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# Inhibition of Rho kinase suppresses capsular contraction following lens injury in mice

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## Abstract:

**PURPOSE:** We investigated the effect of systemic fasudil hydrochloride and an inhibitor of nuclear translocation of myocardin-related transcription factor-A (MRTF-A) on capsular contraction in a puncture-injured lens in mice.

**MATERIALS AND METHODS:** Lens injury of an anterior capsular break was achieved in male adult C57Bl/6 mice under general and topical anesthesia at 1 h after systemic fasudil hydrochloride (intraperitoneal, 10 mg/kg body weight) or vehicle administration. The mice were allowed to heal after instillation of ofloxacin ointment, for 5 and 10 days with daily administration of fasudil hydrochloride or vehicle. In another series of experiment, we examined the effect of systemic administration of an MRTF-A inhibitor (CCG-203971, 100 mg/kg twice a day) on fibrogenic reaction and tissue contraction in an injured lens on day 5 or 10. The eye was processed for histology and immunohistochemistry for SM22, proliferating cell nuclear antigen (PCNA), or MRTF-A. In hematoxylin and eosin -stained samples, the distance between each edge of the break of the anterior capsule was measured and statistically analyzed.

**RESULTS:** A cluster of lens cell accumulation was formed adjacent to the edge of the capsular break on day 5. It contained cells labeled for SM22 and PCNA. The size of the cell cluster was larger in fasudil group of mice than in control mice on day 5. Systemic fasudil or CCG-203971 suppressed an excess contraction of the capsular break at certain time points.

**CONCLUSION:** Systemic administration of fasudil hydrochloride could be a treatment strategy of postoperative capsular contraction following cataract-intraocular lens surgery.

## Keywords:

Lens epithelial cell, mouse, myocardin-related transcription factor-a, rho, tissue fibrosis

## Introduction

Capsular contraction resultant to tissue fibrotic reaction is one of the major causes of decentration of an intraocular lens (IOL) implanted in the capsular bag after cataract removal. Clinicopathological examination using immunohistochemistry shows that a number of myofibroblasts were observed in fibrous tissue formed on the inner surface of the anterior capsule with an IOL.<sup>[1-3]</sup> Epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs)

is the major pathobiological mechanism of the appearance of myofibroblasts in a contracted fibrous lens capsule during healing postcataract surgery. LECs transform into a myofibroblast on activation by exposure to various growth factors and exert a fibrogenic reaction and tissue contraction. Among such growth factors, transforming growth factor- $\beta$  (TGF- $\beta$ ) is a powerful inducer of EMT.

Although TGF- $\beta$  activates multiple cytoplasmic signaling cascades, many of which accelerate the process of EMT, major EMT-related cascades include Smad and Rho kinase (ROCK) signal.<sup>[4,5]</sup>

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During EMT, TGF- $\beta$  dramatically reorganizes cytoskeletal components through the activation of Rho family of GTPases-actin polymerization system.<sup>[6-8]</sup>

As for the EMT in LECs, we reported that Smad3 is a critical factor involved in LEC's EMT in both *in vivo* and *in vitro* conditions.<sup>[9]</sup> The involvement of RhoA-ROCK signal in TGF- $\beta$ -induced  $\alpha$ SMA expression and EMT has also been well studied *in vitro* and *in vivo* conditions.<sup>[10-18]</sup> Although in a limited case in mice, topical administration of a Rho/ROCK inhibitor reportedly suppresses the development of ultraviolet-induced cataract.<sup>[19]</sup>

Rho-induced actin polymerization is mediated by the nuclear accumulation of myocardin-related transcription factors (MRTFs). Two isoforms, MRTF-A and MRTF-B, are normally bound to G-actin. During actin polymerization, free MRTF translocates to the nucleus.<sup>[20]</sup> Recently, it was reported that TGF- $\beta$  induced EMT in Rho/MRTF-A-dependent like other tissue fibrosis models.<sup>[21]</sup>

Fasudil hydrochloride is a selective Rho/ROCK inhibitor that has clinical therapeutic efficacy in patients with cerebral vascular spasm.<sup>[22-24]</sup> In the current study, we investigated the effect of systemic fasudil hydrochloride and an inhibitor of nuclear translocation of MRTF-A on capsular contraction in a puncture-injured lens in mice.

## Methods

Each experiment was approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University (Approval No. 748 and 925) and was conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

### Lens injury model in mouse eyes

Lens injury of an anterior capsular break was achieved as previously reported.<sup>[2,9]</sup> Male adult C57Bl/6 mice were generally anesthetized with an intraperitoneal (ip) pentobarbital sodium (70 mg/kg body weight) and also topically by oxybuprocaine eye drops. A small incision was made in the central anterior capsule with a 26-G hypodermic needle through a corneal incision in one eye (right) after the topical application of mydriatic 1 h after systemic fasudil hydrochloride (ip, 10 mg/kg body weight) or vehicle administration.<sup>[25]</sup> The depth of puncture from the corneal surface was approximately 300  $\mu$ m that was about one-fourth of the length of the blade part of the needle. The mice were allowed to heal after instillation of ofloxacin ointment for 3, 5, and 10 days with daily administration of fasudil hydrochloride or vehicle. The number of eye samples was 3/2, 23/10, or

11/25 for each of the control vehicle group/fasudil group on day 3, 5, or 10, respectively. Eucleated globes were fixed and embedded in paraffin.

In another series of experiment, we examined the effect of systemic administration of an MRTF-A inhibitor (CCG-203971) on fibrogenic reaction and tissue contraction in an injured lens. CCG-203971 blocks nuclear translocation of MRTF-A and suppresses resultant Rho-related tissue fibrogenesis. CCG-203971 (100 mg/kg in 50  $\mu$ l dimethyl sulfoxide) was ip, administered at the time of lens injury and then every 12 h until day 5 or day 10.<sup>[26]</sup> The number of eye samples was 10/7 or 10/6 for each of control vehicle group/CCG-203971 group on day 5 or day 10, respectively.

### Histology and immunohistochemistry

Deparaffinized sections were processed for hematoxylin and eosin (HE) staining and immunohistochemistry as previously reported.<sup>[2,9]</sup>

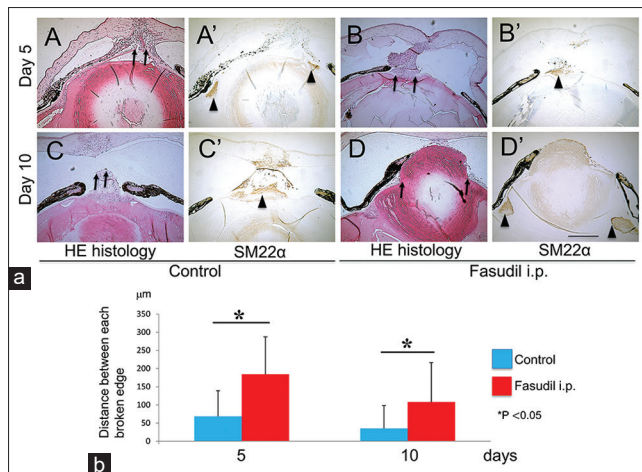
In HE-stained samples, the distance between each edge of the break of the anterior capsule was measured and was statistically analyzed in samples of days 5 and 10. The number of eye samples used in the analysis was 10/10 or 11/25 for each of control vehicle group/fasudil group on day 5 or 10, respectively. In CCG-203971 experiment, the sample size was 10/7 or 10/6 for each of control vehicle group/CCG-203971 group on day 5 or day 10, respectively. For immunohistochemistry, the following antibodies were used: mouse monoclonal anti-MRTF-A antibody (1:100 in phosphate-buffered saline [PBS], Santa Cruz Biotechnology, Dallas, Texas, USA) or rabbit polyclonal anti-SM22 $\alpha$  antibody (1:200 in PBS, Abcam, Cambridge, UK). After secondary peroxidase-conjugated antibody reaction and washing in PBS, the antibody complex was visualized with 3,3'-diaminobenzidine reaction. After nuclear counterstaining with methyl green, the specimens were observed under the regular light microscopy.

Cell proliferation activity was evaluated by immunohistochemistry using mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (1:100 in PBS, Abcam, Cambridge, UK) and measurement of the size of the cell cluster formed at the edge of the broken anterior capsule in each sample of days 5 and 10. The number of eye samples available in the analysis was 23/8 or 8/8 for each of control vehicle group/fasudil group on day 5 or 10, respectively.

## Results

### Contraction of the broken anterior capsule in fasudil-treated mice

A cluster of lens cell accumulation that was immunohistochemically labeled for SM22 was observed



**Figure 1:** Histology and immunohistochemical detection of SM22 in an injured lens. (a) Frames A–D show hematoxylin and eosin-stained histology of a puncture-injured lens in control (A, C) and fasudil hydrochloride-treated mice (B, D) on day 5 (A, B) and day 10 (C, D), respectively. Frames A'–D' indicate the positive expression of SM22 expression (black arrows) in Frames A–D, respectively. The cells in the clusters were considered to be myofibroblasts derived from lens epithelial cells through epithelial-mesenchymal transition. Bar, 100 µm. (b) The distance between each edge (arrows) of the broken capsule was measured and statistically analyzed. The distance between each edge of the broken capsule was statistically significantly larger on both days 5 and 10 in fasudil group of mice as compared with control mice. Significant \* $P < 0.05$  by Mann-Whitney U test

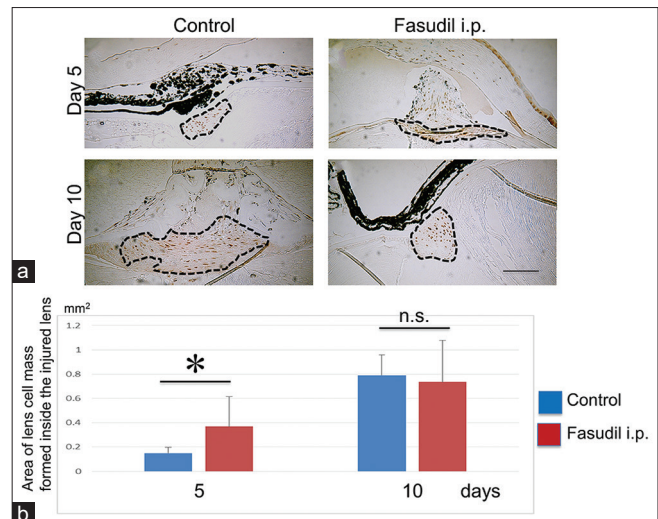
in each edge of the broken anterior capsule [Figure 1a]. The cells in the clusters were therefore considered to be myofibroblasts derived from LECs through EMT. The distance between each edge of the broken capsule was measured and statistically analyzed. The results indicated that the distance between each edge of the broken capsule was statistically significantly larger on both days 5 and 10 in the fasudil group of mice as compared with control mice [Figure 1b].

### Lens epithelial cell proliferation in fasudil-treated mice

A cluster of lens cell accumulation contained a number of PCNA-labeled cells, which indicated that the cluster was formed by cell proliferation [Figure 2a]. The size of the cluster in each sample was determined and statistically analyzed. The results showed that the size of the cell accumulation cluster was larger in a mouse with systemic fasudil hydrochloride as compared with a control mouse on day 5, but not on day 10 [Figure 2b].

### Expression pattern of myocardin-related transcription factor-A in lens cells

MRTF-A was detected in the nuclei of the cells in the cell cluster formed at the edge of the broken capsule of control mice through the healing interval up to day 10 [Figure 3a, c and e]. MRTF-A staining was also detected in the nuclei of the lens cells of fasudil group of mice on day 3 and day 5 [Figure 3b and d]. On the other hand, on day 10, lens cells in the cell cluster did not show nuclear MRTF-A immunoreactivity [Figure 3f].



**Figure 2:** Effects of systemic fasudil hydrochloride on the growth of lens epithelial cells. (a) A cluster of lens cell accumulation contained a number of proliferating cell nuclear antigen-labeled cells in both control and fasudil groups of mice on day 5 and day 10, which indicated that the cluster was formed by cell proliferation. Bar, 10 µm. (b) The size of the cell accumulation cluster was larger in a mouse with systemic fasudil hydrochloride as compared with a control mouse on day 5, but not on day 10. Significant \* $P < 0.05$  by Mann-Whitney U test

### Effect of systemic CCG-203971 administration on postinjury capsular contraction

As shown in Figure 4a, ip administration of CCG-203971 suppressed the nuclear expression of MRTF-A in the lens cells accumulated at the edge of the broken capsule as compared with a control sample. Contraction of the broken anterior capsule was significantly attenuated by systemic CCG-203971 on day 5, not on day 10, postlens injury [Figure 4b and c].

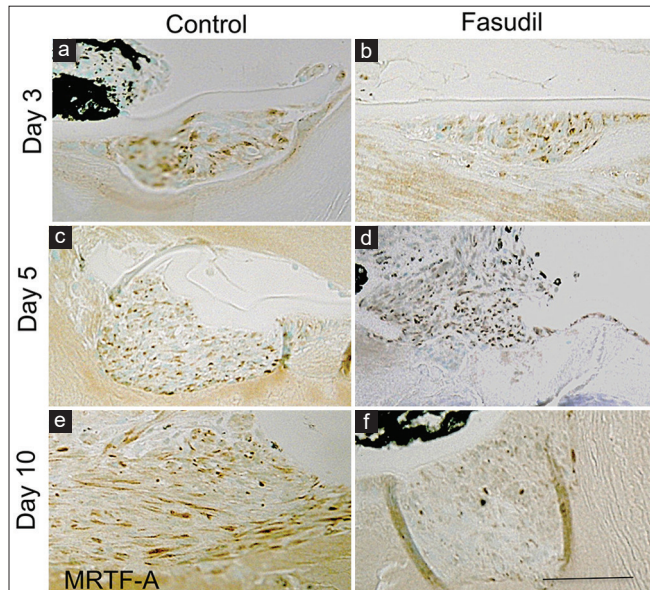
## Discussion

In the current study, we showed that systemic administration of fasudil or of an MRTF-A signal inhibitor, CCG-203971, suppressed contraction of the broken anterior capsule following lens injury in mice.

Rho kinase is reportedly one of the TGF- $\beta$ -activated signaling cascades that involved in EMT in cell types, for example, LEC, in cell culture.<sup>[10,21,27]</sup> Besides Rho signal, mitogen-activated protein kinase, Smad2/3, TAK1/c-Jun N-terminal kinase or p38,  $\beta$ -catenin, and etc., are known to promote EMT process in cell culture.<sup>[28-31]</sup> We cleared that the loss of Smad3 blocks LEC's EMT during healing postpuncture injury in mice.<sup>[9]</sup> However, it was not examined if the inhibition of Rho actually suppresses EMT in a postinjury lens *in vivo*. To clear this question, we conducted the current study using a drug that had been approved to be administered to the patients with cerebral vascular spasm and fasudil hydrochloride. The results showed that systemic fasudil suppressed excess contraction of the injured capsule, although it

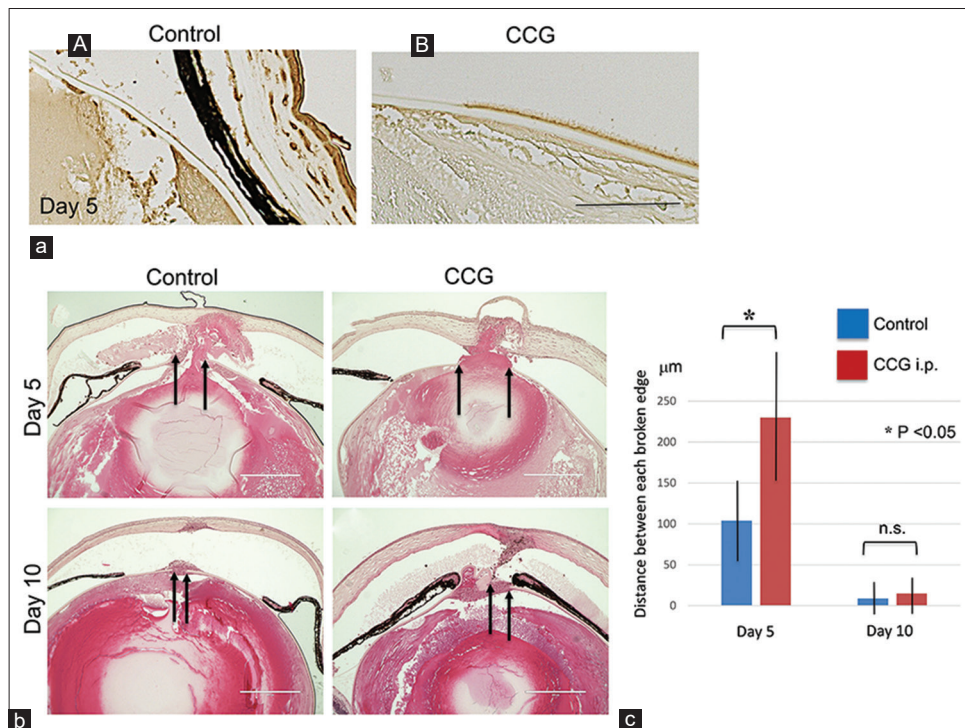
did not block the formation of myofibroblasts of an EMT origin. The phenotype could be explained by the

following previous literatures. Cytoskeletal components are dramatically reorganized during the process of EMT.<sup>[32]</sup> Activation of RhoA/Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK1 and 2) is required for the assembly of actin stress fibers for cell contractile force generation.<sup>[6,33]</sup> Thus, the inhibition of Rho kinase was hypothesized to reduce tissue contraction in an injured lens.



**Figure 3:** Expression pattern of myocardin-related transcription factor-A in lens cells during postinjury interval. Myocardin-related transcription factor-A was detected in the nuclei of the cells in the cell cluster formed at the edge of the broken capsule of control mice through the healing interval up to day 10 (a, c, and e). Myocardin-related transcription factor-A staining was also detected in the nuclei of the lens cells of fasudil-group of mice on day 3 and day 5 (b and d). On the other hand, on day 10 lens cells in the cell cluster did not show nuclear myocardin-related transcription factor-A immunoreactivity (f). Bar, 10 μm

Gupta *et al.* reported that MRTF-A is one of the downstream transmitters that follows Rho-kinase activation during the process of LEC's EMT in cell culture. They showed that the inhibition of nuclear translocation of MRTF-A dramatically suppressed the formation of an αSMA-positive myofibroblast from a cultured LEC.<sup>[27]</sup> We, therefore, here examined if systemic fasudil hydrochloride inhibits the nuclear accumulation of MRTF-A in an injured lens epithelium in mice. Our result showed that during the early phase (on days 3 and 5) of healing postinjury, MRTF-A was detected in the nuclei of LECs accumulated at the site of capsular break in both control and fasudil groups of mice, and then on day 10, nuclear MRTF-A was no longer observed in the cells of a fasudil group mouse, while it was well seen in the cells of a control mouse. We therefore next examined the effect of systemic administration of an MRTF-A inhibitor, CCG-203971, on capsular contraction in an injured



**Figure 4:** Effect of systemic CCG-203971 administration on postinjury capsular contraction. (a) On day 5 postinjury lens epithelial cells adjacent to the capsular break exhibit nuclear myocardin-related transcription factor-A immunoreactivity (A). Systemic administration of CCG-203971 suppressed the nuclear expression of myocardin-related transcription factor-A in lens epithelial cells beneath the broken capsule (B). Bar, 10 μm. (b) H and E staining histology of an injured lens of control and CCG-203971-treated mice on days 5 and 10. Arrows indicate the edge of the broken capsule. Bar, 100 μm. (c) Contraction of the broken anterior capsule was significantly attenuated by systemic CCG-203971 on day 5, not on day 10, postlens injury. Significant \* $P < 0.05$  by Mann-Whitney U test

lens. The results showed that systemic CCG-203971 administration suppressed contraction of the broken anterior capsule on day 5, but not on day 10, in an injured mouse lens.

Taiyab *et al.* reported that the inhibition of Smad3 by adding SIS3, a Smad3 inhibitor, blocks nuclear translocation of MRTF-A and also myofibroblast formation in cultured LECs.<sup>[34]</sup> As mentioned above in the Introduction, the loss of Smad3 abolishes LEC's EMT *in vivo*.<sup>[9]</sup> MRTF-A cascade is considered to be one of the Smad3 downstream components toward EMT *in vivo*-like *in vitro* condition.<sup>[34,35]</sup>

## Conclusion

In the clinical setting, systemic administration of fasudil hydrochloride is a drug legally approved for the treatment of a nonophthalmic disease, i.e., cerebral vascular spasm, which could be an advantage to establish a treatment strategy of postoperative capsular contraction following cataract-IOL surgery.

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Nil.

## Conflicts of interest

The authors declare that there are no conflicts of interests of this paper.

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