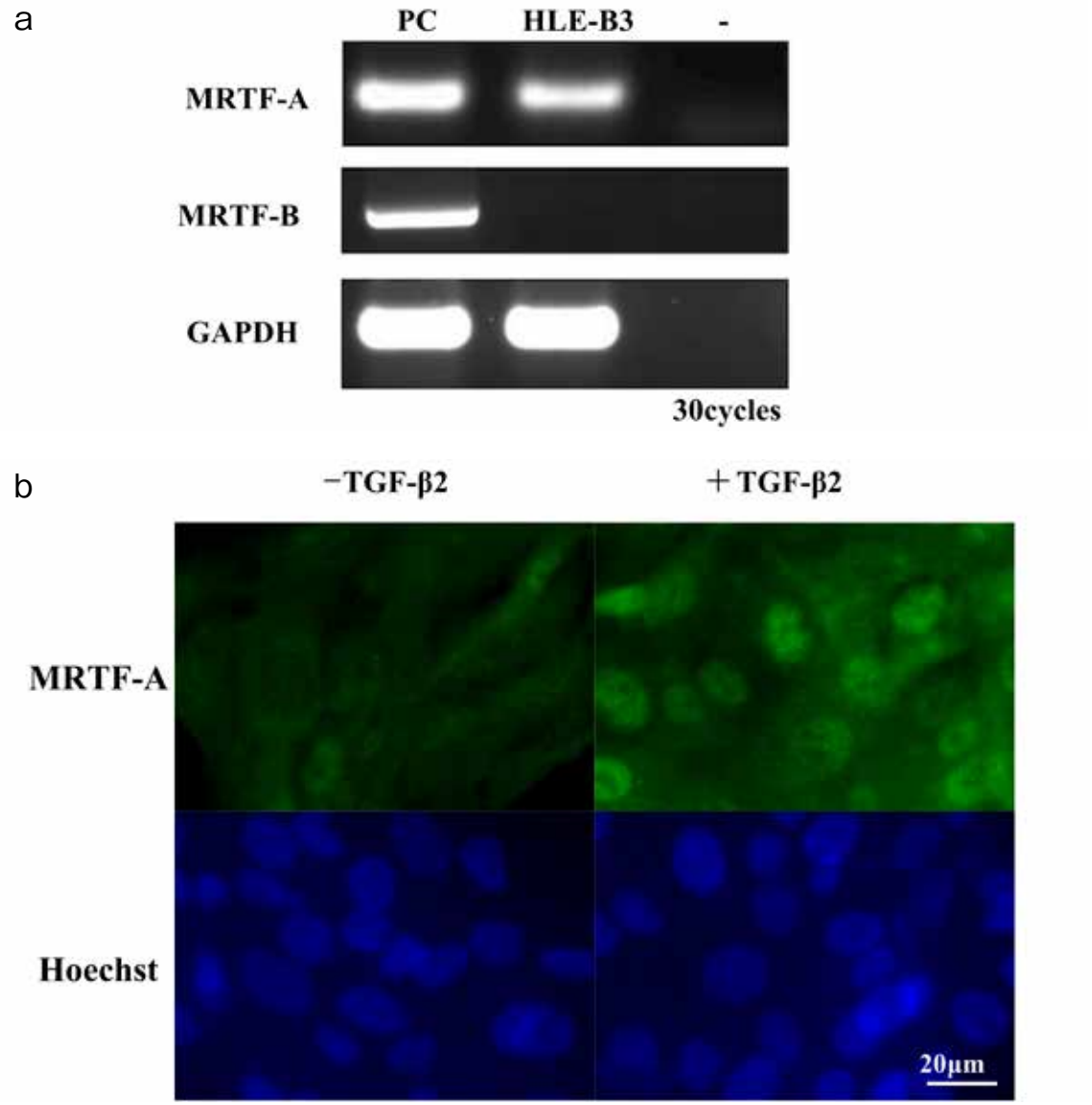

Fig. 1. Translocation of MRTF-A in HLE-B3 cells stimulated by TGF-β2.
a: As a positive control, ARPE-19 cells expressed MRTF-A and MRTF-B mRNA. HLE-B3 cells expressed MRTF-A mRNA, but not MRTF-B mRNA.
b: HLE-B3 cells were cultured with or without 12.5 ng/ml TGF-β2 for 24 h and then treated and immunostained using MRTF-A-specific antibody (green) and a nuclear stain (Hoechst, blue). MRTF-A remained in the cytosol in the absence of TGF-β2, but was translocated to the nucleus after TGF-β2 treatment.

not MRTF-B mRNA (Fig. 1a). Morphological changes of HLE-B3 cells by TGF-β2 were not observed. To examine whether TGF-β2 could promote the nuclear translocation of MRTF-A, HLE-B3 cells were cultured with or without 12.5 ng/ml TGF-β2 for 24 h. MRTF-A remained in the cytosol in the absence of TGF-β2, but was translocated to the nucleus after TGF-β2 treatment (Fig. 1b).

Transfection with MRTF-A siRNA decreased MRTF-A mRNA expression in HLE-B3 cells compared with that in control

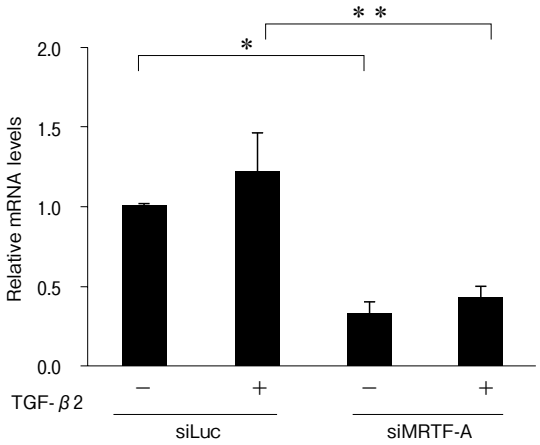

Fig. 2. Effect of TGF-β2 on the MRTF-A mRNA expression.
Transfection with MRTF-A siRNA (siMRTF) decreased MRTF-A mRNA expression in HLE-B3 cells compared with that in control siRNA (siLuc) transfectants. The expression of MRTF-A mRNA was slightly increased by treatment of 12.5 ng/ml TGF-β2, but this increase was not significant. Expression levels were normalized by GAPDH. Data are mean ±SEMs of 3 independent experiments. *$p < 0.0001$, compared with control siRNA, **$p < 0.001$, compared with control siRNA + TGF-β2.

siRNA transfectants. TGF-β2 treatment slightly increased the expression of MRTF-A mRNA, but this increase was not significant (Fig. 2).

To examine whether MRTF-A is related to α-SMA expression in HLE-B3 cells, cells transfected with control or MRTF-A siRNA were cultured with or without TGF-β2 for 24 h. Morphological changes of these cells by TGF-β2 were not observed. TGF-β2 treatment increased α-SMA expression in HLE-B3 cells transfected with control siRNA. This increase was inhibited in HLE-B3 cells treated with MRTF-A siRNA (Fig. 3a).

To examine whether MRTF-A is related type I collagen expression in HLE-B3 cells, cells transfected with control or MRTF-A siRNA were cultured with or without TGF-β2 for 24 h. TGF-β2 increased type I collagen expression in HLE-B3 cells transfected with control siRNA. This increase was partially inhibited in HLE-B3 cells treated with MRTF-A siRNA (Fig. 3b).

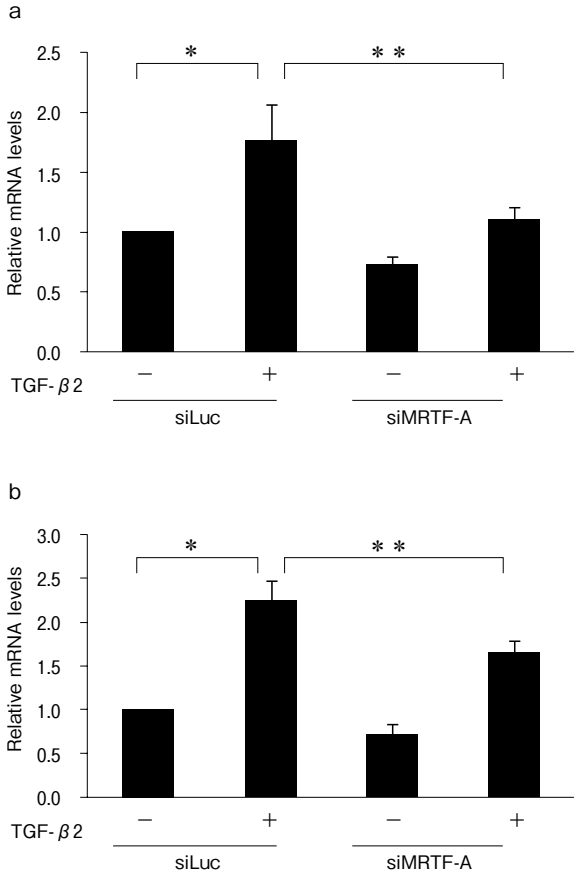

Fig. 3. Roles of MRTF-A in the α-SMA and type I collagen expressions in HLE-B3 cells treated with TGF-β2.
HLE-B3 cells transfected with control siRNA (siLuc) or MRTF-A siRNA (siMRTF) were cultured with or without 12.5 ng/ml TGF-β2 for 24 h. TGF-β2 treatment increased α-SMA (a) and type I collagen (b) expression in HLE-B3 cells transfected with control siRNA (siLuc). These increases were inhibited in HLE-B3 cells treated with MRTF-A siRNA (siMRTF). Expression levels were normalized by GAPDH. Data are mean ± SEMs of 3 independent experiments. *$p < 0.0001$, compared with control siRNA, **$p < 0.001$, compared with control siRNA + TGF-β2.

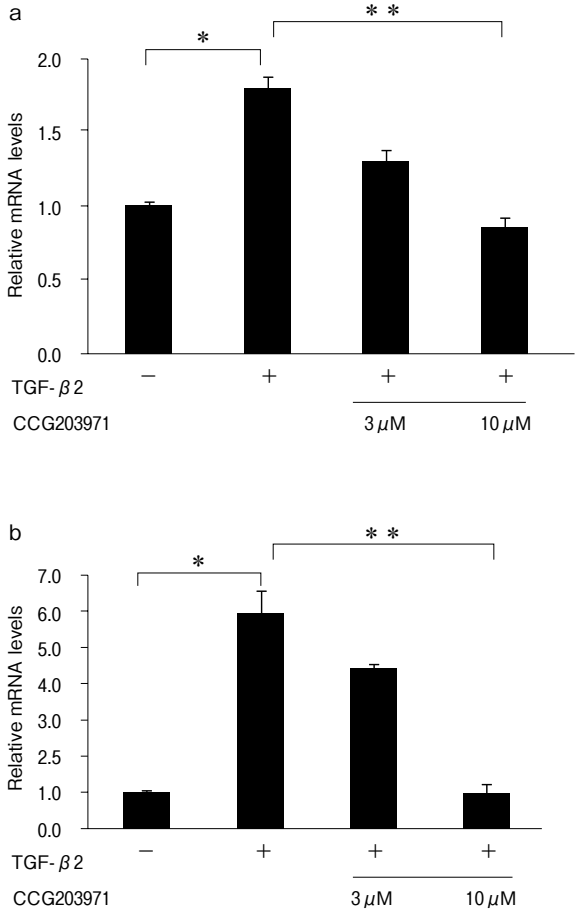

Fig. 4. The effect of CCG-203971 on the α-SMA and type I collagen expressions in HLE-B3 cells treated with TGF-β2.
Treatment with 12.5 ng/ml TGF-β2 increased α-SMA (a) and type I collagen (b) expression in HLE-B3 cells. However, treatment with TGF-β2 and 10 μM CCG203971 abolished these increases. Expression levels were normalized by GAPDH. Data are mean± SEMs of 3 independent experiments. *$p < 0.001$ compared with control (no treatment), **$p < 0.001$ compared with only 12.5 ng/ml TTGF-β2 treatment.

To determine whether CCG203971, a novel inhibitor of MRTF-A [27], could also reduce the expression levels of α-SMA and type I collagen, we cultured TGF-β2-treated HLE-B3 cells with or without CCG203971. Although treatment with TGF-β2 increased α-SMA (Fig. 4a) and type I collagen (Fig. 4b) expression in HLE-B3 cells, CCG203971 abolished these increases (Fig. 4a and b). However, the effects of incubation with 30 μM CCG203971 could not be examined due to cytotoxicity (data not shown).

IV. Discussion

In this study, TGF-β2 promoted nuclear MRTF-A translocation in HLE-B3 cells. TGF-β2 also increased α-SMA and type I collagen mRNA expression, which was repressed following MRTF-A knockdown. The novel inhibitor of MRTF-A, CCG203971, abolished TGF-β2-dependent induction of α-SMA and type I collagen mRNA in HLE-B3 cells. These results suggest that MRTF-A plays a critical role in the EMT of HLE-B3 cells with cultured cells. It is possible that an inhibitor, such as CCG203971, may block type I collagen in production lens epithelial cells produced by TGF-β *in vivo*.

In this study, MRTF-A knockdown and MRTF-A inhibition by CCG203971 repressed type I collagen expression induced by TGF-β2 in HLE-B3 cells. It has not been reported whether MRTF-A plays an important role in collagen expression in TGF-β-induced EMT of human lens epithelial cells. However, in rat renal tubular epithelial NRK-52 cells, the induction of type I collagen mRNA and protein expression by glucose was alleviated by MRTF-A knockdown [31]. Moreover, MRTF-A is a critical mediator of the myodifferentiation of lung [32,33] and cardiac [34] fibroblasts. In these cells, TGF-β promoted the nuclear translocation of MRTF-A, which interacts with SRF to induce α-SMA and type I collagen gene expression. Silencing MRTF-A abolished their induction by TGF-β [33,34].

The Rock inhibitor, Y-27632, inhibited the induction of type I collagen mRNA by TGF-β in retinal pigment epithelial cells [18] and influenced MRTF-A activity [24,34]. After translocation from the cytoplasm to the nucleus, MRTF-A can cooperate with SRF and induce gene expression[32]. This subcellular localization is controlled by monomeric actin (G-actin)[26]. Increased cytoplasmic G-actin concentration promotes G-actin binding to MRTF-A, which retains MRTF-A in the cytoplasm [26,35]. Y-27632 prevents Rho-dependent stress fiber formation, which in turn induces G-actin concentration [15,24]. Thus, Y-27632 reduces the nuclear translocation of MRTF-A in various cells [24] including lens epithelial cells [15]. These findings suggest that the inhibition of TGF-β-dependent expression of type I collagen mRNA expression by Y-27632 may relate to the suppression of nuclear MRTF-A transport.

In this study, the MRFT-A inhibitor CCG203971 suppressed α-SMA and type I collagen expression in HLE-B3 cells. Recently, it was reported that nuclear MRTF-A transport was inhibited by CCG203971 in rat lens epithelial cells [15]. These findings suggest that CCG203971 can potentially serve as a new therapy against anterior capsular cataracts and posterior capsular opacification. CCG203971 is a second-generation inhibitor with far less cytotoxicity than the first-generation inhibitor, CCG1423 [27]. However, in our study, significant inhibition of α-SMA and type I collagen expression in HLE-B3 cells was only obtained using 10 μM CCG203971, as 30 μM CCG203971 showed cytotoxicity. Therefore, further investigations are required before CCG203971 can be used safely as a drug applied topically to the eye.

In this study, TGF-β2 slightly increased the expression of MRTF-A mRNA, but this increase was not significant. In MS-1 endothelial cells, TGF-β promoted α-SMA expression by stimulating the nuclear transport of MRTF-A. Moreover, TGF-β enhanced MRTF-A transcription, which also promoted accumulation of MRTF-A in the nucleus [36]. Further investigation is required to determine how enhanced MRTF-A transcription is related to α-SMA expression induced by TGF-β2 in HLE-B3 cells.

In conclusion, our results show that TGF-β2 promoted the expression of α-SMA and type I collagen in HLE-B3 cells via MRTF-A. These findings suggest that MRTF-A is a critical mediator of type I collagen production in human lens epithelial cells.

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Conflicts of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Myocardin 関連転写因子 A（MRTF-A）は，ヒト水晶体上皮細胞において TGF-β2 の誘導により Ⅰ型コラーゲン産生を調節する

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要旨

トランスフォーミング増殖因子β（TGF-β）で刺激した水晶体上皮細胞において，Myocardin 関連転写因子 A（MRTF-A）がⅠ型コラーゲンの発現に影響を及ぼすかどうかを明らかにするために，ヒトの水晶体上皮細胞 B3 (HLE-B3) に，MRTF-A に対する低分子干渉 RNA（siRNA）をトランスフェクトし，TGF-β2 の有無で培養した．さらにα平滑筋アクチン（α-SMA）およびⅠ型コラーゲン発現を MRTF-A 阻害剤である CCG203971 で検討した．遺伝子発現は real-time PCR によって，MRTF-A の細胞内局在を免疫細胞染色によって検討した．TGF-β2 を添加することによって MRTF-A の細胞質からの核内移行を促進した．HLE-B3 細胞における TGF-β2 の添加は、対照の siRNA においてα-SMA およびⅠ型コラーゲン発現を増加させたが，MRTF-A siRNA では増加しなかった．さらに，CCG203971 は TGF-β2 依存性にα-SMA およびⅠ型コラーゲン誘導を阻害した．今回，HLE-B3 細胞において TGF-β2 が MRTF-A の核内移行を刺激し，α-SMA およびⅠ型コラーゲン発現を促進することを示した．この検討で MRTF-A 阻害剤である CCG203971 が，前嚢下白内障および後嚢下混濁による視力低下を予防し得ることを示唆した．